The reproductive organs, sexual behaviour, and pheromone glands of *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae), and *Phthorimaea operculella* (Zeller) (Lepidotera: Gelechiidae)

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requirements for the Degree of Doctor
of Philosophy in Biological Sciences at
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by

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Abstract

Separate studies concerning reproduction were made of *Costelytra zealandica* (White) (Scarabaeidae: Melolonthinae), and of *Phthorimaea operculella* (Zeller) (Gelechiidae: Gnorimoschemini).

In *C. zealandica* sexual dimorphism was described; the male and female external genitalia were described in more detail than previously; an account was given of the muscles of the external genitalia and of the mechanics of copulation; and the first description was provided of the morphology and histology of the internal reproductive organs. The latter were shown to differ from those of other Scarabaeidae in only a few minor respects. In addition the accessory glands of the female were described and a preliminary identification of the contained bacteria was carried out.

The first account of the sexual behaviour of *C. zealandica* was given and the influences of environmental factors on both this and flight were noted. In addition, sexual attraction was investigated in the field: extracts of females were found to be attractive to males and confirmation was obtained that phenol attracts males. The sex attractant was demonstrated to be most effective when located near the edges of tall edible plants where the beetles swarm.

In *P. operculella*, sexual dimorphism, and the morphology and histology of the internal reproductive organs were described for the first time. The latter were found to show close similarities with those of various other Lepidoptera. In addition, the male and female external genitalia were described in more detail than previously.

The sexual behaviour of *P. operculella* was described for the first time and the time of day when this moth is sexually active was investigated both in the field and laboratory. It was shown that the time when mating occurs is determined by the female releasing her sex pheromone whereas the male will respond at any time although this is maximal when the female is attractive.

An objective and quantitative laboratory bioassay was developed for the female sex pheromone of *P. operculella* and this was used to conclusively demonstrate the presence of a female sex pheromone and to show that it is produced in the middle third of the ovipositor. A histological examination confirmed that the pheromone gland is a dorsal sac-like region of the intersegmental membrane between the 9th and 10th abdominal segments on the ovipositor. The male pheromone glands were shown by histological methods to be located on the hindwings, and an indication was obtained that their scent subdues the escape reactions of females prior to copulation. Finally, the morphology, histology, and ultrastructure of male and female pheromone glands were described and compared with those of other Lepidoptera.

A note on the citation of publications, and the referral to species and their systematic status.

Any publication by more than two joint authors is cited in full the first time it is referred to and thereafter is abbreviated to the form: senior author *et al.* Because of the length of this thesis, the specific name of any organism other than *C. zealandica* or *P. operculella* is given in full only on the first occasion it is referred to in any chapter, and in any section of Chapters 3, 7, and 10. In addition, the systematic status of insect species have often been omitted from the text but are given in Appendix 4.

Erratum. Due to an error in page numbering, 283 follows 281. However, no text has been omitted.

ACKNOWLEDGEMENTS

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CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS USED IN THE TEXT

S.D.	standard deviation
t	Student-t value
P	probability

Addendum

LIST OF ABBREVIATIONS USED IN THE TEXT

*	significantly different at $P<0{\cdot}05$
**	significantly different at $P<0{\cdot}01$
***	significantly different at $P<0{\cdot}001$

Ant	anterior
Ap Post	apophyses posteriores
Ax	nerve axon
B	bacteria
b cop	bursa copulatrix
BM	basement membrane
B pc	basal piece
br	bristle
C	circular
Cc	cyst cells
Csh	Connective tissue sheath
Cul	cuticulin
Cut	cuticle
cv	cavities
Cyx	calyx
D	duct
E Ap	and apparatus
ejdt	ejaculatory duct
end	endocuticle
epi	epicuticle
ER	andoplasmic reticulum
exo	exocuticle
FB	fat body
g	gland
G	Golgi
hyp	hypodermis
IM	intercellular membrane
Int	outer integument
In w	inner wall
IS	intercellular space
I sac	internal sac
L	lipid-like material
Lat	lateral
lm	longitudinal muscle
Lr	lower region
Lum	lumen
m	muscle
M	median
MC	membrane complex
Md	mitochondrion
Md Vel	mitochondrion associated with electron lucid vacuole
Md Vgr	mitochondrion associated with vacuole containing a granular material
Mt	microtubule
N	nucleus
O	ovum
Op	opening
Ovdt	oviduct
Ow	outer wall
p	penis
pc	pore canal
Pg	pheromone gland
Post	posterior
Prm	paramere
P Sc	pheromone scale
P Sc S	pheromone scale socke
R	rectum
Rb	ribosome
res	reservoir
S	secretion
Sc	scale
SD	septate desmosome
sh	sheath
Sp	spine
Sph	spermatophore
spm	spermatozoa
Spth	spermatheca
St	stylus
Stn	sternum
Sves	seminal vesicle
S Vg	sinus vaginalis
T	testis
Teg	tegmen
T fol	testis follicle
tr	trachea
TRC	trichogen
Trg	tergum
Tv	transverse
Ur	upper region
Vdf	vas deferens
Vef	vas efferens
Ve op	conicle opening
Vel	electon lucid vacuole
Vf	vacuole containing a floccular material
Vg	vagina
Vgr	vacuole containing a granular material
Vrb	vacuole with ribosome

LIST OF ABBREVIATIONS USED IN THE TEXT

S.D.	standard deviation
t	Student-t value
P	probability
μ	μm
<	less than
>	greater than

LIST OF ABBREVIATIONS USED IN THE FIGURES

acgl	accessory gland
Ant	anterior
Ap Post	apophyses posteriores
Ax	nerve axon
B	bacteria
b cop	bursa copulatrix
BM	basement membrane
B pc	basal piece
br	bristle
C	circular
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Cul	cuticulin
Cut	cuticle
cv	cavities
Cyx	calyx
D	duct
E Ap	and apparatus
ejdt	ejaculatory duct
end	endocuticle
epi	epicuticle
ER	andoplasmic reticulum
exo	exocuticle
FB	fat body
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Md Vel	mitochondrion associated with electron lucid vacuole
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Mt	microtubule
Mv	microvilli
N	nucleus
O	ovum
Op	opening
Ovdt	oviduct
Ow	outer wall
p	penis
pc	pore canal
Pg	pheromone gland
Post	posterior
Prm	paramere
P Sc	pheromone scale
P Sc S	pheromone scale socket
R	rectum
Rb	ribosome
res	reservoir
S	secretion
Sc	scale
SD	septate desmosome
sh	sheath
Sp	spine
Sph	spermatophore
spm	spermatozoa
Spth	spermatheca
St	stylus
Stn	sternum
Sves	seminal vesicle
S Vg	sinus vaginalis
T	testis
Teg	tegmen
T fol	testis follicle
tr	trachea
TRC	trichogen
Trg	tergum
Tv	transverse
Ur	upper region
Vdf	vas deferens
Vef	vas efferens
Ve op	conicle opening
Vel	electon lucid vacuole
Vf	vacuole containing a floccular material
Vg	vagina
Vgr	vacuole containing a granular material
Vrb	vacuole with ribosomes

CHAPTER 1

Introduction

A considerable amount has been published on the pheromones of certain insects, yet in many instances the relevant basic information on their sexual behaviour, and structure and physiology of the reproductive organs has not been elucidated. This is the situation with the grass grub beetle, *Costelytra zealandica* (White) (Coleoptera, Scarabaeidae, Melolonthinae), the major pasture pest in New Zealand. The decision was made, therefore, to investigate some of these aspects in this insect. However, the adults are only available in any numbers for approximately one month each year and so it was later decided to study another economically important insect, the potato tuberworm moth, *Phthorimaea operculella* (Zeller) (Lepidoptera, Gelechiidae, Gnorimoschemini) during the remainder of the year. This insect was chosen because there were similar gaps in published information concerning it whilst it had the advantages of being readily obtainable throughout the year and being easily cultured in the laboratory.

C. zealandica is widely distributed throughout New Zealand and it also occurs in the Chatham Islands (Given, 1952). It is a very destructive insect, causing most of its economic losses in the larval stage, which eat the roots of pasture plants (e.g. Hoy, 1963; Fenemore, 1966; Radcliffe, 1970, 1971). The adults can also cause local damage to some trees by eating their foliage. Fruit trees in particular can be defoliated, although the attacks are, of course, restricted to the flight season (Appendix 1.1).

In contrast, *P. operculella* has a worldwide distribution (Povolný, 1964), and the larvae are entirely responsible for all the economic losses caused by this insect. In particular they leaf-mine tobacco, and feed inside potato tubers both in the field and in storage, although they will also attack other cultivated Solanaceae (see Chapter 6).

A great deal of research has been carried out on the sex pheromone of *C. zealandica* culminating in its identification as phenol. The first suggestion that females of *C. zealandica* produce a sex pheromone which attracts males upwind towards them, was made by Kelsey (1967), who made this inference from evidence gained in a field experiment. Henzell, Lowe, Taylor, and Boston (1969) then demonstrated in the laboratory that the females produce a sex attractant; they used a"Y-choice olfactometer"and found that males were attracted to crushed females and attempted to copulate with them. Males were not attracted to crushed males and females were not attracted to crushed beetles of either sex. Henzell (1970) then reported evidence that the pheromone could be phenol. He found that an attractive diethyl ether extract of female abdomens contained a substance with properties consistent with phenolic compounds and that one of its gas liquid chromatography peaks had a retention time identical to that of phenol. He also tested some phenolic compounds in the field and found that only pure phenol and phenol dissolved in water were attractive to male beetles. Prior to this, Osborne and Hoyt (1969) found that the adhesive "Pliobond" contained a chemical attractant for males of *C. zealandica* and these authors later identified a phenolic resin as the active component (Osborne and Hoyt, 1970). In addition, they reported that the phenol formaldehyde resin "Durez 12687" was even more attractive to males than "Pliobond", and that phenol and another phenolic compound are present in "Durez". They also found in a field trapping experiment that the proportion of males to females caught in traps baited with pure phenol was higher than the proportion amongst the beetles flying about. These authors therefore suggested that the sex pheromone was either phenol, another phenolic compound, or a mixture of both. Henzell, Taylor, and Lowe (1970) then developed a simple laboratory bioassay for the pheromone. This consisted of dropping samples into containers with male beetles and observing their responses. The containers were the top halves of 2.5 litre jars resting on glass sheets covered with filter paper. These authors tested frozen sections of female beetles in this bioassay and found that the pheromone was located dorsally within the first three abdominal segments. Henzell and Lowe (1970) also used this bioassey together with paper chromatography, thin-layer chromatography, gas-liquid chromatography, and mass spectometry to isolate phenol from females and identify it as the sex pheromone. Firstly, they spotted 0.1, 1.0, and 10.0 μg of phenol onto paraffin blocks and found when these were tested in the bioassay that 80% of the males responded to each quantity of phenol. They then used this method to indicate which fractions of the chemical isolation procedures were active. Each female abdomen was found on chemical analysis to contain between 0.5 and 1.0 μg of phenol, but no phenol was found in any male. In addition these authors found that phenol in concentrations between 10 ppm and 500 ppm in water were attractive to males in field tests. Hoyt and Osborne (1971) subsequently found that bacteria in the "colleterial glands" of the female produce the phenol. This was identified by thin-layer chromatography of cultures of bacteria that were shown to be attractive by field trapping tests. Recently, Osborne and Boyd (1975), working with field traps baited with resin, phenol, and aqueous phenol concluded that an odour synergist is also present in the resin "Durez 12687".

However, despite this research on the sex pheromone of *C. zealandica*, very little detailed information has been published on the sexual behaviour of this insect, or about the structure of its reproductive organs. What has been published is reviewed in Chapters 4 and 3 respectively.

There is a similar lack of information about the sexual behaviour and the structure of the reproductive organs of *P. operculella* but any publications on these are reviewed in Chapters 8 and 7 respectively. However, in contrast to *C. zealandica* very little has been published on the sex pheromone of this moth. Most of this information is in the form of a brief note by Adeesan, Tamhankar, and Rahalkar (1969). These authors first suggested that the females of *P. operculella* released a sex pheromone after observing that males when caged with them "exhibited sexual responses such as clasper extension, fluttering of wings, and spinning flight". Furthermore, caged males showed the same responses when filter paper discs onto which the terminal segments of females had been crushed were introduced amongst them. Acetone, ether, and methylene chloride extracts of the terminal abdominal segments of females were each "attractive" to males. These authors reported that the pheromone gland "is in the form of a dorsal invagination of the intersegmental membrane between the 8th and 9th abdominal segments" and they briefly described its histology. The only other information concerning the pheromone of *P. operculella* is the field trapping studies reviewed in Chapter 8 which confirms that the female produces a sex attractant, and a research report by Rothschild, Bella, and Bartell (1971-1972) noting that the pheromone is an acetate.

The particular aspects selected for this study on both insects therefore included firstly, the structure of their reproductive organs because of their intrinsic interest and also because they constitute background information for the remainder of this study and all future investigations concerning their reproduction; secondly, their sexual behaviour because it is fundamental to all research on their sex pheromones; and thirdly, an attempt to identify and describe their sex pheromone glands. The third aspect involved the use of information gained from the investigation of the sexual behaviour of each insect in order to develop a behavioural bioassay for its sex pheromone. This aspect was chosen because, at the beginning of this investigation, only a preliminary attempt had been made to identify the pheromone glands in *C. zealandica* by Henzell *et al.*(1970), whilst the identification of the sex pheromone gland in *P. operculella* by Adeesan *et al.*(1969) appeared inadequate as no detailed experimental procedure or results were available.

At the beginning of the present investigation, Mr R.F. Henzell of Ruakura Agricultural Research Station, Hamilton, assured me that his male *C. zealandica* readily exhibited sexual behaviour in the laboratory. Few difficulties were therefore anticipated so the decision was made to commence this study despite the major difficulty that the imagines only occur in adequate numbers in the field for approximately one month each year. In addition, when this investigation was started, no method for mass culturing this insect in the laboratory had been published. However, recently some information on this has become available (Wightman, 1972a, 1972b, 1974a, 1974b, 1974c; and Wightman and Farrell,1972). Furthermore it now appears likely that mass culture methods will require considerable space and labour.

In contrast, *P. operculella* may easily be reared in the laboratory and methods for its mass culture have been published by Finney, Flanders, and Smith (1947), and Platner and Oatman (1968). A further advantage is that its life cycle is short, being completed in about one month at room temperature (Chapter 6).

Since the adults of *C. zealandica* are only available seasonally, the investigations on it were started more or less simultaneously. However, after Hoyt and Osborne (1971) found that the female sex pheromone was produced by bacteria within the "colleterial" glands, further attempts to develop a bioassay for determining the site of the pheromone gland were discontinued at the end of the second year of study. Research on this insect was then concentrated on its sexual behaviour and structure of its reproductive organs. None the less a description of the pheromone glands is included with the description of the other internal reproductive organs of the female in Chapter 3. In contrast, the investigations on *P. operculella* approximately followed the sequence given in the relevant chapters. The design of each section was suggested by the results of its predecessor and work could usually be initiated before the previous study was completed.

Most of the literature relevant to this study of *C. zealandica* and *P. operculella* is included in the appropriate chapters and is, therefore, not reviewed here. An account of the published information relating to the seasonal occurrence of *C. zealandica* is, however, included below because it has a bearing on the previous introductory remarks and it is not treated elsewhere.

Few detailed accounts have been published relating to the seasonal occurrence of *C. zealandica*, and it has therefore been necessary to obtain much of the following information from general comments in the introductions of papers concerned with a variety of other subjects. This is also the situation frequently encountered with other aspects of the biology of this insect.

C. zealandica is generally considered to be univoltine (Miller, 1921; Kelsey, 1951; Fenemore, 1966; Osborne and Hoyt, 1969; Farrell and Wightman, 1972) although between 4% and 27% of the

Erratum

Paragraph 4; the last sentence should read: "Possibly a two year life cycle is more frequent in southern populations (Fenemore, 1966).

(Kelsey, 1951) or October (Miller, 1921; Farrell, 1972) until January or early February (Kelsey, 1951). The proportion of imagos found in the soil varies considerably from place to place and from year to year, 50% of the juveniles becoming adult between the 18th October in about one month at room temperature (Chapter 6).

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Adults are generally found in the soil from September (Kelsey, 1951) or October (Miller, 1921; Farrell, 1972) until January or early February (Kelsey, 1951). The proportion of imagos found in the soil varies considerably from place to place and from year to year, 50% of the juveniles becoming adult between the 18th October and 20th November (Fenemore and Perrott, 1970). There is also considerable variation in the pre-emergence period (Kelsey, 1951): this may be as short as a few days (Miller, 1921), but the usual range is from 7 to 10 days (Henzell *et al.*, 1969). The adult lifespan is usually from two to three weeks but may be longer (Kelsey, 1951). Adults usually begin emerging in the first few weeks of November although sometimes they do so at the end of October and some still have not emerged by mid-March (Kelsey, 1967). The duration of the first emergence period varies little at different sites, ranging from 19 to 25 days for 95% of the population (Fenemore and Perrott, 1970).

Most of the beetles fly between late October and January, or possibly February (Cockayne, 1920; Miller, 1924; Helson, 1967; *et seq.*) although the majority do so during a 3 to 4 week period each year (Thomas, 1913; Henzell *et al.*, 1970; Farrell and Wightman, 1972). The largest flights are centred around November and the numbers decline in December (Moodie, 1911; Cockayne, 1911, 1920; Miller, 1921, 1924; Connell, 1933; Kelsey, 1967; Osborne and Hoyt, 1968, 1969). At southern locations flights may start up to two weeks later than at northern ones (Helson, 1967). There is also one record of a major flight occurring in the middle of January (Anon., 1927) but this may have been a species closely related to *C. zealandica*. Certainly some of the *Odontria* species have been collected early in the year, and bear some superficial resemblance to *C. zealandica* although they are usually larger (Given, 1952).

As mentioned above, an important aspect of the present investigations of *C. zealandica* and *P. operculella* was the development of bioassays for each of their sex pheromones. An account of the principles underlying the development of such a bioassay and its design is therefore included here because it applies equally to the research on both insects.

Since a sex pheromone is defined in terms of the behaviour it elicits from other individuals of the same species (Karlson and Butenandt, 1959; Karlson and Lüscher, 1959) then it follows that an essential requirement of a bioassay for a sex pheromone is the inclusion of behavioural responses of the insects concerned. This constitutes the qualitative aspect of the bioassay. Most of the bioassays which have been developed by other research workers do utilise some relevant aspect of the behaviour of the insect being investigated; the only exception being bioassays using the specialised electrophysiological technique known as an electroantennagram. The major use of an electroantennogram is to assist in the chemical isolation of a sex pheromone and to estimate concentrations of pheromone in air. However, the biological activity of a chemical still requires the ultimate assessment in a behavioural bioassay.

A second fundamental requirement for a sex pheromone bioassay is that the live insects used in it are in the appropriate physiological state to react to the pheromone. It is therefore necessary to determine the behaviour of the insect and the environmental conditions under which the behaviour occurs naturally. Attention, of course, must also be paid to physiological variables such as age, previous mating history and circadian rhythms. The importance of some of these factors has been demonstrated for example in a number of noctuid moths by Shorey and Gaston (1965a), Shorey, McFarland, and Gaston (1968a), and Shorey, Morin, and Gaston (1968). Variation amongst these can, in some cases, cause as much as a thousand-fold variation in the threshold response to the pheromone.

Once the importance of these factors has been determined, the bioassay must be designed in such a manner that it does not interfere with the natural habits and idiosyncrasies of the particular species if meaningful results are to be obtained. For example, the methods used by Keller, Mitchell, McKibben, and Davich (1964), and Hardee, Mitchell, Huddleston, and Davich (1966) to bioassay the pheromone of the boll weevil *Anthonomus grandis* Boheman, were probably inadequate in that they did not allow the females freedom of flight when exposed to the male pheromone, and the females were not isolated from one another before the bioassay. According to Hardee, Mitchell, and Huddleston (1967), however, most or all of the adults probably do not come into contact with another boll weevil before their first mating, and Cross and Mitchell (1966) had observed that the females make several short flights towards the male in the field. Therefore, when Hardee *et al.* (1967) designed a new bioassay method which took these factors into account, they obtained greatly improved results from those of the former authors. Other examples of the need to design bioassays around the habits of the insect include those given by Beroza and Jacobson (1963), and Kinzer, Burns and Auclair (1970).

Many factors were taken into account when designing the bioassay used in the present study. Firstly, it was desirable to make the bioassay as objective as possible. This precluded those methods which depend upon observing the sexual behaviour of the insect. It must be stressed, however, that a great deal of valuable research has been carried out by other research workers using observational bioassays (these are listed, for example, in Jacobson, 1972; and Birch, 1974), but these methods are potentially open to considerable subjective error and bias. The conclusion reached was that such methods should be avoided if at all possible except as an additional qualitative check. Fortunately, it appears that although many sex pheromones may serve to excite responding insects before copulation and to evoke a courtship response from them, they are often produced primarily for the purpose of attracting the opposite sex from a distance (Jacobson, 1972). If this holds true for the insect being investigated, and it certainly appeared probable (from the literature cited in Chapters 4 and 8) that it does for *C. zealandica* and *P. operculella*, then the simplest means to achieve objectivity is to use attraction as the key behavioural response. This can be easily measured as for example by using a trapping method. Nevertheless, care must be taken to ensure that any attraction so obtained is not a response to some other odour, such as one emanating from a food, or in the case of a female, from an oviposition site. One way to overcome this error is to test the odour for attractiveness to both males and females and, as an additional check, to use a trapping mechanism which does not harm the insects so that they can be subsequently observed for any obvious sexual behaviour they may exhibit.

One possible advantage of the use of a bioassay that is dependent upon observing behaviour is that the earliest behavioural step in a response sequence can be selected as the bioassay criterion. This allows the detection of the pheromone at the lowest possible concentration. However, the attraction of an insect towards the source of its pheromone is usually one of its first observed responses and therefore a bioassay based on attraction is also likely to be sensitive to relatively low concentrations of pheromone.

A second consideration in developing a bioassay is that ideally it should be capable of replication and give results which are as consistent as possible. The best solution is a laboratory bioassay using laboratory reared insects. Only by doing this can there be any possibility of controlling the numerous factors which may influence behaviour. Care must also be taken to ensure that the laboratory reared animals do not differ genetically from those in the field (see Chapter 6). It is also important that the basic findings of such a bioassay be checked for relevance in the field in case some spurious effect is confusing the results. The bioassay used in the field, however, need only be qualitative; the use of some kind of trapping device is an obvious, simple, and objective solution.

Consistent results are more likely to be obtained from a bioassay if a number of precautions are taken. The use of scrupulously clean equipment for every bioassay prevents the possibility of contamination occurring between successive bioassays. Pheromones are often perceived at very low concentrations in air (e.g. Jacobson, 1972; Birch, 1974) and significant contamination may therefore result from a normally irrelevant effect such as by surface adsorbtion. It also follows that a clean air supply to the bioassay apparatus is necessary. The importance of a pheromone-free air supply has been demonstrated by Shorey, Gaston, and Fukuto (1964) using the noctuid moth *Trichoplusia ni* (Hübner). They compared a bioassay using clean air with one in which the air was recirculated through the bioassay room, and found that contaminated air produced more variable results with attenuation of the males responsiveness, and caused a greater than ten-fold reduction in the sensitivity of the bioassay. It is also good practice to eliminate any extraneous odours from the air which are not normally experienced by the insects when they mate in their natural environment in case these influence the behaviour of the insects.

The use of naive subjects in a bioassay is also desirable: the responsiveness of some male Lepidoptera has been demonstrated to be inhibited after a single recent exposure to their sex pheromone although they recover before the next day (Shorey, *et al.* 1968a). It may therefore be necessary to isolate the insects from individuals of the opposite sex before they are used for bioassay purposes.

Considerable variability can also be introduced into a bioassay by failing to standardise the method used for dispensing a particular pheromone sample. For example, keeping to a strict timing schedule during the preparation of the sample until its insertion into the bioassay apparatus becomes increasingly important the smaller the sample because the relative loss of pheromone and consequent reduction in the amount left in the sample is more rapid. The rate of change in the emission of a pheromone from a substrate will also depend on the material used for dispensing it. For example, the loss of pheromone from an absorbent and adsorbtive material such as filter paper, or from a permeable container such as a plastic bag will be more prolonged than when dispensed from a material such as glass where the pheromone is exposed on the surface. Over a relatively short period of time the pheromone will therefore be emitted at a more constant rate from an absorbent or adsorbtive substrate.

A further consideration is whether to make the bioassay quantitative. Some estimation of the relative amount or activity of the pheromone was considered to be desirable for the present investigations, because of the added information that this would provide. A number of possible situations exist where a quantitative bioassay would be invaluable compared with a purely qualitative bioassay: for example, the pheromone may be produced and stored at different locations in the body of the insect. Alternatively, there may be two components produced in different regions which are both unattractive or only slightly attractive by themselves and which only show their full activity in combination, or one may be a synergist which is itself not attractive. A quantitative bioassay can also give some indication of errors in procedure or whether a particular result may have been influenced by contaminents. It may be added that a quantitative bioassay is essential for isolating or identifying a pheromone because of the estimation it provides of any synergism or losses during purification which could occur. However, no attempt was made at any chemical research in the present study.

One prerequisite for a quantitative bioassay is that there should be a controlled air flow over the insects. The reason for this is the problems associated with the use of still air. If the pheromone is added for example into a closed container with insects, then it may not adequately mix with the air. Also, if the pheromone is first applied to a substrate and then introduced into such a system, its concentration in the air would change as it continued to evaporate. It also follows that the results from a bioassay in still air may be extremely variable.

In summary, the sex pheromone bioassay should preferably depend on a behavioural response which can be measured objectively and which will still permit detection of low concentrations of pheromone. It should not incorporate factors which adversely affect the sexual behaviour of the insect. Standardisation of procedure should be effected by dispensing the pheromone sample in such a way that it is slowly liberated into the air, by using moths of the same age and mated state for each experiment, and by using an uncontaminated air source and clean equipment. Following the same procedure and timing schedule for each bioassay also assists in achieving consistent results. Some form of estimation of the relative amount of pheromone present is also desirable, and this requires that there be controlled air movement in the bioassay apparatus. Finally, adequate controls or checks for the above factors should be carried out or incorporated into the experimental design of the bioassay.

CHAPTER 2

Materials and rearing methods for *C. zealandica*

C. zealandica was usually collected on the farms of Mr. N.O. Goodhue, Marshmeadow Road (Collins Road), Tauwhare, Hamilton R.D.4 and Mr T.P. O'Regan, Te Rahu Road, Te Rahu, Te Awamutu R.D.1. In addition small numbers were also collected from the properties of Mr A.S. Gunn, Craig Road (Collins Road), Eureka, Hamilton R.D.4 and from the Auckland University Limnological Field Station, Lake Rotoiti, Rotorua.

The collection of pupae and third instar larvae and the rearing of adults

The method used was a modification of that of Henzell *et al.*, (1970). The larvae and pupae were collected by digging in 1970 and by following a plough in 1971. They were brought back to the laboratory in a thin layer of fine soil to prevent desiccation and sorted immediately. Those damaged were discarded and the remainder were segregated into larvae, male pupae, and female pupae. The sexes were identified using the morphological characters described in the following section. After having been sorted the insects were placed individually in small cells of miniature ice cube trays and stored in separate plastic boxes for larvae, male pupae, and female pupae. They were then kept in a constant temperature cabinet at $9.5 \pm 0.5^{\circ}C$. This was considered necessary because Henzell *et al.* (1970) noted that when pupae are present and beetle emergence takes place in the field at Ruakura, the average soil temperature at a depth of 10 cm approximates $10^{\circ}C$.

Each ice cube tray had 90 cells with 14 mm sides. In 1970 a layer of plaster of paris 3 to 4 mm thick was poured into the bottom of each cell following the procedure of Henzell *et al.* (1970). These authors found that this reduced wing damage and mortality. In the present study, however, the plaster of paris often became mouldy if the insects died and was difficult to remove, so in 1971 it was replaced with a layer of fine soil of a similar depth.

The trays were placed in polyethylene envelopes for storage. In order to prevent any of the larvae or adults from leaving their cells, they were then stacked in groups with an empty tray at the top and bound together with stout rubber bands. Each tray containing insects was thus covered by the bottom of another tray. The plastic boxes in which the trays were kept were provided with tightly fitting lids and a layer of clean sand wet with 3% to 4% copper sulphate solution. This produced a high humidity inside the boxes; the sand preventing the solution from splashing, and the copper sulphate keeping the water sterile and reducing excess condensation.

The insects were checked every one or two days; dead ones were removed together with the soil or plaster of paris, and new pupae or adults transferred to the appropriate containers.

The results of the rearing procedures are given in Table 2.1 and the cumulative numbers of male and female beetles that had hatched each day in 1971 are shown in Figure 2.1.

The rearing procedure resulted in a total of 117 males and 173 females being produced in 1970, and 294 males and 324 females in 1971. In both years the largest relative losses of immature stages occurred in the larvae, 77.5% dying in 1970 and 94.0% dying in 1971. Only approximately 50% of the pupae died or produced deformed adults overall but losses from individual batches varied from 35.6% to 74.6% with no apparent difference between sexes. The largest losses amongst pupae occurred soon after collection and during adult emergence.

It is of note that the total mortality was less than that obtained by Henzell *et al.* in 1969 and greater than that obtained by Henzell *et al.* in 1970. The former authors lost 70% of their insects whilst the latter lost 36% of 30,000 larvae kept at 8°C to 10°C and 33% of 5,400 larvae kept at 16°C. Deformed individuals were included in the mortality of the present study and this may explain the high overall losses compared with those of Henzell *et al.* (1970). Presumably these authors only collected larvae, although this is not stated, and therefore some losses resulting from handling pupae which

TABLE 2.1

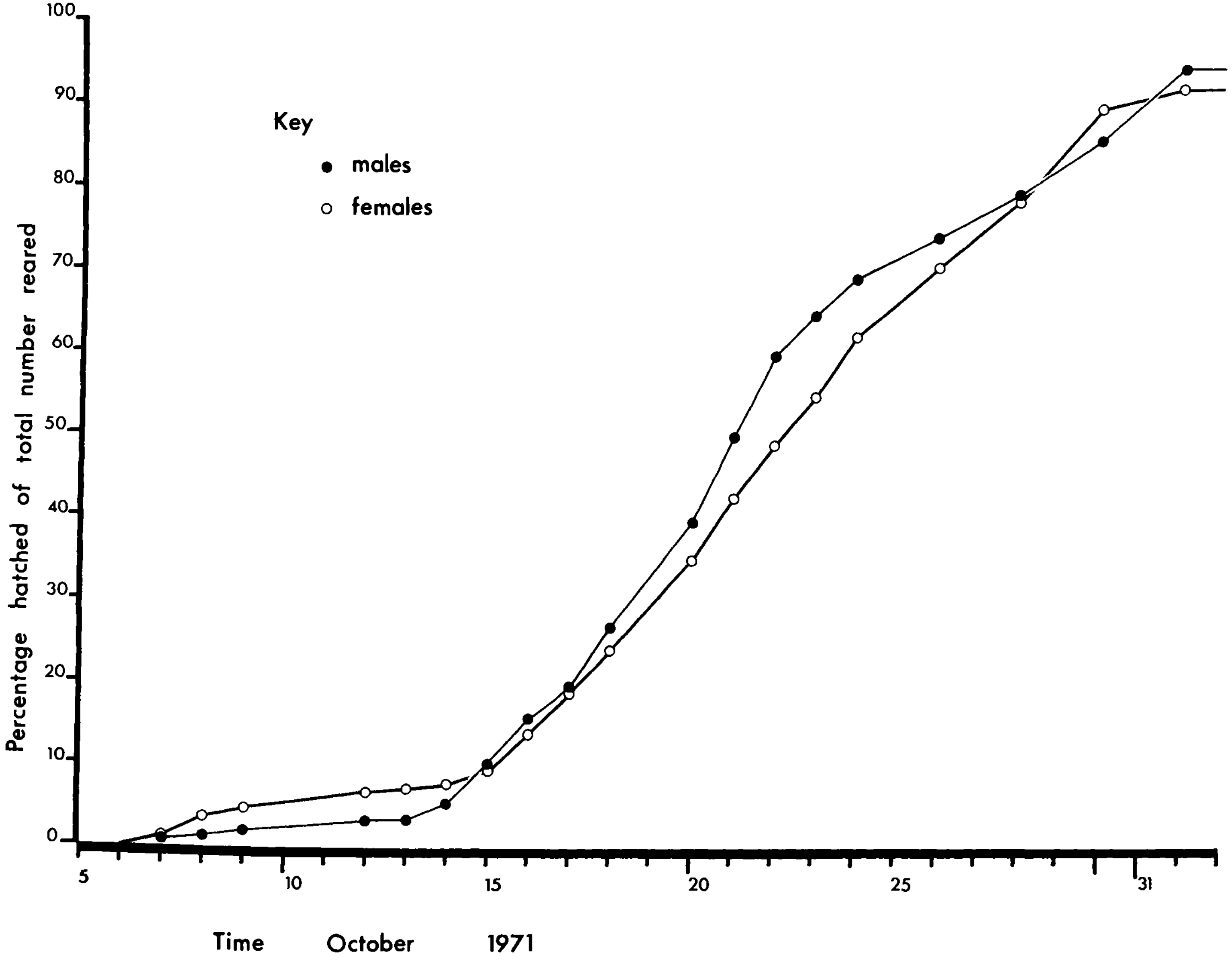
Numbers of beetles reared in the laboratory from field collected larvae and pupae together with the mortality of the larval and pupal stages.

				MALE PUPAE						FEMALE PUPAE						LARVAE				LARVAE & PUPAE		
Date of collection	Collecting locality	Total no. of larvae & pupae collected	No. of teneral adults collected	No. collected	Total no. pupated from larvae	Mortality 1st 5 days (%)	Mortality 1st 10 days (%)	Total mortality (%)	Total no. beetles hatched	No. collected	Total no. pupated from larvae	Mortality 1st 5 days (%)	Mortality 1st 10 days (%)	Total mortality (%)	Total no. of beetles hatched	No. collected	Mortality 1st 5 days (%)	Mortality 1st 10 days (%)	Total mortality (%)	Total mortality 1st 5 days (%)	Total mortality 1st 10 days (%)	Total mortality (%)
26/8/70 - 16/9/70	O'Regan	542	N/R	202	9	N/R	27.72	44.55	117	269	7	N/R	26.02	62.68	173	71	N/R	28.16	77.47	N/R	26.94	53.50
5/10/71	Goodhue	490	0	141	4	14.89	21.99	46.90	77	139	6	17.27	20.14	45.32	76	210	22.86	49.05	95.24	18.98	33.06	68.78
7/10/71	Goodhue	481	0	178	5	15.17	21.35	51.69	86	173	5	9.83	12.12	41.57	104	130	29.23	62.31	92.31	14.97	29.31	60.50
10/10/71	Goodhue	367	8	146	1	28.08	44.52	54.80	66	180	2	22.22	33.33	35.56	116	41	41.46	85.37	95.12	26.70	43.60	49.59
12/10/71	Goodhue	282	4	120	2	2.50	15.83	46.72	65	108	2	8.33	16.67	74.55	28	54	12.96	29.63	92.59	6.74	18.79	67.02
Totals for 1971		1620	12	585	12	15.73	26.15	50.08	294	600	15	15.00	21.33	52.68	324	435	25.29	54.02	94.02	17.41	31.85	61.85

Figure 2.1

The cumulative numbers of male and female *C. zealandica* imagos which hatched from pupae in the laboratory in 1970

***erratum*:** Time should read Date.



Key
● males
○ females
Percentage hatched of total number reared
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
31
Time October 1971

are easily damaged would not have been incurred.

Identification of the sexes of pupae

Male and female pupae were distinguished from one another using the ventral structures at the posterior ends of their abdomens. In the male a conspicuous medial lobe forms an elongated ridge with smaller lobes on each side. These are posterior to what appears to be the 6th visible abdominal sternum but the last sterna are not clearly defined (Figure 2.2). In the female the corresponding area is flatter and there are only two small lobes lying one on either side posterior to the 7th visible sternum (Figure 2.2).

Maintaining adults reared from pupae

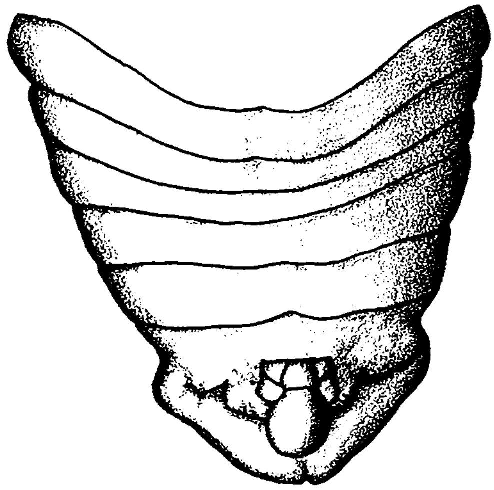
After hatching the adults were transferred to small pots of soil in groups of up to 10 males or 10 females per pot. These were held in a constant temperature cabinet at 15°C under a photoperiod of 15 hours 35 minutes light to 8 hours 25 minutes darkness. Illumination during the light periods was provided from a 200 W incandescent bulb controlled from a timeswitch to switch off at 1930 hours daily in 1970 and 2135 hours daily in 1971. The insects were therefore subjected to an approximate average November photoperiod except that in 1971 the dark phase commenced two hours later than naturally in the field.

The soil pots were clear plastic with volumes of about 58ml diameters at their openings of 52mm, and heights of 38mm. Each had organdie netting fastened over it by means of a push-fit plastic lid with a central 20mm diameter hole. Each pot was half filled with soil to allow the insects to burrow and provided with three or four fresh leaves of *Salix masudana* Koidz (twisted willow) every two or three days for food. The pots were stored in lunch boxes which were provided with a layer of sand wet with 3% to 4% copper sulphate solution in the same manner as were the boxes for larvae and pupae. Mortality records were not kept in 1970 because of the change from using reared beetles to field collected beetles for the bioassays. However, in 1971, 16.4% of the males and 10.5% of the females died within approximately 5 days of hatching while 36.0% of the males and 23.3% of the females died within approximately 10 days of hatching. These results compared favourably with those of Henzell *et al*, (1970) who found that 49% of all the adult beetles kept at 16°C died within 10 days of hatching. Mortality

Figure 2.2

Identification of the sex of
C. zealandica pupae

The entire male and female pupae are shown in lateral view, and their abdomens are shown in ventral view. Note the conspicuous elongated medial lobe at the posterior end of the male's abdomen which presumably represents part of the developing tegmen.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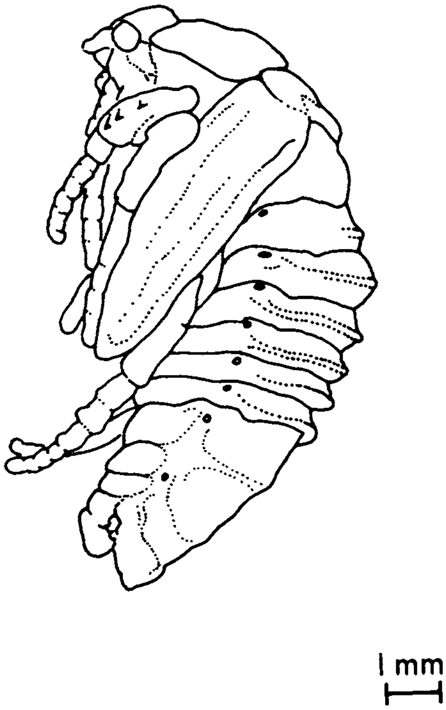
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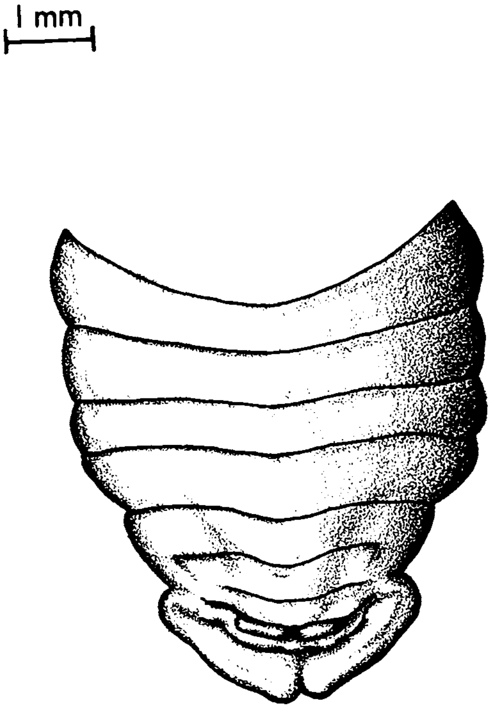
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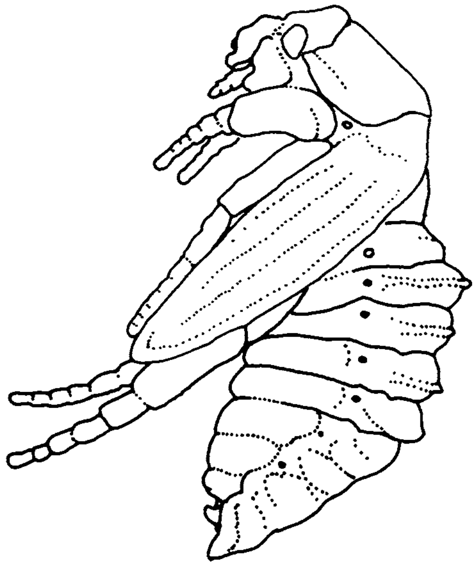


records were not kept for beetles more than 10 days old because they were used when they were between 10 and 15 days old. Using beetles of this age ensured reproductive maturity since Henzell *et al.* (1970) states that "in the field normally a period of 7 - 10 days or longer elapses between final ecdysis and primary beetle emergence". They also found that beetles reared at 8 to 10° C were most likely to respond to female extracts when they were 14 days old. However, these were the oldest beetles tested by these authors. It was therefore likely that the beetles reared at 15°C in the present study reached sexual maturity before they were used because of the higher temperature under which they were reared.

Collection of adults in the field and their maintenance in the laboratory

The beetles were collected at night by knocking them off a hedge of *Berberis vulgaris* L.(barberry) onto a beating tray. They were brought back to the laboratory in large buckets with close fitting lids containing barberry shoots and left in these overnight. Next morning males and females were separated using the identifying characters described in Chapter 3. They were then placed in groups of 100 in translucent white plastic boxes approximately 30cm X 20cm X 10cm high with push-fit plastic lids provided with air holes. These boxes were half filled with soil and kept at the same temperature and photoperiod used for laboratory reared beetles. The boxes were opened every two or three days and fresh shoots of twisted willow were added. Any dead beetles lying on top of the soil were also removed. Water was sprinkled onto the soil to dampen it when necessary.

In 1970, the beetles were collected between 30th October and 3rd November and added together to give a total of 300 males and 200 females. Some of these were then used for bioassay work approximately 5 to 8 days later, and some were used in laboratory mating experiments approximately 16 to 19 days later. Their total mortalities after 16 to 19 days were 10.8% for the males and 1.1% for the females.

In 1971 100 male and 100 female beetles were collected on 29th October while 200 males and 100 females were collected on 4th November. Some of both groups were used for field trapping experiments 2 to 3 weeks later and most of the males plus some of the females collected on 4th November were used after 16 days for bioassay work. Of these latter, 33.3% of the males and 12.5% of the females died before they could be used.

CHAPTER 3

The reproductive organs of *C. zealandica*

Introduction

No thorough study of the reproductive organs of *C. zealandica* has yet been published and only the following information concerning them was available at the start of the present study:- Elliott (1964) described the development of the gonads in the pre-adult stages and also the appearance of the rudiments of the other internal reproductive organs in the larva. Given (1952) studied the external genitalia of the male but he was concerned only with features of taxonomic importance. Kelsey (1965) gave two methods for distinguishing the sex of the adults from external features. More recently Fenemore (1971) published some data on the condition of the internal reproductive organs in relation to the known age and mated state of the beetles, and included some figures of the male and female internal reproductive organs. Fenemore (1971), Farrell (1973), and Wightman and Farrell (1973) included some information on the fecundity of the female. Additional characters for distinguishing the male and female beetles are given by Kain (1972). Duff (1970) determined that the chromosome number was 2n = 20 and investigated karyotype variation between different populations.

Because of the paucity of information on the reproductive organs of *C. zealandica* it seemed desirable to make a thorough study of them especially since this might assist in the identification of the pheromone gland. It also enabled the establishment of a consistent nomenclature and this proved of great value in the description of sexual behaviour.

Methods

For dissection the beetles were anaesthetized with chloroform vapour or carbon dioxide and secured dorsal surface uppermost by embedding their legs in paraffin wax which lined the bottom of a small dish. They were then placed under a dissecting microscope and generally submerged under Clarke's insect saline (Hale, 1958). There were two exceptions to this procedure: those beetles from which parts were required for examination with the transmission electron microscope were dissected under 2.5% glutaraldehyde in Millong's phosphate buffer (pH 7.2); whilst beetles which had already been fixed in hot Bouin's, as described below, were immersed in tap water for dissection.

The histology of the internal reproductive organs was investigated by making complete transverse and **longitudinal** serial sections of excised organs or entire abdomens. Normal histological techniques, as given by Humason (1966), were followed. To reduce damage during transfer from one solution to another each piece of tissue was kept in an upright glass tube with fine netting fixed to the lower end. Zenker's fluid was usually used for fixation, although Helly's, Carnoy's, and Bouin's fixatives, and buffered formalin were also used on occasion. However, Carnoy's and Bouin's fixatives were never relied upon by themselves for giving good cytoplasmic detail because of their acetic acid content. After fixation, the tissue was subjected to the usual washing; dehydration in 50%, 70%, 95%, and 100% ethyl alcohol; and clearing in toluene or benzene. When small pieces of tissue were being processed, eosin was added to the 95% ethyl alcohol to facilitate orientation during subsequent embedding. Soft tissue was vacuum embedded in paraffin wax with ceresin (60°C mp) or Paraplast* (56-57°C mp). Tissue containing sclerotised cuticle was double embedded in 4% celloidin and paraffin wax with ceresin (60°C mp) according to Butt's method (Humason, 1967). The timing schedules which produced the best results with these procedures are given in Appendix 3.1. Post-treatment for mercury consisted of substituting a saturated solution of iodine in 70% ethyl alcohol for the 70% ethyl alcohol during dehydration and later, when staining the sections, including Lugol's solution in the staining series. Most sections were cut at 5 to 6µ, and all were cut between 3µ and 8µ. Egg albumen in water was always used as an adhesive for wax sections to ensure that no cuticle floated off during staining. Gelatin was used to affix double embedded sections to slides. Sections were either stained with Delafield's, Ehrlich's, or Mayer's haematoxylin and counterstained with eosin, or were stained with Mallory-Heidenhain's rapid triple connective tissue stain.

*Fischer Scientific Co., U.S.A.

The external genitalia were examined by dissection and by making whole mounts. These were prepared by the usual maceration technique of boiling in 10% potassium hydroxide followed by washing in water, dehydration in ethyl alcohol, clearing in terpineol, and mounting in D.P.X. or Canada Balsam (Oldroyd, 1958).

The muscles of the external genitalia were examined by dissection. Since they were small and almost transparent, they were usually stained with Mallory's phosphotungstic acid haematoxylin. Firstly, whilst still in Clarke's insect saline, they were lightly fixed by dripping 4% formalin onto them. After a few minutes the fixative was gently flushed away with more saline. The stain was then carefully applied by repeatedly running small amounts of it into the saline near to the muscles and flushing it away with more saline until the muscles were clearly deliminated. Fixation, however, made further dissection difficult because the muscles broke easily. Therefore, the complete series of muscles was examined by sequentially dissecting different beetles to progressively deeper levels before staining.

Tissue was initially prepared for examination with the transmission electron microscope by fixing it in a 2.5% solution of glutaraldehyde in Millonig's phosphate buffer (pH 7.2) for 1 hour and subsequently storing it in this buffer for 1 to 7 days at 4°C. All further preparation was carried out by Miss A.B. Gavey and Dr J.P. Shalcraft, Meat Research Institute, Hamilton. The tissue was post-fixed with 1% osmium tetroxide for 1 hour at room temperature, dehydrated in acetone washes, and embedded in Epon. After trimming the block, the region of interest was located by cutting sections approximately 2µ thick with an ultramicrotome, staining them with 1% toluidine blue, and examining them with the light microscope. When the region was found, thin sections were then cut at approximately 600 Å, stained with uranyl acetate and lead citrate for 5 minutes each, and examined with a Phillips E.M. 200 electron microscope.

Specimens were prepared for scanning electron microscopy by freeze drying them at -35°C and then fixing them to aluminium studs with a conducting silver paint. Further treatment was carried out by Mr E.E. Wheeler, Chief Technician, Department of Pathology, Medical School, University of Auckland, who coated the specimens with a thin film of gold under vacuum. They were then examined with a Phillips Stereoscan scanning electron microscope.

The positions occupied by the genitalia of copulating beetles were determined from pairs which had been collected in the field by knocking them from hedges into a Dewar flask of liquid nitrogen. They were allowed to warm up to -35°C in the laboratory and dropped into boiling Bouin's fluid through a wide diameter reflux condenser. The beetles were recovered when the Bouin's cooled and subsequently washed in running tap water for half an hour before being dissected or sectioned.

It is probable that little movement of the internal organs occurred during fixation. This method did not, however, produce entirely satisfactory histological preparations and so it was used only for determining the positions of the organs during copulation.

Bacteria were cultured using normal aseptic techniques. They were obtained from beetles which had been partially surface sterilised by brief dipping into 70% ethyl alcohol and then shaken dry. The organs containing the bacteria were then dissected, keeping the instruments as sterile as possible. The bacteria were cultured and isolated on nutrient agar at 20°C. It is probable that little contamination occurred using this method of obtaining the bacteria because few different types were found in each of the organs.

All drawings were made from specimens using either graph paper and a microscope equipped with a squared eyepiece graticule, or a microscope fitted with a camera lucidia. Measurements smaller than about 2mm were made with a microscope fitted with a calibrated eyepiece micrometer.

Sexual dimorphism

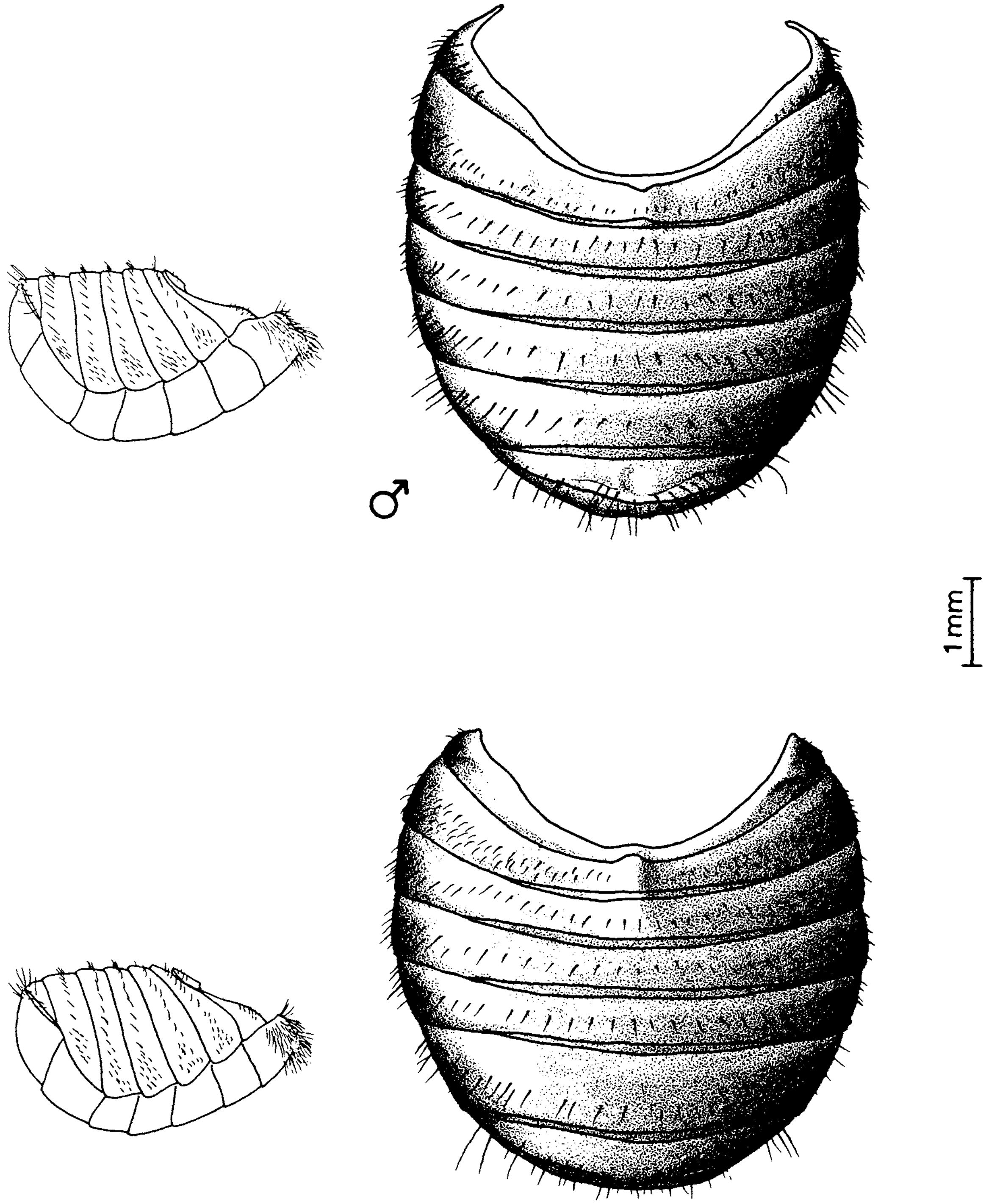
The male and female adult beetles are very similar externally although Kelsey (1965) found that the male could be distinguished from the female by an examination of what he termed the 6th abdominal sternite. This, however, is the 8th sternum, the first abdominal sternum and part of the second being either lost or possibly membranous and therefore indistinct (Imms, 1960; Britton, 1973). In the male the 8th sternum has a conspicuous shallow central depression and a "sulcus" or notch each side of the centre on its posterior margin, but both of these features are absent in the female (Figure 3.1). Kain (1972) confirmed these points but found them difficult to use in the field under poor lighting conditions. He also reported, that in the female the genital styli (Figure 3.19) can be seen when the genital chamber is opened by lateral

Figure 3.1

Sexual dimorphism of the adult
C. zealandica

Lateral and ventral views of the abdomens of male and female.

Note the median depression in the 8th sternum of the male which is absent in the female, and the differences in proportion between the 7th and 8th terga of male and female.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1 mm
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pressure on the abdomen and in teneral or newly emerged beetles the accessory glands in the female or "colleterial glands" can be seen as two dark spherical patches, one on either side of the midline, through the 7th abdominal sternum. Furthermore, he noted that the external genitalia of the male, particularly the parameres (Figure 3.7) are visible through the 8th tergum and that this character, together with the other female characters previously mentioned make determination of the sex easy.

During the present study it was also found that the eggs were occasionally visible through the ventral abdominal walls in some females but this was of limited use in determining sex. The only other external differences that were found were morphometric ones and these were of no practical use in sexing the beetles. All the structures that were measured and their dimensions are summarized in Table 3.1. These show that the antennae, maxillary palpi, all the tarsi, and various abdominal sclerites differ significantly in size between male and female beetles. These differences, though, were mostly too subtle to be recognized by looking at the insect, except for the abdominal sclerites which occupy different proportional areas in the abdomen of male and female.

The internal reproductive organs of the male

The internal reproductive organs of the male *C. zealandica* have not been previously described, although they are figured by Fenemore (1971). However, they have been studied in a number of other male Scarabaeoidea: Bordas (1900) examined those of the melolonthinids *Melolontha melolontha* L., *Hoplia caerulea* Drury, *Hoplia brunnipes* Bonelli, *Hoplia farinosa* L., *Hymenoplia strigosa* Illig., and *Anoxia villosa* Fabr., together with 17 other scarabaeids in the Aphodiinae, Scarabaeinae, and Cetoniinae, two lucanids, and 4 geotrupids. Williams (1945) describes the internal reproductive organs of the scarabaeids *Pinotus carolinus* (L.), *Phyllophaga* sp., *Popillia japonica* Newman, and *Cotinus nitida* (L.); the lucanid *Pseudolucanus capreolus* (L.); the geotrupid *Bolboceerosoma farctum* (Fabr.); and the passalid *Popilius disjunctus* (Illig.). Detailed descriptions also include those of the scarabaeids *M. melolontha* L. by Straus-Dürckheim (1828); *Phyllopertha horticola* L., and *Anomala aenea* Geer by Rittershaus (1927); *Amphimallon majalis* (Razoumowski) by Menees (1963); and *Phyllophaga anxia* (Le Conte)

TABLE 3.1

Dimension of some of the sclerites of male and female *Costelytra zealandica* taken from 15 specimens of both sex.

All measurements in mm.

		Key to opposite diagram	MALE		FEMALE		Student-t value
			MEAN	S.D.	MEAN	S.D.	
Head	length	A	1.93	0.07	1.99	0.12	1.49
"	width	B	2.233	0.115	2.220	0.098	0.34
Prothoracic tergum	length	C	2.187	0.164	2.173	0.144	1.54
"	width	D	3.773	0.205	3.913	0.207	1.86
Mesothoracic tergum	length	E	1.500	0.085	1.48	0.077	0.68
Elytra	length	F	7.107	0.313	7.300	0.342	1.62
Hind wing	length	G	10.647	0.496	10.540	0.582	0.54
7th abdominal tergum	length	H	1.733	0.159	1.833	0.172	1.66
8th abdominal tergum	width	I	3.260	0.282	3.250	0.262	0.09
4th abdominal sternum	length	J	0.429	0.041	0.477	0.038	3.29**
7th abdominal sternum	length	K	0.843	0.075	1.030	0.071	7.00***
8th abdominal sternum	length	L	0.467	0.049	0.503	0.036	2.28*
8th abdominal sternum	width	M	3.876	0.349	4.037	0.326	1.29
Antennal length to last annulus		N	1.113	0.078	1.023	0.089	2.95**
Length of last annulus		O	0.799	0.075	0.635	0.070	6.27***
Maxillary palp	length	P	0.828	0.058	0.783	0.053	2.02**
Prothoracic femur	length	Q_1	1.793	0.093	1.776	0.075	0.56
" tibia	length	R_1	1.910	0.114	1.947	0.112	0.90
" tarsus	length	S_1	2.877	0.094	2.406	0.146	10.52***
Mesothoracic femur	length	Q_2	2.227	0.113	2.189	0.107	0.94
" tibia	length	R_2	1.885	0.112	1.869	0.109	0.41
" tarsus	length	S_2	3.029	0.142	2.519	0.264	6.61***
Metathoracic femur	length	Q_3	2.455	0.109	2.502	0.105	1.19
" tibia	length	R_3	2.298	0.129	2.298	0.139	0.95
" tarsus	length	S_3	3.483	0.164	3.033	0.238	6.02***
Basal plate of tegmen	length	T	2.136	0.123			
	width	U	1.053	0.074			
Paramere	length	W	1.008	0.070			
8th sternite and spicules	length	X	1.281	0.142			
	width	Y	1.060	0.164			
Stylus	length	Z			0.542	0.044	

by Berberet and Helms (1972). In addition, Rittershaus (1927), and Berberet and Helms (1972) give histological descriptions of the internal reproductive organs of *P. horticola* and *A. aenea*, and *P. anxia* respectively; and some histology is available for the cetoniinids *Cetonia floricola* Herbert., and *Cetonia aurata* L.; the geotrupid *geotrupes mutator* Marsham; and the lucanids *Lucanus cervus* L., and *Dorcus parallelipipedus* L. (Bordas, 1900).

The male internal reproductive organs of *C. zealandica* are very similar to those of other Scarabaeoidea. They consist of paired testes connected by vasa efferentia to a pair of vasa deferentia, two vesicules seminales, a ductus ejaculatorius, a pair of accessory glands, and a pair of accessory gland reservoirs (Figure 3.2). This is basically the same arrangement as in all Coleoptera (Bordas, 1900; Imms, 1960; Britton, 1973).

The testes

The testes of *C. zealandica* each consist of 6 follicles which are completely separated although they lie near each other. They are held in place by tracheae close to the integument of the lateral regions of the 6th to 8th abdominal sterna and the lateral edges of their corresponding terga. The follicles are usually positioned so that 5 of them surround the 6th, although sometimes they are arranged in an irregular circle of 6, or a circle of 5 with the 6th internal to another.

Each follicle is an opaque white organ which is approximately spherical in shape and has a maximum diameter of between 0.73mm and 0.13mm (Table 3.2). The poles are generally compressed somewhat; the apical one lying towards the integument and the basal one being attached to the vas efferens. In addition, the equator of the follicle usually has an irregular slightly scalloped outline.

Each follicle is enclosed by a capsule of connective tissue, approximately 2µ in thickness. The end of the vas efferens penetrates this and extends to the centre of the follicle where it expands into a wine-glass shaped opening approximately 80 to 100µ in diameter (Figures 3.3 A, B). Internally, the proximal half to two thirds of the follicle is subdivided radially into 10 to 14 lobes. These are enclosed by a layer of connective tissue less than 1µ thick, and small tracheae also run inward between the walls of adjacent lobes. No attempt

TABLE 3.2

Dimensions of the male internal reproductive organs of *Costelytra zealandica* taken from ten specimens.

All measurements in mm.

		MEAN	S.D.	RANGE maximum	minimum
Maximum diameter of testis follicle		0.942	0.115	1.13 -	0.73
Vas efferens	length	0.767	0.256	1.10 -	0.37
	width	0.038	0.0088	0.05 -	0.03
Vas defferens	length	7.093	0.977	8.27 -	5.33
	width	0.278	0.038	0.33 -	0.10
Reservoir of accessory gland	max. width	0.410	0.077	0.60 -	0.33
Seminal vesicle	max. width	0.290	0.007	0.43 -	0.20
Combined accessory gland reservoir and seminal vesicle	length	1.460	0.210	1.77 -	1.17
Accessory gland	length	60.25	6.365	73	- 52
	width	0.169	0.032	0.23 -	0.07
Anterior part of ejaculatory duct	length	2.06	0.413	2.50 -	1.17
	width	0.187	0.057	0.32 -	0.13
Posterior muscular sheath of ejacularoty duct (outside tegmen only)	length	1.987	0.345	2.73 -	1.80
	width	0.593	0.113	0.87 -	0.47

Figure 3.2

The internal reproductive organs
of the male *C. zealandica*

Dorsal view with the left accessory gland somewhat unravelled.

errata: Ductus ejaculatorus should read:
ductus ejaculatorius

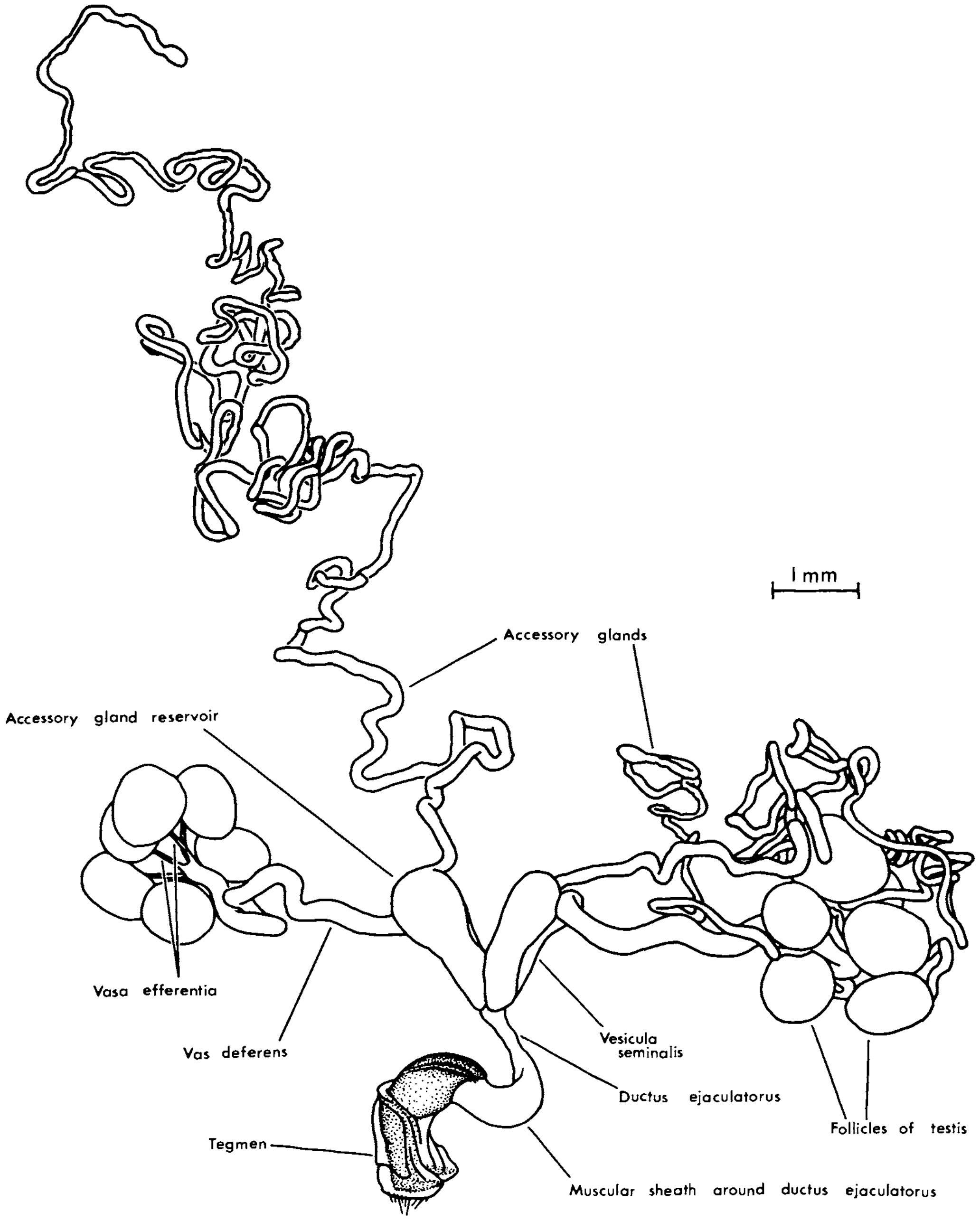

1 mm
Accessory glands
Accessory gland reservoir
Vasa efferentia
Vas deferens
Vesicula seminalis
Ductus ejaculatorus
Follicles of testis
Tegmen
Muscular sheath around ductus ejaculatorus

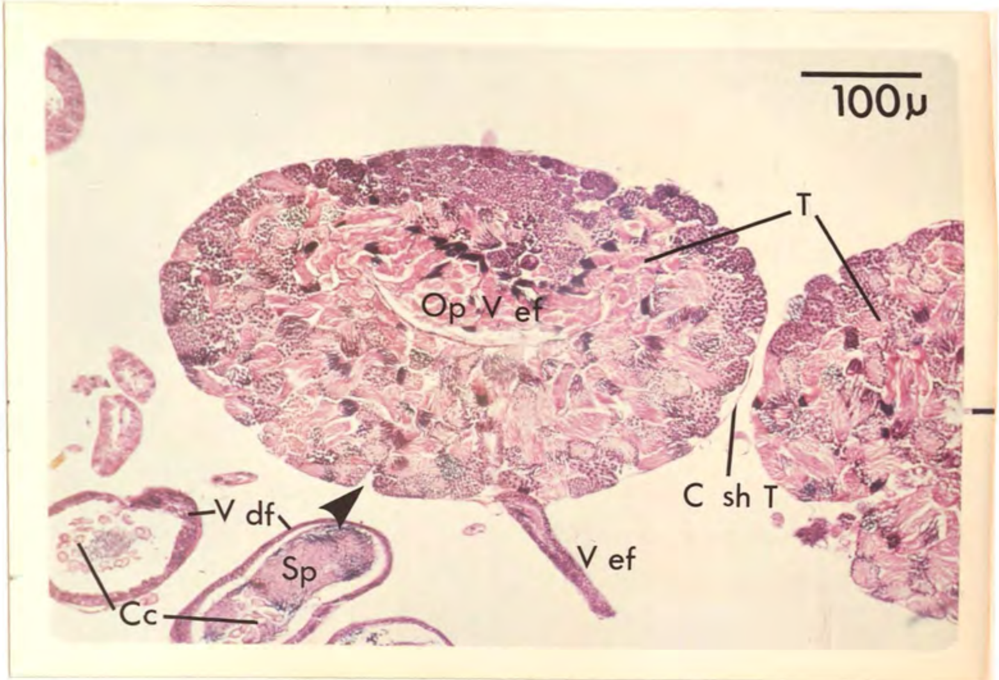
Figure 3.3

Histological micrographs of the male *C. zealandica* reproductive organs.
(All Haematoxylin and eosin)

A. Longitudinal sections through two testes follicles and a vas efferens, and transverse sections through some folds of the vas deferens.
Note the outer connective tissue sheath of the follicle, and a portion of the vas efferens opening. The edges of the follicular lobes are difficult to follow but the clearest can be traced inward from the black arrow. For further details of testis structure see text. Spermatozoa and cyst cells are visible within the vas deferens.

B. A testis follicle, vas efferens and aedeagus in longitudinal section, together with transverse sections through the two accessory gland reservoirs and their associated seminal vesicles.
Note the opening of the vas efferens inside the follicle, the folded internal sac within the muscular sheath of the ejaculatory duct. The basal piece, a paramere, the rectum, and part of the 8th tergum are also visible.

acgl res	accessory gland reservoir
B pc	basal piece
Cc	cyst cell
C sh T	connective tissue sheath of testis follicle
I sac	inner sac
m sh ejdt	muscular sheath of ejaculatory duct
Op V ef	opening of vas efferens
prm	paramere
R	rectum
Spm	spermatozoa
S ves	seminal vesicle
T	testis follicle
Trg 8	tergum 8
V ef	vas efferens
V df	vas deferens


100µ
T
Op V ef
C sh T
V df
Sp
V ef
Cc


acgl
res
S ves
Cc
B pc
V ef
Op V ef
I sac
I sac
m sh ejdt
prm
R
Trg 8
500µ

was made to follow spermatogenesis in the present study but sections of the testes show that the earlier stages consisting of spermatogonia and cysts of synchronously dividing cells are concentrated in a thick disc-shaped region at the apical end where the follicle is not subdivided: later immature cysts tend to be concentrated near the outer surface of the lobes and the remainder of the interior is packed with cysts of mature spermatozoa. These finally become freed from their cysts when they reach the enlarged opening of the vas efferens (Figure 3.3A). The apical end of the follicle is therefore solid while the basal region is partitioned into lobes which are radially disposed about the end of the vas efferens.

The testes are composed of 6 follicles in most Scarabaeidae (Bordas, 1900; Williams, 1945) as they are in *C. zealandica*. However, each testis appears to comprise only 2 follicles in the Passalidae (Williams, 1945; Krause, 1946), and Bordas (1900) notes each has 6 to 8 in *Onthophagus punctatas* Illig., 4-6 in *Anisoplia agricola* L., 10 in *L. cervus*, and 10 to 12 in *D. parallelipipedus*. Williams (1945) also reports that each testis probably only has 4 follicles in *P. carolinus*, and 12 in *P. capreolus* and *C. nitida*, while Rittershaus (1927) similarly notes 12 follicles in each testis of *A. aenea*.

Usually the testis follicles are completely separated from each other in Scarabaeidae and the only exception that was found during the present literature search was the scarabaeinid *Sisyphus schafferi* (L.) in which the follicles of each testis are compressed together and surrounded by a common membrane according to Virkki (1957). It is interesting to note, however, that the follicles of *P. anxia* develop within an epithelial sheath which is later lost in the pupa (Berberet and Helms, 1972), so possibly similar sheaths enclose the follicles in other Scarabaeoidea whilst they are developing, and these are later lost as they mature in all except *S. schafferi*.

The structure of the testis follicle in *C. zealandica* corresponds closely to the septate follicles described by Virkki (1957). These occur in the pleurostict Scarabaeidea and Geotrupidae, whereas the laprostict Scarabaeidae have nonseptate follicles. The follicles of pleurostict Scarabaeidae, however, commonly have an apical invagination and a zone of degenerative cells immediately apical to the funnels of their vasa efferentia, but both of these are not visible in *C. zealandica*.

These are also not described in the follicles of the melolonthinid *P. anxia* by Berberet and Helms (1972). It is interesting to note that usually a large apical cell, or Verson's cell, occurs in each follicle of an insect testis as it develops but these can no longer be found in *C. zealandica*, or in other adult Scarabaeidae according to Virrki (1957).

The external appearance of the testis follicle varies considerably between different families, subfamilies, and species of Scarabaeoidea. Bordas (1900) bases his arrangement of families and subfamilies largely on the follicular morphology of the species they contain, and Virrki (1957) groups the testis follicles into three different morphological types which show progressively more specialization from group I to group III. The follicles of groups II and III have internal "septa", but those of group II are spherical whereas those of group III are "mushroom shaped". Apparently, the Melolonthinae can have follicles of either type because Virrki (1957) gives *Melolontha hippocastani* Fabr. as a group II example and *Amphimallon solstitialis* L. as one of group III. The follicles of *C. zealandica*, however, are intermediate between the two.

Virrki (1957) concludes that the shape of the follicle is presumably due in part to the synchronous growth of numerous spermatocytes which cause the basal region to swell in the direction of the radius and the septa have developed to assist in supplying nutrient and oxygen to the interior of these otherwise solid organs where they are required for meiotic activity and spermiogenesis. Anderson (1950a) also reports a similar testis follicle structure in *P. japonica* to that in *C. zealandica*, but he refers to each follicle as a testis, and each follicle lobe as a follicle. He also notes that groups of spermatocytes produced in the germarium move outward and downward passing along the outer wall of the follicle as they develop, and groups of spermatids usually lie at the inner and lower ends of the follicles. Here spermiogenesis occurs, and the packets of spermatozoa, each still enclosed within two cyst cells, move upward and into the region of the vas efferens funnel where the gametes are released.

The vasa efferentia

The vasa efferentia connect the testis follicles with the ends of the vasa deferentia (Figure 3.2). They are thin transparent tubes with diameters of 0.05mm or less and lengths which vary from about 0.4mm to over 1.0mm (Table 3.2). These measurements are, however, very approximate because the organs are extremely elastic making it difficult to decide what their normal resting positions are.

Each vas efferens is lined with an epithelial layer of cuboidal to columnar cells which vary from about 4 to 10µ in height (Figure 3.4A). These rest on a thick outer basement membrane, and have a smooth well defined border with the lumen. Their intercellular membranes are distinct only if examined by phase contrast and their cytoplasm is densely but fairly evenly basophilic. Also their nuclei are rounded and situated basally close by the cell walls.

Surrounding the vas efferens is a thin layer of muscle fibres which form a reticulum although they are predominantly orientated in a longitudinal to oblique direction.

The lumen of the vas efferens is narrow, less than 40µ across, and free spermatozoa are usually visible within it floating in a clear substance.

The histology of the vasa efferentia of *C. zealandica* resembles that described for *C. floricola* and *C. aurata* by Bordas (1900), *P. horticola* and *A. aenea* by Rittershaus (1927), and *P. anxia* by Berberet and Helms (1972). In all these scarabaeids they are composed of a flattened to columnar epithelium which rests on an outer basement membrane. The nuclei of the epithelial cells can, however, be central as in the cetoniinids and rutelinids, or they can be basal as in *P. anxia* and *C. zealandica*. *C. aurata* also differs from the others in that the cytoplasm of its cells is more granular, and their inner surfaces are irregular and slightly indistinct. In addition, the vasa efferentia of the two rutelinids lack muscles or only have well separated muscle fibres, whereas they are surrounded by a thin muscular layer in the cetoniinids, a "muscularis" in *P. anxia*, and a reticulum of muscle fibres in *C. zealandica*.

Figure 3.4

Histology of the male internal reproductive organs of *C. zealandica*

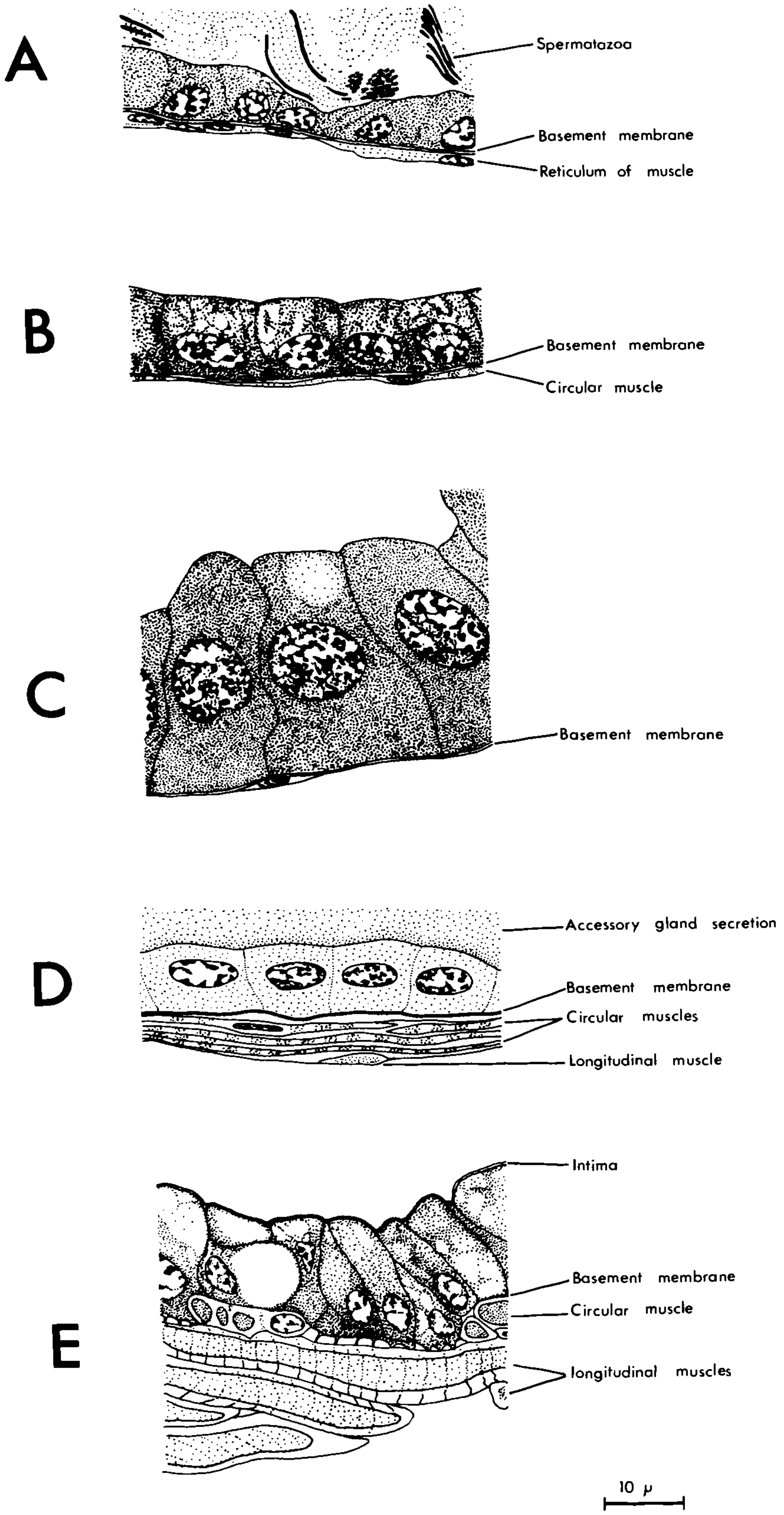
A. Longitudinal section of a vas efferens

B. Longitudinal section of a vas deferens

C. Transverse section of an accessory gland

D. Transverse section of an accessory gland reservoir

E. Transverse section of the anterior region of the ductus ejaculatorius.


A
Spermatazoa
Basement membrane
Reticulum of muscle
B
Basement membrane
Circular muscle
C
Basement membrane
D
Accessory gland secretion
Basement membrane
Circular muscles
Longitudinal muscle
E
Intima
Basement membrane
Circular muscle
longitudinal muscles
10 μ

The vasa deferentia and vesiculae seminales

The distal end of each vas deferens is located medially to the cluster of 6 testis follicles to which it is connected by the vasa efferentia. From this position the vas deferens twists and coils irregularly towards the midline until it comes close to the vas deferens of the opposite side (Figure 3.2). At this point both dilate into seminal vesicles which run parallel to each other without touching and then join the end of the ejaculatory duct. Each seminal vesicle is also bound by a common connective tissue sheath to an accessory gland reservoir which lies parallel and dorsal to it, but the vasa deferentia lie free within the haemocoel, being attached only to tracheae.

The vasa deferentia and seminal vesicles are usually opaque white due to the secretion and spermatozoa within them but sometimes small translucent white regions occur where their contents are less densely coloured.

In length, the vasa deferentia vary between 8.3mm and 5.3mm, and their average diameter is 0.28mm (Table 3.2). The seminal vesicles have maximum diameters of 0.2 to 0.4mm and their mean length is 1.46mm (Table 3.2). Their diameter, however, varies with the quantity of their contents and this depends largely on whether the beetles have copulated recently: immediately after copulation they usually cannot be distinguished from the vasa deferentia, whereas those of unmated beetles or beetles which have not mated for some time are distinctly swollen. For example when 5 field collected male beetles were kept for two weeks without females their seminal vesicles had a mean diameter of 0.30mm (S.D. = 0.071mm) and this was significantly larger ($P<0.01$) than those in beetles which had just been collected (Table 3.2).

Histologically, the vasa deferentia and seminal vesicles are very similar to each other. Both are lined with an epithelium of flattened to columnar cells which vary in height from 6 to 25μ depending on how much they become flattened by the volume of secretion and spermatozoa inside the organs (Figure 3.4B). The epithelium of the seminal vesicles also becomes thrown into longitudinal folds when they are nearly empty (Figure 3.5A). Most of these folds occur laterally and ventrally, and few form dorsally where the accessory gland reservoirs are attached. The epithelial cells rest on an outer basement membrane, have well defined rounded or bulging inner walls (Figure 3.5B),

Figure 3.5

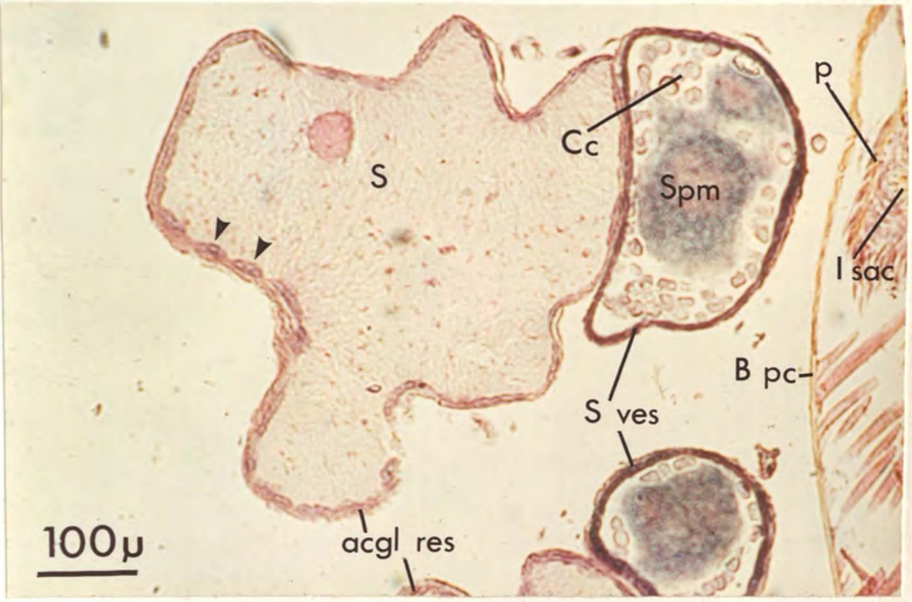
Histological micrographs of male
C. zealandica reproductive organs

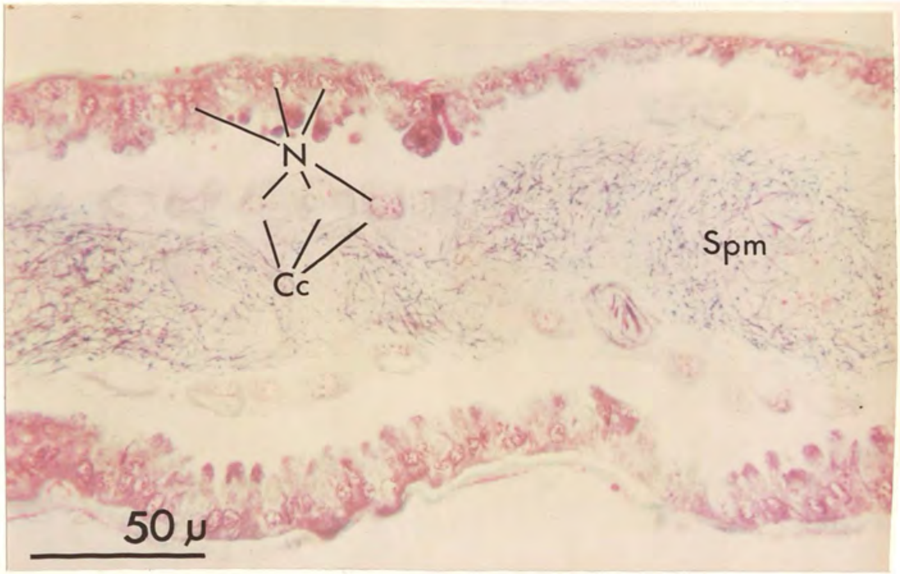
(All haematoxylin and eosin)

A. Transverse section through a seminal vesicle and accessory gland reservoir showing the longitudinally folded epithelium of the accessory gland reservoir (black arrows), and cyst cells surrounding spermatozoa. Portions of the other seminal vesicle and accessory gland reservoir are also visible towards the lower edge of the field and parts of the basal piece, a penis sclerite, and the internal sac are visible in longitudinal section to the right.

B. Longitudinal section of a vas deferens showing the spermatozoa, cyst cells, and the rounded apical surfaces of the vas deferens epithelium projecting into the lumen of the organ. The colour reproduction is not accurate and the cytoplasm of the vas deferens epithelial cells, and the chromatin of the cyst nuclei should be deep blue.

acgl res	accessory gland reservoir
B pc	basal piece
Cc	cyst cell
I sac	inner sac
N	nuclei
P	penis sclerite
S	secretion of accessory gland
Spm	spermatozoa
S ves	seminal vesicle


p
Cc
S
Spm
I sac
B pc
S ves
100μ
acgl res


N
Spm
Cc
50 μ

and indistinct intercellular membranes. Their cytoplasm is as basophilic as that of the epithelium lining the vasa efferentia, but it differs from this in staining unevenly as darker branching and anastomozing strands which run through a lighter staining matrix. No distinct vacuoles are visible within these cells but the strands become longer and sparser towards the apical surface of the cells.

The vasa deferentia are surrounded by a sparse layer of circular to oblique muscle fibres but no longitudinal fibres are visible. This circular muscle increases in thickness around the vesiculae seminales until it is composed of at least three layers. Scattered longitudinal muscle fibres also appear in the connective tissue sheath which binds the seminal vesicles to the accessory gland reservoirs.

In section, the lumens of the vasa deferentia and vesiculae seminales are packed with a tangled mass of spermatozoa which lie separated from each other and suspended within a non staining material (Figure 3.5b). Around the outside of the spermatozoa is a scattered layer of large rounded cells which vary from about 20 to 30µ in diameter. These are completely separated from each other and occur particularly frequently in the seminal vesicles. Their rounded nuclei are large, approximately 8 to 10µ in diameter, and stain lightly, while their cytoplasm is eosinophilic. Presumably these are old cyst cells which have originated from the testes because there are apparently no cells in the walls of the vasa deferentia and vesiculae seminales from which they could have been derived. Similar cyst cells have also been reported in the vasa deferentia of the rutelinid *P. japonica* by Anderson (1950a). According to him they form envelopes about groups of developing spermatozoa, and when the mature spermatozoa are released the cyst cells round off and degenerative changes are initiated in them. They also pass down the reproductive tract of the male *P. japonica* with the spermatozoa and finally disintegrate in the female tract. Since they are rich in glycogen, Anderson (1950a) suggested that they supply nutrient when they break down for the spermatozoa stored in the "seminal receptacle".

Generally the vasa deferentia of Scarabaeoidea are long tubes which increase in diameter proximally before they join the ductus ejaculatorius. This expansion is often quite marked and forms a seminal vesicle as in *C. zealandica*. Similar structures also occur for example in *M. Melolontha* according to Straus-Dürckheim (1828); most of the Scarabaeoidea examined by Bordas (1900); and in *B. farctum*, *Phyllophaga* sp, *P. japonica*, and *C. nitida* as reported by Williams (1945). In addition, Straus-Durckheim (1828), Bordas (1900), and Rittershaus (1927) report that the vas deferens of the Scarabaeoidae described by them are also complexly folded and coiled with the abdomens of the beetles. The latter author refers to the coiled regions as "Der Nebenhoden" or Epididymus, but this is distinct from the "Epididymus" as used by Williams (1945) which refers to a swelling of the distal region of the vas deferens in the lucanid *P. capreolus*.

The histology of the vasa deferentia and vesiculae seminales of *C. zealandica* resembles that of the other Scarabaeoidae which have been investigated. The vasa deferentia of *G. mutator*, *C. floricola*, *C. aurata*, *L. cervus* and *D. parallelipipedus*, according to Bordas (1900), are composed of a cuboidal or columnar epithelium which rests on a very thin basement membrane and is surrounded by a thin layer of circular muscle. The epithelial cells have more or less smooth apical surfaces, large spherical nuclei, and an even granular cytoplasm which differs from that of *C. zealandica* in being vacuolated apically. The seminal vesicles of these species also have similar histological structures to their respective vasa efferentia, and therefore represent dilations of them, but the epithelium of the seminal vesicles of *D. parallelipipedus* differs from the others in being flattened. Rittershaus (1927) also reports that the vas efferens, "Nebenhoden", and vas deferens of *P. horticola* and *A. aenea* cannot be differentiated histologically because they show only minor differences. The "Nebenhoden", however, like the vasa efferentia, lacks muscles or only has individual separated fibres, whereas the "vas deferens" is surrounded by an increasingly thick layer of circular muscle. Possibly the muscles surrounding the vas deferens of *C. zealandica* are similar to those in *P. anxia*, but Berberet and Helms (1972) only describe them as a "muscularis".

The accessory glands and accessory gland reservoirs

The accessory glands form a pair of long thin irregularly coiled tubes which are expanded proximally into two short reservoirs before they join the ejaculatory duct (Figure 3.2). Both pairs of organs are never as densely coloured as are the vasa deferentia or vesiculae seminales: the accessory glands are usually transparent or sometimes a light translucent white because of their contents, whilst their reservoirs are a denser translucent white due to their contents and to their increased diameters.

The accessory glands lie free within the haemocoel except for their tracheal supply, and they form a tangled mass around the other organs in the posterior half of the abdomen. When dissected out they have a mean diameter of 0.17mm and vary in length from 52 to 73mm (Table 3.2). Their reservoirs are slightly curved ventrally into banana-like shapes: they run parallel to each other touching medially, and ventrally each is attached to a seminal vesicle as already described (Figure 3.5A).

Both pairs of organs are located medially or towards the right hand side of the beetle's abdomen and underneath the rectum in the 5th to 6th visible segments. Here they lie at an angle with their anterior ends directed anterio-ventrally and their junction with the ejaculatory duct posterio-dorsally.

The diameter and length of the reservoirs depends on the time since copulation: those of 5 beetles measured immediately after copulation had a mean diameter of 0.68mm (S.D. = 0.05mm) and a mean length of 1.96mm (S.D. = 0.11mm), whereas those of 5 beetles which had been kept separately in the laboratory for two weeks after copulating were smaller, having a mean diameter of 0.37mm (S.D. = 0.21mm), and a mean length of 1.60mm (S.D. = 0.30mm).

In cross-section the accessory glands are round to oval; they are lined with an epithelium of columnar cells which vary in height from about 10.5 to 90µ although they are generally within the range of 65 to 80µ (Figure 3.4c). These cells rest on an outer basement membrane and are arranged approximately radially about the lumen of the gland which varies in cross-section from rounded or oval to flattened. Often it also has up to 3 or 5 short extensions which incompletely divide the epithelium into longitudinal ridges. The apical walls of the cells are smooth, their intercellular membranes are indistinct, and their cytoplasm is densely and evenly basophilic with only occasional small vacuoles. The nuclei are central, have diameters of about 10µ, and are packed densely with granular chromatin. No muscles are visible surrounding the accessory glands.

The histological structure of the accessory gland reservoirs (Figure 3.4D) is in total contrast to that of the accessory glands. The epithelial cells vary from flattened to columnar and from about 4.5 to 12μ in height depending largely on the amount of secretion the reservoirs contain. When relatively empty this epithelium also becomes folded longitudinally in a similar fashion to the seminal vesicles. However, the folds now occur predominantly in the dorsal and lateral walls of the organ. The epithelial cells rest on a basement membrane, their intercellular membranes are indistinct unless examined under phase contrast, and their apical walls are also sometimes difficult to distinguish from the secretion. In addition, the cytoplasm of these cells is eosinophilic, while their nuclei are usually oval with maximum diameters of 7 to 10μ, and are centrally situated. Closely enveloping the reservoirs are from 1 to 5 or more layers of circular or slightly oblique muscle, and outside this they are each enclosed by a connective tissue sheath containing scattered longitudinal muscle fibres which binds the reservoirs to the seminal vesicles as previously described.

Male Scarabaeoidea generally possess one pair of accessory glands although there are exceptions; for example *C. nitida* and the genus *Cetonia* have 3 pairs, and *P. disjunctus* has 2 pairs according to Williams (1945), and Bordas (1900). The glands are also usually thin coiled tubes, which are much longer than the beetles body, and which also dilate into reservoirs near their junctions with the ejaculatory ducts. These reservoirs, however, can sometimes be hardly noticeable expansions as they are in *P. horticola* L. (Rittershaus, 1927) and *P. anxia* (Le Conte) (Berberet and Helms, 1972), or there can be more than one to each accessory gland as in *L. cervus* L. (Bordas, 1900) and *A. aenea* Geer (Rittershaus, 1927).

The storage function of the accessory gland reservoirs in *C. zealandica* has been investigated previously by Fenemore (1971). He found that unmated primary emerged males, in almost all cases have fully distended reservoirs, and that their contents become markedly depleted immediately following copulation. However, they slowly recover afterwards to a fully distended condition in 20 to 30 days. It seems fairly certain that Fenemore (1971) is in fact describing the accessory gland reservoirs although he refers to them as seminal vesicles because he clearly labels the accessory gland reservoirs as seminal vesicles in his figures. In the present study, these structures have been demonstrated histologically to be accessory gland reservoirs although seminal vesicles do exist as described above, but they are of smaller diameter and are bound to the ventral surfaces of the accessory gland reservoirs. It is therefore also possible that Fenemore (1971) is referring to both accessory gland reservoirs and seminal vesicles as the "seminal vesicles". Certainly both have been found in the present study to show corresponding changes in size in relation to copulation.

The accessory glands and reservoirs of *C. zealandica* show histological similarities with those of other Scarabaeoidea. They resemble, most closely, those of another melolonthinid *P. anxia* described by Berberet and Helms (1972). In this insect the epithelium of the accessory glands is composed of columnar cells with large basal nuclei. The cells also rest on a basement membrane, and only the reservoirs are surrounded by a "muscularis".

The most detailed study of scarabaeoid accessory glands was by Anderson (1950b) who investigated them in the rutelinid *P. japonica*. He reported that they are divided histologically into 4 regions: region I is apical and here the gland is almost a solid cylinder of columnar cells arranged radially around a small irregular lumen. They rest on a very thin basement membrane and their nuclei are basal. In region II the glands increase in diameter but the cells remain the same size; the lumen only expanding. Further down, in region III, the cells increase in size and produce an apocrine secretion by apically budding off globules of secretion packed cytoplasm. In the lower region IV, the lumen increases in diameter, and the epithelial cells decrease in height to squamous or cuboidal. They show practically no secretory activity. Regions III and IV are also surrounded by an inner circular sheet of muscle and an outer layer of separated longitudinal muscle fibres. According to Anderson (1950b) the cells in regions I, II, and III have perinuclear basophilic regions from which run basophilic strands to the apices of the cells. The upper levels of the gland produce a mucus-like protein-polysaccharide ground substance and many smaller droplets of lipid; full apocrine cells in the middle region secrete mucus globules, and the lower levels apparently do not contribute to the secretion but serve as expanded reservoirs for it. He finally concludes that a "secretion product consisting of these compounds may be considered as admirably adapted to its several functions of lubrication, sperm-suspension, and spermatophore formation". It appears that the accessory gland reservoirs of *C. zealandica* are therefore very similar histologically to region IV in *P. japonica*, but the accessory glands differ in that they cannot be subdivided into three regions histologically. However, these regions in *P. japonica* show only very slight differences and it is therefore still quite possible that the accessory glands of *C. zealandica* are subdivided functionally but this requires a histochemical demonstration. It is also of note that the accessory glands of Rutelinae may not always be subdivided because Rittershaus (1927) makes no mention of this in *P. horticola* and *A. aenea*, although similar subdivisions may occur which he considered were not sufficiently marked to mention. He reports that their accessory glands are circular or nearly so in cross section, their epithelial cells contain granular protoplasm and are columnar, and their lumens are constricted into irregular clefts. In addition, there are occasional protoplasmic projections or "protoplasmatishe Fortsatze" in the lumens which arise from the cells and he considers that they are indicative of strong secretory activity. The accessory glands of these rutelinids are surrounded by a few muscle fibres but these thicken around the reservoirs and form a distinct inner circular layer and an outer layer containing separated longitudinal muscle fibres. He notes also that the reservoirs are lined by flattened epithelial cells.

Finally, the histology of the accessory glands of two lucanid genera, *Lucanus* and *Dorcus*, have been described by Bordas (1900) and they also show some similarities to those of the above Scarabaeidae. He reports that they are composed of a secretory epithelium containing elongate, cylindrical cells, and that the protoplasm of these cells is granular basally and compact and fibrillar apically. These cells are described as liberating their secretion by rupture, and globules of this product are frequently seen to be attached to the cells by a pedicel. In their secretory activity these cells therefore show some resemblances to the corresponding cells in the rutelinids considered previously.

The ductus ejaculatorius

The ejaculatory duct forms a long tube to the internal sac of the external genitalia (Figure 3.2). It is divided into an anterior first region and posterior second region: the first region varies from 1.17 to 2.50mm long, and is uniformly thin for most of this length with a mean diameter of 0.19mm (Table 3.2), although it expands slightly at its anterior end where it is joined by the seminal vesicles and accessory glands. The second region is completely enclosed by a muscular sheath (Figure 3.6A) which will be described in more detail in the section dealing with the muscles of the male external genitalia. Two large tracheae (Figure 3.6A;tr) pass beneath the muscular sheath dorso-laterally at its anterior end and run back on either side of the ejaculatory duct supplying the tissues within the sheath;their entries mark the junction between first and second regions. Posteriorly, a third or so of the second region and its muscular sheath also enter the anterior end of a cylinder formed by the basal piece of the tegmen and its ventral intrinsic muscles. The muscular sheath and second region of the ejaculatory duct is only slightly thicker anteriorly than the first region, but posteriorly it increases in diameter until it enters the basal piece and here it has a mean maximum diameter of 0.59mm. The free portion, which lies outside the basal piece, also has a length varying from 1.8 to 2.7 mm (Table 3.2).

Most of the ejaculatory duct and its muscular sheath is coloured an opaque white although it is less intense than that of the vesiculae seminales and the vasa deferentia. The posterior ventral surface of the muscular sheath, however, usually has some brown and yellow-brown areas where the respective colours of the outer and inner walls of the inner sac show through.

The ejaculatory duct normally lies characteristically twisted and coiled within the abdomen when the external genitalia are withdrawn, but it untwists and straightens out when the external genitalia are extended for copulation and then the ejaculatory duct shows its true relationship to the rest of the beetles body. Therefore, in order to avoid confusion, the paired terms anterior-posterior, and ventral-dorsal are applied to this organ in the following account as if it is straightened out during copulation.

Figure 3.6

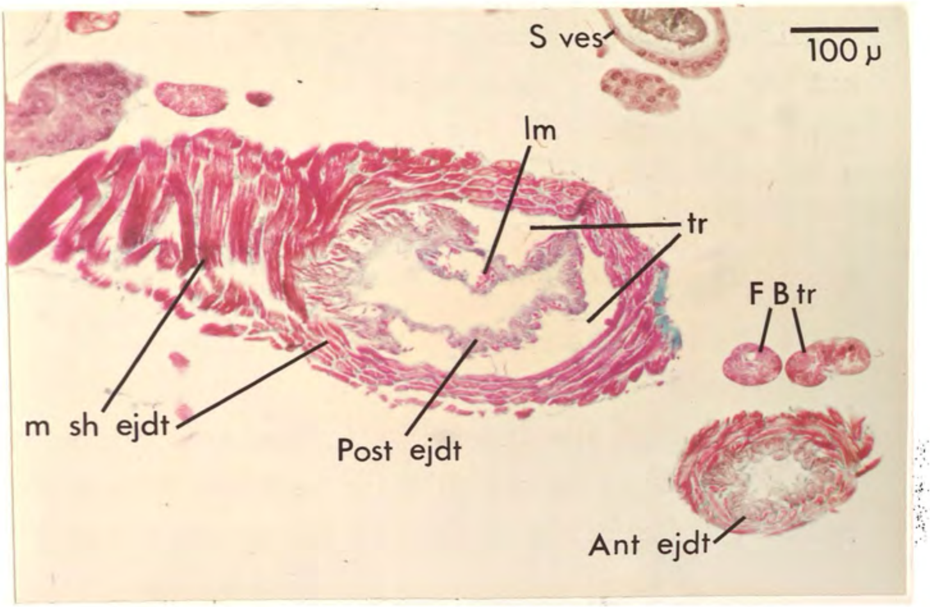
Histological micrographs of male
C. zealandica reproductive organs

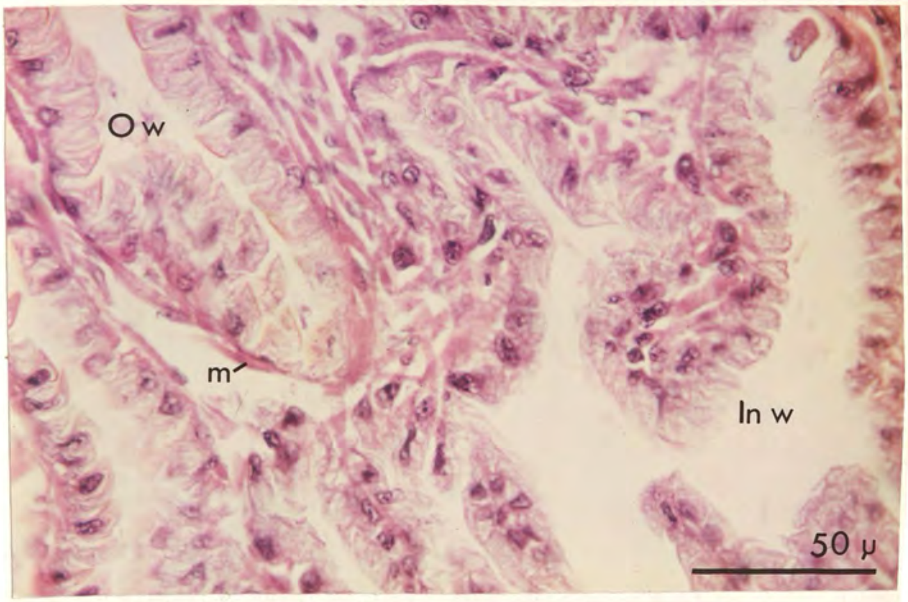
A. Section through the anterior region of the ejaculatory duct and the posterior region where it is surrounded by the muscular sheath.
Note the longitudinal muscle fibres around the posterior end of the ejaculatory duct. Tracheae surrounded by fat body and a portion of a seminal vesicle are also visible.

(Mallorys triple connective tissue stain).

B. Section through the internal sac showing its inner wall, outer wall with flap-like cuticular projections (their exocuticle is yellow), and muscular plexus of the internal sac.

Ant ejdt	anterior region of ejaculatory duct
FB tr	fat body surrounding trachea
In w	inner wall of internal sac
lm	longitudinal muscle
m	muscle
m sh ejdt	muscular sheath of ejaculatory duct
O w	outer wall
Post ejdt	posterior region of ejaculatory duct
S ves	seminal vesicle
tr	tracheae


S ves
100 μ
lm
tr
F B tr
m sh ejdt
Post ejdt
Ant ejdt


O w
m
In w
50 μ

When in the withdrawn position, the anterior end of the ejaculatory duct initially runs posteriorly from its junction with the accessory glands and seminal vesicles, then it bends to the beetle's left through a curve of 80° to 120°. Near the end of this curve it also starts to bend ventrally and at about this point it joins the second region. This ventral bend is continued until the ejaculatory duct is directed posteriorly once again, except that now it lies ventrolaterally to the left in the beetle's abdomen. The ejaculatory duct also becomes twisted whilst it is being bent, so that its dorsal surface is rotated through about 90° to the beetle's right before the organ enters the basal piece. The latter is also rotated the same amount as described below in the section on the male external genitalia.

The entire ejaculatory duct is lined with an epithelium of cuboidal to columnar cells which rest on an outer basement membrane, have distinct intercellular membranes, and secrete a thin intima (Figure 3.4E). This epithelium is thrown into longitudinal folds by the surrounding muscle layers (Figure 3.6A) so that the cells are frequently compressed or stretched out of shape, but where they are not distorted they vary from 5 to 12.5µ in height. Also when the cells are compressed the basement membrane often becomes secondarily folded longitudinally into small wrinkles which are up to about 3µ in height and 3 to 5µ apart, and the intercellular membranes often assume complex patterns. Within the epithelial cells, the nuclei are basal and the cytoplasm appears to be secretory being basophilic and staining in darker strands like that in the cells of the vesiculae seminales and vasa deferentia. It also contains numerous distal vacuoles which are occasionally enlarged to occupy most of the space within the cell (Figure 3.4E).

Closely surrounding the epithelial lining in the first region of the ejaculatory duct is a single layer of scattered longitudinal muscle fibres enclosed within an outer thick layer of circular muscle (Figure 3.6A). At the anterior end of the ejaculatory duct where it expands slightly, the layers of circular muscle thicken and extend a short distance over the posterior ends of the seminal vesicles and accessory gland reservoirs to form a sphincter which regulates the passage of fluids into the ejaculatory duct. In the second region of the ejaculatory duct, the layers of longitudinal and circular muscle are separated from each other by a fluid filled space (Figure 3.6A). The longitudinal muscle also stays closely applied to the epithelium whilst the circular muscle becomes thickened to form the muscular sheath. Within this space the ejaculatory duct is free to move about, but the fluid is entirely separated from the blood by the muscular sheath. The fluid within the space is probably used to assist in everting the inner sac during copulation as described below in the section dealing with the mechanics of copulation.

The ejaculatory duct is short in most Scarabaeidae but it is comparatively long in *C. zealandica* and *M. melolontha* L. (Strauss-Dürckheim, 1828). Also its morphological appearance is relatively simple in Scarabaeidae being a tubular organ of either uniform diameter or increasing in diameter posteriorly. Detailed information is, however, only available for *M. melolontha* L. (Straus-Dürckheim, 1828); *L. cervus* L. and *D. parallelipipedus* L. (Bordas, 1900); *P. horticola* L. (Rittershaus, 1927); and *P. anxia* (Le Conte) (Berberet and Helms, 1972). From these descriptions, it appears that the anatomical structure of the ejaculatory duct of *C. zealandica* corresponds closely with those of the melolonthinids *M. melolontha* and *P. anxia*. In these species it also consists of an anterior tubular region and a posterior region enclosed within a muscular sheath but separated from it by a fluid filled cavity. This sheath is termed "le constricteur du canal ejaculatoire" by Straus-Dürckheim (1828) and "the erection fluid pump" by Berberet and Helms (1972). The anatomical similarity with *C. zealandica* is particularly close for *M. melolontha*, extending even to the manner in which the ejaculatory duct is folded within the muscular sheath. In contrast, the ejaculatory duct of the rutelinid *P. horticola* has no part which corresponds with the first region in *C. zealandica*, and instead the entire organ is enclosed within a muscular sheath, termed a "Muskelmantel" by Rittershaus (1927). This is also separated from the ejaculatory duct by a fluid filled space so that it resembles the second region of *C. zealandica*. The comparative structure and function of the muscular sheath in the above scarabaeids is discussed in the sections on the muscles of the male external genitalia and the mechanics of copulation.

Straus-Dürckheim (1828) gives no histological information on the ejaculatory duct of *M. melolontha* but some is given for *P. horticola* by Rittershaus (1927), and *P. anxia* by Berberet and Helms (1972). These latter joint authors, however, note only that the first region of the ejaculatory duct of *P. anxia* is "composed of epithelium, a chitinous intima and an outer muscularis which consists primarily of circular muscles". Rittershaus (1927), on the other hand, gives a full description for *P. horticola*: she reports that there are three parts to the ejaculatory duct, but the third is the inner wall of the internal sac and is not considered here. The first part which lies outside the tegmen is lined with an epithelium of cuboidal cells and is folded inside a layer of longitudinal muscle. The second part lies inside the "copulatory apparatus"; its epithelial lining is also folded and is apparently secretory. Rittershaus (1927) notes that this region is lined with cuticle which is mostly thin but reaches a considerable thickness in places and is penetrated by fine canals; she does not mention a cuticular lining in the first part of the duct. Outside the epithelium of the second part there is also an inner layer of longitudinal muscle and an outer layer of circular muscle, and it therefore differs in the latter respect from that of *C. zealandica*.

The ejaculatory ducts of the Lucanids *L. cervus* and *D. parallelipipedus* differ a lot from those of the Scarabaeidae mentioned above. Morphologically that of *L. cervus* is thickened anteriorly and narrows posteriorly, whilst that of *D. parallelipipedus* is thin both anteriorly and posteriorly but it has a thickened middle region. In both insects the thickenings of the ducts are due to an increase in the thickness of the muscular layers only. Histologically, the ejaculatory duct of *L. cervus* is composed of the following layers, in order from the outside inward: a very thin "peritoneale" membrane; a thick "manchon" of muscle comprising external circular fibres, several middle oblique fibres, and 4 to 5 inner layers of longitudinal muscle; a very thin basement membrane; a columnar epithelium with very large central nuclei and cytoplasm which is granular basally and fibrous apically; and an indistinct chitinous intima. The ejaculatory duct of *D. parallelipipedus* is very similar to this except that the muscular layers are thinner where the duct inters the tegmen and the thin intima is produced into numerous bristles. The muscle layers of both lucanids are therefore arranged in the same order as those of the scarabaeids but differ from them in not having the circular muscles in the posterior part of the duct developed into a muscular sheath separated by a space from the underlying longitudinal muscles.

The histological structure of the ejaculatory duct of *C. zealandica* therefore shows similarities with those of other scarabaeids, and differs from lucanids in the development of a posterior muscular sheath. Amongst the Scarabaeidae, however, the most notable differences are with *P. horticola* which lack an intima in the anterior region, and have a circular layer of muscle outside the longitudinal muscle and within the muscular sheath.

Considered overall the anatomy and histology of the internal reproductive organs of male Scarabaeidae are similar and only show minor variations. The organs are morphologically simple except for the ejaculatory duct and this shows slight differences in the details of its association with the muscular sheath in the species investigated. However, the ejaculatory duct of *C. zealandica* is almost identical anatomically with that of *M. melolontha* making the resemblance between the reproductive organs of these melolonthinids particularly close. As far as the histology is concerned, the differences between the species are also only slight, the most important being variations in muscle layers, the development of a connective tissue sheath around each seminal vesicle and accessory gland reservoir in *C. zealandica*, and the apparent lack of a cuticular lining in the first part of the ejaculatory duct of *P. horticola*.

The male external genitalia

There are no elements of the genital complex of male Coleoptera that can be referred directly to the gonopods and the genitalia therefore consist of so called phallic structures only (Snodgrass, 1935; Lindroth and Palmén, 1970).

In the male *C. zealandica* all the cuticular structures which originate embryologically posterior to the 8th abdominal segment lie hidden in the adult within a genital chamber, except when in use during copulation. This genital chamber is invaginated between the 8th tergum and sternum which normally lie close to each other bounding the posterior end of the abdomen. The genital chamber can therefore only be seen if these sclerites are prised apart. It is then visible as an oval depression in the central third of the transparent articular membrane which stretches between the 8th tergum and sternum.

The terminology of Lindroth and Palmén (1970) has been followed in this account, but in order to avoid confusion the terms used by Snodgrass (1935) have also been included because these have been in wide use. Also, the terms used by other authors whose works have a bearing on this study have been noted where these differ from those of Lindroth and Palmén (1970), and Snodgrass (1935).

The genital chamber takes the shape of a shallow oval pit with a transverse mound centrally located on its anterior surface. A further sac-like invagination of the genital chamber arises from the mid-dorsal surface of the central mound and the anus opens into the genital chamber mid-dorsally.

The 9th sternite (Figure 3.7 A, B) supports the central mound in the genital chamber; it lies with its flat surface roughly parallel to the frontal plane of the insect and it forms a curving bar which passes transversely through the central mound. Short bristles arise from the posterior region of the ventral surface of this sternite and project posteriorly towards the opening of the genital chamber (Figure 3.7 A).

The anterior lateral edges of the genital chamber are supported by the posterior ends of the spicules (Figure 3.7 A,B). These ends are dorso-ventrally orientated C-shaped rods which lie on either side of the 9th sternite and curve around its lateral tips. The anterior parts of the spicules extend inward anteriorly from the ventral parts of the C-shaped rods and they eventually curve towards each other and join anteriorly. Slightly thickened cuticle is stretched across between the anterior parts of the spicules from both their dorsal and ventral edges to form a thin flat pouch-like invagination of the genital chamber. The flat dorsal and ventral walls, however, lie close to each other so that the opening is represented only by a fine slit which is situated ventral to the 9th sternite. The whole structure therefore forms a flat apodeme with thinner dorsal and ventral surfaces. It has a mean maximum width of 1.06mm and a mean length of 1.28mm (Table 3.1). This complete structure is termed the "spiculum gastrale" by Snodgrass (1935) and the "lateral ventral plate" and "inner ventral

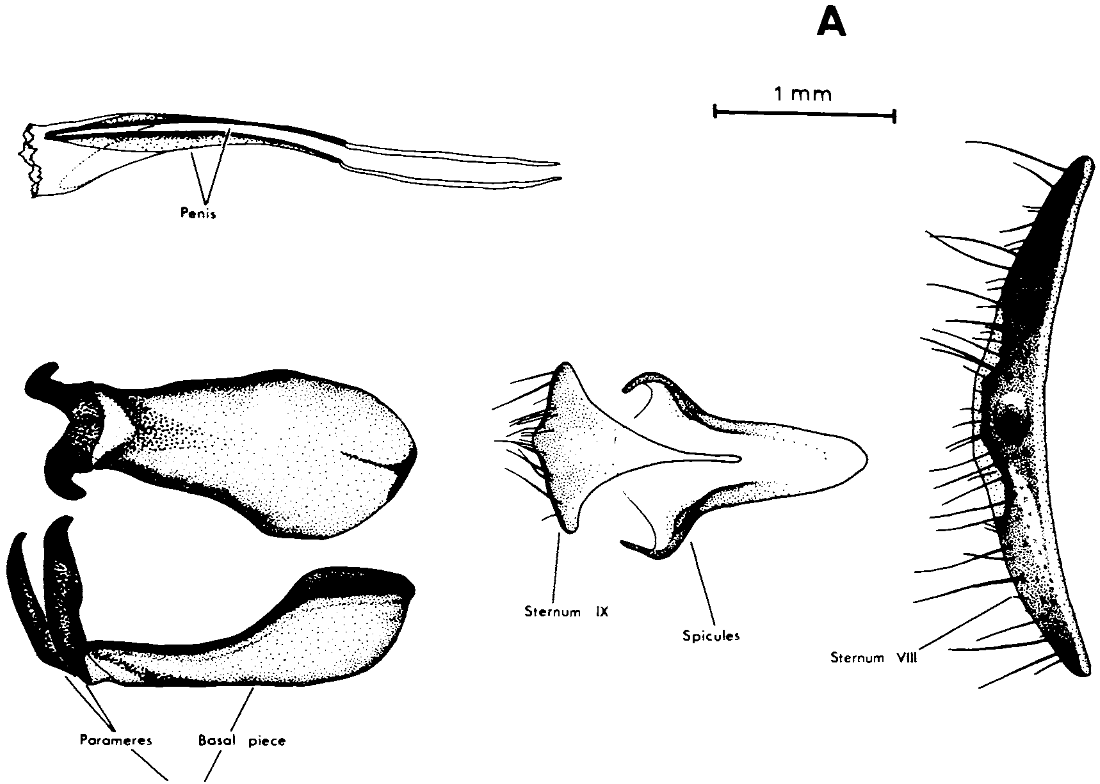
Figure 3.7

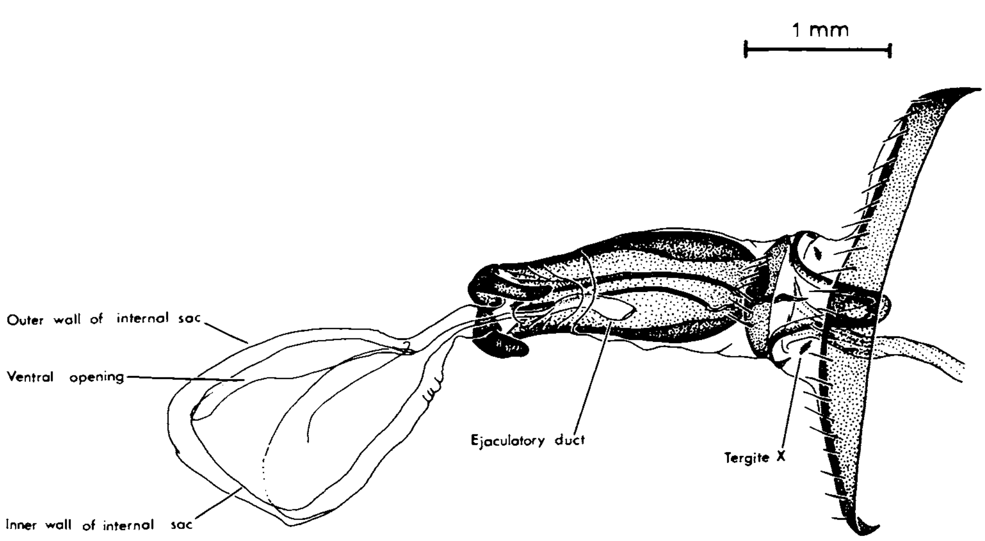
The external genitalia of the male *C. zealandica*

A. 8th sternum and sclerites of the external genitalia separated from one another.

Ventral view of 8th sternum
Dorsal view of 9th sternite and spicules
Dorsal and lateral view of tegmen
Dorso-lateral view of penis sclerites with the internal sac removed.

B. Whole mount of the external genitalia and 8th sternum after maceration with potassium hydroxide. Some folds of the internal sac and ejaculatory duct omitted for clarity. Ventral view.


A
1 mm
Penis
Sternum IX
Spicules
Sternum VIII
Parameres
Basal piece
Tegmen


B
1 mm
Outer wall of internal sac
Ventral opening
Inner wall of internal sac
Ejaculatory duct
Tergite X

plate" by Given (1952). It appears to occur in all male scarabaeid beetles; its anterior portion can either be tongue-shaped as in all the New Zealand Melolonthinae (Given, 1952), or produced into a thin rod as for example in *M. melolontha* L. (Straus-Dürckheim, 1828) and *A. majalis* (Razoumowski) (Butt, 1944).

In *C. zealandica* the posterior dorsal surface of the apodeme which incorporates the spicules is itself invaginated again to form a thin walled flattened sac which tapers to a point anteriorly at its distal end. It lies parallel to the apodeme with the spicules and its dorsal wall eventually extends posteriorly to unite with the anterior edge of the 9th sternite (Figure 3.7 A). A similar invagination can be seen in Figure 5, plate 5 of Given's monograph on the New Zealand Melolonthinae (Given, 1952) but he does not name it.

The sac-like invagination of the genital chamber which opens dorsal to the 9th sternite extends inward and terminates where it joins the tegmen anteriorly (Figures 3.9, 3.10 A). The tegmen forms the functional copulatory organ and consists of three sclerites; a basal piece and two parameres (Figure 3.7 A). The basal piece is the largest sclerite and is shaped like an inverted shoe horn. Snodgrass (1935) terms it the "phallobase" and Given (1952) the "basal shield". The parameres, which are termed by Given (1952) the "genital claspers" are two curved prongs which are approximately mirror images of each other and articulate with the posterior lateral corners of the basal piece.

The tegmen encloses the penis, the end of the ejaculatory duct and the inner sac which are described below. However, the term aedeagus is applied to the combined tegmen, penis and inner sac, and normally, when at rest, it lies twisted to its left and rotated onto its right side (Figures 3.10 A, B). During copulation, however, it becomes rotated and untwisted until it occupies a position where it shows its true morphological relationship with the rest of the beetle's body. Also it is extended so that about a third of it projects posteriorly out of the abdomen (Figures 3.8 A, 3.10 C). In the following account, therefore, the terms dorsal and ventral, anterior and posterior, and left and right are applied to the aedeagus as if it were in use and directional changes due to twisting and rotation are ignored.

Figure 3.8

The morphology of the male external genitalia of *C. zealandica*

A. A scanning electron micrograph of the ventral surface of a male with fully extended tegmen and internal sac. The male was quick frozen with liquid nitrogen *in copula*, and the female was dissected away later.

B. A higher magnification scanning electron micrograph of the area of the internal sac indicated by the black square in Figure 3.8 A. This photograph shows the posteriorly directed cuticular flaps which invest the outer wall of the internal sac.

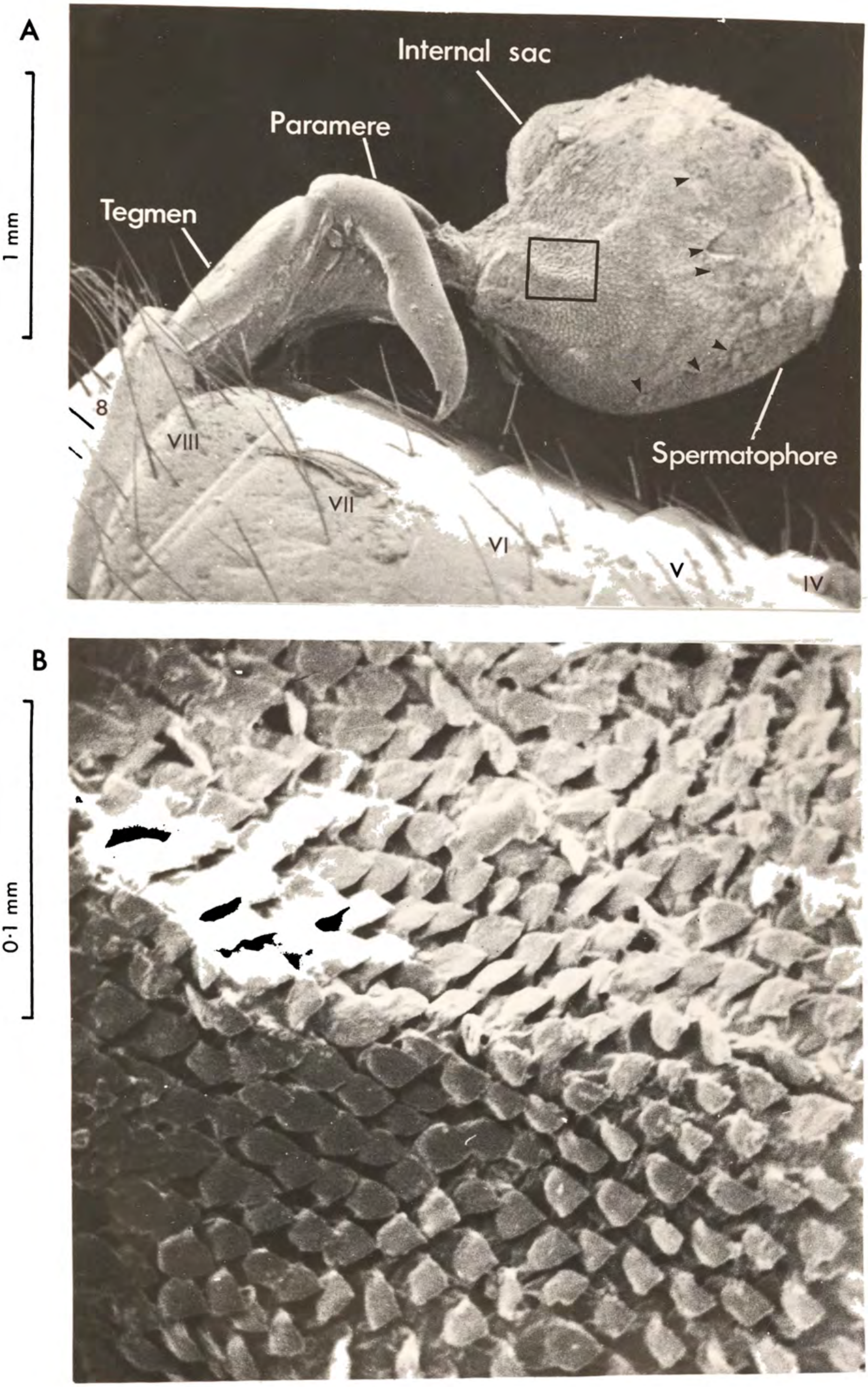
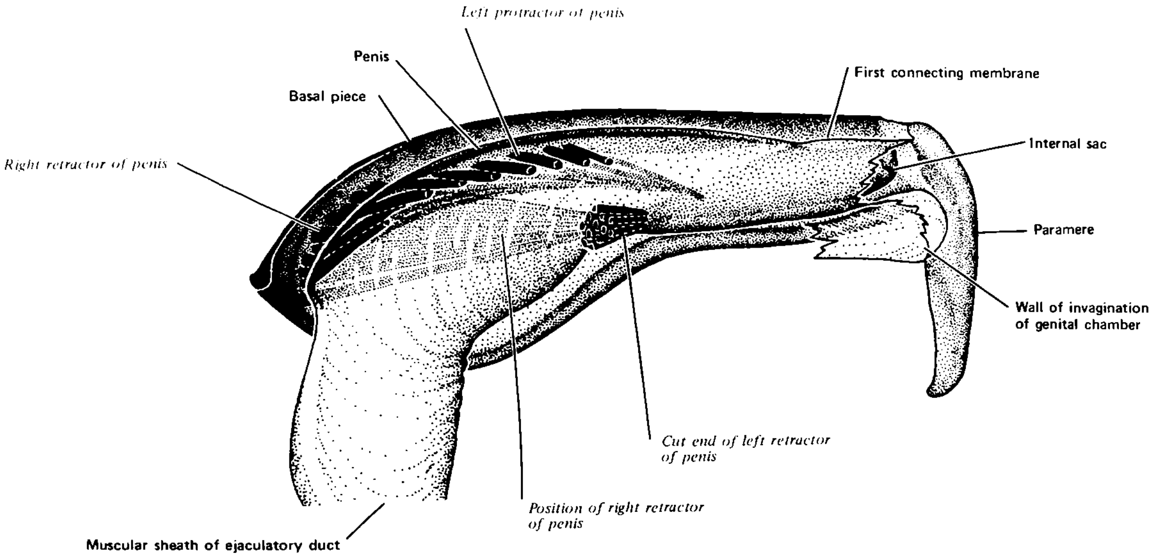

A
1 mm
Internal sac
Paramere
Tegmen
Spermatophore
8
VIII
VII
VI
V
IV
B
0·1 mm

Figure 3.9

Intrinsic phallic muscles of *C. zealandica*

Upper: Aedeagus viewed from the left, with left side of tegmen removed. The right ventral flexor of the paramere and the right dorsal extensor of the paramere have been omitted for clarity.

Lower: Sagittal section of the tegmen viewed from the left, with the ejaculatory duct, penis, and associated structures removed. The right retractor of the penis has also been removed but normally it lies medially to the right ventral flexor of the paramere.


Left protractor of penis
Penis
Basal piece
First connecting membrane
Internal sac
Right retractor of penis
Paramere
Wall of invagination of genital chamber
Cut end of left retractor of penis
Position of right retractor of penis
Muscular sheath of ejaculatory duct

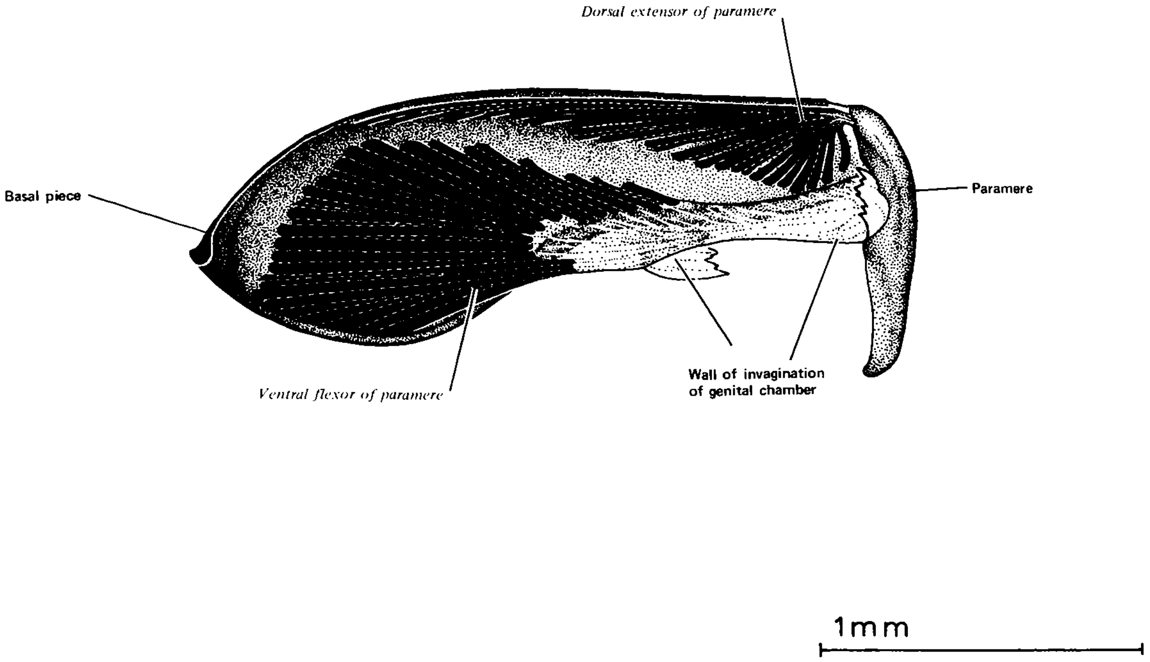

Dorsal extensor of paramere
Basal piece
Paramere
Wall of invagination of genital chamber
Ventral flexor of paramere
1mm

Figure 3.10

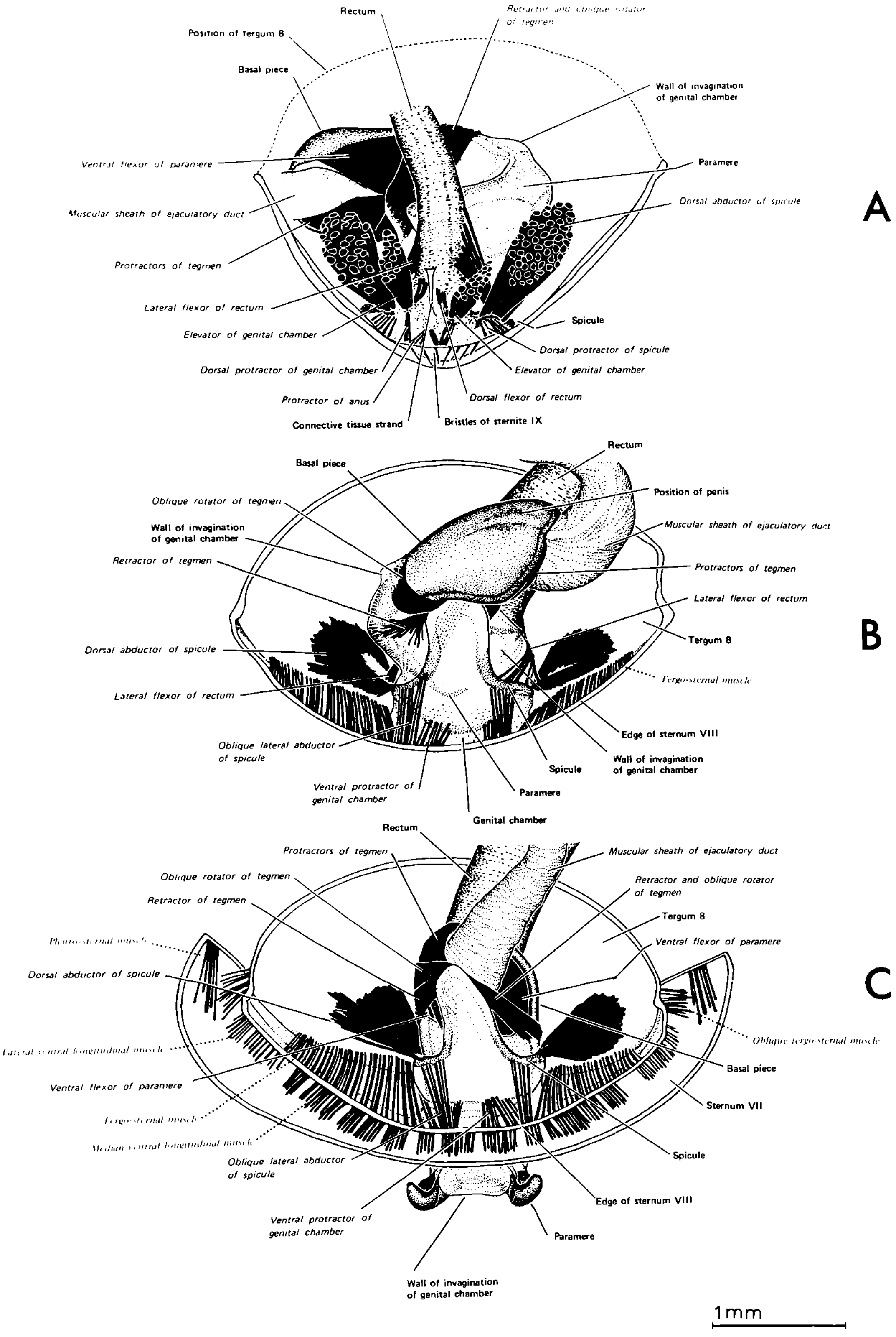
Extrinsic phallic muscles and anal muscles of *C. zealandica*

(All muscles are labelled in italics, and the abdominal muscles are also shown by dotted indicator lines.)

A. Retracted external genitalia *in situ* with tergum 8 removed. View from posterio-dorsal with sternum VIII omitted for clarity.

B. Ventral view of retracted external genitalia *in situ* with sternum VIII removed.

C. Ventral view of external genitalia in the position occupied during copulation. Sternum VI has been removed and the internal sac has also been omitted for clarity (c.f. Figure 3.8A).


A
Rectum
Retractor and oblique rotator of tegmen
Position of tergum 8
Basal piece
Wall of invagination of genital chamber
Ventral flexor of paramere
Paramere
Muscular sheath of ejaculatory duct
Dorsal abductor of spicule
Protractors of tegmen
Lateral flexor of rectum
Spicule
Elevator of genital chamber
Dorsal protractor of spicule
Dorsal protractor of genital chamber
Elevator of genital chamber
Protractor of anus
Dorsal flexor of rectum
Connective tissue strand
Bristles of sternite IX
B
Rectum
Basal piece
Position of penis
Oblique rotator of tegmen
Muscular sheath of ejaculatory duct
Wall of invagination of genital chamber
Retractor of tegmen
Protractors of tegmen
Lateral flexor of rectum
Tergum 8
Dorsal abductor of spicule
Tergo-sternal muscle
Lateral flexor of rectum
Edge of sternum VIII
Oblique lateral abductor of spicule
Wall of invagination of genital chamber
Spicule
Ventral protractor of genital chamber
Paramere
Genital chamber
C
Rectum
Protractors of tegmen
Muscular sheath of ejaculatory duct
Oblique rotator of tegmen
Retractor and oblique rotator of tegmen
Retractor of tegmen
Tergum 8
Ventral flexor of paramere
Pleuro-sternal muscle
Dorsal abductor of spicule
Oblique tergo-sternal muscle
Lateral ventral longitudinal muscle
Basal piece
Ventral flexor of paramere
Sternum VII
Tergo-sternal muscle
Median ventral longitudinal muscle
Spicule
Oblique lateral abductor of spicule
Edge of sternum VIII
Ventral protractor of genital chamber
Paramere
Wall of invagination of genital chamber
1mm

Twisting and rotation of the withdrawn aedeagus occurs commonly amongst the Coleoptera according to Lindroth and Palmén (1970) who term this movement "torsion". In *C. zealandica* it appears to form a means whereby the aedeagus can be packed compactly into the end of the abdomen without interfering with other internal organs.

The basal piece averages 2.13mm in length and 1.05 in width (Table 3.1). Dorsally it is joined to the wall of the genital chamber invagination at a point approximately two thirds along from its anterior end, whereas ventrally the wall of the invagination is folded posteriorly to join the lateral edges of the basal piece and some of the anterior surfaces of the parameres thus forming a tube with the basal piece. The posterior third of the tegmen therefore is contained within the invagination of the genital chamber and the anterior two thirds project into the body cavity.

The parameres average 1.0 mm in length (Table 3.1). They articulate by condyles with the posterior lateral tips of the basal piece from which the greater part of them project ventrally. Dorsal to the condyles they give off extensions which follow the cross-sectional shape of the basal piece and abut against each other dorsally in the mid-line. These extensions form levers to which attach the muscles responsible for extending the parameres posteriorly as described in the section on the mechanics of copulation.

All gaps between the parameres and the basal piece are bridged by thin articular membrane similar to that which forms the walls of the genital chamber. In addition, the genital chamber membrane folds around at the posterior end of the tegmen where it is attached to the dorsal extensions of the parameres and becomes invaginated within the tegmen for a short distance before uniting with the penis and its internal sac. This invagination corresponds to the "first connecting membrane" of Lindroth and Palmén (1970) and the "endophallic chamber" of Menees (1963).

The penis in *C. zealandica* is represented by two long thin sclerites (Figure 3.7 A) which lie alongside each other on the dorsal surface of the muscular sheath surrounding the ejaculatory duct. These sclerites extend only as far as the anterior end of the basal piece and then continue anteriorly as two thin straps of flexible cuticle to the anterior end of the muscular sheath. Posteriorly, the penis is broadly joined by thin cuticle to the internal sac which normally lies folded within the muscular sheath.

The internal sac forms the functional intromittant organ of the male and during copulation it fills the bursa copulatrix of the female. It is most easily described when it is extended; it then forms a globular double walled organ with the opening directed posteriorly and slightly ventrally so that it roughly resembles a wine goblet (Figures 3.7 B, 3.8 A). The entire outer surface of the internal sac is covered by small triangular scale-like flaps of slightly stiffened cuticle which are usually directed with their sharp tips pointing posteriorly. The cuticular wall of the internal sac is folded in at the edges of the opening to form the internal wall which has no scale-like ornamentation. The inner wall follows the shape of the outer wall and terminates anteriorly where it meets the posterior end of the ejaculatory duct at what corresponds to the anatomical gonopore. When withdrawn the internal sac collapses and is folded mostly within the posterior ventral part of the muscular sheath. Sometimes it also projects a small distance posteriorly past the penis and even out of the tegmen. The folds are complex and variable but there are always a number of prominent anterior-posterior pleats and the anatomically posterior and ventral regions of the organ tend to lie anterior to the remainder.

The penis is termed the "aedeagus" by Snodgrass (1935) and the internal sac the "endophallic chamber" or "vesica". As already noted, however, the term aedeagus is used by Lindroth and Palmén (1970) to include both the tegmen and penis.

In Scarabaeidae the penis is always reduced (Britton, 1973) but the tegmen, which is well developed, varies considerably in shape and this has been shown to be of taxonomic importance (e.g. Given, 1952). The external genitalia of *C. zealandica*, however, are relatively simple compared with some of the variations which occur in other Scarabaeidae as described, for example by Sharp and Muir (1912).

The musculature of the external genitalia of the male

In this section only the structure of the musculature is described because functional considerations are treated in the final section of this chapter.

The musculature of the anus and rectum was examined as well as that of the external genetalia, since they were closely associated together and this enabled a comparative approach.

The genital muscles of the male may conveniently be divided into extrinsic muscles which lie outside the tegmen, and intrinsic muscles which lie entirely within it. The extrinsic muscles are attached to the basal piece of the tegmen, the surface of the genital chamber, the spicules, and the 8th sternite, whilst the intrinsic muscles are attached to the basal piece, the parameres, the surface of the genital chamber, the penis, and the internal sac.

The first description of the genital musculature of a scarabaeid was by Straus-Dürckheim who published the anatomy of *Melolontha melolontha* L. in 1828. Since then the complete genital musculature has been described for only two other male Scarabaeidae, the melolonthinid *Amphimallon majalis*(Razoumowski) (Menees, 1963) and the geotrupid *Geotrupes stercorosus* Scriba (Hieke, 1966), although Rittershaus (1927) described the intrinsic musculature of *Phyllopertha horticola* L. and *Anomalia aenea* Geer together with the extrinsic muscles which are attached to the tegmen.

Straus-Dürckheim named the muscles according to the sclerites they insert upon, but the nomenclature of these has since changed. Later workers used the terminology of Snodgrass (1935). In the present work the terminology of Lindroth and Palmén (1970) has been used, although this has necissitated a further change in the naming of the muscles. At the same time nomenclature according to supposed function has been adopted as far as possible.

Intrinsic muscles of the phallus

Dorsal extensors of the parameres (Figure 3.9).

These two fan shaped muscles originate on the medial dorso-lateral tips of the parameres and run inside the basal piece to insert on its inner posterior-dorsal and lateral surfaces.

Ventral flexors of the parameres (Figures 3.9, 3.10 A, C).

These are two large muscles which are attached to the inner anterio-lateral surface of the basal piece and join the membranous wall of the genital invagination at its most anterior lateral connection with the basal piece. These muscles are visible as the most ventral ones in the basal piece running on either side of the ejaculatory duct sheath.

Protractors of the penis (Figure 3.9).

This pair of thin flat muscles originates anterio-laterally on the penis sclerites and is inserted on the lateral edges of the basal piece about midway along it.

Retractors of the penis (Figure 3.9).

From their origins ventro-laterally on the first connecting membrane where it joins the muscular sheath of the ejaculatory duct these paired muscles insert anteriorly in the basal piece medially to the ventral flexors of the parameres. They run as flat slightly tapering sheets dorsally to the ventral flexors of the parameres. They can usually be seen running around both sides of the ejaculatory duct without having to dissect the basal piece, but are partially obscured by the ventral flexors of the parameres.

Muscular sheath of the ejaculatory duct (Figures 3.2; 3.9; 3.10 A, B, C).

This complex sheath of circular or diagonal muscles forms a tube around the posterior part of the ejaculatory duct. However, it is only attached to it anteriorly and joins the penis dorsally, and the internal sac posteriorly. Two prominent muscles included here with the muscular sheath are a thickening of circular fibres at the level of the anterior ends of the penis sclerites, and a pair of muscles which originate from the anterior tips of the penis sclerites and run posteriorly and obliquely over the outer surface of the muscular sheath, inserting near its anterior end. The first of these muscles forms a sphincter, the last compresses the ejaculatory duct.

Retractor of the internal sac (not figured)

This comprises a large diffuse series of muscle fibres which originates on the dorsal surface of the internal wall of the internal sac and inserts dorsally within the muscular sheath on the anterior internal walls of the penis sclerites, and also on the posterior part of the ejaculatory duct where it folds back upon itself.

Muscular lining of the internal sac (Figures 3.3 B; 3.6 B).

This is a thin dense plexus of muscle fibres which covers the internal surfaces of the inner and outer walls of the internal sac. These fibres form a reticulum although near the junction with the ejaculatory duct they tend to be circularly arranged proximally to the cuticle, and longitudinally orientated distally.

Extrinsic muscles of the phallus

Protractors of the tegmen (Figures 3.10 A, B, C).

These two muscles originate on the anterior edge of the basal piece slightly towards its right hand side and run close together around the right side of the ejaculatory duct to insert on the anterio-lateral sides of the triangular apodeme arising dorsally to the spicules.

Retractors of the tegmen (Figures 3.10 A, B, C).

From their origins on the walls of the invagination of the genital chamber posterio-laterally to its junction with the tegmen, these two muscles run to join the spicules anteriorly along their dorso-lateral edges.

Oblique rotators of the tegmen (Figures 3.10 A, B, C).

This pair of muscles originates dorso-laterally on the walls of the invagination of the genital chamber and close to its junction with the tegmen, and inserts dorso-laterally on the anterior ends of the spicules anteriorly to the retractors of the tegmen.

Dorsal abductors of the spicules (Figures 3.10 A, B, C).

These comprise a large bilaterally symmetrical pair of muscles which originates on the posterior dorso-lateral edges of the spicules and radiates posterio-dorsally to posterio-laterally, inserting on the 8th tergum.

Oblique lateral abductors of the spicules (Figures 3.10 B, C)

Each of these bilaterally symmetrical pair of muscles originates laterally on a posterio-ventral side of a spicule and joins the 8th sternum near its attachment with the genital chamber membrane.

Dorsal protractors of the spicules (Figure 3.10 A)

These comprise a very fine short pair of muscles each of which originates dorsally and posterio-laterally on the dorsal arms of the spicules and inserts on the 8th tergum where it joins the genital chamber.

Ventral protractors of the genital chamber (Figures 3.10 B.C).

Originating on the genital chamber between the posterior ventral tips of the spicules this small bilaterally symmetrical pair of muscles runs to insert on the 8th sternum near its attachment with the genital chamber membrane, medially to the insertions of the protractors of the spicules.

Anal muscles of the male

Lateral flexors of the rectum (Figure 3.10 A).

These paired muscles originate on the posterior lateral tips of the spicules and run anteriorly to insert laterally on the rectum.

Dorsal flexors of the rectum (Figure 3.10 A).

Each of this pair of muscles originates on the anterior tips of the two sclerotised remnants of the 10th tergum, and passes anteriorly to insert on the posterior dorso-lateral surface of the rectum.

Elevators of the genital chamber (Figure 3.10 A).

These comprise large paired muscles which originate on the anterior tips of the two remnants of the 10th tergum posteriorly to the dorsal flexors of the rectum, and insert on the 8th tergum.

Dorsal protractors of the genital chamber (Figure 3.10 A).

These small paired muscles originate anteriorly on the remnants of the 10th tergum but posteriorly to the elevators of the genital chamber, and insert on the 8th tergum where it joins the genital chamber.

Protractors of the anus (Figure 3.10 A).

Each of these two muscles is very fine and they originate close together on the genital chamber dorsal to the anus. They then pass obliquely to insert on the 8th tergum medially to the point where the protractors of the genital chamber insert on it.

The muscles of the external genitalia and anus of the male *C. zealandica* and other Scarabaeoidea are compared in Table 3.3. Homologous muscles, or those which are attached to the same sclerites or area of cuticular membrane in these scarabaeoids are shown opposite each other in Table 3.3, whereas muscles which are probably homologous

Table 3.3

The muscles of the external genitalia of various male Scarabaeoidea.

Species (family;subfamily)	*Costelytra zealandica* (White) (Scarabaeidae; Melolonthinae)	*Melolontha melolontha* L. (Scarabaeidae; Melolonthinae)	*Amphimallon majalis* Razoumowski (Scarabaeidae; Melolonthinae)	*Phyllopertha horticola* L. and *Anomala aenea* Geer (Scarabaeidae; Butelinae)	*Geotrupes stercorosus* Scriba (Geotrupidae; Geotrupinae)
Author	The present study	Straus-Durckheim (1828)	Menees (1963)	Rittershaus (1927)*	Hieke (1966)
INTRINSIC PHALLIC MUSCLES	Dorsal extensors of the parameres	L'Extenseur de la pince	Dorsal flexor of the phallobase		M. phallobasoapodemo-phallobasicus superior
	Ventral flexors of the parameres	Le Flechisseur de la pince	Ventral flexor of the phallobase	Die sweite Muskel der ventral platte ((Der Attraktor der Ventral platte))	M. phallobasoapodemo-phallobas medialis M. phallobasoapodemo-phallob inferior
	Protractors of the penis	L'Extracteur de la verge	Protractors of the aedeagus and endophallic chamber	Die Protraktoren des "Penis"	((M. phallobaso-phalloapodemal
	Retractor of the penis	L'Intracteur de la verge	Retractor of the aedeagus and endophallic chamber	Die Retraktoren des Median Lobe	M. phallobasoapodemo-phallicus
	Retractor of the internal sac	La Muscle ejaculateur	-	Die Retraktoren des Inneren Sackes	((M. phallo-ductalis))
	Muscular lining of the internal sac	Le Constricteur du prepuce	-		M. endophallicus
	Muscular sheath of the ejaculatory duct	((Le Constricteur du canal ejaculatoire))	-	Der Konstriktor des Rutenkanals (or "Ringmuskelschicht")	-
	-	-	-	-	M. phallobasoapodemo-phalloapo superior
	-	-	-	-	M. phallobasoapodemo-phalloapo inferior
	-	-	-		M. phallobasoapodemo-phalloapod basalis
	-	-	-	-	M. phallobaso-phallicus
	-	-	-	-	M. phallobasoapodemalis
	-	-	-	-	M. phallobasicus externus
	-	-	Constrictor of aedeagus and ensophallic chamber	-	M. phallobasicus internus
EXTRINSIC PHALLIC MUSCLES	Protractors of the tegmen	L'Extracteur de l'etui de la verge	Protractor of the apodeme of the phallobase and genital chamber	Protraktoren der Rutenkapsel	M. tergoapodemo-phallobasoapodemalis medialis
	Retractors of the tegmen	((Le Pretracteur de la gaine de la verge))	Oblique rotator of the genital chamber	Protraktoren der Rutenkapsel	M. paratergo-phallicus
	Oblique rotators of the tegmen	Le Pretracteur de la gaine de la verge	Retractors of the genital chamber		M. tergoapodemo-phallobaso-apodemalis lateralis
	Oblique lateral abductors of the spicules	((L'Elevateur de la piece anale inferieure))	Oblique lateral abductors of the spiculum gastrale		M. antecosto-antecostalis uronotum lateralis
	Ventral protractors of the genital chamber	((Le Rotateur du cloaque))	Ventral protractors of the genital chamber and spiculum gastrale		M. urotergo-sternalis anterior
	Dorsal abductors of the spicules	((L'Abaisseur de la piece anale inferieure)) ((Le Retracteur anterieure de la piece anale inferieure)	Oblique protractor of genital chamber		M. antecosta-antecostalis urosterni
	Constrictors of the genital chamber	((Le Transverse du cloaque))	Constrictors of the genital chamber		M. tergo-paratergalis
					M. phallo-rectalis
	Dorsal protractors of the spicules	Le Retracteur posterieur de la anale inferieure	Dorsal abductors of the spiculum gastrale		M. uronoto-antecostalis
	-	Le Retracteur de la gaine de la verge	-		
ANAL MUSCLES	Lateral flexors of the rectum	Le Flechisseur lateral du rectum	Depressor of rectum		-
	Dorsal flexors of the rectum	L'Abaisseur du rectum	Dorsal flexors of the rectum		M. Urotergo-rectalis
	Elevators of the genital chamber	L'Elevateur du cloque ou de l'anale superieure	((Elevator of rectum))		-
	Protractors of the genital chamber	Le Retracteur du cloaque	-		-
	Protractors of the anus	((Le Dilatateur de l'anus))			-

*NOTE: Rittershaus (1927) described only the intrinsic phallic muscles and those which directly move the tegmen. Blank spaces opposite the other muscles therefore do not necessarily signify that there are no corresponding muscles in *P. horticola* and *A. aenea*.

with ones in *C. zealandica* are indicated by double brackets. The latter are either those whose positions are not perfectly clear from their descriptions but which appear to correspond to the ones they are tabulated with, or else they have insertions close to the muscles they possibly correspond to, and they are therefore possibly derived from them.

The names of the muscles as given by the original authors have been retained in Table 3.3. Apart from Hieke (1966), who names them according to their attachments, all the other authors use a similar terminology to that followed in the present study. Differences in the names given to homologous muscles by these authors can be attributed to the terminology of the phallic structures which they used, to differences in the proportions of the phallic structures, and to the fact that the exact points of attachment of homologous muscles can vary in different insects. They can therefore occupy different positions relative to other structures and consequently have different functions.

Rittershaus (1927) is the only author who has listed any homologous muscles in the Scarabaeidae. She notes that the following muscles in the two rutelinids described as "Die Protraktoren des Penis", "Die Retraktoren des Median lobe", "Der Konstriktor des Rutenkanals", and "Die Retraktoren des Innes Sackes" correspond to the muscles described by Straus-Durckheim (1828) in *M. melolontha* as indicated in Table 3.3. This is therefore in agreement with the conclusions reached in the present study. Rittershaus (1927), however, does not attempt to homologise "Der paarige Attraktor der Ventral platte" and "Der zweite Muskel der Ventral platte" with any muscles in *M. Melolontha* because this insect does not possess ventral plates in its tegmen. A possible homology proposed here, is that both muscles in the rutelinids correspond with the ventral flexors of the parameres in *C. zealandica* and their homologues in the other melolonthinids. This can be explained if it is allowed that the single group of muscle fibres which comprises each ventral flexor of the paramere has become split into anterior and posterior groups by the movement apart of their points of attachment to the basal piece. This seems reasonable because the points of attachments of the muscles can themselves be moved relative to their homologues in other beetles. The converse to this, where two muscle pairs fuse into a single pair may of course have occurred

Erratum

The first sentence should read:

Nevertheless, the two pairs of muscles in the rutelinids, which are possibly homologous with the ventral flexors of the parameres in *C. zealandica*, are associated with the possession of ventral plates in the rutelinids, and this may represent some functional refinement for the moving of their genitalia.

As can be expected, Table 3.3 shows that the number of muscles shared by any two of the beetles depends on how closely related they are. Considering firstly the intrinsic muscles; those of the 5 Scarabaeidae are the same except for the ventral muscles of the tegmen dealt with above and the muscles of the internal sac which in *A. majalis* are different from the other Melolonthinae and Rutelinae. This latter difference can be correlated with the different mechanism employed in *A. majalis* for everting the internal sac and this is considered below under the section dealing with the mechanics of copulation. The musculature of the internal sac of *G. stercorosus* is also difficult to homologise with that of the Scarabaeidae, but otherwise this geotrupid possesses the same intrinsic muscles and 6 or 7 additional pairs. It therefore shows a greater degree of difference with the Scarabaeidae than the 5 representatives of this family do between themselves. Two of the intrinsic muscles of *G. stercorosus* the "M. phallobasoapodemo-phallobasicus medialis" and "M. phallobaso-apodemo-phallobasicus inferior", are possibly both homologous with the ventral flexors of the parameres in *C. zealandica*, being perhaps related through a similar intermediate form as shown by the Rutelinae with their two homologues.

Rittershaus (1927) describes only a single pair of extrinsic muscles in the rutelinids, the "Protraktoren der Rutenkapsel". These are, however, the only extrinsic muscles attached to the tegmen and they therefore represent both the "protractors of the tegmen" and "retractors of the tegmen" in *C. zealandica*, and their homologues. This therefore adds support to the previous view that muscles can either split or that two can combine. The muscles of the other Scarabaeoidea are easily homologised with one exception: *G. stercorosus* possesses an extra pair of muscles, the "M. phallo-rectalis". The extrinsic muslces are attached to the spicules which are considered to be derived from part of the 9th sternum by Menees (1963) in *A. majalis*, or derived from the 9th segment and not necessarily from its sternum alone by Hieke (1966) and Lindroth and Palmén (1970), and it is probably this shared depending on which of these represents the more primitive state. Nevertheless, the split ventral flexors of the parameres represented as two pairs of muscles in the rutelinids may perhaps be the result of a functional refinement associated with possession of ventral plates.

As can be expected, Table 3.3 shows that the number of muscles shared by any two of the beetles depends on how closely related they are. Considering firstly the intrinsic muscles; those of the 5 Scarabaeidae are the same except for the ventral muscles of the tegmen dealt with above and the muscles of the internal sac which in *A. majalis* are different from the other Melolonthinae and Rutelinae. This latter difference can be correlated with the different mechanism employed in *A. majalis* for everting the internal sac and this is considered below under the section dealing with the mechanics of copulation. The musculature of the internal sac of *G. stercorosus* is also difficult to homologise with that of the Scarabaeidae, but otherwise this geotrupid possesses the same intrinsic muscles and 6 or 7 additional pairs. It therefore shows a greater degree of difference with the Scarabaeidae than the 5 representatives of this family do between themselves. Two of the intrinsic muscles of *G. stercorosus* the "M. phallobasoapodemo-phallobasicus medialis" and "M. phallobaso-apodemo-phallobasicus inferior", are possibly both homologous with the ventral flexors of the parameres in *C. zealandica*, being perhaps related through a similar intermediate form as shown by the Rutelinae with their two homologues.

Rittershaus (1927) describes only a single pair of extrinsic muscles in the rutelinids, the "Protraktoren der Rutenkapsel". These are, however, the only extrinsic muscles attached to the tegmen and they therefore represent both the "protractors of the tegmen" and "retractors of the tegmen" in *C. zealandica*, and their homologues. This therefore adds support to the previous view that muscles can either split or that two can combine. The muscles of the other Scarabaeoidea are easily homologised with one exception: *G. stercorosus* possesses an extra pair of muscles, the "M. phallo-rectalis". The extrinsic muslces are attached to the spicules which are considered to be derived from part of the 9th sternum by Menees (1963) in *A. majalis*, or derived from the 9th segment and not necessarily from its sternum alone by Hieke (1966) and Lindroth and Palmén (1970), and it is probably this shared origin which is reflected in the similarity of the extrinsic muscles of Scarabaeoidea.

The anal muscles of the Melolonthinids correspond with each other reasonably closely, the three insects sharing 5 or possibly 6 pairs of muscles, and *M. melolontha* and *C. zealandica* sharing a further two pairs. These latter muscles are very small and lie close to the internal surface of the genital chamber so that Menees (1963) may have considered them to be part of the muscular layers which surround the rectum. The Rutelinid *G. stercorosus*, however, again shows the greatest difference between any of the Scarabaeoidea sharing only two pairs of muscles in its anal region with those of the Scarabaeidae and having a further one which is difficult to homologise at all because of the well developed 9th tergum of *G. stercorosus*.

The present investigation concerning the musculature of the external genitalia of *C. zealandica* adds nothing towards what is already known about the relationship between the phallic structures of male Scarabaeidae and the embryonic segmentation of their abdomens. The most detailed publication relating to this is that of Menees (1963) who studied the morphology, musculature, embryology, and postembryonic development of the phallic organs, genital chamber, and spicules of *A. majalis*. He concluded that the phallic organs and spicules are entirely developed from the 9th sternum. Hieke (1966), and Lindroth and Palmén (1970) also regard the spicules as being derived from the 9th segment but not necessarily from the 9th sternum alone. However, Lindroth and Palmén (1970) note that, apart from the spicules, no further elements belonging to abdominal segments seem to be incorporated in the aedeagus of Coleoptera. Hieke (1966) from his study of the musculature of the aedeagus of *G. stercorosus* found that it was impossible to tell from this alone what the relationship was between the copulatory apparatus and the 9th and 10th abdominal segments, and the same conclusion also applies to the present study of *C. zealandica*. Whether or not the origin of the phallic structures in *C. zealandica* differs from those of *A. majalis* could only be determined when a comparable investigation to that of Menees (1963) is carried out, but it seems reasonable, in the meantime, to follow Menees' (1963) interpretations of them because both insects are Melolonthinae. The extrinsic muscles which run between the spicules and the 8th segment can therefore be considered as derivatives of the 8th and 9th intersegmental muscles while the other extrinsic muscles, together with the intrinsic muscles, can be considered as derived from the 9th sternal muscles.

The internal reproductive organs of the female

The internal reproductive organs of the female *C. zealandica* have not been previously described although they are figured by Fenemore (1971). They have, however, been described for a number of other Scarabaeoidea: Williams (1945) reported their morphology in *Pinotus carolinus* (L.), *Bolbocerosoma farctum* (Fabr.), *Eucanthus lazarus* (Fabr.), *Phyllophaga* sp., *Popillia japonica* Newman , *Ligyrus gibbosus* (De G.), *Dynastes tityus* (L.), *Cotinus nitida* L. , and *Popilius disjunctus* (Illig.). Other detailed descriptions include those of *Melolontha melolontha* L. by Straus-Dürckheim (1828); *Aphodius fimetarius* L. by Stein (1847); *Phyllopertha horticola* L. and *Anomala aenea* Geer by Rittershaus (1927); *Scarabaeus sacer* L. and *Scarabaeus semipunctatas* Fabr. by Heymons (1930); *Haliocapris gigas* Fabr. by Dattar Gupta and Kumar (1963); *Amphimallon majalis* (Razoumowski) by Menees (1963); and *Phyllophaga anxia* (Le Conte) by Berberet and Helms (1972). Some histology is also available for *S. sacer* and *S. semipunctatus* (Heymons, 1930), *H. gigas* (Dattar Gupta and Kumar, 1963), and *A. majalis* (Menees, 1963), while Rittershaus (1927) gives good histological descriptions for *P. horticola* and *A. aenea*.

The internal reproductive organs of the female *C. zealandica* consist of paired ovaries and lateral oviducts, a median oviduct, vagina, bursa copulatrix, spermatheca, spermathecal gland, and two pairs of accessory glands (Figure 3.11).

The ovaries

The ovaries each consist of 6 telotrophic ovarioles which are translucent white in colour for most of their length. However, within each ovariole a series of oocytes can be seen which increase in diameter posteriorly and become progressively whiter and more opaque as they mature.

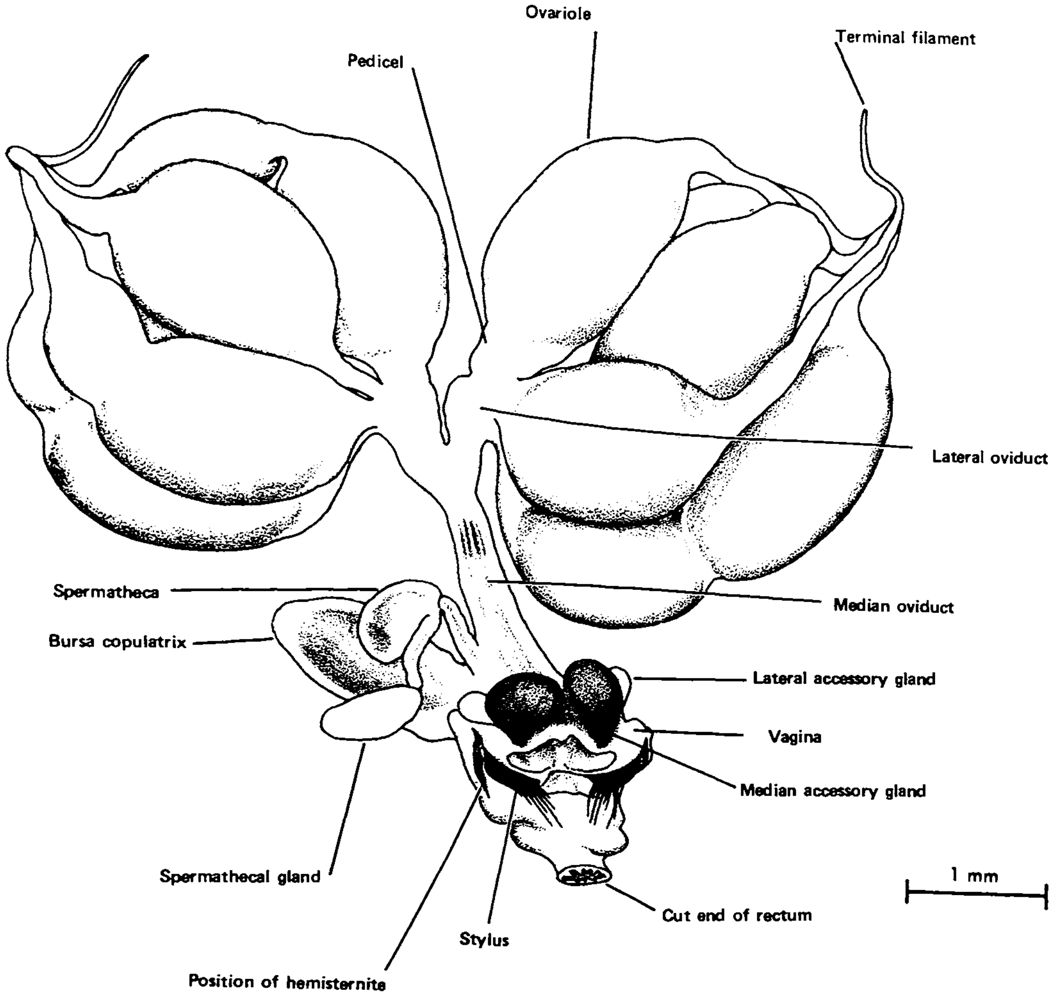
Each ovariole comprises a terminal filament, germarium, vitellarium, and pedicel. Each terminal filament fuses with those of the other 5 ovarioles to form a common terminal filament for the

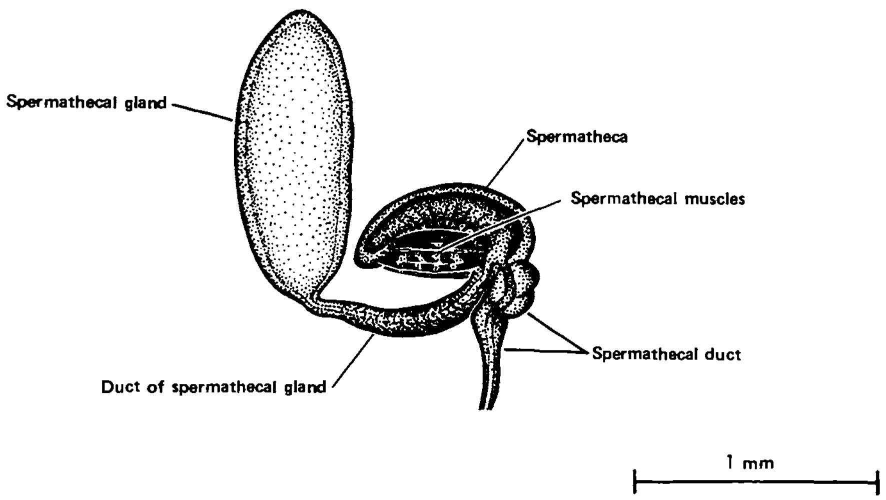
Figure 3.11

The internal reproductive organs
of the female *C. zealandica*

Upper Ventral view of the entire organs with terminal filaments of the ovaries separated from each other.

Lower Detail of spermatheca and spermathecal gland.


Ovariole
Pedicel
Terminal filament
Lateral oviduct
Spermatheca
Median oviduct
Bursa copulatrix
Lateral accessory gland
Vagina
Median accessory gland
Spermathecal gland
1 mm
Cut end of rectum
Stylus
Position of hemisternite


Spermathecal gland
Spermatheca
Spermathecal muscles
Spermathecal duct
Duct of spermathecal gland
1 mm

ovary. This runs dorsally and medially within the abdomen and it eventually joins with the end of the other terminal filament from the opposite ovary in a small dilation. The terminal filaments therefore form a thin transverse band of tissue through the abdomen which runs over the rectum and is apparently attached only to the ovaries and tracheae. Posteriorly, the terminal filament of each ovariole increases gradually in width and joins the germarium at a slight swelling. This junction is, however, best distinguished histologically. The vitellarium follows the germarium and forms the largest part of the ovariole. As already noted oocytes can be seen within it which increase in size as they move down it, but its anterior end cannot be easily distinguished from the germarium except for a slight constriction and usually 2 to 4 small oocytes can be seen which are followed by one or two very much larger proximal ones. The pedicel connects the ovariole to the calyx of a lateral oviduct and it either forms a short constricted tube behind the last oocyte or else it becomes distended with mature ova if the lateral oviducts are also filled. Finally, the 6 ovarioles of each ovary are often enclosed within a thin common connective tissue sheath, but this sometimes ruptures so that the ovarioles lie free within the abdomen except for their connections with the terminal filaments and pedicels.

The ovaries vary considerably in size depending on the degree of development of the oocytes. When these are all immature the ovaries occupy ventral positions within the 5th to 7th abdominal segments, and each ovariole together with its terminal filament can be as small as 2.7mm long and 0.3mm in maximum width (Table 3.4). A single ovary can, however, contain 12 or more mature ova, and then it occupies most of the ventral and lateral space within the abdomen between segments 2 and 7, with each ovariole and its terminal filament reaching up to 4.7mm in length and 1.3mm in width (Table 3.4).

A full histological investigation of the ovaries was not attempted in this investigation but the following observations were made: the terminal filament of each ovariole consists of a central core surrounded by a connective tissue sheath. The core is rounded in cross-section and has a diameter of 6 to 7μ for most of its length except where it expands to 12 to 15μ near the germarium. It consists of a strand of eosinophilic cytoplasm with a distinct outer cell membrane, and a central row of round closely packed nuclei 3 to 5μ in diameter.

TABLE 3.4

Dimension of the female internal reproductive organs of *Costelytra zealandia* taken from ten specimens.

All measurements in mm.

		MEAN	S.D.	RANGE maximum minimum
Ovariole and terminal filement	length	3.387	0.609	4.67 - 2.67
	max. width	0.603	0.295	1.33 - 0.33
Lateral oviduct	length	0.780	0.209	1.13 - 0.47
	max. width	0.413	0.093	0.60 - 0.27
Median oviduct	length	1.847	0.388	2.47 - 1.33
	max. width	0.370	0.029	0.40 - 0.33
Bursa copulatrix	length	2.353	0.495	3.20 - 1.60
	max. width	0.910	0.144	0.60 - 0.37
Duct of spermatheca	length	0.717	0.069	0.83 - 0.60
	max. width	0.165	0.021	0.20 - 0.13
Spermatheca	length	0.637	0.090	0.77 - 0.50
	max. width	0.427	0.056	0.53 - 0.37
Duct of spermathecal gland	length	0.590	0.089	0.43 - 0.70
	max. width	0.123	0.027	0.17 - 0.10
Spermathecal gland	length	0.910	0.146	1.23 - 0.70
	max. width	0.485	0.069	0.40 - 0.57
Median accessory gland	max.diameter	0.600	0.085	0.77 - 0.50
Lateral accessory gland	max.diameter	0.287	0.059	0.40 - 0.20

However, no basement membrane or intercellular walls are visible.

The connective tissue sheath which encloses the terminal filament is continuous with the outer sheath of the ovariole, and both have the same structure. It consists of a thin layer of connective tissue with occasional nuclei and tracheae, closely applied to it, and an outer reticulum of widely spaced muscle fibres which are predominantly orientated circularly. This sheath also forms a transverse septum between the terminal filament and the germarium, completely separating them. In addition, the 6 terminal filaments of an ovary are also enclosed within the common connective tissue sheath of the ovary which has the same structure as the outer sheath around each ovariole. It constricts past the ends of the germaria so that it gathers the terminal filaments of the ovarioles together into a compact bundle forming the common terminal filament of the ovary.

The germarium is a dense mass of cells approximately 800 to 950µ long by 100 to 150µ in diameter which tapers and rounds off anteriorly, and constricts slightly before joining the vitellarium posteriorly. Histologically it is divided into distinct anterior and posterior regions both of which are enclosed within an outer basement membrane approximately 0.5µ in thickness and an outer connective tissue sheath around the ovariole.

The anterior region is 700 to 850µ long and constitutes the largest part of the germarium. Histologically, it has a fairly uniform structure, being occupied largely by trophic cells, with occasional interstitial cells between them. The trophic cells are mostly packed closely together so that their walls appear octagonal in section, but posteriorly and towards the centre of the anterior region they become more loosely packed and somewhat rounded even though they do not become entirely separate from each other. The nuclei of these trophic cells are round or slightly oval, have diameters of 6 to 8µ, and have prominent nucleoli. They also occupy most of the cells except for thin outer regions of slightly basophilic cytoplasm. At the extreme apical tip of the germarium, 4 to 6 cells are also found which are similar to trophic cells except that their nuclei stain much deeper. Possibly these are trophic cells in early prophase but apart from this, no mitotic activity was seen in the trophic cells in any of the ovarioles examined during this study. The interstitial cells of the anterior region have oval or distorted nuclei with diameters of 2 to 3μ, and they stain deeper than the trophic nuclei. The cytoplasm of these cells also stains less deeply basophilic than that of the trophic cells. The interstitial cells occur most frequently next to the basement membrane, and it is possible that here there is a very thin epithelium but the cell boundaries cannot be followed.

The posterior region of the germarium is only 50 to 100μ in length (Figure 3.12), and the histological change between it and the anterior region is quite abrupt. Large numbers of prefollicular cells appear amongst the interstitial cells around the periphery of the posterior region. These prefollicular cells resemble interstitial cells except that their nuclei are larger and have diameters of 5 to 9μ. In addition, the prefollicular cells move inwards posteriorly towards the centre of the germarium until they almost completely pack its posterior end. They also become progressively flattened in the transverse plane of the organ. The central portion anterior to this consists largely of loosely packed cytoplasm, a confusion of cell membranes, and a relatively large number of interstitial nuclei compared with the anterior region. Amongst this are scattered cells resembling trophic cells except that their nuclei are less densely staining, together with young oocytes which have very irregular shapes, dense basophilic cytoplasm, and larger nuclei. These nuclei eventually enlarge into germinal vesicles containing globules of eosinophilic material as the oocytes grow, and the oocyte cytoplasm becomes densely packed with basophilic granules. When the oocytes reach 14 to 19μ in diameter they also acquire a nutritive cord which connects them with the trophic tissue. This appears as an anterior extension of the oocyte which is 4 to 7μ in width near the oocyte but it tapers, becomes less densely staining, and harder to follow until it disappears after 10 to 15μ. As the oocytes continue to grow, they pass down into the vitellarium where they become surrounded by a single layer of follicular cells, but they retain their nutritive cord connection with the vitellarium for some time.

The vitellarium is bounded by an outer basement membrane continuous with that of the germarium, and it is also enclosed within the outer connective tissue sheath of the ovariole. A very thin epithelial layer of extremely flattened cells probably rests on the inner surface of the basement membrane but it is not visible at the magnification available. It is, however, indicated by occasional small nuclei which lie against the basement membrane. The follicular epithelium lies within this and forms a single layer of cells around the oocytes and solid interfollicular plugs between each of them.

As the oocytes pass into the vitellarium they round off and grow in size. They also lose their nutritive cords when they attain a diameter somewhere between 100 and 200μ. The nutritive cords resemble those of young oocytes in the germarium except that they are much longer and usually only 3 to 4μ thick. They are also extremely difficult to follow after 10 to 20μ because their cytoplasm stains progressively less intensely until it resembles that of the follicular cells. The furthest a nutritive cord was followed in the present study was about 80μ, and this originated from an oocyte 35 to 50μ in diameter which was just passing into the vitellarium. Once the nutritive cord is lost the cytoplasm of the oocyte retains its uniformly basophilic and densely granular appearance until it reaches approximately 250μ in diameter. An eosinophilic layer then appears next to the follicular epithelium and eosinophilic globules, approximately 1 to 10μ in diameter, subsequently form throughout the cytoplasm. The germinal vesicle also migrates towards the periphery of the oocyte halfway along and eventually the eosinophilic globules pack the cytoplasm by the time the chorion is being secreted

The follicular epithelium varies in appearance with the size of the oocytes it encloses. Around oocytes 50 to 80μ in diameter it is composed of columnar cells 10 to 14μ in height with oval nuclei approximately 7μ long by 5μ in diameter which are perpendicular to the ovum. Some follicle cells also divide around small oocytes but cell division ceases as the oocytes grow. The follicle cells also flatten out while their nuclei enlarge until by the stage when the chorion is being secreted the follicle cells are flattened to about 5μ in height and they are almost completely filled by their nuclei which are approximately 4μ high and 12 to 15μ in diameter. In the interfollicular plugs the follicle cells are frequently elongated or distorted but in all other respects they are identical to those around ova. The cells in the centre of a plug may also have less densely staining cytoplasm around nuclei than elsewhere.

The follicular epithelium also forms an ovariole plug behind the proximal follicle. The structure of this varies, however, depending on whether or not any ovulation has occurred. In ovarioles from which eggs have not been liberated the follicular epithelium forms a single layer of cells around the last ovum. The epithelial lining of the pedicel also follows this inward and joins the basement membrane of the vitellarium leaving a small area about 30µ in diameter where the follicle cells are apparently open to the lumen of the pedicel. In ovarioles which have ovulated and which have nearly mature ova in their last follicles, the follicular epithelium forms a solid plug of tissue approximately 25µ long by 30µ in diameter. This has the same appearance as an interfollicular plug except that it joins the infolded pedicel epithelium posteriorly. Most of the follicle cells therefore degenerate and become resorbed after ovulation, but the corpus luteum was not investigated during the present study.

The pedicel forms a tubular extension of the calyx of a lateral oviduct and its histological structure is identical to that of the lateral oviducts described below.

The only investigations by other authors which have any bearing on the ovaries *C. zealandica* are those relating to egg production: Fenemore (1971) found that out of a total of 153 virgin insects kept in the laboratory none laid eggs although small numbers of fully developed eggs were found in the reproductive systems of 22 of them. The development of fully formed eggs proceeded more rapidly after mating but the highest mean number found in three groups of beetles collected from different localities was 6.9, and the maximum number within any one female was 25. In addition, the maximum mean number of eggs laid by mated beetles after 30 days was 10.0, and the highest number laid by a single individual was 24. However, Kain in a personal communication (Radcliffe and Payne, 1969) reported that *C. zealandica* could lay between 4 and 32 eggs during the first oviposition, and 2 to 16 during the second. Fenemore (1971) also found that females contained an average of between 9.1 and 25.6 fully formed eggs without outer envelopes in the ovaries with a maximum number in any one female of 42. This suggests that the ovarioles generally contain 1 or 2 large ova although on occasion they can contain 4 or more.

The ovaries of *C. zealandica* show no unusual features when compared with other Scarabaeoidea. According to Robertson (1961) the ovaries of most Scarabaeoidea including the Melolonthinae are composed of 6 ovarioles, the exceptions being the Bolbocertini (Geotrupidae) with only 1 or 2 ovarioles, the Passalidae with 2, the Trogidae and Hoplini with 5, the Rutelinae with 5 or 6, *A. fimetarius* (Aphodiinae) with 7, and the Lucanidae with 12. In addition Heymons (1929, 1930) notes that the Scarabaeini are exceptional in having only a single ovary consisting of one ovariole.

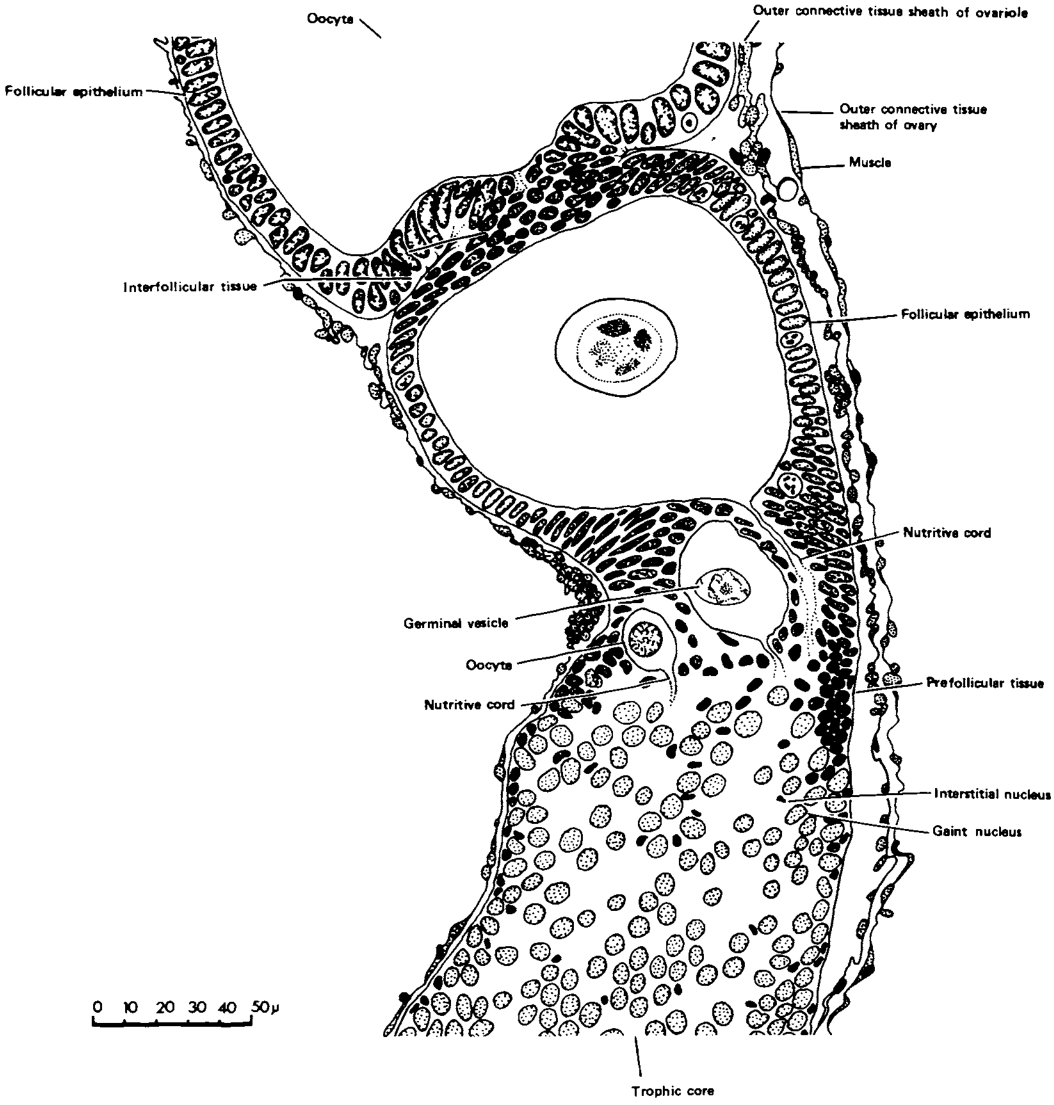
In Scarabaeidae, the ovaries show minor variations in the points of attachment of the terminal filaments and the presence or absence of an outer connective tissue sheath of the ovary. The terminal filaments in *P. anxia* as described by Berberet and Helms (1972) appear to resemble those described above in *C. zealandica*. They loop dorsally over the midgut, to join together and no mention of any other attachment is made. This contrasts with the terminal filaments of *A. majalis* which are attached medially to the venter of the 2nd abdominal tergum (Menees, 1963). No mention is also made by other authors of an outer connective tissue sheath enclosing each ovary as is frequently present in *C. zealandica*, although each ovary of *P. anxia* develops within an outer connective tissue sheath in the larvae but the sheath is later lost before the adult emerges (Berberet and Helms, 1972).

Histologically the ovarioles of *C. zealandica* are similar to those of other Coleoptera-Polyphaga as reported by Schlottman and Bonhag (1956) except that the nurse cells do not consist of elongated cysts of polynucleated masses. The nutritive cords of Scarabaeoidea, however, are either difficult to observe or else do not occur. Those in *C. zealandica* appear to be similar to those in *Passalus cornutus* Fabr. as reported by Krause(1946). In this passalid they are only found with smaller developing oocytes, and they cannot be followed very far into the germarium. Similar nutritive cords are also reported to occur in *P. horticola* and *A. aenea* by Rittershaus (1927). In contrast Dattar Gupta and Kumar (1963) make no mention of nutritive cords in *H. gigas*, and Berberet and Helms (1972) state that there are none in *P. anxia* although the trophic cells still remain in the germarium.

Figure 3.12

Longitudinal section through an ovariole where the posterior region of the germarium joins the anterior end of the vitellarium.

erratum: "Gaint nucleus" should read
"Trophic nucleus"


Oocyte
Outer connective tissue sheath of ovariole
Follicular epithelium
Outer connective tissue sheath of ovary
Muscle
Interfollicular tissue
Follicular epithelium
Nutritive cord
Germinal vesicle
Oocyte
Prefollicular tissue
Nutritive cord
Interstitial nucleus
Gaint nucleus
Trophic core
0 10 20 30 40 50μ

The lateral oviducts

The lateral oviducts in *C. zealandica* form two short flattened ducts which are translucent white and run from the ovaries medially and obliquely posteriorly to the median oviduct. Each forms a wide calyx where the ovarioles join, whereas it usually narrows before reaching the median oviduct (Figure 3.11). The entire organ can, however, be very distended by mature ova which are stored in it prior to oviposition so that it is very variable in size. Its length ranges from 0.5 to 1.1mm and its width from 0.3 to 0.6mm (Table 3.4).

The lateral oviducts are histologically identical to the pedicels of the ovarioles, and all have a uniform structure. They are lined by an epithelium of cuboidal to flattened cells which vary between 3 and 8µ in height (Figure 3.13 A). These cells rest on an outer basement membrane, have indistinct intercellular membranes, and smooth apical walls.

Their cytoplasm is granular and lightly basophilic, and their nuclei are oval with maximum diameters of up to about 10µ. The epithelium is also folded in a predominantly longitudinal direction with the walls of each fold being closely compressed so that the basement membrane comes into contact with itself in the centre of the fold. Within the lumen of the lateral oviducts and ovariole pedicels, there is a large quantity of secretion which appears in sections as a strongly eosinophilic homogenous or compact finely granular mass which encloses occasional large non-staining spaces (Figure 3.14 A). This secretion probably originates from the epithelial cells of the lateral oviducts and pedicels although they show no obvious signs of secretory activity such as vacuoles or brush borders.

The epithelium of the lateral oviducts is surrounded by two layers of muscle fibres. The outer circular layer is continuous with the outer sheath of the ovary, and when this occasionally ruptures it becomes retracted along the lateral oviduct so that it no longer encloses the upper part. The inner muscle layer is often up to three or four muscle fibres in thickness and these are orientated for the most part longitudinally.

The lateral oviducts of *C. zealandica* are histologically similar to those of other Scarabaeidae. Rittershaus (1927) reports that

Figure 3.13

Histology of the female internal reproductive organs of *C. zealandica*

A. longitudinal section of the calyx of a lateral oviduct

B. transverse section of posterior region of the median oviduct near its anterior end

C. transverse section through the anterior of the bursa copulatrix

D. transverse section of the duct of the bursa copulatrix

E. transverse section of a lateral accessory gland

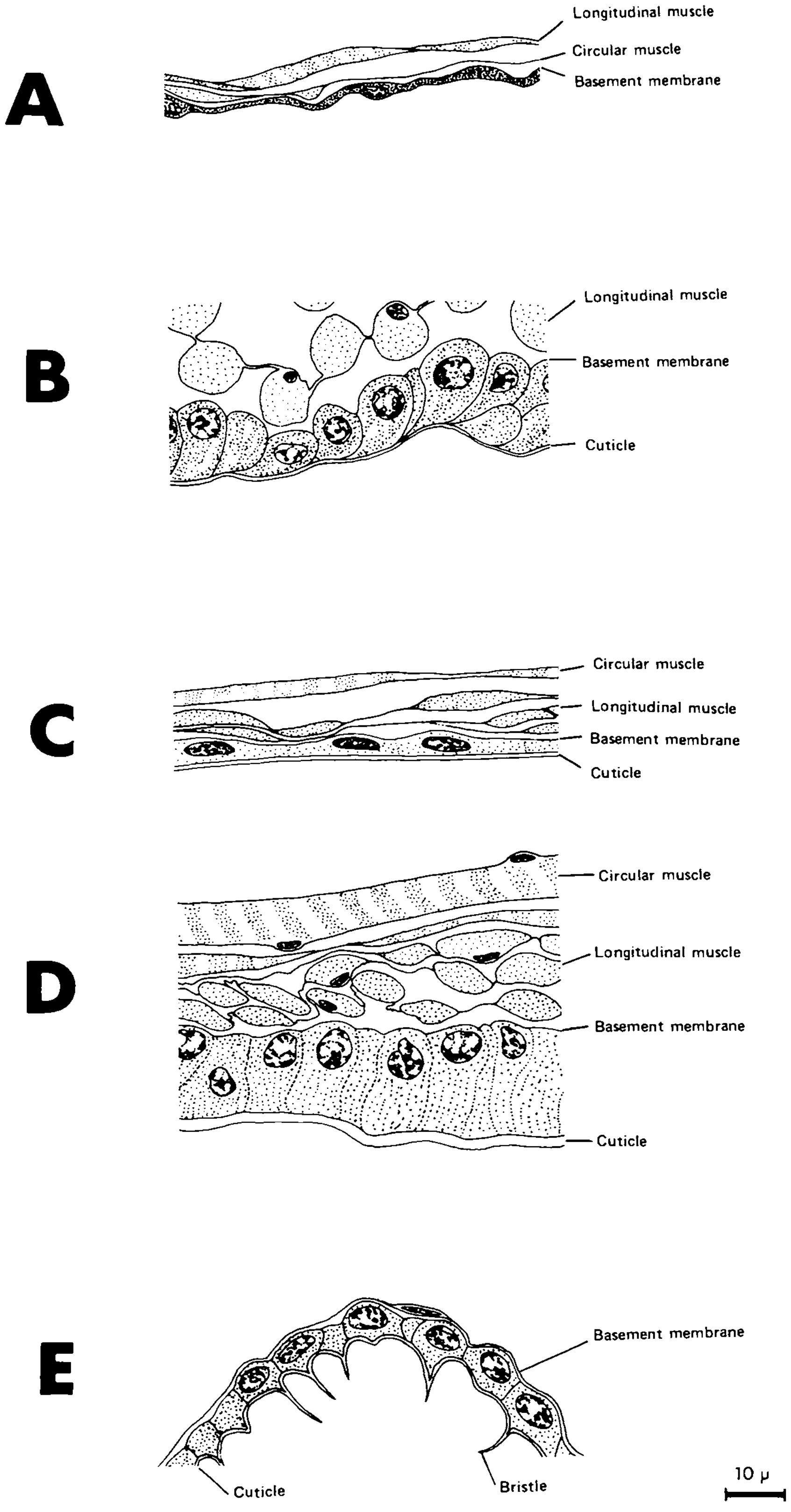

A
Longitudinal muscle
Circular muscle
Basement membrane
B
Longitudinal muscle
Basement membrane
Cuticle
C
Circular muscle
Longitudinal muscle
Basement membrane
Cuticle
D
Circular muscle
Longitudinal muscle
Basement membrane
Cuticle
E
Basement membrane
Cuticle
Bristle
10 μ

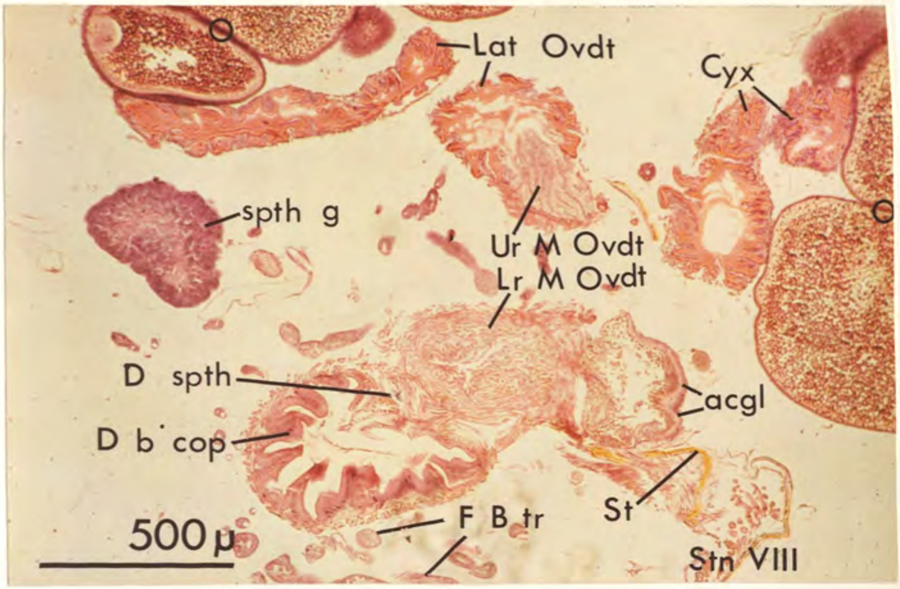
Figure 3.14

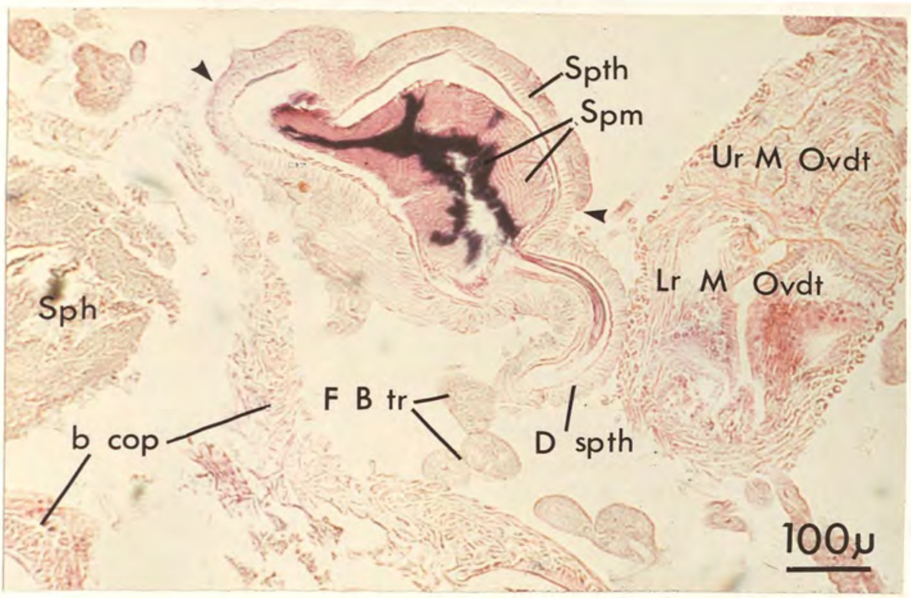
Histological micrograph of female
C. zealandica reproductive organs
(All haematoxylin and eosin)

A. A section through the lower part of the reproductive organs at low magnification. Part of the two ovaries, the calyxes and lateral oviducts, and upper region of the median oviduct are visible in longitudinal to oblique section, while the lower region of the median oviduct, a median and a lateral accessory gland, the duct of the bursa copulatrix, and the spermathecal duct appear in transverse section. In addition, this section also passes through the edge of the spermathecal gland but not through its lumen. The spermathecal gland is shown at higher magnification in Figure 3.18, whilst the accessory glands, median oviduct, and the ducts of the burse copulatrix and spermatheca are also shown in Figure 3.15.

B. Longitudinal section through the spermatheca and part of its duct showing the compressor muscle and the spermatozoa arranged with their heads pointing towards the centre of the lumen and their tails towards the walls of the organ. The layer of cells which covers the convex surface of the spermatheca is also visible between the black arrows. Also apparent in this field are oblique sections through the junction between upper and lower regions of the median oviduct, part of the wall of the bursa copulatrix, and a part of a spermatophore.

acgl	accessory gland
b cop	bursa copulatrix
Cyx	calyx
D b cop	duct of bursa copulatrix
D spth	duct of spermatheca
F B tr	fat body surrounding tracheae
Lat Ovdt	lateral oviduct
Lr M Ovdt	lower region of median oviduct
m	muscle
O	ova
sph	spermatophore
Spm	spermatozoa
spth	spermatheca
spth g	spermathecal gland
St	stylus
Stn V111	8th sternum
Ur M Ovdt	upper region of median oviduct


O
Lat Ovdt
Cyx
spth g
O
Ur M Ovdt
Lr M Ovdt
D spth
acgl
D b cop
St
F B tr
500 μ
Stn VIII


Spth
Spm
Ur M Ovdt
Lr M Ovdt
Sph
F B tr
b cop
D spth
100μ

the lateral oviduct or "Eileiter" of *P. horticola* and *A. aenea* is composed of a folded epithelium of flattened cells which are elongated longitudinally, have smooth apical surfaces, and characteristically the amount of cytoplasm in them is smaller than the volume of their nuclei. In this latter report, however, the lateral oviducts of these rutelinids differ from those of *C. zealandica*, which have cells containing two to three times as much cytoplasm as nucleus. In *P. anxia*, Berberet and Helms (1972) noted that the epithelium is highly folded and composed of cuboidal cells. It also has a very similar appearance in section to that of *C. zealandica* (Figure 44; Berberet and Helms, 1972).

The muscle layers which surround the lateral oviducts in different Scarabaeidae appear to be quite variable. Rittershaus (1927) describes well developed inner circular and outer longitudinal muscle layers in *P. horticola*, and *A. aenea*; Dattar Gupta and Kumar (1963) report an inner longitudinal, middle circular, and outer longitudinal layers in *H. gigas*; and Berberet and Helms (1972) note only a layer of circular muscles in *P. anxia*.

The median oviduct

The median oviduct runs ventrally from the union of the lateral oviducts in the 7th segment to the vagina in the 8th segment. It tends to be more rounded in cross-section than the lateral oviducts and its internal walls are folded longitudinally. In addition, it is a deeper white than the lateral oviducts for most of its length except for the middle region which is a light brown. The length of the median oviduct averages 1.85mm and its width varies between 0.33 to 0.40mm (Table 3.4).

Histologically, the median oviduct consists of two distinct regions, the first of which occupies the anterior third or so of the organ. This is lined with an epithelium which is compactly folded longitudinally so that it occludes the lumen (Figures 3.14 A, B). No cuticular lining is present here, and the epithelial cells are cuboidal, have heights of 20 to 30µ, rest on a thin basement membrane, have indistinct intercellular walls, and have smooth apical surfaces (Figure 3.13 B). They contain a slightly basophilic cytoplasm which is granular and concentrated in the basal half to third of the cells whilst their apical regions are packed with large irregular spaces containing a non-staining clear material. The nuclei are rounded to oval with maximum diameters of 7 to 8µ and they are also basal. This epithelium is surrounded by two layers of muscles anteriorly, which are continuous with those around the lateral oviducts and consist of an inner longitudinal layer and an outer circular layer. Posteriorly, however, these muscle layers merge into a complex thick reticulum of muscle fibres which run predominantly circularly and act as a sphincter to regulate the passage of mature ova and secretion down the median oviduct.

The posterior two thirds of the median oviduct is lined with an epithelium which is loosely folded longitudinally to give a complex crumpled cross-sectional pattern, and the lumen of the duct is not occluded (Figures 3.14 A, 3.15 A). The epithelial cells secrete a cuticular intima approximately 1µ thick, rest on a basement membrane which is often difficult to distinguish because of the folding, and have indistinct intercellular membranes. Their cytoplasm is eosinophilic and occupies approximately the same volume in each cell as the nucleus, which is flattened and has a maximum diameter of up to 10µ. Two well developed layers of muscle surround the epithelium; the inner is longitudinal and reaches 60µ in thickness in places whilst the outer is circular and has a maximum thickness of about 50µ. Scattered longitudinal muscle fibres also run between the circular muscles and give the appearance of a widely spaced longitudinal layer outside the circular layer, while other muscle fibres branch off to the bursa copulatrix and spermathecal duct.

The histology of the median oviduct of Scarabaeidae appears to be variable although the only other detailed descriptions are those for *P. horticola* and *A. aenea* by Rittershaus (1927). She reports that their median oviduct or "Eiergang" consists of two regions both of which have a longitudinally folded epithelial lining and a chitinous intima. The relatively large cells in the anterior region also have large rounded nuclei but they secrete a thin intima. This is produced into numerous fine hairs which project posteriorly in *A. aenea*, whereas these hairs are scarce in *P. horticola*. In contrast to this the intima of the posterior region is smooth and becomes thicker posteriorly while the cells which secrete it are poor in cytoplasm and have small flattened nuclei. The entire median oviduct is surrounded by a thick layer of

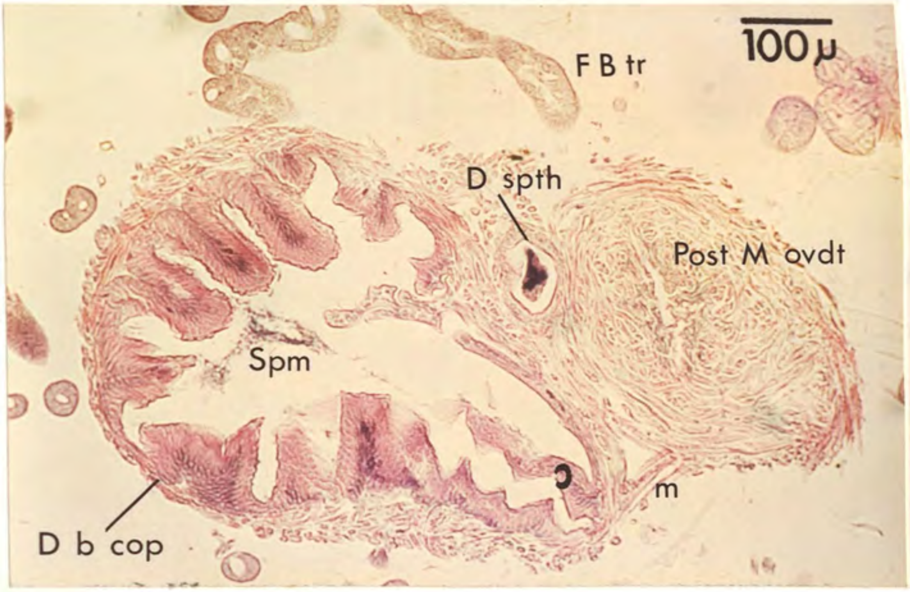
Figure 3.15

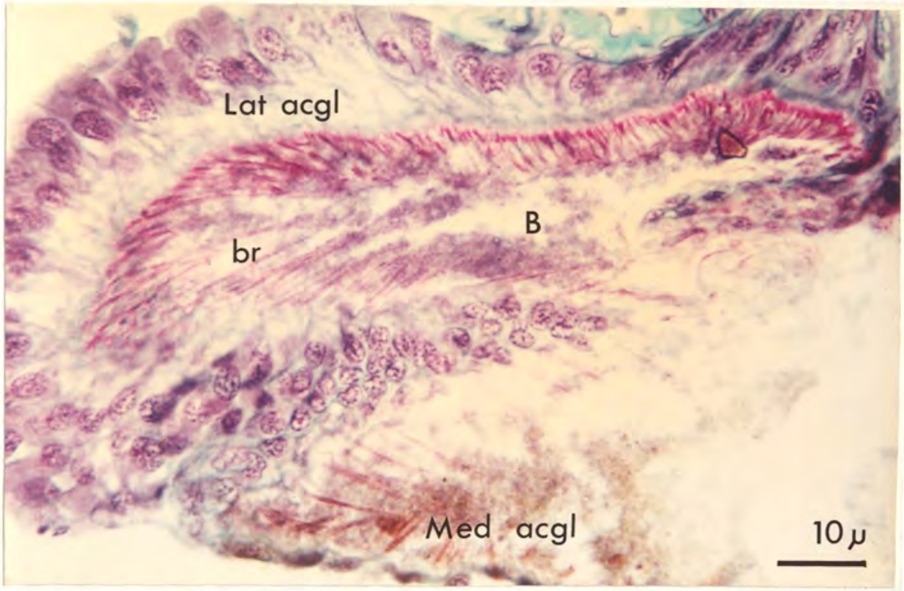
Histological photomicrographs of the female *C. zealandica* internal reproductive organs.

A. Transverse section through the posterior region of the median oviduct and the duct of the bursa copulatrix anterior to the junction of the spermathecal duct. Note the muscle fibres running between the median oviduct and duct of the bursa copulatrix, and the fat body surrounding tracheae.
(Haematoxylin and eosin)

B. Transverse section through a lateral accessory gland and medial accessory gland, showing the deeply staining intima of the lateral gland, and the bristles and bacteria in both glands.
(Mallorys triple connective tissue stain and fast green)

B	bacteria
br	bristles
D b cop	duct of bursa copulatrix
D spth	duct of spermatheca
F B tr	fat body surrounding trachea
Lat acgl	lateral accessory gland
m	muscle
M acgl	medial accessory gland
Post M ovdt	posterior region of median oviduct


100µ
F B tr
D spth
Post M ovdt
Spm
m
D b cop


Lat acgl
B
br
Med acgl
10µ

circular and oblique muscle fibres sandwiched between inner and outer layers of individual longitudinal muscle fibres which also increase in number posteriorly. The anterior regions of the median oviducts of these rutelinids therefore differ considerably from the anterior region in *C. zealandica*, but the posterior regions are remarkably similar.

The median oviduct of *P. anxia* also appears to differ from that of *C. zealandica*. According to Berberet and Helms (1972) it is "composed of a highly folded layer of cuboidal epithelial cells which is invested by a layer of circular muscles". It is also lined with a chitinous intima which increases in thickness posteriorly. Presumably the entire duct is therefore lined with cuticle in contrast to that of *C. zealandica*.

The only other information concerning the median oviduct of Scarabaeidae is a description of the muscles which surround it in *H. gigas* by Dattar Gupta and Kumar (1963). These authors report that they consist of inner longitudinal, middle circular, and outer longitudinal layers which therefore show some similarity to those around the median oviduct of the rutelinids described by Rittershaus (1927). These muscle layers in *H. gigas* and the rutelinids are therefore similar in some respects to those in *C. zealandica* except that the outer longitudinal fibres are very widely spaced in *C. zealandica* and appear to be part of the circular muscle layer.

The vagina

The vagina forms a terminal sac-like dilation of the reproductive tract which lies close to the 7th and 8th sterna and is somewhat flattened anterio-posteriorly (Figures 3.11, 3.20). It opens posteriorly between the styli into the genital chamber and its lateral edges are strengthened by hemisternites as described in the following section dealing with the external genitalia of the female. In addition, the vagina is joined anteriorly by the bursa copulatrix, anterio-ventrally by the median oviduct, and latero-ventrally by the two pairs of accessory glands.

Except for the hemisternites and styli, the vagina is lined with unsclerotised cuticle which varies from 2 to 20µ in thickness, being thinnest laterally and thickest dorsally. This cuticle is also distinctly layered. The epithelium which secretes it is identical to that which forms the median oviduct, consisting of flattened cells, resting on a basement membrane, with no visible intercellular membranes, relatively little eosinophilic cytoplasm, and oval to flattened nuclei with maximum diameters of 6 to 8μ. The various muscles which overlie the epithelium are described in the section on the muscles of the external genitalia of the female.

In other Scarabaeidae the histology of the vagina is generally similar to that in *C. zealandica*. Berberet and Helms (1972) term the vagina the "genital chamber" in *P. anxia* and describe it as being "a large cavity with walls that are composed of a thick muscularis primarily of circular muscles which invest the epithelium and very thick intima". Rittershaus (1927) also reports that in *P. horticola* and *A. aenea* it has a very thick smooth intima and strongly developed muscles. The vaginas of these rutelinids, however, possess remarkable unicellular glands with canals that penetrate the intima. These are particularly numerous between the "Vaginalpalpen", but there is no evidence at all of similar glands in *C. zealandica*.

The bursa copulatrix and spermatophore

The bursa copulatrix is an elongate sac which narrows posteriorly into a duct with internal walls folded in a longitudinal direction (Figure 3.11). This duct leads to the vagina immediately dorsal to its junction with the median oviduct. The colour and size of the bursa copulatrix depends on whether it contains a spermatophore or not. When empty it is translucent white, somewhat flattened, and as small as 1.6mm in total length and 0.37mm in maximum width, but when it contains a freshly deposited spermatophore it is dense white and its distal region becomes distended, reaching a maximum total length of 3.2mm and a diameter of 0.60mm (Table 3.4). The spermatophore, however, gradually changes colour and shrinks: after one to two days it starts shrivelling and turning slightly yellow or brown, and eventually it becomes a small hard lenticular or thimble-shaped mass which is a deep yellow-brown and occupies the extreme distal end of the bursa copulatrix. Additional spermatophores are also frequently deposited proximally to the older ones so that the bursa copulatrix may have a variety of colours between white and deep yellow-brown.

The distal region of the bursa copulatrix is lined with an epithelium which is thrown into complex folds when there is only a small amount of spermatophore material within the organ. The epithelial cells are flattened with heights of between 1 and 7μ, they rest on a thin basement membrane, have indistinct intercellular membranes, and secrete a cuticular lining of 0.5 to 1μ thick (Figures 3.13 C, 3.14 B). Their nuclei are oval to flattened and reach a maximum diameter of about 10μ and they have relatively little eosinophilic cytoplasm. Two layers of muscle surround the epithelium and these are arranged in inner longitudinal and outer circular layers, but they become progressively oblique anteriorly although the muscle fibres in the two layers always cross at an angle.

The proximal region of the bursa copulatrix, which forms the duct to the vagina, is lined with an epithelium which is thrown into about 10 large longitudinal folds (Figures 3.13 A, 3.15 A). Dorsally and laterally this epithelium is composed of columnar cells which vary in height from 15 to over 22μ (Figure 3.13 D). They rest on a thin outer basement membrane and secrete a cuticular intima which is approximately 2μ thick along most of the duct but becomes thinner anteriorly and thicker posteriorly. The cytoplasm of these cells is eosinophilic and stains mainly in long strands which run vertically to the intima and are easily confused with the intercellular membranes. Also, the nuclei are rounded or slightly oval, have diameters of 5 to 8μ and lie close to the basal walls of the cells. In contrast the epithelium along the ventral side of the proximal region of the bursa copulatrix is histologically identical to that lining the distal region, being composed of flattened cells, 1 to 5μ thick with small amounts of eosinophilic cytoplasm and oval to flattened nuclei.

The entire proximal region of the bursa copulatrix is, however, surrounded by two muscle layers which become thinner ventrally. They consist of an inner layer of longitudinal muscle which reaches up to 50μ in thickness and an outer circular layer up to 30μ thick. Oblique muscle fibres are also scattered irregularly through both these layers, and ventrally some muscle fibres also branch from both layers to join the median oviduct or spermathecal duct.

As already noted, the size and consistency of the spermatophore depends on the length of time it has been in the bursa copulatrix. When newly formed it is a soft white mass which adopts the shape of the available space within the bursa copulatrix, but it gradually shrinks, hardens, and changes colour to yellow or brown. In histological section a freshly made spermatophore consists of a non-staining ground substance within which are suspended small irregular eosinophilic granules and spermatozoa. Both are more or less scattered evenly throughout the spermatophore except near the boundaries of large swirls of ground substance and here they become more concentrated. Most of the spermatozoa are also separate but occasionally bundles of them are visible near the edges of swirls. Spermatozoa which have turned yellow or brown have no eosinophilic material within them. In sections they consist of numerous large globules of clear material bounded by a material which stains grey in haematoxylin and eosin, and which has clusters of small deeply staining basophilic bodies suspended within it.

In Scarabaeoidea a sac-like bursa copulatrix similar to that in *C. zealandica* is commonly present, although Williams (1945) notes that one is not found in *P. carolinus*, *E. lazarus* and possibly *B. farctum*. Rittershaus (1927) also reports that two additional small sac-like structures, or "Ausstulpungen", arise from the base of the bursa copulatrix of *A. aenea*.

Histologically, the bursa copulatrix of *C. zealandica* closely resembles those of *P. horticola* and *A. aenea* as reported by Rittershaus (1927). However, she divides them each into three regions but the proximal one nearest the vagina has the same histological structure as the vagina and is therefore an extension of it. This proximal region is followed by a thick duct or neck piece which leads into a distal sac. Both have distensible, highly folded walls consisting of secretory epithelial cells with lightly staining cytoplasm and large chromatin poor nuclei. The intima is thin in the distal sac but becomes thicker in the duct where it also has widely spaced bristles pointing towards the vagina. The duct is surrounded by both longitudinal and circular muscle fibres, the circular ones being especially numerous, and both decrease to a thin layer around the sac-like distal region. The paired evaginations or "Ausstulpungen" of *A. aenea* have walls which are folded proximally and become progressively smoother distally where the epithelium is secretory. These organs are lined with a thick intima and numerous bristles which become shorter and sparser nearer the bursa copulatrix, and it is surrounded by a thick layer of muscle.

Berberet and Helms (1972) note only that the bursa copulatrix of *P. anxia* is a dorsal invagination of the vagina which has a thinner "muscularis" and intima than the vagina. According to Heymons (1930), the bursa copulatrix of *S. sacer* and *S. semipunctatus* is reduced to a shallow bag with folded walls. Two of these folds which are lateral shield a ventral forked groove which leads to the mouth of the spermathecal duct ("Canalis copulatrix") and possibly also serve as anchoring points for the male's tegmen during copulation. These folds are possibly homologous with those in the duct of the bursa copulatrix of *C. zealandica* but in this insect they do not come into contact with the tegmen during copulation.

The spermatophores of Scarabaeidae are generally similar to that in *C. zealandica* with the exception that in *S. semipunctatus* according to Heymon (1930) the spermatophore forms a long brownish tubular structure which lies coiled up within other fluid secretions of the male organs in the bursa copulatrix of the female.

The histological appearance of the spermatophore of *C. zealandica* closely resembles that of *M. melolontha* as reported by Landa (1960). According to this author it originates by the simple mixing of the contents of the "genital duct" and three components from the accessory glands of the male. These eventually become differentiated into islands of hyaline and granular substance. At this stage the spermatozoa separate from their bundles and become active until they have metabolised all the hyaline substance about 24 hours later.

Fenemore (1971) is the only author who has published any information on the bursa copulatrix of *C. zealandica.* He reported similar colour changes in the spermatophore to those described in the present investigation and observed distension of the bursa copulatrix following copulation and slow shrinkage afterwards. However, he noted that in "most instances the bursa never returned to its former virgin size even up to 20 - 30 days after mating ... but in some individuals it did return to a condition indistinguishable from that of unmated females by that age". This suggests that some digestion and absorbtion of the spermatophore takes place very slowly. In this respect it is interesting to note that Landa (1960) reported that the spermatophore of *M. melolontha* is digested within 5 days of copulation. The epithelium of the bursa copulatrix of both these melolonthinids must therefore be secretory and absorptive, and there must also be pores through the intima to allow the passage of liquids through it. It is also likely that the spermatophores of these insects are composed of materials which have a high nutritive value since Anderson (1950b) reported that the accessory glands of the male *P. japonica* produce lipid, a mucus, and a mucus-like protein-polysaccharide, so that their digestion and absorption may provide an important source of nutrients for the female during the production of eggs. A similar function has also been attributed to the spermatozoa and seminal fluid received by female insects that are fertilised by haemocoelic insemination (Hinton, 1963).

The spermatheca and spermathecal gland

The spermatheca and its gland lie between and slightly to the left of the bursa copulatrix and median oviduct, with the gland anterior to the spermatheca. Muscle fibres from the bursa copulatrix and median oviduct and tracheae hold the posterior end of the spermathecal duct in position, but the other parts of these organs are held in place by tracheae only.

The spermatheca is a transparent elongated sac which is filled after copulation with an opaque white fluid containing spermatozoa (Figure 3.11). Two prominent muscles lie on either side of it and stretch from one end to the other bending it into a C-shaped curve. These probably form a pump for sucking up spermatozoa or expelling them from the spermatheca. One end of the spermatheca is also joined by a transparent duct which leads to the dorsal surface of the duct of the bursa copulatrix near the vagina. This spermathecal duct is filled with an opaque white fluid like that in the spermatheca. It is also twisted once or twice almost into a helix close to the spermatheca but it runs more or less straight for the remainder of its length. The total length of the spermatheca averages 0.64mm, whilst its maximum width, including the muscles, averages 0.43mm, and the spermathecal duct averages 0.72mm in length and 0.17mm in diameter (Table 3.4).

The spermathecal gland is an elongate completely transparent sac which averages 0.91mm in length and 0.49mm in maximum width (Table 3.4). One end drains into a thin transparent duct which joins the concave side of the spermatheca close to its junction with the spermathecal duct (Figure 3.11). The duct to the spermathecal gland averages 0.6mm in length and 0.12mm in width (Table 3.4) but it tends to get slightly thicker just before joining the spermatheca.

The spermatheca is formed by an epithelium of columnar cells which vary between 8 and 22μ in height (Figures 3.14 B, 3.16 C). The tallest cells occur on the concave surface of the organ where they are squashed together sideways and become elongated. All the cells rest on an outer basement membrane, have distinct intercellular walls, and secrete an intima which is 3 to 4μ thick. In addition, their cytoplasm is eosinophilic, and their nuclei which are basal, vary from rounded to oval, and have diameters of 6 to 8μ. On the convex surface and sides of the spermatheca this epithelium is surrounded by another outer layer of cells which closely resemble those of the fat body, whilst the remainder is either not covered or attached to the compressor muscles. The outer cells are for the most part arranged in a single layer but they can be two to three cells thick in some places. They vary from almost cuboidal to irregular, have large central nuclei about 15μ in diameter, and their cytoplasm is densely packed, granular, and slightly basophilic. Occasional small vacuoles approximately 2 to 3μ in diameter are also scattered through their cytoplasm. Some of these vacuoles are filled with an eosinophilic substance whilst others enclose a space where fat or oil has possibly been removed during the embedding procedure. In addition, some of the cells around the edges of this outer layer are enclosed by a thin sheet of connective tissue but this does not extend over the majority of them.

The lumen of the spermatheca, after copulation, becomes filled with spermatozoa. Initially these are disorganised but eventually they arrange themselves with their heads side by side, pointing inwards around a central clear space and with their tails towards the walls of the organ (Figure 3.14 B). Generally, the fluid in which the spermatozoa are suspended does not stain with haematoxylin and eosin, but there is sometimes a narrow layer of homogeneous material between the spermatozoa and the intima which stains a very light reddish blue. This possibly originates in the spermathecal gland since it stains the same as its secretion.

The spermathecal duct is formed by an epithelium the cells of which are identical to those that form the spermatheca, except that their

Figure 3.16

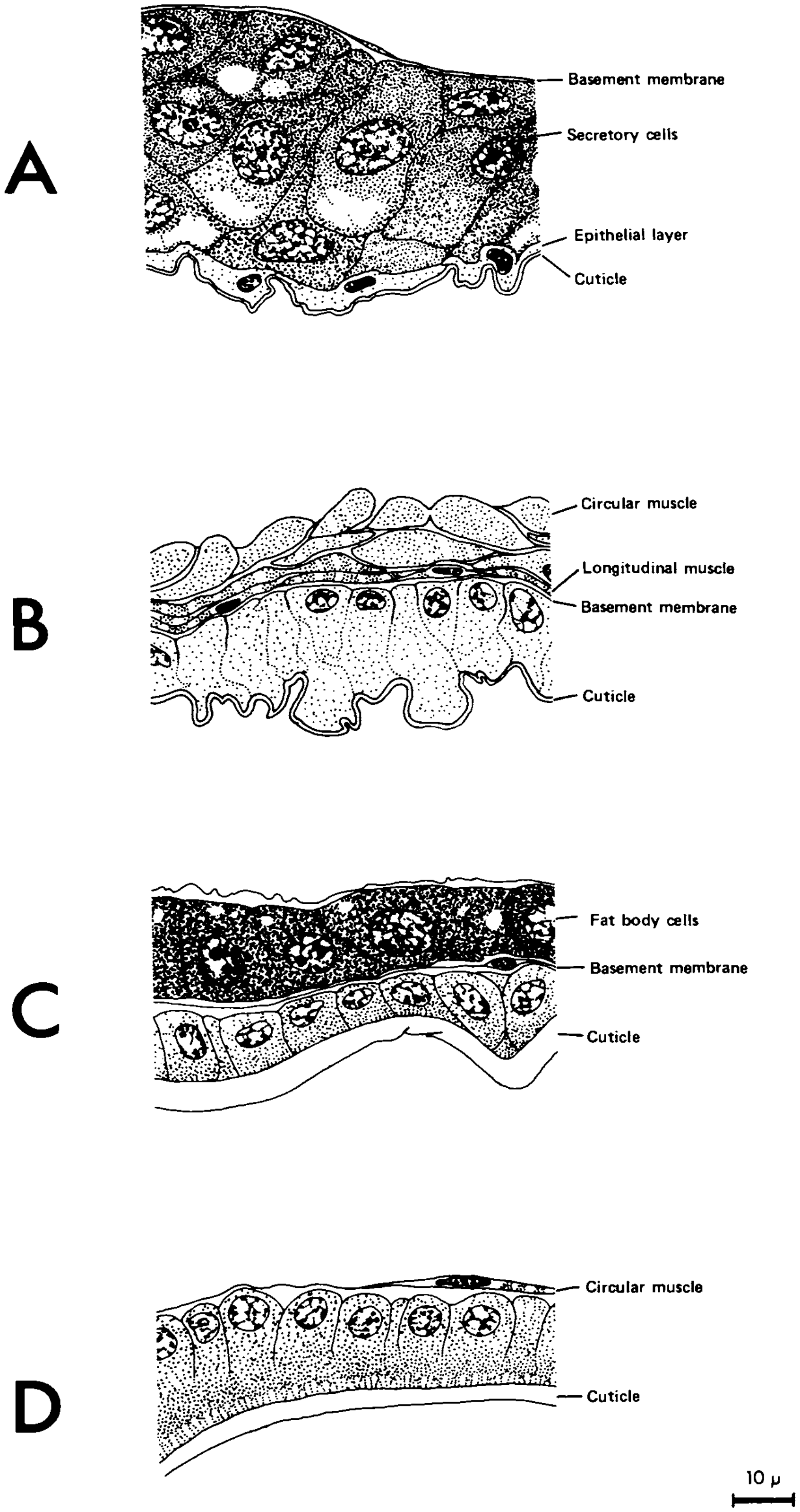
Histology of the spermatheca and spermathecal gland of the female *C. zealandica*

A. Transverse section of the spermathecal gland

B. Longitudinal section of the spermathecal gland duct

C. Longitudinal section of the convex surface of the spermatheca

D. Transverse section of the spermathecal duct about halfway along its length.


A
Basement membrane
Secretory cells
Epithelial layer
Cuticle
B
Circular muscle
Longitudinal muscle
Basement membrane
Cuticle
C
Fat body cells
Basement membrane
Cuticle
D
Circular muscle
Cuticle
10 µ

apical cytoplasm often appears as fine strands which run vertically to the cell surface (Figures 3.15 A, 3.16 D). These cells also have more rounded basal surfaces than those of the spermatheca and their heights are more uneven. The cuticular lining of the duct varies a lot in thickness: it is thickest where the duct is twisted close to the spermatheca and here it varies around the diameter of the duct from 2 to 8µ. Where it is thickest it also has a distinctly lamellar structure but no exocuticle is apparent. The intima becomes gradually thinner posteriorly and for the most part it varies around the duct from 1 to 4µ thick. Around the outside of the anterior part of the spermathecal duct irregular clusters of the same type of cell which surrounds the spermatheca also adhere to its basement membrane. However, these clusters become scarcer posteriorly until there are none around the last half of the duct. No muscle surrounds the anterior region of the duct, but a few widely spaced muscle fibres appear over the posterior half which run in all directions and increase in number towards the duct of the bursa copulatrix. Some of these muscles also run across to the median oviduct and to the bursa copulatrix.

The duct of the spermathecal gland is lined by an epithelium composed of the same type of cell as that of the spermatheca (Figure 3.16 B). However, the intima is thinner, being less than 0.5µ thick, and the epithelium is thrown into large longitudinal folds by an outer layer of circular muscle. This muscle is approximately 12µ thick over most of the length of the duct but it increases to about 20µ in thickness near the spermatheca where it forms a sphincter which compresses the epithelial folds and completely blocks the lumen. This sphincter controls the flow of liquid from the spermathecal gland.

The spermathecal gland has an entirely different histological structure to its own duct, the spermatheca, or the spermathecal duct. It is lined with a cuticular intima approximately 0.5µ thick and this is surrounded in turn by an inner epithelium and an additional outer layer of gland cells (Figure 3.16 A, 3.17). The inner cells are flattened and vary from approximately 5µ to less than 1µ in height. They rest on an outer basement membrane, their intercellular walls cannot be distinguished, and they have a relatively small quantity of slightly basophilic cytoplasm.

Figure 3.17

Histological micrograph of the spermathecal
gland of *C. zealandica*

A portion of the spermathecal gland is shown in cross section with the lumen towards the left lower region of the field. Note the small tubule (indicated by a black arrow) in one of the outer cells, and the small nuclei (n) between the outer cells.

Lum	lumen of gland
n	nucleus


10μ
n
n
Lum

Their nuclei, however, are rounded to flattened and occupy a large proportion of the space within the cells even though they are small and have maximum diameters of about 6µ.

The outer layer of cells around the spermathecal gland varies from approximately 30 to 70µ in thickness. It consists of columnar gland cells which probably form a single layer, but they are usually distorted sideways instead of being arranged vertically to the epithelium below them, so that they almost always give the appearance in sections of being two to three cells thick. The walls of these cells are very distinct and their nuclei are usually basal, rounded, and large, having diameters of 13 to 16µ. Their cytoplasm is for the most part basophilic and granular. This is densely packed basally, but it often encloses irregular clear spaces apically, and in some cells a small cylindrical eosinophilic region can be distinguished, usually close to the nucleus and apical to it (Figure 3.17). Each of these regions is about 5µ in diameter and appears to be bent into a U-shape with arms about 5µ long directed towards the apical surface of the cell. In addition, each of these regions has a central tube within it which is about 1µ in diameter, and the basophilic cytoplasm surrounding the eosinophilic region is often slightly denser than elsewhere for 2 to 4µ and has a radiating appearance. These outer gland cells are surrounded by a thin connective tissue sheath which probably also penetrates between each of the cells as an extremely thin layer to the basement membrane of the inner epithelium because small elongate nuclei, approximately 6µ in length by 1.5 to 3µ in width can occasionally be seen between the outer gland cells. However, an investigation using the electron microscope is necessary before this structural arrangement can be confirmed.

The spermatheca and spermathecal gland of *C. zealandica* resemble those in other Scarabaeidae except for differences in size and proportion. A C-shaped spermatheca is also found in many other Coleoptera according to Stein (1847), and Heymons (1930), and this shape is due to the compressor muscles. Stein (1847) notes that the compressor muscle is single in the aphodiinid *A. fimetarius*, and termed it the "Kompressor muskel", but Heymons (1930) pointed out that it is paired in *S. sacer* and *S. semipunctatus*. and termed them the "Musculus compressor". Rittershaus (1927) terms them the "Kompressionsmuskulatur" in *P. horticola* and *A. Aenea*, and Berberet and Helms (1972) report a spermathecal compressor muscle in *P. anxia*, but Straus-Dürckheim (1828) and Menees (1963) make no mention of them in *M. melolontha* and *A. majalis* respectively. Williams (1945) also does not note these muscles in any of the Scarabaeoidea investigated by him but he reports that they occur in the coccinellids *Hippodamia convergens* (Guer.) and *Coccinella novemnotata* (Hbst.), and in the cerambycid *Prionus laticollis* Drury. However, he does not mention them in the coccinellid *Epilachna varivestis* Mals. or in the cerambycid *Romaleum rufulum* (Hald.) so it is therefore possible that this muscle is sometimes not visible by morphological examination.

Other Coleoptera possessing a spermatheca with a compressor muscle and a spermathecal gland similar to those in the Scarabaeidae mentioned above include the elatrid *Ctenicera aeripennis destractor* (Brown); many of the Scolytidae (Chararas, 1956), including *Dendroctonus monticolae* Hopk. (Cerezke, 1964); and amongst the Curculionidae, *Curculio caryal* (Horn) (Bissell, 1937), three species of *Polydrosus* (Lenkowa, 1949), *Brachyrhinus sulcatus* (Fabr.) (Cram, 1958), *Anthonomus grandis* Boheman (Burke, 1959), and three species of *Graphognathus* (Stone, Herman and Brady, 1971).

The spermathecal compressor muscle in *C. zealandica* may be used both for forcing spermatozoa from the spermatheca into the vagina through the spermathecal duct and posterior end of the bursa copulatrix, and also for sucking spermatozoa into the spermatheca. These actions could be brought about in the following way: contraction of the muscle would increase the bend of the spermatheca thereby reducing its internal volume and hence increasing the pressure of its contents, whereas relaxation of the contracted muscle has the reverse effect. The spermatheca is probably returned to its original shape largely through the resilience of its relatively thick cuticular lining with some assistance from the tissues surrounding it, but this recovery action is likely to be weak compared to the compressive effect of the contracting muscle. The ejection of spermatozoa, as suggested by Snodgrass (1935) is therefore the most likely use of this muscle, although Heymons (1930) considers that its sucking action is the primary cause of spermatozoa entering the spermathecae of *S. sacer* and *S. semipunctatus*. Migration of spermatozoa into the spermatheca of insects has been reviewed by other authors including Heymons (1930), and Davey (1965), and both muscular and chemotactic effects may play some part. With respect to the Coleoptera, Stein (1847) suggested that the spermatozoa were pushed up into the spermatheca by peristaltic contractions of the vagina, whilst Harnish (1915) considered that in chysomelids chemical attraction of spermatozoa results in their migration to the spermatheca, and that this process lasts a number of hours. On the other hand, Heymons (1930) considered that the spermathecal gland secretion of the scarabaeinids examined by him may influence spermatozoa over a short distance only, whereas Rittershaus (1927) suggested that in *P. horticola* and *A. aenea* the spermathecae are filled with spermatozoa by a combination of muscular movements and active swimming by the spermatozoa in response to chemotactic attraction. Landa (1960) considered that in *M. melolontha* the spermatozoa leave the bursa copulatrix actively in response to the secretion from the spermathecal gland and they are not expelled by pressure in the bursa copulatrix. This author demonstrated this by observing that spermatozoa travelled up a glass capillary tube filled with secretion from the spermathecal gland when this was placed in an artificial spermatophore made from the contents of the reproductive system of a male. Possibly, therefore, in *C. zealandica* spermatozoa get into the spermatheca by a combination of their own chemotactic movements and suction from the spermathecal compressor muscle, assisted perhaps by some pressure in the bursa copulatrix created by its surrounding muscles. Movement of spermatozoa in response to chemotactic stimuli are suggested from the observations that in recently copulated females spermatozoa become concentrated in the bursa copulatrix near the opening of the spermathecal duct with their heads pointing towards its opening. In addition, the spermatozoa also eventually become organised inside the spermatheca with their heads pointing towards the centre of the lumen but leaving a clear space in the centre which extends to the posterior end of the organ.

Exactly the same organisation of spermatozoa has been reported in the spermathecae of *P. horticola* and *A. aenea* by Rittershaus (1927) but the reverse occurs in the scarabaeinids examined by Heymons (1930) where the spermatozoa become organised with their heads towards the lining of the spermatheca. In all the above insects the spermatozoa form a tangled mass inside the spermathecae soon after copulation and eventually reorganise themselves later. Muscular movements may therefore be important in all these Scarabaeidae because Heymons (1930) points out that the initial disorganisation of the spermatozoa suggests a turbulent mixing which could result from active suction. Chemical attraction, however, does not explain why the spermatozoa become organised within the spermatheca of *C. zealandica* and the rutelinids whereas it can in the scarabaeinids. In the former insects this could perhaps be explained by the secretion of the accessory gland being attractive to spermatozoa and acting only in the presence of some other factor which is essential for movement of spermatozoa. Two possibilities seem likely: firstly, this factor may be produced by the spermatheca in which case spermatozoa could enter the spermatheca entirely by chemotactic attraction, and secondly, this factor may be present only in the material of the spermatophore, in which case the spermatozoa could swim to the opening of the spermathecal duct in response to the attractant issuing from it and they would then have to be sucked into the spermatheca together with some of the spermatophore material. Once in the spermatheca the spermatozoa could only swim into the secretion from the spermathecal gland as far as the factor diffused into it. Both of these possibilities may also be acting simultaneously, and the factor may be some chemical which neutralises an inhibitor of sperm movement present in the spermathecal gland secretion.

Histologically, the spermatheca of *C. zealandica* is similar to that described by Rittershaus (1927) for *P. horticola* and *A. aenea*, and by Heymons (1930) for *S. sacer* and *S. semipunctatus*. In the two rutelinids it is lined with a wavy intima and the epithelial cells of the convex and concave surfaces differ. On the convex side they are cuboidal and have large rounded nuclei, but they differ from those in *C. zealandica* in having large apical vacuoles and a striated appearance or "stubchensaum" next to the intima. Those on the concave side are columnar and have elongated nuclei which lie with their long axes towards the lumen of the gland. In addition, the convex surface is surrounded by a layer of large plasma rich cells. According to Heymons (1930) the corresponding epithelial cells in the two scarabaeinids are cuboidal on the convex surface and columnar on the concave one, but their nuclei are centrally placed. They secrete a thick cuticular intima with spiral strengthening ridges, and some of the spermathecal gland cells adhere to its convex surface near its distal end. In *P. anxia*, Berberet and Helms (1972) only note that the spermatheca is composed of a cuboidal epithelium and that it is lined with a thick intima.

The spermathecal ducts of the rutelinids described by Rittershaus (1927), the scarabaeinids described by Heymons (1930), and *P. anxia* as reported by Berberet and Helms (1972) all resemble the anterior part of this duct in *C. zealandica*, having a thick intima and no surrounding muscles. However, in the scarabaeinids this duct differs from the others in that it is surrounded, except for its extreme anterior end, by a thick multiple layer of very large round or pear-shaped gland cells. Heymons (1930) reports that these cells have granular cytoplasm and large round or irregular nuclei. They are also each connected to the spermathecal duct by a fine canal or "chitinoses Sekretröhrchen" which has a funnel-shaped opening into the cytoplasm near the nucleus. Each of these canals either connects individually to the spermathecal duct or else it joins others before doing so. These secretory cells are therefore organised in a similar fashion to the secretory cells in the spermathecal glands of some other Scarabaeidae as described below.

The structure of the spermathecal gland of *C. zealandica* is also similar to those of the rutelinids and scarabaeinids described by Rittershaus (1927) and Heymons (1930) respectively, being composed of an inner epithelial layer which secretes a thin intima and an outer layer of secretory cells which is more than one cell thick in some places. These secretory cells are also larger than the epithelial cells and have larger nuclei. In *S. sacer* and *S. semipunctatus*, Heymons (1930) reports that the glandular cells are large and have granular cytoplasm. They make up an irregular layer forming several lobes surrounding a central duct and some of these cells also adhere to the end of the spermatheca as previously mentioned. However, Heymons (1930) does not note any fine structural details such as he does in the gland cells around the spermathecal ducts and it is therefore possible that these cells are more similar to the cells on the outside of the spermatheca of *C. zealandica*, but confirmation of this will have to await investigation with the electron microscope.

The histology of the spermathecal glands of *P. horticola* and *A. aenea*, as reported by Rittershaus (1927) is particularly close to that in *C. zealandica*. In these rutelinids the inner epithelial cells are thin and have little cytoplasm whereas the secretory cells have large chromatin rich nuclei which are central to basal in position, and their cytoplasm is basophilic except for a round eosinophilic mass in each cell. This mass is homogeneous and generally situated close to the nucleus. Rittershaus (1927) also notes that the secretion in the lumen of the gland is eosinophilic and concluded that the homogeneous masses within the secretory cells was material which had not yet been secreted. Furthermore his histological figure of the spermathecal gland of *A. aenea* (Figure 56; Rittershaus, 1927) shows occasional small circular structures in the centres of the eosinophilic masses, and some fine ducts passing from the lumen of the gland into some of the secretory cells. A similar histology has also been reported for the spermathecal glands in the curculionid genus *Polydrosus* by Leńkowa (1949). The inner epithelial cells of these insects are, however, columnar and the outer secretory cells are loosely packed although apically each is produced into a thin "process" which passes through the inner epithelium to the lumen of the gland. Leńkowa (1949) considers that the secretion of these cells reaches the lumen through these "processes". This histological structure in these Coleoptera is also similar to that which occurs in the spermathecal glands and accessory gland of some Lepidoptera, as reviewed in the section on the male internal reproductive organs of *P. operculella* in Chapter 7.

The structure of the above mentioned gland cells strongly suggests that they may have a similar organisation to that of the spermathecal bulb of *Periplaneta americana* (L) as reported from investigations using the electron miscroscope by Smith (1968), and Gupta and Smith (1969). The bulb consists of a cuticular lining surrounded by two distinct layers of cells. The inner epithelium is continuous with the hypodermis, and the cells comprising it are covered with a tough basement membrane outside which lie visceral muscle fibres. Outside this again is a layer of secretory cells, each of which flanks one of the inner epithelial cells. A prominent cavity is invaginated into each secretory cell from its apical surface. This cavity is lined with microvilli and the secretions of the cell are released cyclically into it. Also, it is provided with a central extracellular duct made up of a loose feltwork of fibrils and the secretion penetrates into this. The second half of the duct along which the secretion passes to the lumen of the gland is provided by the underlying epithelial cell, but here the tube is probably cuticular and it is composed of alternating concentric cylindrical layers with an epicuticular lining. Whether or not this is the same structure as is present in *C. zealandica* will, however, have to await examination with the electron microscope.

It is perhaps of interest that the histology of the spermathecal gland of *P.anxia* may be different from that of the Coleoptera described above. Berberet and Helms (1972) note only that it is "composed of a thick epithelium of somewhat disorganised cells" and their micrograph of it (Fig. 49; Berberet and Helms, 1972) does not clarify whether it is different or not.

Finally, it can be noted that the most likely function for the spermathecal gland secretion is to provide an exogenous nutrient source for the spermatozoa in the spermatheca(Davey, 1965) and it may possibly also produce an attractant for the spermatozoa as previously mentioned.

The histological structure of the spermathecal gland duct in the rutelinids described by Rittershaus (1927), and *P.anxia* described by Berberet and Helms (1972) is very similar to that of *C. zealandica* being composed of an epithelial layer invested by a multiple layer of circular muscle fibres and lined with intima. Berberet and Helms (1972) suggest that contractions of these muscles could force secretions through the duct from the spermathecal gland and this may also occur in *C. zealandica*. However, these muscles also form a sphincter in this latter insect which controls the flow from the gland. In contrast to these above insects, the duct of the spermathecal gland in the scarabaeinids described by Heymons (1930) differs in that it has no muscular layer surrounding it. It also possesses a unique small muscle which runs to the duct of the bursa copulatrix or "canalis copulatrix" and branches to the distal end of the spermatheca. Heymons (1930) suggests that this latter muscle acts as an adductor muscle fixing the spermatheca and its gland in place during violent muscular movements of the gut.

The accessory glands

The 4 accessory glands are round organs which have wide openings into the vagina (Figure 3.11). They are grouped together in pairs which lie on either side of the median oviduct along the anterio-ventral edge of the vagina. The lateral accessory glands are smaller than the median ones with mean diameters of 0.60mm and 0.29mm respectively (Table 3.4) and both vary in colour from white in newly emerged adults to yellow brown or light brown in older individuals. Frequently the medial glands are, however, darker than the lateral ones, but the colour is due mainly to their contents because the walls of the accessory glands are translucent white. These contents are also usually a creamy fluid although they can be almost solid in the medial glands when brown.

Each pair of accessory glands forms two sac-like evaginations of the vagina. They are composed of a columnar epithelium which is continuous between the two glands of each pair and generally varies between 18 and 25μ in thickness (Figure 3.15 B). The cells comprising it are tallest in the lateral sides of the lateral glands where they are compressed together sideways, and they become progressively lower towards the medial and anterior edges of the medial accessory glands where they eventually grade into vaginal epithelium. Elsewhere, around the edges of the accessory glands, the histological change to vagina is rapid.

The epithelial cells of the accessory glands have rounded basal surfaces which lie on a very thin outer basement membrane (Figures 3.13 E, 3.15 B). This is usually only clearly visible when it is stretched from the bulge of one cell to another without following the indentation between them. The intercellular membranes are clearly distinguishable basally but become indistinct apically, the cytoplasm is eosinophilic, and the nuclei are relatively large, having diameters of 8 to 10μ. They also occupy most of the basal space within the cells and consequently often become somewhat cuboidal. The nuclei remain large where the cells become lower in the medial glands and the cytoplasm reduces in volume instead. The epithelial cells secrete a cuticular lining less than 1μ thick and this is produced into tapering spines mostly 30 to 50μ long which are characteristic of these cells. Each cell apparently only has a single spine arising from its apical surface as a wide slightly curving cone. This narrows to less than 1μ in diameter approximately one third of the way down the spine, and after this it is relatively straight and tapers only slightly to its tip. In addition, the cuticle is eosinophilic between the bases of the spines, where they are densely arranged on the lateral walls of the lateral glands (Figure 3.15 B). Finally, clusters of fat body cells often adhere to the basement membrane of the accessory glands but they are not always associated with them as is the case with those on the convex surface of the spermatheca.

The fluid in the lumens of the accessory glands can be seen to contain numerous bacteria and their decomposing products under the light microscope (Figure 3.15 B) and in electron micrographs (Figure 3.18 A, B). These bacteria are packed particularly densely in the spaces between the spines, which form a dense feltwork presumably functioning to prevent all the bacteria from being lost from the widely opening glands.

The general appearance of the accessory glands of *C. zealandica* has been described previously by Hoyt and Osborne (1971) who termed them "colleterial glands". These authors also reported that the "interior wall of each gland is lined with many thin, bifurcate projections ..." but none of the bristles were found to fork in any of the 20 or so specimens examined during the present study.

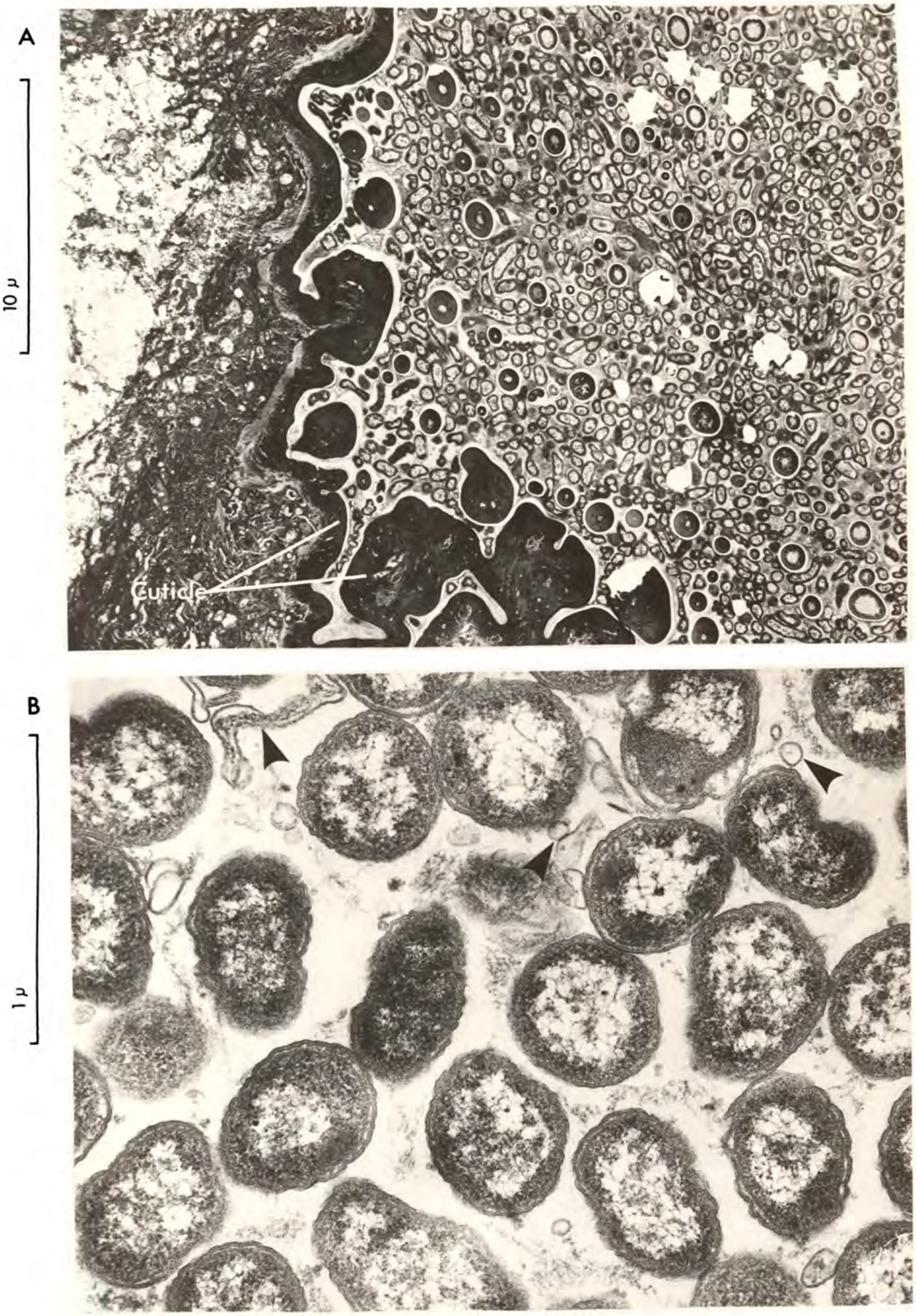
The accessory glands are bilobed or double organs in *P. horticola* and *A. aenea* according to Rittershaus (1927) and Williams (1945), and they therefore resemble those of *C. zealandica*. In most other Scarabaeoidea they either form a pair of simple sac-like organs, as in *M. melolontha* L. (Straus-Durckheim, 1828; Stein, 1847); *A. aenea* Geer (Rittershaus, 1927); *Phyllophaga* sp., *P. japonica* Newman, *L. gibbosus* (De G.), *C. nitida* L. (Williams, 1945); *P. anxia* (Le Conte) (Menees, 1963); and *A. majalis* (Razoumowski) (Berberet and Helms, 1972); or else they are lacking as in *S. sacer* L., *S. semipunctatus* Fabr. (Heymons, 1930); *B. farctam* (Fabr.), *E. lazarus* (Fabr.), and *P. carolinus* (L.) (Williams, 1945). Few histological descriptions of these organs are, however, available, and the most detailed is that of Rittershaus (1927) for *P. horticola* and *A. aenea*. The accessory glands of these rutelinids, however, show only slight similarities with those in *C. zealandica*. The accessory glands of *P. horticola* are the most complex: each is composed of an outer secretory sac-like part which drains into a large medial bubble-shaped storage organ near its opening into the vagina. The secretory part is composed of a single layer of gland cells with large round chromatin rich nuclei, and well developed plasma bodies.

Figure 3.18

Electron micrographs of the accessory glands of *C. zealandica*

A. A low magnification electron micrograph showing the epithelial cells on the left, the cuticular lining in the centre, and the lumen on the right, together with cuticular spines which are indicated by the white arrows towards the top right of the field. Numerous fine canals are visible which penetrate the cuticle; probably the secretion of the epithelial cells passes through these into the lumen of the gland.

B. A high magnification electron micrograph through the fluid in the lumen of the accessory glands clearly showing the bacteria. Some decomposing bacterial membranes, some of which are indicated by black arrows, are also visible.


A
10 μ
Cuticle
B
1 μ

Distally, their intercellular membranes frequently lose contact basally while the cuticular lining is lamellated and forms a honeycomb of wide tubes which project into the lumen, each tube arising from a single cell. The secretion inside these tubes is also composed of fine granules whereas in the lumen of the organ it forms large lumps. The storage part is composed of cells with basal nuclei and plasma bodies which are drawn out into long rods. Most of the intima is provided with bristles, but the distal end of the **organ projects** back into its centre as a conicle mound and this is very densely covered with bristles which fork at their tips. Granules of secretion are stored amongst the latter bristles and presumably the forking tips of the bristles prevent it from escaping. The entire accessory gland is covered by muscle, but this is thin over the secretory region and thick over the bristle mound. These muscles evert the mound before and during copulation, and when evertion occurs the bristles are spread so that the secretion is exposed. Rittershaus (1927) presumes a similar action is involved in taking up the secretion as well. She also considers that the secretion of these glands plays some role during sex recognition and suggests that the spreading of the bristles during eversion increases the surface area of the secretion allowing greater contact with the air for volatization. The functions and structure of the accessory glands of *A. aenea* are similar to those of *P. horticola* except that they are not bilobed and they have no conicle mound. Instead the secretory cells comprise the outer wall of the organ and each cell is provided with a single bristle, whilst the densely packed forked bristles arise from the wall next to the vagina. Simple bristles also arise from the secretory cells of the accessory glands of *M. melolontha*, *Amphimallon*, and *Cetonia* according to Stein (1947) and Rittershaus (1927). The accessory glands of these insects are also not differentiated into secretory and storage areas. They are therefore possibly similar to the accessory glands of *C. zealandica*, but further detail is not available. The accessory glands of *P. anxia* are, however, quite different from those of *C. zealandica*. Berberet and Helms (1972) report that each is composed of "thick epithelium that partially surrounds a central region filled with refractive, basophilic rods," and that two ducts extend from the central region and open into the genital tract near the vulva. They report that the eggs are apparently coated with accessory gland secretions as they pass through the vagina, but they did not determine the function of the "refractive rods".

The bacteria of the accessory glands

The majority of bacteria present in the accessory glands were gram negative coccobaccilli although a few gram negative rods were also scattered amongst them. When examined under the electron microscope (Figure 3.18 B) the walls of these bacteria appear to be composed of three electron dense layers, and this corresponds well with their gram-negativity: according go Glauert and Thornley (1969) gram-positive bacteria have walls composed of one or two electron dense layers, depending on the stain used, whereas gram-negative bacteria generally have walls consisting of three dense layers.

Since no identification of the bacteria in the accessory glands of *C. zealandica* was published by late 1973, a preliminary identification was made by Miss N.R.C. Davies, Principal Technologist, Waikato Hospital, Hamilton using 4 female beetles. The coccobaccilli in the accessory glands included an *Enterobacter* and an *Acinetobacter* species, whilst the rods were probably *Yersinia pseudotuberculosis* (Pfeiffer) or a similar species (Appendix 2.12). In addition, the bacteria in the gut were also investigated because the anus opens close to the accessory glands and it is possible that some of these bacteria are contaminants. Species of *Staphylococcus*, *Micrococcus*, and *Acinetobacter* were found together with the same bacterium which is found in the accessory gland. This is probably *Y. pseudotuberculosis*. It is interesting to note that Bauchop and Clarke (1975) recently investigated the hind-gut microbiology of the third instar larva of *C. zealandica*. They obtained two small flagellate protozoa and 12 different bacterial isolates of which 10 were identified to genera. These were gram-positive and gram-negative rods, none of which were found in the adult gut in the present study. This seems to indicate entirely different floras in larval and adult guts. The guts of larvae and adults are certainly distinct morphologically; the hind-gut being especially different. The *Staphylococcus* and *Micrococcus* in the gut of *C. zealandica* adults may have been normally present there, have entered it fortuitously with the food, or have been present in the body cavity. Buchanan and Gibbons (1974) list three species of *Staphylococcus*:

S. saprophyticus (Fairbrother) is common in air, soil, and dust, as well as in other situations. The other two species normally inhabit the skin and some mucous membranes of warm blooded animals, although *S. aureus* Rosenbach has been isolated from the gut of *M. melolontha* L. (Steinhaus, 1963). The larvae of *C. zealandica* will ingest soil (Allison, 1969) but whether the adults do is not known. According to Buchanan and Gibbons (1974) *Acinetobacter calcoaceticus* (Beijerinck) is the only species described for this genus. It is present in soil, water, and both diseased and healthy animals so it seems probable, therefore, that the same species of *Acinetobacter* is present in both the gut and accessory glands of *C. zealandica*. *Y. pseudotuberculosis* produces infections in man, birds, sheep, and other animals, including sporadic infections in cows, and it has also been isolated from specimens of soil and dust (Dubos, 1958; Buchanan and Gibbons, 1974). If this is the bacterium in *C. zealandica* then it could have been acquired by contamination from cow dung since the beetles were collected from a dairy farm. The larvae will eat cow dung in the laboratory (Wightman, 1972a), but it is not known whether the adults eat cow dung, or whether the bacteria can survive in the gut from larva to adult. Two species of *Enterobacter* are known: *E. cloacae* (Jordan) and *E. aerogenes* Hormaeche and Edwards (Buchanan and Gibbons, 1974); both occur in soil, water, and in the faeces of man and other animals. It is therefore possible that any of these bacteria could have originated as contaminants from the soil. Nevertheless, the observation that *Enterobacter* was only found in the accessory glands suggests that it is the organism which produces the phenol although there is also the possibility that the phenol producing bacteria exist both in the gut and the accessory glands. This would be a possible explanation to account for the observation of Henzell *et al.* (1970) that the attractive region is located in the dorsal half of the first three abdominal segments of *C. zealandica*. Further research perhaps also involving attractancy tests with bacterial cultures is necessary to determine which bacteria produce the phenol, and whether these bacteria are limited to the accessory glands.

The production of phenol by bacteria has been reported before by various authors listed by Gunsalus and Stanier (1961). The bacteria involved are *Escherichia coli* (Migula), *Clostridium pseudotetanique* (Prevot), and *Clostridium tetani* (Flugge), together with "*Bacillus* *phenolologenes*" which has an uncertain synonymy. All are found in soil or faeces and this constitutes the only very tenuous link with the bacteria found in *C. zealandica*. According to Gunsalus and Stanier (1961) these bacteria utilise tyrosine as the starting point in the production of phenol, and Hoyt and Osborne (1971) have suggested that a similar pathway may be followed by the phenol producing bacteria in *C. zealandica*. It is interesting to note that hardening and darkening of the insect cuticle results from tanning by phenolic substances derived from tyrosine (Hackman, 1974; Wigglesworth, 1974). These substances accumulate in the cuticle and most which have been isolated are 3,4-dihydric phenols. Although the haemocytes may be involved in tyrosine metabolosm (Arnold, 1974) it is evident that the epidermal cells certainly are. Also the accessory glands of *C. zealandica* are epidermal derivatives since they are lined with cuticle and therefore it seems even more likely that they may secrete tyrosine or some derivative of it which the bacteria could convert to phenol.

The possibility that the accessory glands themselves can produce phenol has not yet been ruled out. No reference to the production of phenol by insects is, however, known to the writer, but the Strongylosomid millipede *Oxidus gracilis* (Koch) does (Blum, MacConnell, Brand, Duffield, and Fales, 1973). The defensive exadate of this millipede contains 1 to 5% phenol. However, the secretion of various other phenolic substances from ectodermal glands of insects is well known and this has been reviewed by Gilmour (1961), and Weatherston and Percy (1970b).

An interesting aspect concerning phenol in *C. zealandica* is its concentration. Henzell and Lowe (1970) stated that each female "contains between 0.5 and 1.0μg of phenol, which corresponds to approximately 10 to 20 parts per million by weight". Hoyt and Osborne (1971), however, found that symbiotic bacteria in the accessory glands of the female produce the phenol. If the volume of the accessory glands is determined from the measurements of their maximum diameter assuming that the glands are spherical and have a density of 1.0, then the concentration of phenol within these glands can also be determined if it is assumed that all the phenol is present within them, and that it is distributed evenly throughout the gland and its epithelial walls. The results of these calculations give an average concentration of 3.0g/1 with possible limits of 0.9 to 7.2g/l. It should also be noted that the assumptions made would in fact tend to make the concentration lower so that these values can be regarded as minimum ones. Phenol, however, is a well known disinfectant and it is used as the comparative standard for determining the Rideal-Walker coefficient of disinfectants: a 1% solution of phenol being sufficient to kill all the *Salmonella typhi* (Schroeter) under standardised conditions in between 5 and 7.5 minutes (Anon., 1934, with amendments 1943, 1951, 1953, 1963). Obviously the concentration of phenol within these accessory glands is a powerful disinfectant and it therefore follows that the bacteria are unusually tolerant to this chemical, or that phenol is present elsewhere in the body of the beetle.

The digestive tract offers one possibility of an alternative site for phenol. *C. zealandica* is phytophagous (Appendix 1.1) and some plants are known to contain phenol. This source seems unlikely though since phenol has been reported in only 5 plants of scattered systematic distribution (Harborne, 1964) and *C. zealandica* is unlikely to come into contact with any of them. A more likely source is that the phenol is produced in the gut as a result of digestion or maceration of the plants. It could conceivably be produced by decarboxylation of *p*-hydroxybenzoic acid which occurs commonly in plants (Harborne, 1964): the decarboxylation being accomplished by a fairly non specific enzyme such as peroxidase. However, it seems unlikely that this would produce more than a trace of phenol if in fact such a reaction occurred. A third possible source is the bacteria in the gut as described above.

Summary

The internal reproductive organs of the female *C. zealandica* are morphologically simple and show similarities with those of other Scarabaeidae. Histologically, most of the organs also have a counterpart in another scarabaeid, but particular close similarities occur with the lateral oviducts of *P. anxia*, the spermatheca and spermathecal gland of *A. aenea* and *P. horticola*, and the bursa copulatrix of *P. horticola*. *C. zealandica* is, however, unusual in that its ovaries are clearly telotrophic and each is usually enclosed within a connective tissue sheath, that its median oviduct has an anterior region which is histologically the same as the lateral oviducts, and that the accessory glands are histologically uniform instead of being differentiated into regions, but these are only minor differences.

The external genitalia of the female

The nomenclature of the female external genitalia used in the following description is that of Lindroth and Palmén (1970).

The external genitalia of the female, like those of the male, lie withdrawn and out of sight in a genital chamber. This is formed as a shallow invagination in the articular membrane between the 8th abdominal sternum and tergum at the posterior end of the body. The opening to this genital chamber can usually only be seen if the 8th sternum and tergum are parted and then it appears as an oval hole occupying approximately the medial third of the membrane. Ventrally the genital chamber extends anteriorly above the 8th sternum to form a pocket but elsewhere it is shallow.

The lateral edges of the genital chamber are supported by a pair of long thin S-shaped sclerites, the hemisternites (Figure 3.19 A, B). These are considered to represent the divided 9th sternum (Lindroth and Palmén, 1970). A flattened stylus articulates with the medial edge of each hemisternite mid-way along it and runs medially along the anterior wall of the genital chamber (Figure 3.19 A, B). However, the styli from either side do not meet in the mid-line and there is a gap, ventral to which the vulva opens from the vagina. The styli, which have a mean length of 0.54mm (Table 3.1) project as flattened plates posteriorly into the genital chamber from their attachments to the hemisternites and the genital chamber wall. A fringe of bristles also arises from their ventral surfaces near their posterior edges and project towards the opening of the genital chamber.

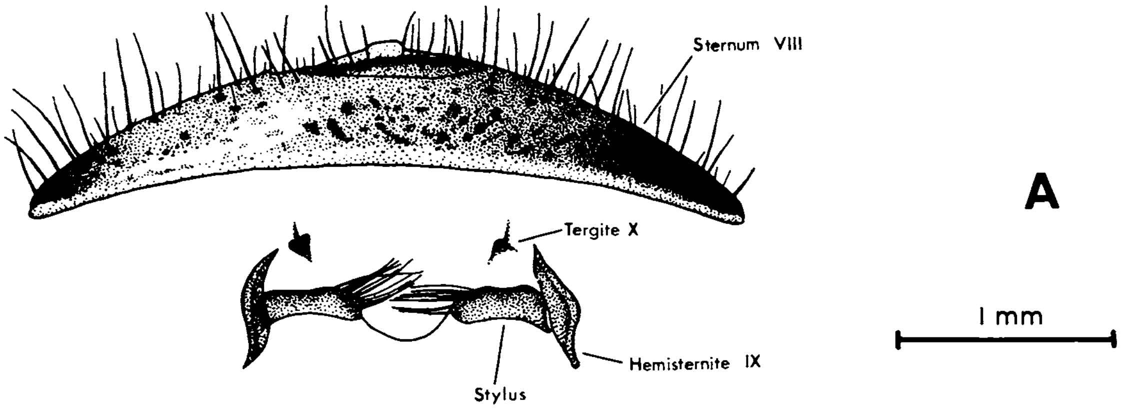
The anus opens into the genital chamber dorsally above the gap between the styli. On either side a thin rod-shaped sclerite is attached to the wall of the genital chamber and orientated in an anterio-posterior direction. Heymons (1930) calls these sclerites the "paranal pieces" but they are termed the 10th tergites here since they are considered to represent remnants of the 10th tergum by Menees (1963) and Hieke (1966). These authors also note that they may incorporate part of the 9th tergum.

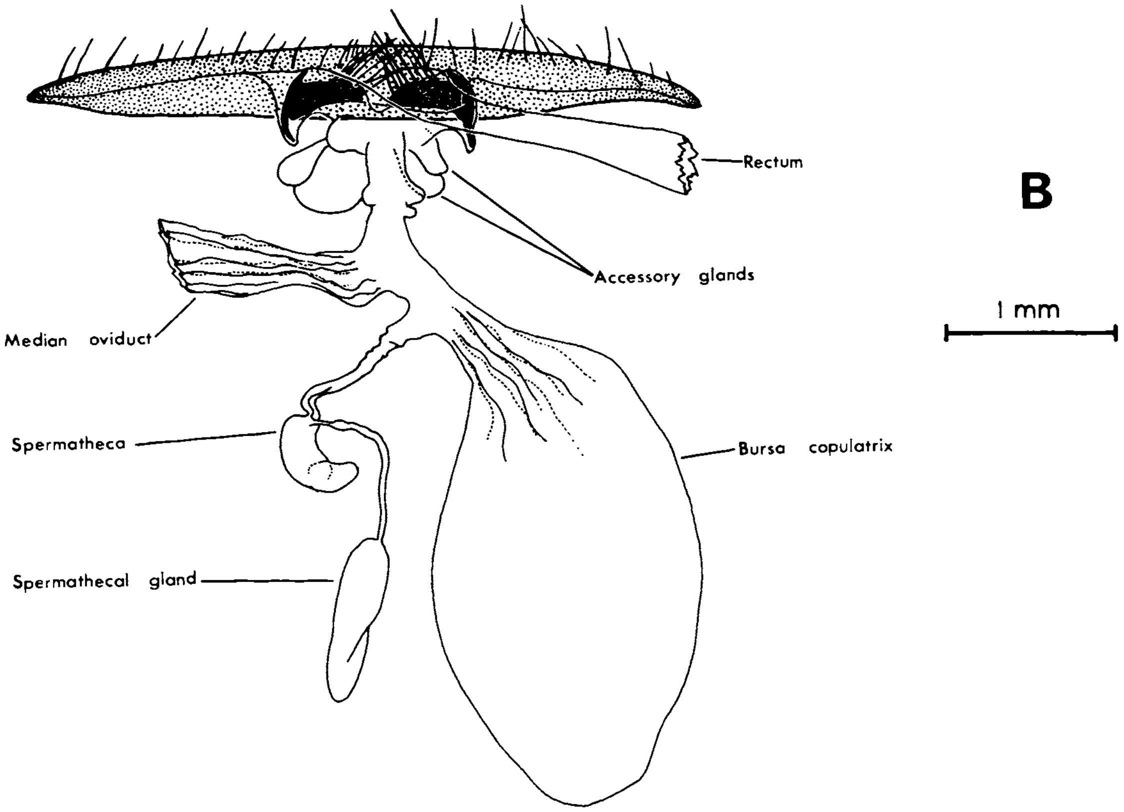
<u>Figure 3.19</u>

The external genitalia of the female *C. zealandica*

A. Ventral view of the 8th sternum and dissected out sclerites of the external genitalia

B. Whole mount of the female reproductive organs and 8th sternum after maceration with potassium hydroxide. Some of the folds in the intima of the bursa copulatrix and median oviduct omitted for clarity. Ventral view.


Sternum VIII
A
Tergite X
1 mm
Hemisternite IX
Stylus


Rectum
B
Accessory glands
1 mm
Median oviduct
Spermatheca
Bursa copulatrix
Spermathecal gland

The external genitalia of female insects in general are simpler and less varied than those of the male (Mackerras, 1973) and this appears to hold for the Scarabaeoidea. For example, the female external genitalia of *C. zealandica* resemble those in the 6 representatives from the Scarabaeoidea described by Tanner (1927); *Melolontha melolontha* L., (Straus-Dürckheim, 1828); *Phyllopertha horticola* L. and *Anomala aenea* Geer (Rittershaus, 1927); *Amphimallon majalis* (Razoumowski) (Menees, 1963); and *Phyllophaga anxia* (Le Conte) (Berberet and Helms, 1972). Finally, it can also be noted that Tanner (1927) considered that the external genitalia of female Scarabaeoidea are amongst the most highly specialised of beetles, since they lack an ovipositor.

The musculature of the external genitalia of the female

The only descriptions of the genital musculature of female scarabaeid beetles are those of *Melolontha melolontha* L., by Straus-Dürckheim (1828) and of *Amphimallon majalis* (Razoumowski) by Menees (1963). Menees (1963) named the muscles as far as possible according to their supposed function and this practice has also been followed here.

Ventral protractor of the genital chamber (Figure 3.20 C).

This is a series of short fine muscle fibres which originate along the ventral wall of the vagina between the ventral tips of the hemisternites and posterior to the accessory glands. These muscle fibres then run posteriorly to join the 8th sternum near its junction with the ventral edge of the genital chamber.

Ventro-lateral protractors of the genital chamber (Figure 3.20 B, C).

These originate on the ventro-lateral edges of the hemisternites and run posterio-ventrally as a pair of large muscles which inserts on the 8th sternum near its junction with the lateral walls of the genital chamber.

Ventral elevators of the genital chamber (Figure 3.20 a, B, C).

This is a pair of large muscles which originates in the middle of the lateral edges of the hemisternites and runs dorso-laterally to join the 8th tergum near its lateral posterior border.

Figure 3.20

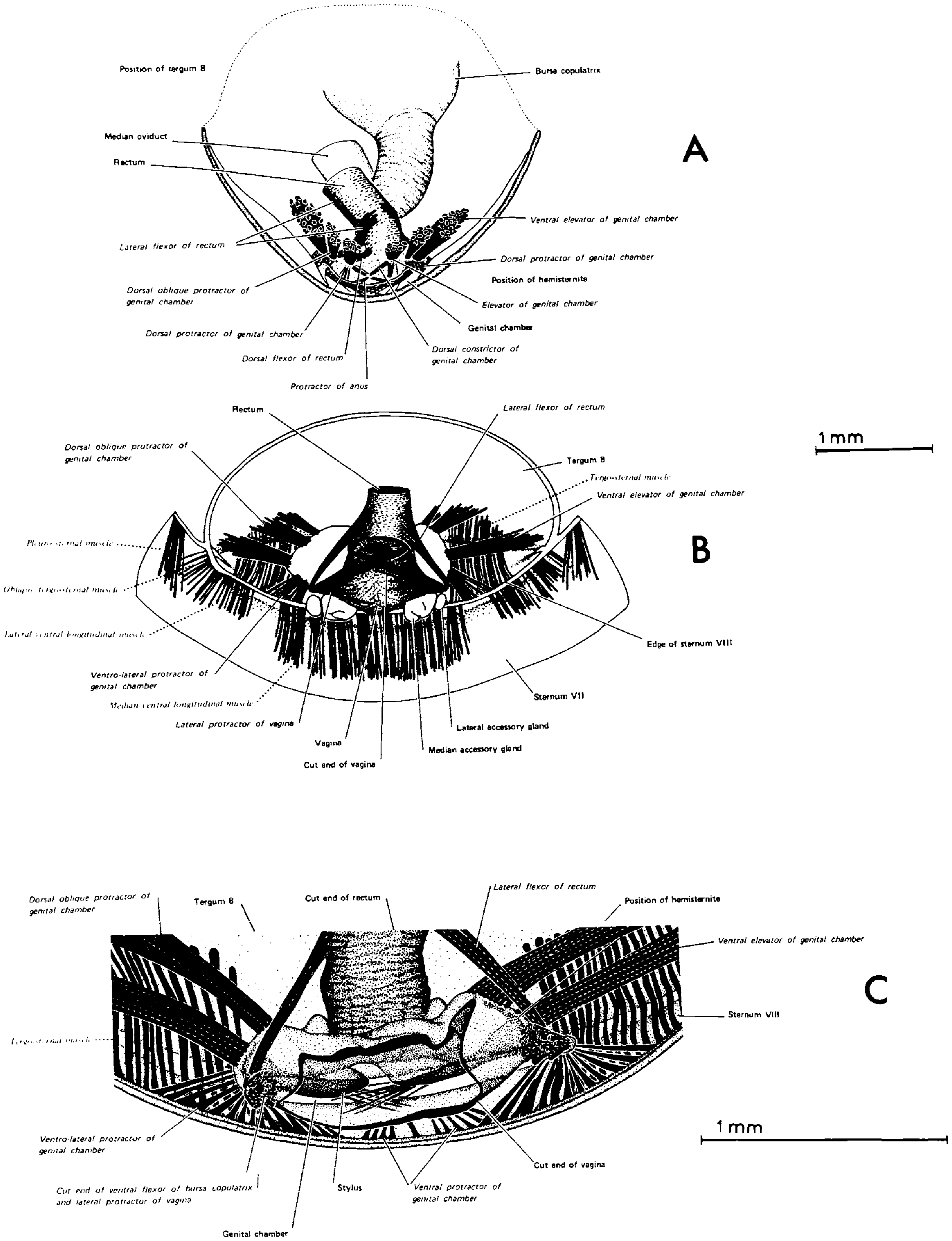
Muscles of the external genitalia of the female *C. zealandica*

(All muscles are named in italics, and the abdominal muscles are also indicated by dotted lines.)

A. Dorsal view of the female external genitalia *in situ*, with tergum 8 removed. Sternum VIII has been omitted for clarity.

B. The female external genitalia *in situ*, viewed from antero-ventrally.

C. The female external genitalia *in situ*, viewed from antero-ventrally. The antero-ventral portion of the vagina has been cut off removing the median oviduct and bursa copulatrix.


Position of tergum 8
Bursa copulatrix
Median oviduct
Rectum
A
Ventral elevator of genital chamber
Lateral flexor of rectum
Dorsal protractor of genital chamber
Position of hemisternite
Dorsal oblique protractor of genital chamber
Elevator of genital chamber
Genital chamber
Dorsal protractor of genital chamber
Dorsal constrictor of genital chamber
Dorsal flexor of rectum
Protractor of anus
Rectum
Lateral flexor of rectum
1 mm
Dorsal oblique protractor of genital chamber
Tergum 8
Tergo-sternal muscle
Ventral elevator of genital chamber
Pleuro-sternal muscle
B
Oblique tergo-sternal muscle
Lateral ventral longitudinal muscle
Edge of sternum VIII
Ventro-lateral protractor of genital chamber
Sternum VII
Median ventral longitudinal muscle
Lateral protractor of vagina
Lateral accessory gland
Vagina
Median accessory gland
Cut end of vagina
Dorsal oblique protractor of genital chamber
Tergum 8
Cut end of rectum
Lateral flexor of rectum
Position of hemisternite
Ventral elevator of genital chamber
C
Sternum VIII
Tergo-sternal muscle
1 mm
Ventro-lateral protractor of genital chamber
Cut end of vagina
Cut end of ventral flexor of bursa copulatrix and lateral protractor of vagina
Stylus
Ventral protractor of genital chamber
Genital chamber

Dorsal oblique protractors of the genital chamber (Figure 3.20 A, B, C).

From their origins on the lateral edges of the hemisternites near their dorsal tips, these two muscles run laterally to join the 8th tergum medial to its junction with the ventral elevators of the genital chamber.

Dorsal protractors of the genital chamber (Figure 3.20 A).

These originate on the dorsal tips of the hemisternites posteriorly to their dorsal oblique protractor muscles and run as a pair of small muscles obliquely in a lateral direction to insert on the 8th tergum by its junction with the lateral edge of the genital chamber.

Lateral protractors of the vagina (Figure 3.2o B, C).

These are two large muscles which originate on the ventral tips of the hemisternites anterior to the ventro-lateral protractors of the genital chamber. These muscles fan out and join the ventral surface of the vagina and posterior end of the median oviduct close to the median line.

Ventral flexors of the bursa copulatrix (not figured)

Each of this pair of muscles forms a dorsal branch of a lateral protractor of the vagina. It originates at the same point as the lateral protractor but inserts on the dorso-lateral wall of the vagina close to its junction with the bursa copulatrix.

Dorsal protractors of the vagina (not figured).

This is a pair of short muscles which originates on the dorsal tips of the hemisternites and runs posteriorly to insert on the dorso-lateral walls of the vagina.

Dorsal muscular plexus of the vagina (not figured).

This plexus comprises a thin mat of fine muscle fibres which either originate and insert on the dorsal wall of the vagina and run at various angles over its dorsal surface or originate on its dorsal surface and insert near the junction of the bursa copulatrix.

Ventral muscular plexus of the vagina (not figured).

This is an incomplete sheet of longitudinally orientated fine muscle fibres which originate and insert on the ventral wall of the vagina between and anterior to the accessory glands.

Retractors of the median accessory glands (not figured).

Each of these originates on the anterior base of a median accessory gland and runs anteriorly a short distance to insert on the ventral wall of the vagina. They comprise two series of very fine muscle fibres which could alternatively be included in the ventral muscular sheath of the vagina.

Anal muscles of the female

Lateral flexors of the rectum (Figure 3.20 A, B, C).

Each of these two long muscles originates on the ventral tip of a hemisternite, anteriorly to a ventral protractor of the genital chamber and inserts on two areas of the rectum. The posterior fibres of each of these muscles run dorsally over the rectum and are attached to it medially and dorsally; the anterior fibres branch from the posterior ones when close to the rectum, run anteriorly along the rectum and attach to it laterally.

Dorsal flexors of the rectum (Figure 3.20 A).

These originate on the anterior tips of the remnants of the 10th tergites and run anteriorly as two muscles which insert on the posterior dorso-lateral area of the rectum.

Elevators of the genital chamber (Figure 3.20 A).

These are medium sized muscles which originate on the anterior tips of the 10th tergites, posterior to the dorsal flexors of the rectum and insert on the 8th tergum medially to the dorsal oblique protractors of the genital chamber.

Dorsal protractors of the genital chamber (Figure 3.20 A).

Each of these two small muscles originates on a 10th tergite posteriorly to an elevator of the genital chamber and then runs laterally to insert on the 8th tergum where it joins the dorso-lateral edge of the genital chamber.

Protractors of the anus (Figure 3.20 A).

These comprise a very fine pair of muscles which originate close together in the dorsal mid-line of the genital chamber near the anus and runs parallel to the dorsal protractors of the genital chamber and inserts on the 8th tergum where it joins the genital chamber.

Dorsal constrictors of the genital chamber (Figure 3.20 A).

These originate laterally on the anterior tips of the 10th tergites and insert on the dorsal wall of the genital chamber anteriorly by the insertions of the protractors of the anus. They are very small muscles.

A comparison of the genital muscles of the female *C. zealandica* with those of the melolonthinids *M. melolontha* and *A. majalis* as described by Straus-Dürckheim (1828) and Menees (1963) respectively is given in Table 3.5. These are apparently the only other female Scarabaeoidea whose genital muscles have been described, and the names used by the original authors are given in Table 3.5.

All three species have 6 pairs of genital muscles which are easily homologised, and *C. zealandica* has a further 4 which are not easily related to any in the other species. One of these, the dorsal protractor of the vagina can possibly be homologised with "le retracteur oblique de l'oviductus" and "la dilatateur de l'anus" of *M. melolontha*, and the "short oblique elevator of the genital chamber" of *A. majalis*. In *C. zealandica* one end of the muscle is attached to a spicule as apparently is "le retracteur oblique de l'oviductus" and "la dilatateur de l'anus" in *M. melolontha*; the other end of these muscles is attached to the dorsal surface of the vagina, the 10th tergite, and the rectum respectively. In *A. majalis* the "short oblique elevator of the genital chamber" runs from the dorsal part of the genital chamber near the 10th tergite to the median part of the genital chamber and it therefore resembles "le retracteur oblique de l'oviductus" in *M. melolontha*. However, the homologies between all these muscles are tenuous, and involve migration of the attachments so that they are perhaps more easily regarded as being unrelated. The dorsal muscular plexus and ventral

Table 3.5

The muscles of the female external genita

Erratum

Insert below the table heading:

"Muscles of doubtful homology are indicated by double brackets and are placed next to their most probable homologues in *C.zealandica*"

GENITAL MUSCLES	Ventro-lateral protractor of the genital chamber	L'Abaisseur de l'anale inferieure	Ventr
	Ventral elevators of the genital chamber	L'Elevateur de la piece anale inferieure	Dorsa
	Dorsal oblique protractor of the genital chamber	L'Elevateur de la piece anale inferieure	Dorsa
	Dorsal protractor of the genital chamber	Le Retracteur (posterieur) de l'anale	Dors
	Lateral protractors of the vagina	((Le court Retracteur droit de l'oviductus))	Later
	Ventral flexors of the bursa copulatrix	Le long Retracteur droit de L'oviductus	Protr
	Dorsal protractors of the vagina	((La Dilatateur de l'anus)) ((Le Retracteur oblique de l'oviductus))	((Sh
	Dorsal muscular plexus of the vagina	((Le Sphincter de la vulve))	Const
	Ventral muscular plexus of the vagina	((Le Sphincter de la vulve))	
	Retractors of the median accessory glands	-	
ANAL MUSCLES	Lateral flexors of the rectum	Le Flechisseur lateral du rectum	Later
	Dorsal flexor of the rectum	L'Abaisseur du rectum	Depr
	Elevators of the genital chamber	L'Elevateur du cloaque	Eleva
	Dorsal protractors of the genital chamber	Le Retracteur du cloaque	
	Protractors of the anus	-	
	Dorsal constrictor of the genital chamber	-	
	-	Le Transverse du cloaque	((Con
	-	-	Dorsa
	-	-	Short

Table 3.5

The muscles of the female external genitalia of various Melolonthinae.

Species	*Costelytra zealandica* (white)	*Melolontha melolontha* L.	*Amphimallon majalis* Razoumowski
Author	The present study	Straus-Durckheim (1827)	Menees (1963)
GENITAL MUSCLES	Ventral protractor of the genital chamber	L'Abaisseur du cloaque Le Rotateur du cloaque	((Ventral protractor of the genital chamber))
	Ventro-lateral protractor of the genital chamber	L'Abaisseur de l'anale inferieure	Ventral protractor of the genital chamber
	Ventral elevators of the genital chamber	L'Elevateur de la piece anale inferieure	Dorsal-oblique elevator of the genital chamber.
	Dorsal oblique protractor of the genital chamber	L'Elevateur de la piece anale inferieure	Dorsal elevator of the genital chamber
	Dorsal protractor of the genital chamber	Le Retracteur (posterieur) de l'anale	Dorsal-oblique retractor of the genital chamber
	Lateral protractors of the vagina	((Le court Retracteur droit de l'oviductus))	Lateral protractors of the oviduct
	Ventral flexors of the bursa copulatrix	Le long Retracteur droit de L'oviductus	Protractor of the vagina
	Dorsal protractors of the vagina	((La Dilatateur de l'anus)) ((Le Retracteur oblique de l'oviductus))	((Short oblique elevator of the genital chamber))
	Dorsal muscular plexus of the vagina	((Le Sphincter de la vulve))	Constrictor of the vagina
	Ventral muscular plexus of the vagina	((Le Sphincter de la vulve))	-
	Retractors of the median accessory glands	-	-
ANAL MUSCLES	Lateral flexors of the rectum	Le Flechisseur lateral du rectum	Lateral flexor of the rectum
	Dorsal flexor of the rectum	L'Abaisseur du rectum	Depressor of the rectum
	Elevators of the genital chamber	L'Elevateur du cloaque	Elevator of the anus
	Dorsal protractors of the genital chamber	Le Retracteur du cloaque	-
	Protractors of the anus	-	-
	Dorsal constrictor of the genital chamber	-	-
	-	Le Transverse du cloaque	((Constrictor of the rectum))
	-	-	Dorsal constrictor of the vulva
	-	-	Short depressor of the vulva

plexus of the vagina of *C. zealandica* are included for completeness, and they are probably largely regarded as belonging to the internal genitalia by the other authors so that they are therefore not described by them. Possibly this is also the case with any homologes which may exist for the very small retractors of the median accessory glands of *C. zealandica*.

Apart from the three anal muscles shown in Table 3.5 which are easily homologised between the three insects, drawing any relationships between all the others is difficult. The dorsal protractors of the genital chamber of *C. zealandica* correspond to "le retracteur du cloaque" of *M. melolontha*, but the protractors of the anus, and the dorsal constrictor of the genital chamber have no corresponding muscles. "Le Transverse du cloaque" of *M. melolontha* appears to correspond to the "constrictor of the rectum" of *A. majalis*, but the latter insect also has another two muscles with no evident homologes. Attempts to relate many of these anal muscles would involve recognising which muscle attachments have migrated, and this is best left until enough scarabaeids have been investigated to show intermediate conditions.

Lastly it can be noted that, as a consequence of the hemisternites being derivatives of the 9th sternite, the muscles which run from it to the 8th segment are intersegmental in origin while the others which are attached to it are 9th sternal muscles.

The mechanics of copulation

The behaviour of the beetles during mating is described in detail in Chapter 4. The male is the active partner and searches for the female. When one is found the tegmen is usually extended so that the parameres and about a quarter of the basal piece project posteriorly from the end of the abdomen. The male then mounts the female and eventually inserts his tegmen into her genital chamber, thus becoming firmly attached to her. This attachment is so secure that males are often carried about in spite of having lost tarsal contact with the substrate.

The involvement of the musculature of the female is very much less than that of the male. It appears to be restricted to gripping the tegmen between the 8th sternum and 8th tergum, using the sterno-tergal muscles. It has been suggested, however, (Menees, 1963) that in ***Amphimallon* *majalis*** (Razoumowski) the female genital chamber muscles actively assist the tegmen to grip. However, this scarabaeid differs from *C. zealandica* in that the male's parameres are fused to the basal piece and are therefore possibly not as efficient. In *C. zealandica* the parameres push up the walls of the female's genital chamber to form pockets dorsally on either side of the rectum. The parameres then hook into these when they are flexed anteriorly.

The tegmen is extended to project out of the abdomen principally by its protractors although their action is assisted to a minor extent by the dorsal protractors of the spicules, the oblique lateral abductors of the spicules, and the ventral protractors of the genital chamber. All these tend to compress the genital chamber in a posterior direction. The major action of these latter muscles is, however, the adjustment of the angle at which the tegmen is held and expansion of the genital chamber, to facilitate extension of the tegmen.

Straightening and untwisting of the tegmen from its withdrawn position is achieved by its oblique rotators and retractors. These act by pulling on the walls of the invagination of the genital chamber. The protractors of the tegmen also automatically assist this movement when they contract because of their assymetrical attachment to the right hand anterior end of the basal piece. This attachment also tends to counteract detortion during the final stages of evertion.

Insertion of the parameres into the female is assisted by their being swung posteriorly through an angle of 30° to 40°. This results from the action of their dorsal extensor muscles. Once inside the female genital chamber they are swung ventrally and slightly anteriorly by their ventral flexors which act by pulling on the genital chamber wall where it lies ventral to the tegmen and attaches to the bases of the parameres. The parameres then hook into and grip the female and their action is further assisted by the basal piece which is held at a slight angle ventrally. The basal piece is held firmly by the oblique lateral rotator and lateral retractor muscles and by the tergo-sternal muscles of the 8th abdominal segment which tend to trap the tegmen between the sternum and tergum and act as a fulcrum for the more anterior muscles to act about.

The gripping action of the parameres consists entirely of an anterio-posterior movement; they do not approximate their ventral tips in a scissor-like action because they are not equipped with muscles to do so. However, the medial point of attachment of their dorsal extensors does tend to rotate them and close their tips when they are swung posteriorly. This assists their entry into the female and exit from the male.

Once the tegmen has been inserted into the female, intromission is accomplished by the male everting his internal sac into the bursa copulatrix. The internal sac expands to completely fill the bursa copulatrix although frequently the space is somewhat restricted distally by old spermatophores that have shrunken and hardened. The new spermatophore is then produced completely enclosed within the internal walls of the internal sac and when this has been completed the internal sac opens posteriorly and slightly ventrally as its walls are retracted dorsally and posteriorly from between the spermatophore and bursa copulatrix.

The following account of the mechanism by which the internal sac is extended is largely speculative since few of the muscles concerned act on rigid sclerites. It is, however, most probably accomplished by a combination of its own muscular movements and by hydrostatic pressure from fluids pumped into it. The muscular movements probably consist of peristaltic waves of contraction brought about by the muscular sheet which lines the internal sac, but these movements have only been observed in living material dissected under Clarke's insect saline. Under these conditions the peristaltic waves are initiated at the posterior lip of the internal sac and pass anteriorly over its surface, one at a time, as narrow ripples. As each contraction passes under the spines covering the outer wall they are momentarily raised and swung to point anteriorly before resuming their normal positions facing posteriorly. If these actions also occur during intromission then they possibly assist by levering and gripping. While the internal sac is being extended fluid is pumped between its inner and outer walls from the space between the ejaculatory duct and the muscular sheath surrounding it. This movement of fluid is caused by contractions in the muscular sheath which constrict its diameter and also compress it lengthwise anteriorly to the penis sclerites. A small amount of compression in the posterior region is also brought about by contractions of the dorsal protractor muscles of the penis which attach to the anterior end of the two sclerites and bend it ventrally whilst also sliding it posteriorly a small way within the tegmen. The short first connecting membrane, however, prevents the penis from being slid out of the tegumen. Final expansion of the internal sac within the bursa copulatrix is most probably assisted by the semen and spermatophore precursor fluids being pumped into it from the ejaculatory duct. These gel to form the spermatophore before the internal sac opens and is withdrawn.

Withdrawal of the internal sac is accomplished by contractions of its retractor muscle, which pulls it into the muscular sheath, and the penis retractor muscle, which pulls the penis and the ventral edge of the internal sac into the basal piece. In addition, retraction and folding of the internal sac is probably also assisted to some extent by its muscular lining, and especially by those fibres which are orientated longitudinally in it.

Withdrawal of the tegmen is accomplished after relaxation of its protractor muscles and ventral flexors of the parameres. The retractors of the tegmen and its oblique rotators then pull the invaginated wall of the genital chamber inward followed by the tegmen. However, in order for the tegmen to be withdrawn it must first twist and rotate because the parameres project ventrally from the basal piece and they cannot be swung posteriorly very much. At the same time the assymetrical attachment of the tegmen protractor muscles impart a torque as they are being stretched ensuring that the tegmen rotates onto its right-hand side and twists to its left. This action is also probably assisted by the slightly uneven action of the tegmen retractors and oblique rotators.

The functional significance of the external genitalia of the male can now be appreciated. The apodeme between the spicules is stiffened to take compressive and rotative forces from the retractors and oblique rotators of the tegmen while the tapering apodeme dorsal to it and anterior to the 8th sternite remains thin walled since it only experiences tenstion from the protractors of the tegmen. The inverted shoe-horn shape of the basal piece forms a rigid tube for supporting the beetle and forming a strong attachment to the female while it is expanded anteriorly to form an attachment for its extrinsic and intrinsic muscles, especially the large ventral flexors of the parameres which are responsible for attachment to the female. On the other hand the penis is reduced to thin sclerites which provide struts from which the retractor of the internal sac can act while at the same time allowing the muscular sheath of the ejaculatory duct to constrict to evert the internal sac.

The mechanics of copulation in the Scarabaeoidea have been previously described in full only for *A. majalis* by Menees (1963). The basal piece of this insect undergoes similar movements to that in *C. zealandica*, brought about by corresponding muscles (listed in Table 3.3). The parameres in *A. majalis* are fused to the basal piece and this is divided into anterior and posterior parts. During copulation the anterior part is bent dorsally by the "dorsal and ventral flexors of the phallobase" and this functions, presumably, in aiding attachment to the female. Therefore, despite this structural difference, the tegmen of *A. majalis* is still operated by corresponding muscles in a similar fashion to that in *C. zealandica*. In contrast, however, the anatomy of the penis and ejaculatory duct in *A. majalis* show major differences to those in *C. zealandica*, and the mechanism by which the internal sac enters the bursa copulatrix is very different. Menees (1963) attributes this to "paired protractors of the endophallus" which pull this chamber posterior to the "phallotreme opening" (the opening between the parameres). Seminal fluid is then squeezed into the bursa copulatrix by the combined action of the "flexors of the phallobase, retractors of the aedeagus and retractors of the endophallus".

The mechanics of copulation have also been briefly described for *Phyllopertha horticola* L. and *Anomala aenea* Geer by Rittershaus (1927) and show some similarity to what occurs in *C. zealandica*. In *P. horticola* and *A. aenea* the copulatory apparatus is shifted to the right when at rest and must be turned through 90° to function. There are, however, only two muscles, the "Protraktoren der Rutenkapsel", which attach to the connecting membrane or wall of the genital chamber to the right of the tegmen and insert on the spicules. The left muscle of this pair is responsible for twisting and pulling on the tegmen to protact it, while the right only pulls it. These muscles may also assist in retracting the tegmen since specific retractors are lacking. The parameres are also fused to the tegmen and there are no muscles for bending or stretching them although they are moved passively when the inner sac is advanced. This is achieved as in *C. zealandica* by blood pressure within the "Ringmuskelschicht" or muscular sheath. Accompanying this is a rhythmic pumping movement of the ventral plate against the tegmen. After intromission, the inner sac is withdrawn by contraction of the "Retraktoren des Inneren Sackes".

The anatomy and movements of the muscular sheath, penis, and internal sac in *Phyllophaga anxia* (Le Conte) as described by Berberet and Helms (1972) are almost identical to that described above for *C. zealandica*. Berberet and Helms (1972) term the muscular sheath the "erection-fluid pump" and consider that it acts in the following manner during copulation: "... the fluid contents of the erection-fluid pump are forced into the aedeagus by contraction of the pump, which results in eversion of the endophallus into the genital chamber of the female". In addition, these authors describe a retractor muscle within the muscular sheath of *P. anxia* similar to that in *C. zealandica* and report that its function is also to retract the internal sac after formation of the spermatophore.

Finally, it can also be noted that the basal pieces of *Melolontha melolontha* L. and *Geotrupes stercorosus* Scriba are also probably protracted and retracted in a similar manner to those in *C. zealandica*, but this is only speculation drawn from the anatomical descriptions given by Straus-Dürckheim (1828), and Hieke (1966) respectively. Rittershaus (1927) notes that to effect copulation the parameres of *M. melolontha* are moved against each other and they are actively pumped rhythmically up and down while being inserted into the female. The functioning of the parameres in *G. stercorosus* is not clear although according to Hieke (1966) they possess muscles for moving them. The ejaculatory duct of *G. stercorosus* was not treated by Hieke (1966) but the aedeagus and ejaculatory duct of *M. melolontha* was described by Straus-Dürckheim (1828) and they are very similar to the corresponding structures in *C. zealandica*. It is therefore probable that eversion of the internal sac is accomplished by a similar mechanism. One anatomical difference between *C. zealandica* and *M. melolontha* is that the two penis sclerites in *M. melolontha* bend ventrally and fuse to form a ventral strut which projects out of the tegmen and this may be of direct assistance during intromission.

CHAPTER 4

Mating and flight behaviour of *C. zealandica*, and the environmental factors that affect them

Introduction

The initial aim of this investigation was to develop a laboratory bioassay for the sex attractant of *C. zealandica* using behavioural responses of the males, and then to use this to locate the site of pheromone production in the female. A knowledge of mating and the conditions under which it occurs were therefore basic to this work but there was little detailed information even though research had been carried out for some years on the sex attractant of this beetle.

It was well established that the adult beetles are active above ground at dusk and during the night. They have been reported to fly at dusk usually for less than an hour but possibly for up to a few hours and then to alight and feed on the foliage of plants where they may remain for some hours or even continue eating all night (Connell, 1933; Helson, 1967; Hilgendorf, 1902; Kelsey, 1952, 1968; Miller, 1921, 1924; Moodie, 1911; Osborne and Hoyt, 1969; Radcliffe and Payne, 1969; Thomas, 1913). Some of the beetles may fly again at dawn (Hilgendorf, 1902) but during the day they all burrow into the soil or hide under dense foliage, rocks or other objects (Cockayne, 1911; Miller, 1924; Connell, 1933; Kelsey, 1951; Helson, 1967). Few authors, however, note when mating behaviour and copulation occur: Thomas (1913) sometimes observed pairing on the evening of the flights, Miller (1924) noted that it usually occurred after the beetles had settled and Henzell *et al.* (1969) mentioned that mating occurred at dusk when there was still some light. Copulating beetles have been found on foliage after the flights (Kelsey, 1951; Osborne and Hoyt, 1969) and Kelsey (1968) found that more than 99% of the females collected from the ground and plants during the hour after the flights had mated. Beetles have also been observed attempting to mate with "Pliobond" adhesive for up to three hours after the beginning of the flights (Osborne and Hoyt, 1969) so possibly males can begin to mate with females during this period as well. In addition, the information referred to below often carries the implication that sexual behaviour occurs at dusk.

Mating in *C. zealandica* has not been described in detail yet even though Henzell *et al.* (1970) described a behavioural bioassay in which they referred to the responses of males to a dummy containing an active fraction as "attempting to copulate with it ... or with one another". The following observations have, however, been published: Males will attempt to mate with crushed or sectioned females (Henzell *et al*, 1969, 1970) but they will not attempt to mate with females that have been dried or washed with lipid solvents (Osborne and Hoyt, 1969). Live female beetles often attract two or three and sometimes more males (Kelsey, 1967) and it has been shown by various authors that the females use phenol as a pheromone and that the males are attracted to it by either flying or walking (see Chapter 1). Phenol will attract males and induce them to attempt to copulate with other males, paraffin dummies, dried beetles stuck down with "Pliobond" adhesive and small pieces of dried "Pliobond" (Osborne and Hoyt, 1969, 1970; Henzell *et al*, 1969). Copulating beetles have been found partially or completely buried in sand (Fenemore and Perrott, 1970); on or partially beneath the soil, within the litter layer, and on the walls of an insectary (Radcliffe and Kain, 1971); in turf and pasture (Kelsey, 1951; Radcliffe and Payne, 1969); and on trees (Osborne and Hoyt, 1969; *et seq.*), hedges, and shrubs where they can be very abundant (Kelsey, 1951). Beetles have also been found to mate when both sexes were collected together in a net or kept together in a container (Kelsey, 1951).

Individual beetles have been observed to emerge from the soil, mate, feed and tunnel back into the soil each night over a period of up to 32 days (Kelsey, 1951). After emerging from the soil males usually fly after resting for a short time on the surface (Fenemore and Perrott, 1970) but the behaviour of females changes with their age. Initially they mate close to their primary emergence sites (Kelsey, 1951; Fenemore, 1966; Radcliffe and Payne, 1969, *et seq.*). They almost invariably remain stationary on first breaking through the surface (Fenemore and Perrott, 1970) and then mating usually occurs within a few minutes (Fenemore, 1970). After copulation they burrow beneath the soil again without feeding or flying and lay their eggs (Radcliffe and Payne, 1969; Fenemore and Perrott, 1970). On subsequent emergences the females may fly and eat although some unmated females may fly with those that have laid their eggs (Radcliffe and Payne, 1969). Kelsey (1968) also found that most females that fly have already mated although he observed that if they had not mated by the time they had climbed pasture plants they invariably flew to trees where mating then occurred (Kelsey, 1951). Additional evidence that females do not fly on their first emergence was obtained from the slow reinfestation rates of plots cleared of larvae (Fenemore, 1965, 1970) and this is also one possible explanation for the variations in the proportions of males and females caught on foliage which range from less than 25% females (Kelsey, 1972) to approximately 50% females (Kelsey, 1968; Radcliffe and Payne, 1969) even though the true sex ratio is close to 50% females (Farrell, 1972).

This change in the behaviour of the females is also shown in the seasonal flight patterns; flights early in the season consist almost entirely of males (Fenemore, 1966; Fenemore and Perrott, 1970) although there is no evidence from soil sampling or emergence cages that males develop earlier then females (Fenemore, 1966). Kelsey (1951), however, noted that males emerged earlier than females although this conclusion may have been drawn from his light trapping records which showed that the catches usually contained less than 5% females during the early half of the emergence periods but that the percentage then increased over the last half until they amounted to nearly 25% of the total collections. It appears, however, that males are caught most frequently in light traps (Helson, 1967; Kelsey, 1968) and that light traps do not give a reliable indication of the beetle numbers or of the sex ratio of the emerging adults (Kelsey, 1968).

In addition to these seasonal changes, males also begin to emerge and fly earlier in the evenings than do the females (Kelsey, 1951; Fenemore and Perrott, 1970) and it has been suggested that this contributes to females being mated and laying their eggs near their emergence sites after emerging for the first time (Kelsey, 1951; Fenemore, 1966).

Nothing has been published specifically on the environmental conditions under which mating occurs although there is some data in relation to flight and emergence. These are discussed here because of their association with mating.

The beetles usually fly close to the ground (Miller, 1924) but have also been found to alight on trees (Hilgendorf, 1902; Kelsey, 1951; Osborne and Hoyt, 1969). They fly in any direction when the wind speed is below 54m per minute and invariably upwind when it is up to about 270m per minute (Kelsey, 1957, 1968). Cold and windy weather seems to be unfavourable for flight (Miller, 1924; Connell, 1933; Osborne and Hoyt, 1968; *et seq.*) and in Canterbury large flights do not occur if the temperature is lower than 9.4°C, during light or heavy rain, or if the wind speed exceeds 160 to 270m per minute (Kelsey, 1951). Flight is significantly correlated with increasing temperature and decreasing wind speed (Kelsey, 1968) although some beetles may fly on any nights even in adverse weather (Kelsey, 1951). Flight activity is reduced on moonlight nights (Connell, 1933; Miller, 1924) and Kelsey (1968) considered that the females would be influenced by the intensity of moonlight and might fly away from it although he found no evidence of this in a field oviposition trial. Wind and temperature influence emergence but to a lesser extent than they do flight; beetles emerge when the grass temperature is down to 2.2°C and the wind speed is as high as 965m per minute but high day temperatures, relative humidities and rainfall during the day do not appear to have any influence on emergence or flight (Kelsey, 1968).

As none of the above studies have been specifically designed to provide detailed information on mating, it was necessary to investigate it more fully in the field before establishing a laboratory bioassay. This work was directed primarily towards acquiring basic information on sexual behaviour, including the time of day and the environmental conditions when mating occurs. Some aspects of flight were also studied because of its association with sexual behaviour and this included the use of sticky traps for demonstrating the arrival pattern of flying males to an attractant source and whether the position of the females on the vegetation in the field affects their relative attractiveness for males. Solutions of pure phenol were used as the attractant in these sticky traps in preference to female extracts because phenol is considered to be the pheromone (Chapter 1) and known quantities of it could be applied consistently, whereas female extracts could vary from batch to batch and most probably change chemically with time.

Methods

Most of this investigation was concerned with the behaviour of beetles that flew near and settled on a 2 m high hedge of ***Berberis vulgaris*** L. (Barberry) dividing two fields on Mr H.C. Goodhue's farm near Hamilton (Chapter 2). This hedge was chosen because it was noted that beetles became concentrated on top of it every evening when flight occurred; and its length and uniformity allowed replicate experiments to be performed along it simultaneously. Observations were also made on the beetles when they were on the ground or on other plants both at this location and on Mr T. O'Regan's farm near Te Awamutu (Chapter 2).

The beetles could usually be observed in the dim natural light when they were on the hedge by moving one's position until the beetles were silhouetted against the sky. This could not be done, however, with beetles on the ground or on pasture plants and therefore artificial lighting was used more frequently in these positions. Artificial lighting was provided mostly by a torch although sometimes a darkroom safe light fitted with a 100 W bulb and a Kodak 1A filter (red) were also used.

Photographs of the beetle's behaviour were taken with a Nikon F camera fitted with a Micro-nikkor 55 mm F. 3.5 lens mounted on an aluminium support next to a Zeiss Ikatron S1 electronic flash (Figure 4.1). An L-shaped frame was mounted in front of the camera in such a way that the arms of the L lay along two of the edges of its field of view to aid photography in dim light; a reflecting plate was also fitted to the L-frame so that the subject was illuminated from both sides. The camera was then prefocused to the frame and the output of the flash was adjusted by taping tissue paper over it until the maximum depth of field was obtained for the film used. Photographs were then quickly and accurately taken after composing the subject within the arms of the L-frame.

The time spent by the beetles in copulation was determined by collecting pairs of beetles from the hedge, placing them in a covered aquarium on the grass nearby and then examining them at intervals of 6 minutes or less using a torch. All the copulating pairs were collected within a three minute period from a region of the hedge previously cleared of all copulating beetles to ensure they had all mated at about the same time. The aquarium they were placed in was provided

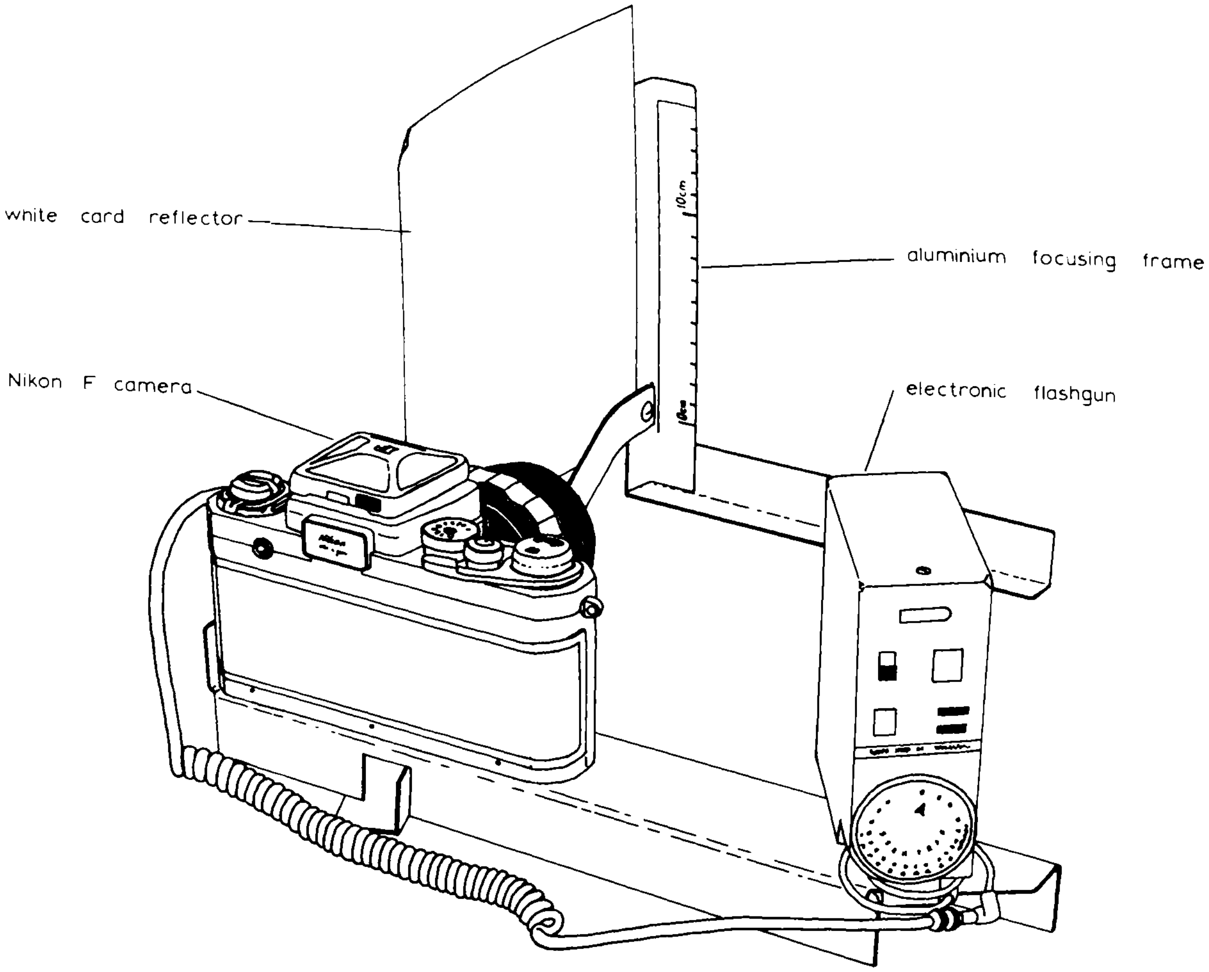
Figure 4.1

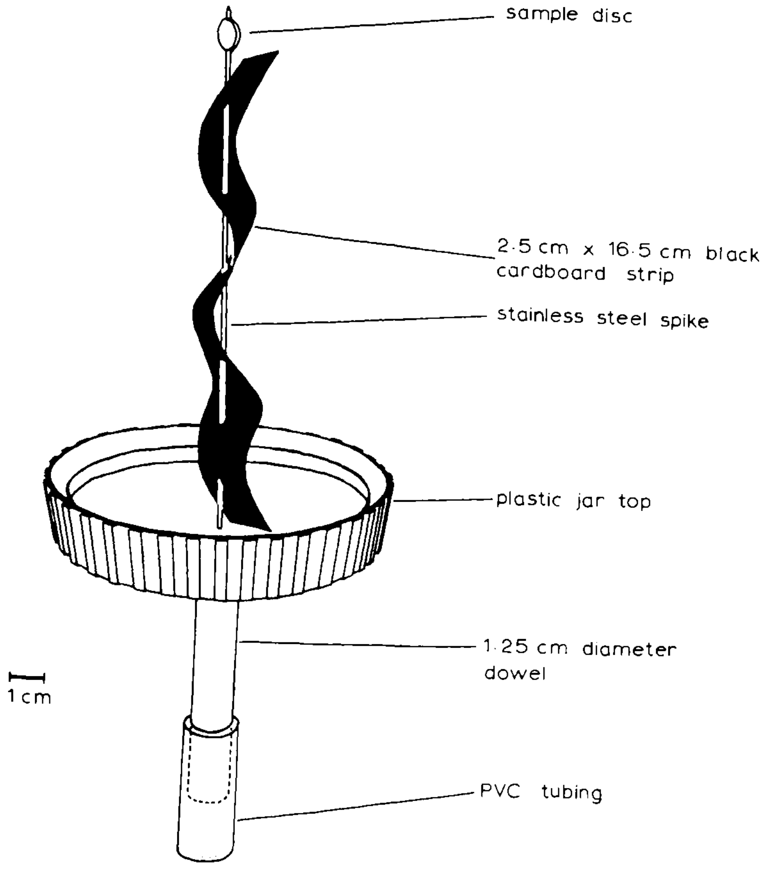
The equipment used for close-up photography
of *C. zealandica* adults in dim light

The Nikon F. camera was prefocused to the plane of the L-shaped aluminium frame and the output of the Zeiss Ikatron S1 electronic flash was adjusted by taping tissue paper over it to obtain the maximum depth of field in the photographs. Subjects were photographed when the arms of the L-frame were positioned around them.

A sticky trap used for investigating
attraction of *C. zealandica* in the field

The surfaces of the black cardboard strip and inside of the plastic lid were coated with "Stickem special" to snare any beetles that alighted on them. The short length of P.V.C. tubing on the end of the handle was used to push over the top of a piece of dowel previously positioned in the field, to set the trap.


white card reflector
aluminium focusing frame
10cm
Nikon F camera
electronic flashgun


sample disc
2·5 cm x 16·5 cm black cardboard strip
stainless steel spike
plastic jar top
1·25 cm diameter dowel
1 cm
PVC tubing

with 10 freshly cut shoots of barberry and lined with blotting paper so that the beetles could walk about and eat.

Samples of copulating beetles were also taken at different times at night to investigate what happened internally while they copulated. Each sample was taken by knocking about 10 copulating pairs off the hedge and into a Dewar flask of liquid nitrogen and subsequently storing them in netting bags under liquid nitrogen until they were transported back to the laboratory. The frozen beetles were then dropped into about 1 litre of boiling alcoholic Bouin's (Humason, 1967) for about 1 minute and dissected under cold 70% alcohol.

Changes in the relative numbers of beetles that flew during the dusk flights were determined by taking flash photographs of a small region of hedge at intervals during the dusk flight periods. The camera was mounted on a gatepost in such a way that it pointed along the hedge and the electronic flash was set 2 m from the hedge and 2 m above the ground so that it illuminated a short length of the hedge next to the gate. The flash output and the camera were adjusted so that any light from the sky did not affect the film to ensure that the image photographed was only illuminated by the flash. The top and side of the hedge and any insects flying near it were therefore silhouetted against a dark background in the photographs (Figure 4.2) and this enabled the beetles to be easily identified and counted.

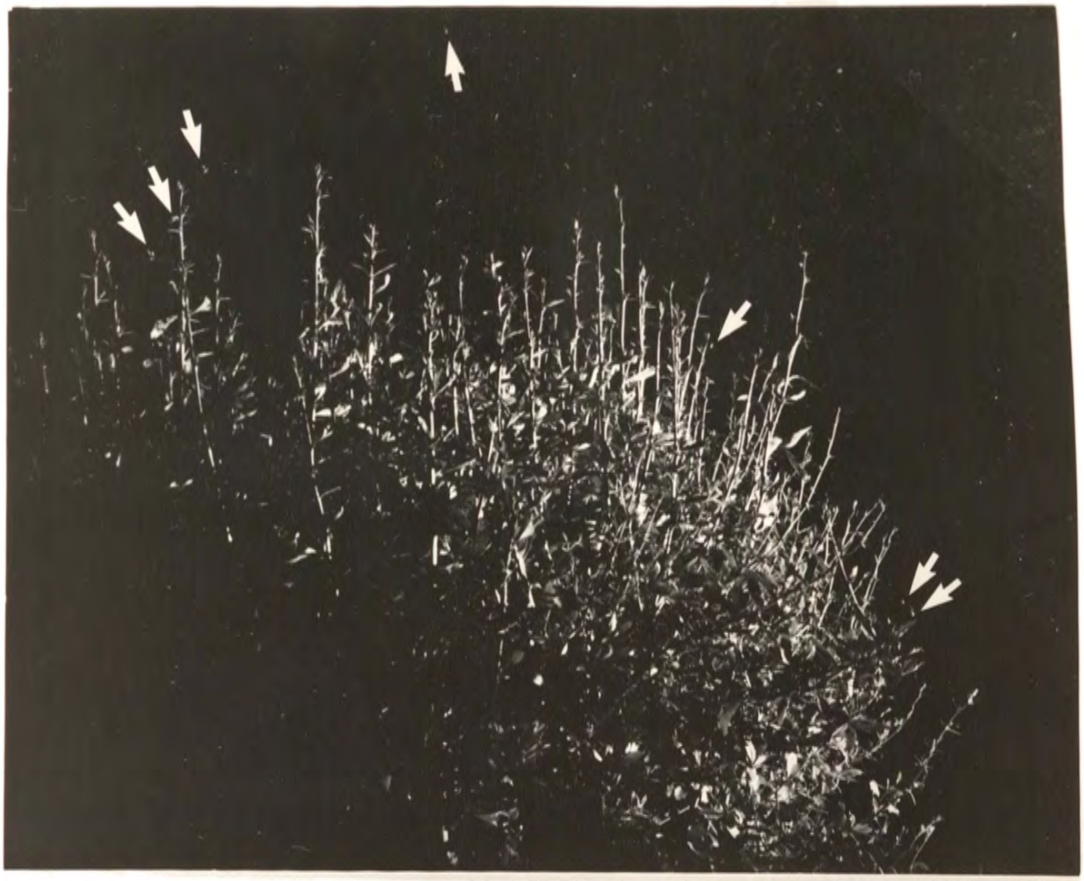
The sticky traps were used to determine the relative numbers of beetles that were attracted to phenol solutions at different times or at different positions in the field and to test whether beetles or their extracts were attractive or not. Usually some of the traps were baited with samples and the remainder were baited as blanks so the attractiveness of the samples could be found by comparing the numbers of beetles caught in sample and blank traps.

The various samples used in the traps were 50 µl or 100 µl of phenol solutions, 1 ml of a beetle extract in an organic solvent, crushed whole beetles, and live beetles. All the liquids were applied to discs of Whatman seed test paper 13 mm in diameter so that they could be placed on the traps, while the beetles were crushed between filter papers and the live beetles were contained within brass gauze cages 20 mm long and 20 mm in diameter. The corresponding blanks used with these samples were 50 µl or 100 µl of water, 1 ml of the organic solvent, clean filter paper or empty cages.

Figure 4.2

Two examples of the photographs taken to determine the relative numbers of *C. zealandica* flying

The upper photograph was taken at 1914 hours on the 5th November, 1972 and the lower one two minutes later. They show the top and southern side of the barberry hedge as illuminated by the electronic flash (near position A in Figure 4.3). *C. zealandica* are indicated by white arrows and numerous small psychodid flies appear as small white specks. Moths were also occasionally photographed but are not shown here. In practice the number of flying beetles was determined directly from the negatives by examining them under a binocular microscope at low magnification. The size, shape, and especially the raised elytra, when visible, enabled the flying beetles to be clearly distinguished from the other insects.



The phenol solutions were freshly prepared each fortnight from "Analar" phenol and glass distilled water and the extracts of beetles were made with "Analar" chloroform or "Analar" diethyl ether the same day they were used. Each extract was made by dropping 100 male or female beetles that had been collected the previous evening into about 50 ml of the solvent and then grinding them up in it. The solvent was then filtered through a Pyrex number 3 sintered glass filter and the remains of the beetles were recovered, ground up in solvent again and filtered. This was repeated three times and then all the solvent was concentrated to about 5 ml in a rotary evaporator at less than 40°C and under reduced pressure. The final extract of 10 ml was then made up by adding the required amount of solvent to the concentrate.

The sticky traps were made from the plastic screw-on lids of 1 litre plastic jars with sharpened stainless steel spikes projecting from the centres of their inner surfaces and with wooden dowelling handles fixed to the centres of their outer surfaces (Figure 4.1). Irregularly twisted strips of black cardboard 0.5 mm thick and 25 mm wide by 105 mm long were threaded over most of the lengths of the steel spikes leaving the sharpened points exposed for impaling the samples. The cardboard strips and the insides of the lids were coated with "Stickem special"[1]. Short lengths of P.V.C. tubing were fastened to the ends of the handles so the traps could be set quickly by pushing the hollow ends of P.V.C. tubing over the tops of dowels previously positioned in the field. The irregularly twisted cardboard strips therefore projected upwards when the traps were set and resembled shoots of vegetation in outline. When not in use or during transportation, the lids of the sticky traps were screwed into their plastic jars so that the sticky surfaces were enclosed and any beetles on them were protected against accidental loss.

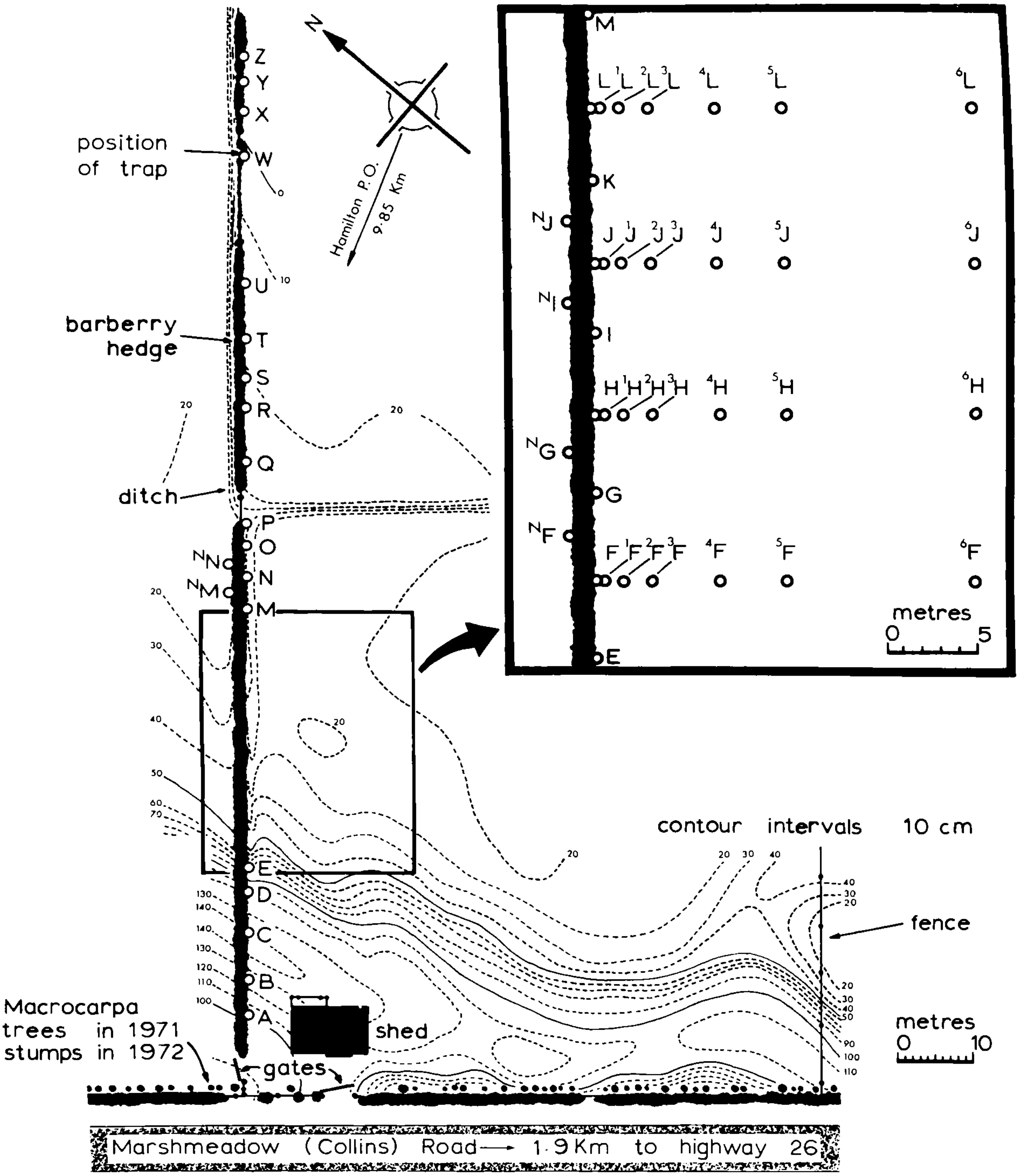
Fifteen of these sticky traps were used in 1971 and 25 were used in 1972 but this was the maximum number that could be operated simultaneously. The positions occupied by the traps in the field are shown in Figure 4.3. Those positions along the south side of the hedge were permanently established by fastening dowels onto fenceposts bordering the hedge. These dowels were initially adjusted so that the tops of the traps were level with the top of the hedge and the hedge was then cut back to its original height for more

[1] Michel & Pelton Co. California

Figure 4.3

Map of the field in which *C. zealandica*
was caught in sticky traps.

The traps were set in the positions indicated by circles. These positions are identified by letters. In addition, a row of Macrocarpa trees (*Cupressus macrocarpus* Gord.) about 6 to 10m high ran alongside the road in 1971, and these were cut down before the beetles began to emerge in 1972.


N
Hamilton P.O. 9·85 Km
position of trap
barberry hedge
ditch
Z
Y
X
W
U
T
S
R
Q
P
O
N
M
ᴺN
ᴺM
E
D
C
B
A
shed
Macrocarpa trees in 1971 stumps in 1972
gates
fence
contour intervals 10 cm
metres
0 10
metres
0 5
L ¹L ²L ³L ⁴L ⁵L ⁶L
K
ᴺJ
J ¹J ²J ³J ⁴J ⁵J ⁶J
ᴺI
I
H ¹H ²H ³H ⁴H ⁵H ⁶H
ᴺG
G
ᴺF
F ¹F ²F ³F ⁴F ⁵F ⁶F
20
30
40
50
60
70
90
100
110
120
130
140
Marshmeadow (Collins) Road → 1·9 Km to highway 26

than 1m on either side of the traps whenever it grew about 0.25 m higher than the traps. The other trapping positions were prepared each time they were needed by driving dowels of the required lengths into the ground.

The traps were only set on evenings when the wind did not blow directly from one trapping position to another in case the samples interferred with each other. Usually the traps were arranged in repeating sequences of samples and blanks when they were put out in the field but they were moved along one position when they were set on subsequent evenings so that traps with the same baits were not set in the same position each time. This made the application of the samples and blanks much simpler so that the traps could be prepared just before they were set.

Liquid samples and blanks were always applied to the traps in the field just before they were set to ensure that they did not dry up before the beetles started flying. Phenol solutions and water blanks were dispensed with a 100 µl microsyringe[1] and the extracts of beetles and solvent blanks were dispensed with an automatic pipette[2] in multiple applications of 0.1 ml allowing time for the solvent to dry between each application. Crushed beetles were also prepared in the field before they were put on the traps but live beetles were placed in their cages in the laboratory and transported to the field in them. When live beetles were used in the traps these were set out between 30 to 40 minutes before the start of the flights to allow time for the beetles to settle down. All other traps were usually set a few minutes before the first beetles started flying although occasionally this was done when up to a dozen or so were already flying. Once the traps were set they were then left alone until the end of the flights unless otherwise stated and they were only removed when no more beetles could be seen or heard flying. Before removing them plastic jars were screwed onto the traps to contain any snared beetles as described above; then they were taken back to the laboratory where the beetles were removed, cleaned with kerosene so their sex could be determined and then counted. After this the traps were prepared for future use by either replacing the "Stickum" removed with the beetles or completely cleaning them in kerosene followed by hot water and detergent and fitting new cardboard strips with fresh "Stickum". However, this cleaning was only done once every 7 to 10 days because

[1] . Hamilton microliter syringe, Hamilton Company, California.

[2] . Scharz Mann Biopette, New York.

psychodid flies became snared in the traps had started to rot and smell.

Meterological data was recorded during the peak periods of flight activity or recorded continuously while the beetles were being observed. Temperature was taken at grass height with a mercury thermometer or a thermohygrograph[1]. Humidity was also taken with the thermohygrograph or with a wet and dry bulb thermometer swung above the grass. Light intensity was taken with a lux meter[2] orientated vertically upward and wind speed was obtained with a stopwatch and an anemometer[3] placed on a fence post.

Results

General observations.

When adult *C. zealandica* occurred in the field they burrowed in the soil or hid underneath low objects during the day and they were only observed above ground at dusk, during the night,and at dawn. If the conditions were favourable most of the beetles flew when they emerged at dusk, but they settled before darkness on the ground or on vegetation including pasture, trees,and shrubs. They were seldom observed to fly during the night and only a few flew at dawn. At dusk many of the beetles flew towards the tops of tall plants where they settled in dense concentrations as described below but during the night they walked about and became dispersed over the vegetation. The beetles were also commonly found eating foliage and copulating at dusk and at the beginning of the night although the number involved in these activities diminished as the night progressed until they were rarely observed by the morning. In addition, most of the beetles returned to the ground and burrowed back into it during the night and usually only some of those that had settled on taller plants remained above ground until dawn.

Mating behaviour and Copulation

Most of these observations were made during the dusk flights when there was still sufficient daylight left for the beetles to be easily seen and relatively fewer beetles were observed mating at night

[1] Ota Keiki Seisakusho Co. Ltd., Japan.

[2] Bruno Lange type S60, Germany.

[3] Isuzu, Japan.

partly because this occurred less frequently at night and also because it was more difficult to observe without disturbing the beetles.

Mating occurred on plants or on the ground and invariably it was the males who found the females. The females appeared to be attractive to males and those that were observed before they mated either rested or ate foliage for a short time before the males arrived. They did not, however, show any distinguishing behaviour patterns or postures to indicate when they were attractive and so this could only be inferred from the reactions that males showed to them: these females adopted any posture between a level stance and one in which their bodies were held at an angle with their mouthparts close to the substrate and with their abdomens raised. Often their elytra were also slightly raised when they were in this angled posture and sometimes one or both of their metathoracic legs lost contact with the surface and were slowly waved about at intervals (Figures 4.4 A,B; 4.5 A,B). However, this angled posture with raised elytra was not adopted by females exclusively because 24 males and 59 females were identified in this stance.

Male beetles usually appeared to search for females both at dusk and during the night but occasionally mating followed an apparently accidental encounter with a female.

At dusk most males were observed to mate after they had first flown towards the females. They approached the females by flying slowly upwind towards them while constantly facing into the wind and alternately flying from one side to the other. This flight behaviour is subsequently referred to here as hovering and it was also shown by all beetles that were about to settle. The only observed difference was that males took relatively longer in approaching and alighting near females than other beetles did when they were settling to rest or feed. Males were also frequently observed to fly towards females when there were few other beetles about and this suggested that the males were being attracted while in flight to the females. The following experiments were therefore

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of aqueous phenol in concentrations of up to 60 g/l to determine the concentration ranges over which it was an effective attractant in these

Erratum

Last paragraph, line 2:

"... night half the " *should read* "... night three of the " partly because this occurred less frequently at night and also because it was more difficult to observe without disturbing the beetles.

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First of all the sticky traps were given a preliminary test on 4 nights to test their effectiveness. On each night half the traps were baited with 50 µl of water and the rest were baited with 50 µl of aqueous phenol in concentrations of up to 60 g/l to determine the concentration ranges over which it was an effective attractant in these

Figure 4.4

Photographs of mating in *C. zealandica*

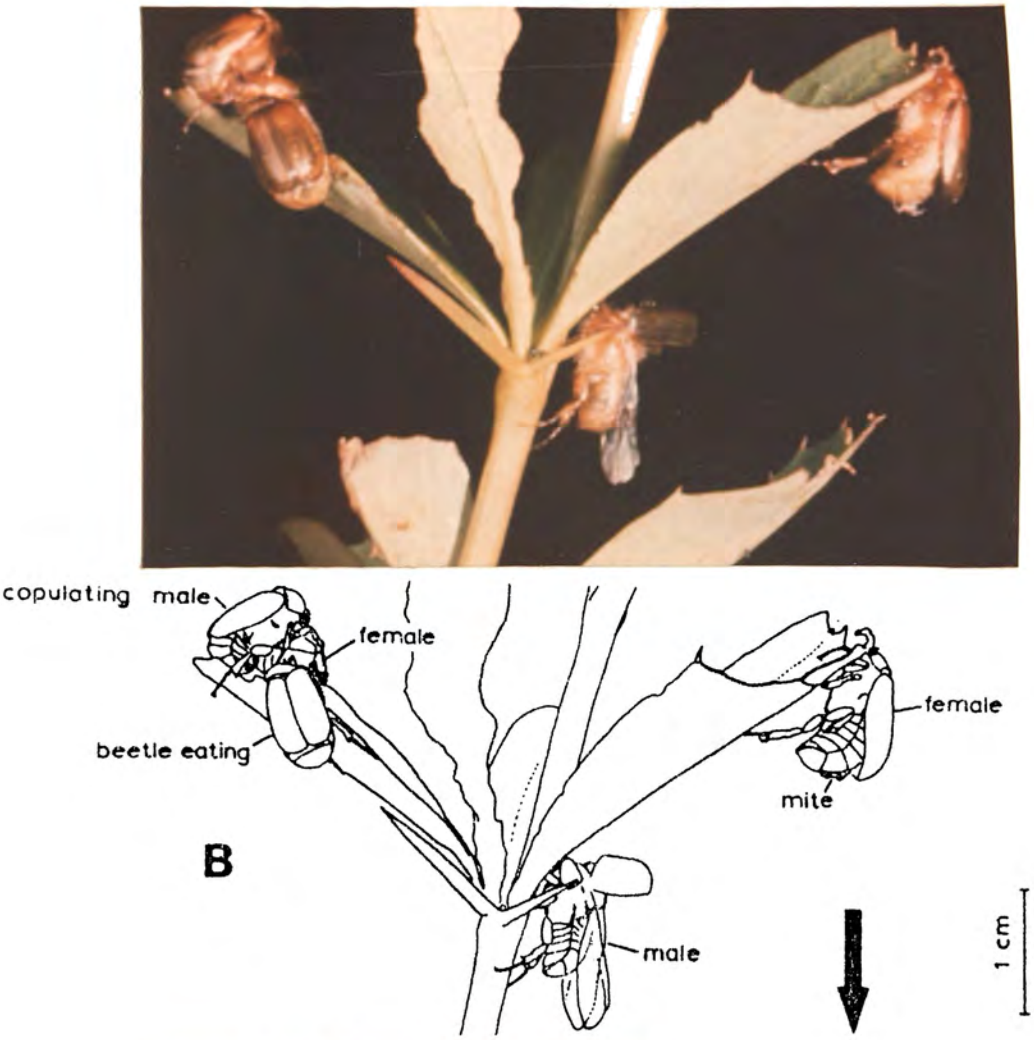
All photographs were taken of beetles on a barberry hedge during the dusk flight period.

A. A female with slightly raised elytra eating barberry.

B. A group of beetles on a shoot of barberry including one copulating pair, two females eating, and a male running about searching for the female identified by slightly raised elytra and a mite on her abdomen. The male has just stopped beating his wings but his elytra are still raised.

C. A photograph of part of the same shoot of hedge as in B above taken shortly after the male had found the female with the mite. He has climbed onto her back, his tegmen is extended, and he is in the process of moving backwards to position the end of his abdomen behind that of the female. He is also in the process of folding his wings.

A



B

copulating male
female
beetle eating
female
mite
male
1 cm

C

mite
tegmen

Figure 4.5

Photographs of mating in *C. zealandica*

All photographs were taken of beetles on a barberry hedge during the dusk flight period.

A. Beetle eating with slightly raised elytra.

B. Beetle at rest with slightly raised elytra.

C. A male beetle running about and beating his wings, searching for the female lower down the shoot and with one of her wings not completely folded.

D. A male beetle photographed on the back of a female. He is moving about beating his wings.

A

B

C

D

traps. The traps were then placed in position on top of the hedge and left there during the dusk flight period.

The results of this experiment are given in Figure 4.7 and Table 4.1 and showed that the traps were effective and that their catches were to some extent quantitative. Those traps baited with phenol in concentrations of 0.06 g/l or higher caught more male beetles than traps baited with water and generally more males were caught the higher the concentration of phenol used (Figure 4.7). However, relatively few females were caught in any of the traps and they only showed a slight increase in numbers with increasing concentration of phenol (Table 4.1). This, therefore, indicated that phenol was attractive to males and that it was probably not an attractant for females. It was also apparent that the best concentration of phenol to use in these sticky traps was probably 6 g/l because the numbers of beetles caught in traps baited with this were sufficiently large to demonstrate that attraction had occurred when compared to the catches of blank traps and yet they were not too large for sexing and counting conveniently. Most subsequent work with the traps was therefore done using 6 g/l phenol solution as the attractant.

Observation of the traps showed that they caught only those beetles that had flown there and that most of these had hovered towards them and alighted on the twisted pieces of cardboard coated with 'Stickem'. Once caught they then struggled and slowly slipped downward until they eventually reached the inverted lids of the traps. Each beetle carried some 'Stickem' down with it so that later arrivals became less heavily coated and were more likely to be able to crawl off the other beetles in the lids and fall from the traps. This was observed to start occurring occasionally after about 40 to 50 beetles had accumulated in the lids and then the traps became increasingly inefficient as more beetles were caught even though the lids could hold between 120 to 150 dead beetles. However, the numbers of beetles caught in traps baited with 6 g/l phenol were generally within the efficiency range and so no further modification of the traps was necessary.

The next experiment with the sticky traps was designed to demonstrate conclusively whether or not flying male beetles were attracted to phenol. This experiment was performed initially on two nights early in the flight season and then it was repeated again later

TABLE 4.1

Numbers of beetles caught in traps containing a range of phenol samples.

DATE	SAMPLE; g/l PHENOL	0	0.02	0.06	0.2	0.6	2	6	20	60
5/11/71	TRAP POSITION	E J O	F K P		G L Q		H M R		I N S	
	MALE CATCH	7 4 0	6 3 2		12 3 9		9 15 20		36 33 6	
	FEMALE CATCH	0 1 0	2 0 0		0 2 0		0 0 1		0 2 2	
8/11/71	TRAP POSITION	H M R		I N S		E J O		F K P		G L Q
	MALE CATCH	0 0 0		0 0 0		0 1 0		0 3 1		6 2 3
	FEMALE CATCH	0 0 0		0 2 0		1 0 0		1 1 0		3 0 1
9/11/71	TRAP POSITION	G L Q		H M R		I N S		E J O		F K P
	MALE CATCH	11 15 12		7 9 5		36 34 23		38 40 31		38 76 77
	FEMALE CATCH	0 1 1		0 2 0		3 2 0		0 0 1		2 0 1
11/11/71	TRAP POSITION	H M R			G I J	F K L	E O S	N P Q		
	MALE CATCH	6 10 5			2 9 8	15 16 33	26 8 4	16 10 31		
	FEMALE CATCH	0 0 0			0 1 0	0 0 1	1 2 1	2 0 1		

Figure 4.6

Photographs of mating in *C. zealandica*

All photographs were taken of beetles on a barberry hedge during the dusk flight period.

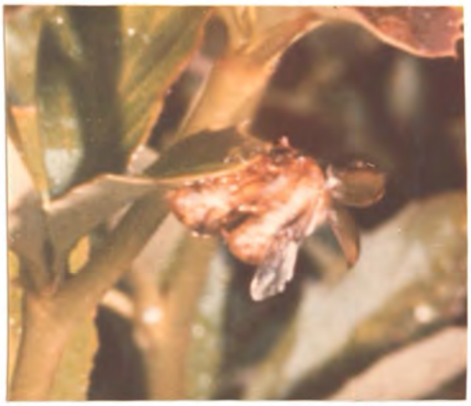
A. A pair of copulating beetles and a male flying towards them.

B. A male beetle climbing over the back of a female he has just run towards. His tegmen is extended and he has just stopped beating his wings.

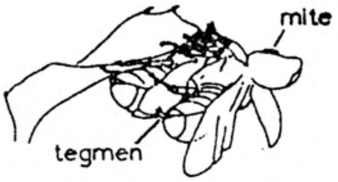
C. A male beetle climbing onto a pair of copulating beetles and beating his wings.

D. A male beetle on the back of a copulating male. He is moving backward with extended tegmen and unsuccessfully attempting the manoeuvre shown in Figure 4.4 C. Also his wings are in the process of being folded.

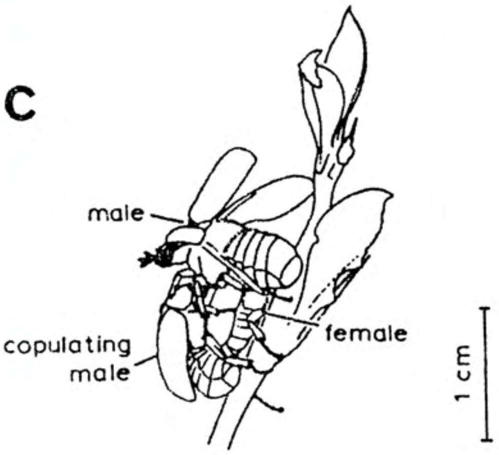
A



B


mite
tegmen

C


male
copulating male
female
1 cm

D

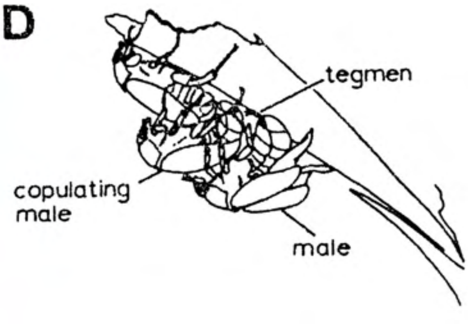
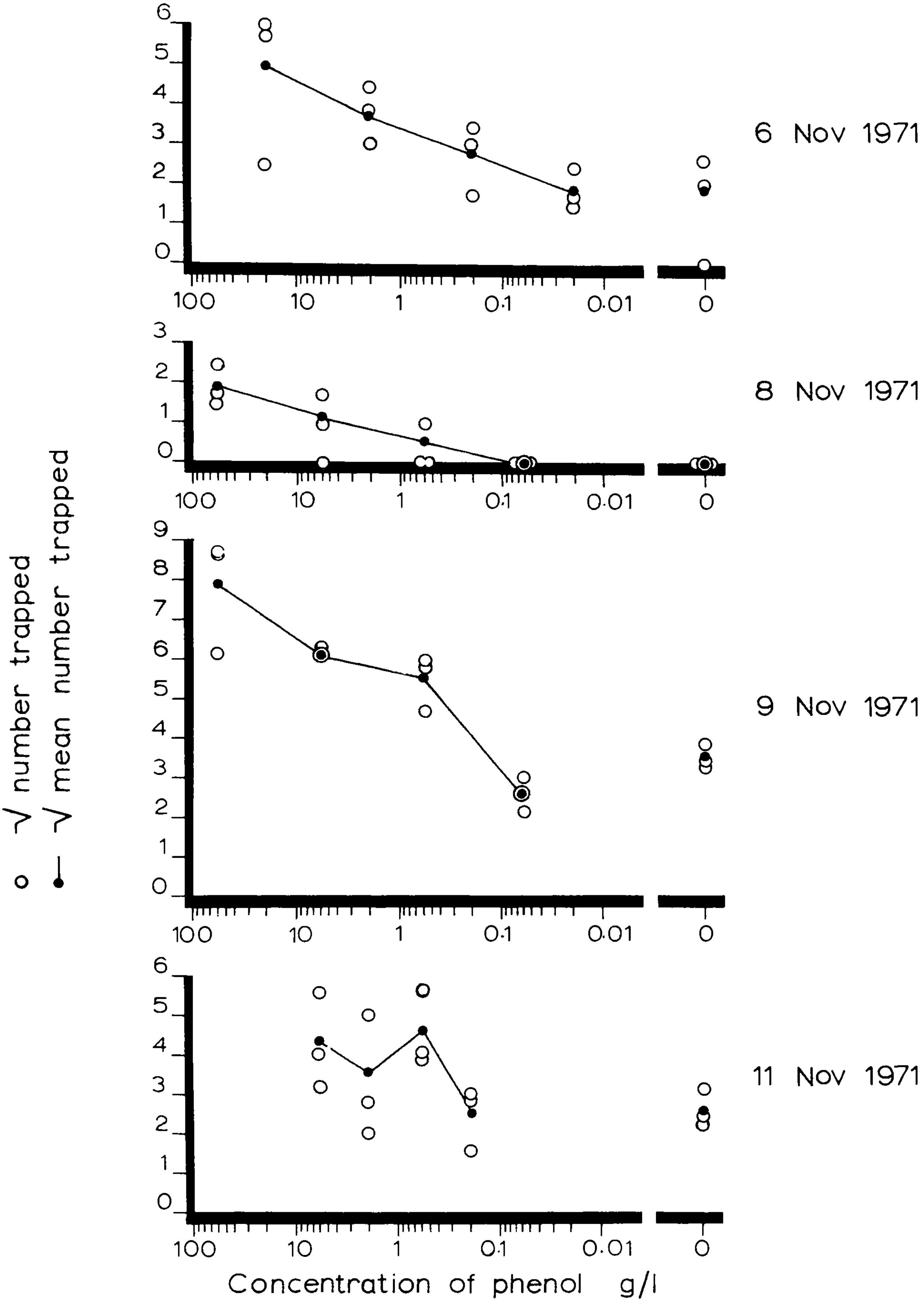

tegmen
copulating male
male

Figure 4.7

The relationship between the concentration of phenol used in the sticky traps and the number of male beetles caught in them

The data is taken from Table 4.1.

○ √ number trapped
●— √ mean number trapped
6
5
4
3
2
1
0
6 Nov 1971
3
2
1
0
8 Nov 1971
9
8
7
6
5
4
3
2
1
0
9 Nov 1971
6
5
4
3
2
1
0
11 Nov 1971
100
10
1
0·1
0·01
0
Concentration of phenol g/l

on when relatively fewer beetles flew to find what effects this had on the catches. On each of these nights half the traps were baited with water and the rest were baited with 6 g/l of phenol, then the catches were compared after they had been placed on top of the hedge over the dusk flight periods.

The mean numbers of male beetles caught each night in the traps baited with phenol were always greater than the mean numbers caught in the traps baited with water, and the differences between these catches were significant on all of the nights except the last when a mean of only 2.1 beetles were caught in the phenol baited traps (Table 4.2). Few females were caught in any of the traps although there was a tendency for slightly greater numbers of them to be caught in traps baited with phenol than in those baited with water, and these differences were significant on one occasion (Table 4.2). This experiment therefore showed conclusively that males were attracted to phenol, that males could respond to an attractant by flying towards it, and that phenol was also a slight attractant for female beetles.

Once the males had been shown to fly towards phenol another series of experiments was performed with sticky traps placed on top of the hedge over the dusk flight periods to find if the females could attract the males. Initially, the traps were fitted with brass cages on the 18th November, 1971 and these were either left empty or else they had two live virgin, two to three week old male or female beetles placed inside them. The numbers of beetles caught in either the male or the female baited traps did not indicate significant attraction when they were compared with the numbers caught in the blank traps although a greater mean number of males were caught in the female baited traps than in the male baited traps and both these contained more males on the average than the blanks did (Table 4.3). It was possible, though, that one pair of females had shown attraction because 16 males were caught in one of the traps baited with females and this was twice as high as any of the other traps caught (Appendix 2.1). This suggested that if all the females did contain the attractant then they could control whether or not it was released. This possibility was investigated by testing squashed beetles when the traps were next put out on the 20th November. On this occasion some of the traps were again left blank and the rest were each baited with one squashed virgin male or female beetle. However, few beetles flew that night and therefore few were caught in any

TABLE 4.2

Differences in the numbers of beetles caught in traps containing 50μl of either water blanks or 6 g/l phenol samples.

DATE	SAMPLE	6 g/l PHENOL	0 g/l PHENOL	6 g/l PHENOL		0 g/l PHENOL		SIGNIFICANCE LEVEL BETWEEN MEANS (t-test)
15-11-71	TRAP POSITION	E G I K M O Q S	F H J L N P R	MEAN	S.D.	MEAN	S.D.	
	Male catch	5 6 20 10 12 2 14 6	2 5 3 5 3 1 4	9.38	5.83	4.14	2.61	0.05
	Female catch	2 1 3 0 2 1 2 0	0 0 0 1 0 0 0	1.38	1.06	0.14	0.38	0.05
16-11-71	TRAP POSITION	E G I K M O Q S	F H J L N P R					
	Male catch	24 10 11 17 24 23 6 17	6 5 1 7 10 9 8		7.88	6.57	2.99	0.005
	Female catch	4 2 0 1 1 0 1 0	4 0 2 0 0 1 1	1.13	1.36	1.14	1.46	NS
28-11-71	TRAP POSITION	E G I K M	F H J L					
	Male catch	8 6 6 8 6	4 1 0 1	6.80	1.09	1.50	1.73	0.001
	Female catch	1 0 2 1 1	0 1 0 1	1.00	0.71	0.51	0.58	NS
15-12-71	TRAP POSITION	F H J L N P R	E G I K M O Q S					
	Male catch	1 1 4 2 4 2 1	0 0 0 4 3 4 1 2	2.14	1.35	1.75	1.75	NS
	Female catch	0 0 0 0 1 0 0	0 0 0 0 0 0 0 0	0.14	0.38	0.00	0.00	NS

TABLE 4.3

Differences between the mean numbers of beetles caught in traps containing 50µl samples of 6 g/l phenol solution, blanks as empty cages, and live or squashed beetles. Means from Appendix 2.1.

DATE	SAMPLE	MEAN OF MALE CATCHES	S.D. OF MALE CATCHES	SIGNIFICANCE LEVEL BETWEEN MALE CATCHES (t-test)	MEAN OF FEMALE CATCHES	S.D. OF FEMALE CATCHES
18-11-71	Blank	4.80	3.11	blanks & male samples N.S.	0.55	0.40
	2 live virgin males	6.25	2.06	blanks & female samples N.S.	1.00	0.82
	2 live virgin females	8.75	4.99	Male & female samples N.S.	0.75	0.96
27-11-71	Blank	8.00	3.50	blanks & male samples N.S.	0.75	0.96
	10 live males	5.25	1.89	blanks & female samples N.S.	0.25	0.50
	10 live females	4.00	1.63	male & female samples N.S.	0.50	1.00
	6 g/l phenol	15.33	4.04	blanks & phenol samples 0.05	1.67	0.58
20-11-71	Blank	0.00	0.00	blanks & male samples N.S.	0.00	0.00
	1 squashed virgin male	0.80	1.30	blanks & female samples N.S.	0.60	0.89
	1 squashed virgin female	0.00	0.00	male & female samples N.S.	0.00	0.00

of the traps (Table 4.3) so no conclusions could be drawn from this experiment. There were also no more virgin beetles left after this although one further attempt was made on the 27th November to find if live beetles were attractive in the field using beetles which had been collected in the field two to three weeks previously and kept segregated in separate containers of males and females. This time phenol baited traps caught significantly more males than the blanks (Table 4.3) showing that attraction had occurred that night, but there were no significant differences between the catches of blank traps and the male or female baited ones. Possibly the beetles disturbed each other and this prevented them from releasing their attractant, but whatever the reason this experiment did show that there were probably too many difficulties involved in using live beetles and therefore extracts of beetles were used in the subsequent experiments instead.

To start with diethyl ether extracts of male and female beetles were tested on the 29th and 30th November and on the 1st December, 1971. The extracts were freshly prepared each day from beetles collected the previous night. On each of these occasions some of the traps were left blank, some were baited with 6 g/l of phenol and the remainder were baited with the extracts. The amount of extract applied to each trap on the first night contained the equivalent of 4 beetles and on the next two nights contained the equivalent of 10 beetles. These experiments were unfortunately performed late in the flight season when few beetles flew: all the catches were, therefore, low and there were no significant differences between any of them except for the phenol baited ones on the 30th November which were significantly more attractive than the blanks (Table 4.4). However, it was still possible that the female extracts were attractive because traps beited with it caught more than twice as many males as the blanks on the last two nights (Table 4.4). These experiments were therefore continued in 1972. This time, however, chloroform extracts of beetles collected in the field the previous night were used and none of the traps were baited with phenol solutions. Instead approximately a third of them were baited with chloroform blanks and the remainder were baited with extracts containing the equivalent of 10 males or 10 females. The results of all these experiments are given in Table 4.5 and show that those traps baited with female extracts always caught a higher mean number of males than the blanks did while those traps baited with male extracts either caught more

TABLE 4.4

Differences between the mean numbers of beetles caught in traps containing either 6 g/l phenol or diethyl ether blanks or diethyl ether extracts of whole beetles. Individual trap catches given in Appendix 2.2

DATE	SAMPLE	MALE CATCHES		SIGNIFICANT LEVEL BETWEEN MALE CATCHES (t-test)	FEMALE CATCHES	
		MEAN	S.D.		MEAN	S.D.
29/11/71	Blank	2.25	1.71	blanks & male extracts N.S.	0.00	0.00
	4 male equivalent	2.00	0.82	Blanks & female extracts N.S.	0.25	0.50
	4 female equivalent	2.00	1.56	male & female extracts N.S.	0.25	0.50
	6 g/l phenol	5.33	6.11	blanks & phenol samples N.S.	0.00	0.00
30/11/71	Blank	2.25	2.63	blanks & male extracts N.S.	0.00	0.00
	10 male equivalent	2.50	2.65	blanks & female extracts N.S.	0.75	0.96
	10 female equivalent	5.75	4.99	male & female extracts N.S.	0.25	0.50
	6 g/l phenol	8.67	6.11	blanks & phenol samples N.S.	1.00	1.00
1/12/71	Blank	0.50	0.58	blanks & male extracts N.S.	0.00	0.00
	10 male equivalent	1.33	2.31	blanks & female extracts 0.05	0.33	0.58
	10 female equivalent	2.00	1.00	male & female extracts N.S.	0.67	1.15
	6 g/l phenol	3.60	2.70	blanks & phenol samples N.S. (0.06)	0.40	0.89

TABLE 4.5

Differences between the mean numbers of beetles caught in traps containing chloroform blanks and chloroform extracts of whole beetles.

Individual trap catches given in Appendix 2.3

DATE	SAMPLE	MALE CATCHES		SIGNIFICANT LEVEL BETWEEN MALE CATCHES (t-test)	FEMALE CATCHES	
		MEAN	S.D.		MEAN	S.D.
23-11-72	Blank	3.78	2.54	blanks & male samples N.S.	0.44	0.53
	10 male equivalent	3.38	2.88	blanks & fem. samples N.S.	0.00	0.00
	10 female equivalent	4.13	2.30	male & female samples N.S.	0.13	0.35
24-11-72	Blank	3.89	2.03	blanks & male samples N.S.	0.22	0.44
	10 male equivalent	3.50	3.02	blanks & fem. samples 0.001	0.13	0.35
	10 female equivalent	10.13	4.22	male & female samples 0.005	0.25	0.71
27-11-72	Blank	2.86	2.85	blanks & male samples N.S.	0.38	0.74
	10 male equivalent	3.63	2.97	blanks & fem. samples N.S.	0.25	0.71
	10 female equivalent	7.25	7.83	male & female samples N.S.	0.13	0.35
28-11-72	Blank	2.25	1.67	blanks & fem. samples 0.005	0.00	0.00
	10 male equivalent	7.73	4.91	blanks & male samples N.S.	0.25	0.45
	10 female equivalent	5.13	4.33	male & female samples N.S.	0.00	0.00

males or less males on the average than the corresponding blanks. On two of these nights the traps baited with female extracts also caught significantly more males than the blanks while the traps baited with male extracts never had significantly different catches from the blanks. In addition, the numbers of females caught in all the traps were low and showed no tendencies for either male or female extracts to be attractive to them.

When considered together, all the above experiments with beetle extracts demonstrated that female beetles contained an attractant for the male beetles and that the males could respond by flying towards it. They did not, however, demonstrate that the female beetles do in fact use this substance to attract flying males but at least they did not preclude the possibility of this occurring. In this way they lent support to the previous conclusion drawn from observations of their behaviour, that females could be attractive to flying males.

After male beetles had flown towards the females they were usually observed to land within 10 cm or so of them but some occasionally landed directly on the females and immediately started orientating themselves for copulation as described below. Those that landed near the females ran about searching for them, going up and down shoots if they were on vegetation or turning in irregular spirals or figures of eight if they were on the ground. While they ran they also frequently beat their wings intermittently (Figures 4.4 B, 4.5 C) making short buzzing sounds lasting less than about 0.25 second. If they did not find any females within 10 to 15 seconds they usually took flight again and either began hovering towards the same females or else flew elsewhere. Occasionally some males also appeared to mate at dusk without having flown specifically towards their mate a short time beforehand. Such individuals were observed to behave like those of the males that copulated during the night, running about searching for the females and also sometimes periodically beating their wings after they had walked to within about 5 cm or so of them.

As soon as a male found a female he usually climbed immediately onto her and then moved about until they were aligned in the same direction (Figures 4.4 C, 4.5 D, 4.6 B). Occasionally a male first positioned himself in the opposite direction to the female but he usually moved around after one or two seconds when he could not copulate successfully. When the male was on top of the female he also touched her frequently with his palps and often beat his wings periodically (Figures 4.4 D, 4.5 B). Soon after contacting the female the male usually extended his tegmen so it projected from his abdomen (Figures 4.4 C, 4.5 B) and it was always extended by the time he had aligned himself with her body. After this the male moved backwards (Figure 4.4 C) until the top of his abdomen was just posterior to the female's abdomen and then he moved his body around slightly until he managed to copulate with the female (Figures 4.4 B, 4.6 A). Most males achieved copulation within 1 or 2 seconds after first touching the females unless they positioned themselves in the wrong direction initially or if there were other males with the females as described below. On one occasion, however, a male was observed to attempt to copulate for about 50 seconds with a female whose abdomen was covered by a leaf, the female then moved and the male was able to copulate with her.

Once the beetles were copulating they remained together for variable periods of time until the males withdrew their tegmens and walked away. However, the length of time spent in copulation was difficult to measure directly in the field because the beetles moved about. Therefore it was estimated by taking 25 pairs of beetles from the hedge soon after they had started copulating and placing them in a covered aquarium nearby where they were subsequently observed at intervals of 2 to 8 minutes using a torch. The temperature inside this aquarium remained within 12.0 ± 0.5°C while the observations were being made and the periods spent copulating by the beetles varied considerably; the first beetles started separating after only 7.5 ± 3.5 minutes, half had separated after about 50 minutes and there were still two pairs copulating after 90 minutes when the observations were discontinued (Figure 4.8 A). Two beetles were also observed to copulate a second time 81 minutes after they were collected. The copulation times fell into a skewed frequency distribution having a mean after logarithmic transformation of log 1.419 minutes and a standard deviation of log 0.429 minutes, corresponding to a mean time of 28 minutes spent in copulation. However, all these times were only considered to be approximations because of the possibility that the beetles' behaviour was affected by the treatment even though every care was taken to ensure that they were transferred gently and that minimum use was made of the torch.

Figure 4.8

Histograms of the copulatory activity of *C. zealandica* during the night

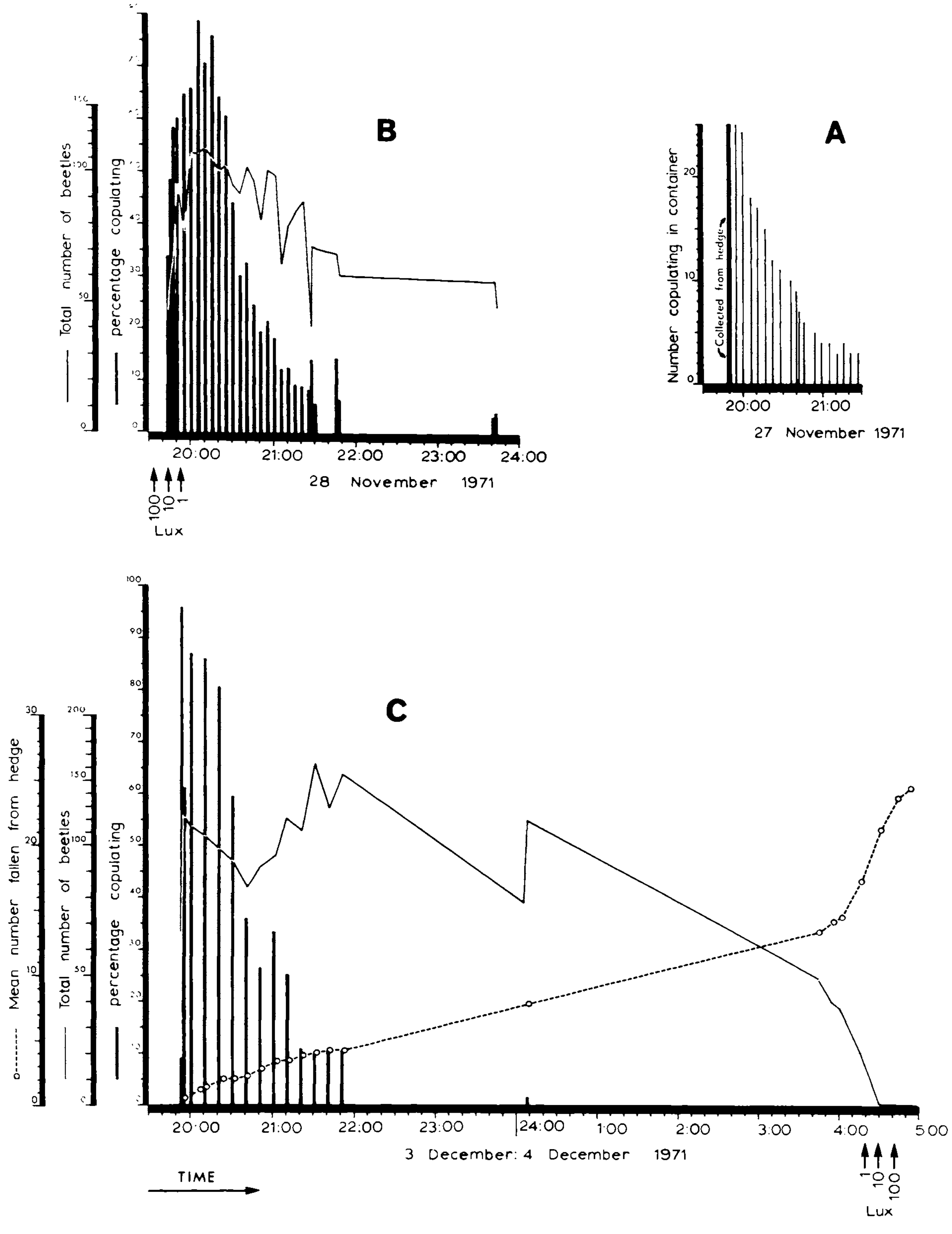
A. The duration of copulation. The beetles were collected *in copula* and the numbers still paired were determined at intervals during the following hour and 40 minutes by observation.

B. The proportions of beetles found copulating on a barberry hedge at different times during the beginning of the night of the 28th November, 1971. Each estimate was made from counts of the numbers of single and copulating beetles during a two minute period. The total numbers of beetles counted are also indicated.

C. The proportions of beetles found copulating on a barberry hedge at different times during the night of the 3rd to 4th December, 1971. These estimates were made in the same way as for B above and the total numbers of beetles counted are also indicated. In addition, the cumulative numbers of beetles which fell into large trays filled with detergent and water during the night are also shown.

Meterological data

Date	Time	Temperature °C	Relative Humidity %	Wind speed km/h
27/11/71	1743 2010	12.0 12.0	100 87	
28/11/71	1940 1945 2000	11.5 11.4 10.6	89 93 90	} 0.075
3-4/11/71	1946 2031 2045 2137	17.3 16.5 17.0 16.5	96 96 95 72	} >0.01

B
Total number of beetles
percentage copulating
0
50
100
150
0
10
20
30
40
20:00
21:00
22:00
23:00
24:00
28 November 1971
100
10
1
Lux
A
Number copulating in container
Collected from hedge
0
10
20
20:00
21:00
27 November 1971
C
Mean number fallen from hedge
Total number of beetles
percentage copulating
0
10
20
30
50
100
150
200
30
40
50
60
70
80
90
100
20:00
21:00
22:00
23:00
24:00
1:00
2:00
3:00
4:00
5:00
3 December: 4 December 1971
TIME
1
10
100
Lux

Female beetles were frequently found with two or occasionally more males on them although only one male successfully copulates at a time with each female. Sometimes these males arrived before the females had begun to copulate but more commonly they were found on females that were already copulating. In both these cases the males were observed to climb over the females and over the other males with their **tegmens** extended and they often attempted to copulate alongside each other, with the anterior of the female (Figure 4.6 C) or on top of another male (Figure 4.6 D). Usually these males flew away or walked off after one or two minutes if they were unsuccessful and only occasionally did they try for as long as 5 to 10 minutes. Many of the males that walked away remained nearby eating or resting and this occurred most frequently towards the end of the flight periods. These males could form clusters of up to 5 or sometimes more beetles around the copulating pairs.

The female beetles often walked about or ate foliage while they were copulating but the males remained dorsal to the females the whole time and were carried around in this position. Most of the males also became quite firmly attached to the females by their genitalia because they often folded their legs against their bodies while they were being carried about and copulating beetles could usually be picked up by holding onto the males alone. However, the males were seldom observed to eat whilst copulating because they rarely twisted from their dorsal positions; those that did eat usually only managed this when the females moved so that leaves were conveniently placed by their mouths.

On only one occasion was a male ever found copulating with another male, and this occurred in a container half filled with soil where approximately 100 males were being kept isolated from females for bioassay work as described in Chapter 2. The mating males were discovered in the morning 13 days after they had been collected and they remained fastened together in the usual copulatory position until they died after two and four days respectively for the upper and lower males. Usually, however, males did not show any signs of mating behaviour when they came into contact with other males except when these males were already on top of or next to attractive females.

Estimates of the relative proportions of beetles copulating at different times during the night were made on the hedge late in the flight season of 1971, on the 28th November and the 3rd to 4th December and early the next flight season on the 7th to 8th November, 1972. The results of this are given in Figures 4.8 B and C and 4.9 C respectively while the records of the meterological conditions on these nights are included in the explanations to the appropriate figures.

The proportion of beetles copulating on the 28th November, 1971 increased over the flight period and reached a maximum 5 to 10 minutes after they had stopped flying when about 75% were paired. After this the numbers copulating rapidly diminished until only about 10% were paired an hour later and less than 5% a further two hours later (Figure 4.8 B). During the night the beetles also became progressively harder to find because they dispersed over the vegetation or fell to the ground and this was reflected in the total numbers of beetles counted. However, these totals only gave approximate relative estimates because they were obtained as counts per unit time and they would therefore probably be exaggerated at the beginning of the night when most of the beetles were copulating and concentrated together near the top of the hedge. Relative changes in the total numbers of beetles on the hedge were therefore estimated on the next two nights from the numbers that fell into trays of detergent and water placed under the hedge.

The relative proportions of beetles that copulated during the night of the 3rd to 4th December followed approximately the same pattern except that the proportion copulating reached a maximum of about 80% five minutes or so before the last beetles had stopped flying (Figure 4.8 C). Most of the females on the hedge probably copulated on both the above nights in 1971 because about 40% of the beetles collected from the hedge in beating trays just after the dusk flights were females during the latter part of November (Table 4.6) and this was close to the proportion of females observed as copulating pairs. It was also likely that relatively few of the beetles mated after the peak copulation periods on these nights because the rate at which copulating pairs diminished could be accounted for by the variations in copulation times determined previously.

During the night of 7th to 8th November 1972 the relative numbers of copulating beetles again increased to a maximum of about 75% five to ten minutes after the last ones had settled, but the proportion copulating diminished more slowly than on the previous nights because 45% were copulating an hour after the peak period and about 10% were still copulating another 4 hours later (Figure 4.9 C).

TABLE 4.6

Sex ratio of beetles collected in beatings from the hedge just after the flights had finished.

DATE	SEX RATIO (% males)	TOTAL NO. OF BEETLES COLLECTED
16-11-71	51.4	661
1-11-72	59.6	220
7-11-72	67.9	187
14-11-72	62.0	179
21-11-72	60.9	115
23-11-72	65.4	67
26-11-72	54.4	125
27-11-72	57.9	95
28-11-72	62.1	58
" "	60.0	35
" "	63.8	47

Figure 4.9

Histograms of the copulatory activity of *C. zealandica* on a barberry hedge during the night of the 7th to 8th November, 1971

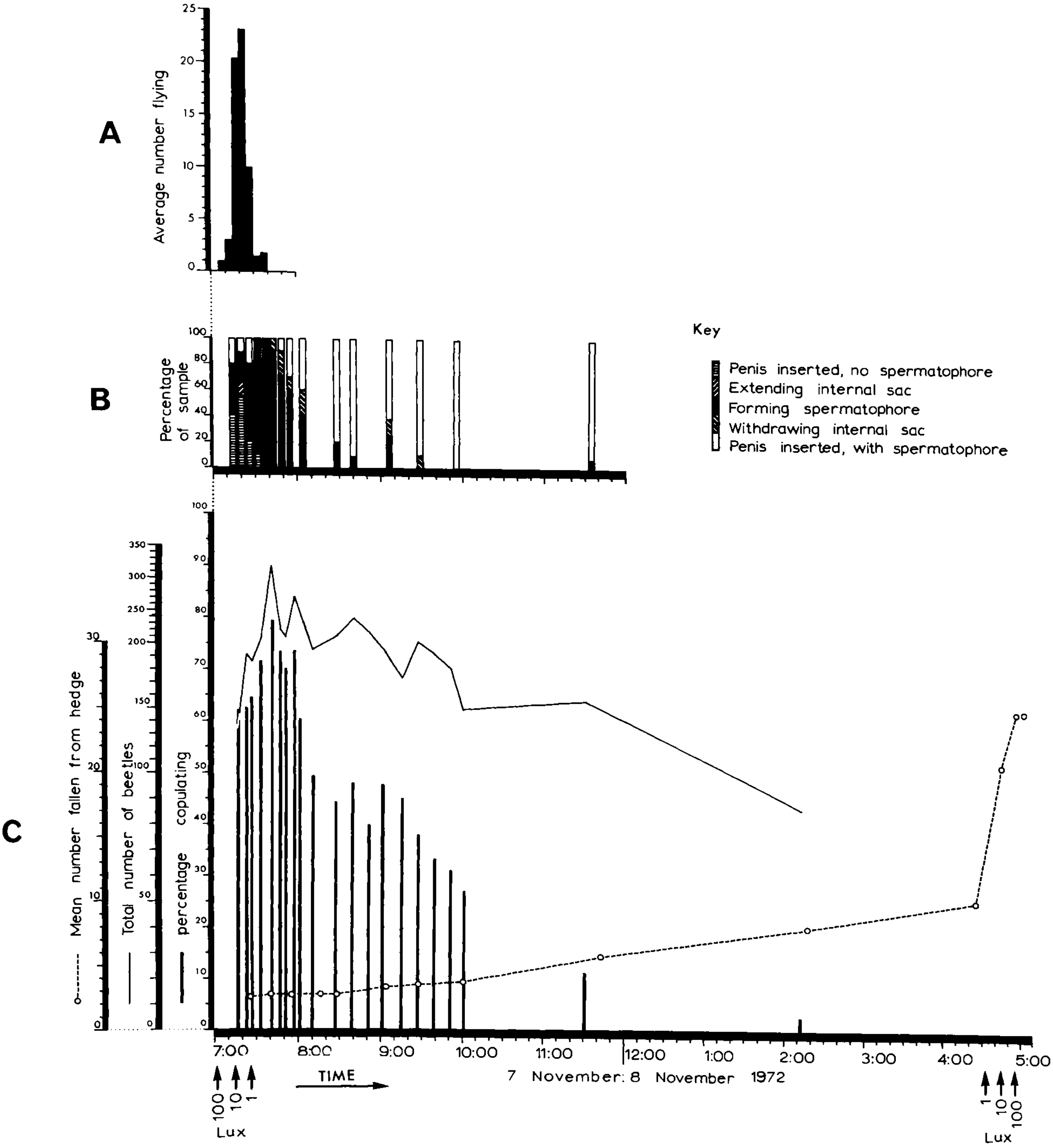
A. The average number of beetles photographed flying during a series of 5 minute periods at dusk. The original data is given in Figure 4.13.

B. The proportions of copulating male beetles which were extending their internal sacs, forming spermatophores, or withdrawing their internal sacs at different times during the beginning of the night. Each analysis was made from about 10 copulating pairs collected in liquid nitrogen.

C. The proportions of beetles found copulating at different times during the night together with the total numbers of beetles counted and the rate at which the beetles fell from the hedge. These estimates were made in the same ways as for Figures 4.7 B and 4.7 C above.

Meterological data
(No detectable wind.)

TIME	TEMPERATURE °C	RELATIVE HUMIDITY %
1900	18.5	63
2000	17.5	72
2100	17.2	74
2200	16.0	78
2300	15.2	82
2400	15.2	80
0100	15.7	81
0200	15.7	79
0300	15.3	80
0400	15.0	81
0500	15.1	79
0530	15.0	79


A
Average number flying
0
5
10
15
20
25
B
Percentage of sample
0
20
40
60
80
100
Key
Penis inserted, no spermatophore
Extending internal sac
Forming spermatophore
Withdrawing internal sac
Penis inserted, with spermatophore
C
Mean number fallen from hedge
Total number of beetles
percentage copulating
0
10
20
30
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60
70
80
90
100
7:00
8:00
9:00
10:00
11:00
12:00
1:00
2:00
3:00
4:00
5:00
TIME
7 November: 8 November 1972
100
10
1
Lux
1
10
100
Lux

Probably most of the females mated on this night as well because 32% of the beetles were females from a total of 187 beaten from the hedge after the flight period and this was slightly less than the proportion of females observed copulating. Possibly this discrepancy indicated that paired beetles were more likely to be counted than single ones although it could also be due to sampling error. During this night, samples of copulating beetles were also quick frozen at intervals so their reproductive organs and the positions occupied by their genitalia could be examined later in order to find how many of the beetles mated late in the night. In the first of these samples 20% to 40% of the copulating males had their internal sacs extended within the bursa copulatrixes of the females and were producing spermatophores. The remainder did not have their internal sacs fully extended or had them completely withdrawn in their tegmens. Of these, 40% to 60% of the total males were attached to females without spermatophores and 20% were attached to females containing fresh spermatophores (Figure 4.9 B). The proportion of males producing spermatophores then increased as more beetles copulated until all the males in the samples were doing this by the time when the maximum numbers copulated. However, these estimates were only approximate because of the small size of the samples and it was therefore likely that some males were at other stages of copulation although most were exchanging spermatophores. When the relative numbers of copulating pairs were also considered over this period, it appeared that the males usually began producing spermatophores within 5 to 10 minutes from the beginning of copulation and then they spent an average of half an hour or more completing the exchange. Some males, however, completed fertilisation within about 5 to 10 minutes because they were found on females with fresh spermatophores near the beginning of the flight when relatively few beetles had begun to copulate. After the maximum numbers had copulated increasingly more of the males were found attached to females containing spermatophores although some were still found producing spermatophores after about 4 hours of darkness. Probably most of the males attached to females containing fresh spermatophores had produced them because the volume of the contents in the males' reproductive organs appeared subjectively to be much less than in males that had not mated. If this was the case then few of the beetles mated after dusk on this night as well and the comparatively high proportions of beetles found copulating at this time were therefore probably due to their having stayed paired for longer. However, there was still the possibility that some of these males had copulated previously and were in the process of copulating again because there was no way of determining the number of fresh spermatophores in females and newly formed spermatophores were soft and could merge together.

Erratum

Paragraph 2, line 4:

"... fell at a faster rate ..." *should read* "... fell more frequently ..."

from the hedge at a steady rate

none

and

4.9 C). However, no male beetles were observed to be attracted to females or to mate with them at dawn and no copulating beetles were found during any of the two minute counts just before and at dawn (Figures 4.8 C and 4.9 C) although an occasional pair was found by searching a large length of hedge in the time between these counts. In addition, mating behaviour was never observed at dawn on any morning and it also appeared from the following experiments with sticky traps that the males were not attracted to phenol at dawn either. In these experiments half the traps were always left blank while the remaining ones were baited with larger quantities of phenol than was used at dusk to increase the chances of detecting attraction. The traps were then placed in position before dawn and left there during the flight periods. For the first experiment on 25th November, 1971, the traps were placed at the top, half way up, or near the ground on both sides of the hedge and 50 µl of 60 g/l phenol was used in each of the baited traps. No beetles were caught in any of them (Table 4.7). Next, on the 8th November, 1972 the sticky traps were placed on top of the hedge or at various distances up to 20 m from it at hedge height or near the ground. The baits used on this occasion were 100 µl samples of 6 g/l phenol solution but again no beetles were caught in any of the traps (Table 4.7). Finally, on 9th November, 1972 the traps were either placed on top of the hedge or near its base and 100 µl samples of 6 g/l phenol were again used as the baits. Two male beetles were caught this time but they were both in separate blank traps on top of the hedge and all the other traps were empty (Table 4.7). These three experiments therefore confirmed that the males were not attracted to females at dawn because they did not fly towards phenol at this time.

It therefore appears from all the above observations and to their having stayed paired for longer. However, there was still the possibility that some of these males had copulated previously and were in the process of copulating again because there was no way of determining the number of fresh spermatophores in females and newly formed spermatophores were soft and could merge together.

The beetles were found to fall from the hedge at a steady rate during the nights if the 3rd to 4th December 1971 and the 7th to 8th November 1972 until about half had fallen off by dawn. Those that remained fell at a faster rate or occasionally flew away until none were left on the hedge by the time the sun had risen (Figures 4.8 C and 4.9 C). However, no male beetles were observed to be attracted to females or to mate with them at dawn and no copulating beetles were found during any of the two minute counts just before and at dawn (Figures 4.8 C and 4.9 C) although an occasional pair was found by searching a large length of hedge in the time between these counts. In addition, mating behaviour was never observed at dawn on any morning and it also appeared from the following experiments with sticky traps that the males were not attracted to phenol at dawn either. In these experiments half the traps were always left blank while the remaining ones were baited with larger quantities of phenol than was used at dusk to increase the chances of detecting attraction. The traps were then placed in position before dawn and left there during the flight periods. For the first experiment on 25th November, 1971, the traps were placed at the top, half way up, or near the ground on both sides of the hedge and 50 µl of 60 g/l phenol was used in each of the baited traps. No beetles were caught in any of them (Table 4.7). Next, on the 8th November, 1972 the sticky traps were placed on top of the hedge or at various distances up to 20 m from it at hedge height or near the ground. The baits used on this occasion were 100 µl samples of 6 g/l phenol solution but again no beetles were caught in any of the traps (Table 4.7). Finally, on 9th November, 1972 the traps were either placed on top of the hedge or near its base and 100 µl samples of 6 g/l phenol were again used as the baits. Two male beetles were caught this time but they were both in separate blank traps on top of the hedge and all the other traps were empty (Table 4.7). These three experiments therefore confirmed that the males were not attracted to females at dawn because they did not fly towards phenol at this time.

It therefore appears from all the above observations and

TABLE 4.7

Numbers of beetles caught at dawn in blank and phenol baited traps placed at various positions on a hedge or in a field.

All distances and heights in metres.

TRAPS ON BOTH SIDES OF THE HEDGE EACH BAITED WITH A 50ul SAMPLE

DATE																
25/11/71	Trap position	F	${}^{N}F$	${}^{N}G$	H	I	${}^{N}I$	J	${}^{N}J$	K	L	M	${}^{N}M$	N	${}^{N}N$	O
	Height of trap from ground	2	.25	1	1	2	2	.25	1	1	2	1	.25	2	2	2
	Concentration in g/1 of sample	60	0	60	0	0	0	60	0	60	0	0	60	60	60	60
	CATCH	No beetles caught in any trap														

TRAPS AT DIFFERENT POSITIONS IN THE FIELD EACH BAITED WITH A 100ul SAMPLE

8/11/72	Trap position	E	${}^{1}F$	${}^{1}F$	${}^{4}F$	${}^{4}F$	${}^{6}F$	${}^{6}F$	G	${}^{1}H$	${}^{1}H$	${}^{4}H$	${}^{4}H$	${}^{6}H$	${}^{6}H$	I	${}^{1}J$	${}^{1}J$	${}^{4}J$	${}^{4}J$	${}^{6}J$	${}^{6}J$	K	${}^{1}L$	${}^{4}L$	${}^{6}L$
	Distance of trap from hedge	Height of trap from hedge																								
	0	2							2							2							2			
	0.5		2	.25						2	.25						2	.25						2		
	6.5				2	.25						2	.25						2	.25					.25	
	20						2	.25						2	.25						2	.25				.25
	Concentration in g/1 of sample	0	6	6	6	6	6	6	6	0	0	0	0	0	0	0	6	6	6	6	6	6	6	0	0	0
	CATCH	No beetles caught																								

TRAPS ON THE SOUTHERN SIDE OF THE HEDGE
EACH BAITED WITH A 100ul SAMPLE

9/11/72 .	Trap position	A	B	C	D	E	F	G	H	I	J	K	K	L	L	M	M	N	N	O	O	P	P	Q	Q	R
	Height of trap from ground	2	2	2	2	2	2	2	2	2	2	2	.25	2	.25	2	.25	2	.25	2	.25	2	.25	2	.25	.25
	Concentration of g/1 of sample	0	6	0	6	0	6	0	6	0	6	0	0	6	6	6	6	6	0	6	6	6	0	6	6	0
	CATCH (all male)	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

experiments that most *C. zealandica* mate during the dusk flight periods. They then mate progressively less frequently as the night progresses and mating only occurs rarely during the second half of the night and never at dawn.

Flight behaviour

C. zealandica showed a crepuscular flight activity pattern; large numbers of beetles flew at dusk for periods of between 15 to 40 minutes (Appendix 2.4) but relatively few flew at dawn and they were only rarely observed to fly at night.

At dusk the beetles were observed flying either at any height up to about 3 or 4 m above the ground, in rapid more or less straight paths and in any direction or else hovering slowly upwind close to the ground or vegetation. However, the transition between these two types of flight was smooth and progressive, so that it was difficult to decide at what point the change occurred,although most of the beetles that were hovering appeared to be within about 0.5 m above plants or the ground or less than about 1 m downwind from elevated vegetation.

When hovering the beetles constantly faced into the wind and flew to and fro approximately horizontally and at right angles to it covering a distance of about 0.3 m or less in each direction. Their flight paths therefore tended to follow an irregular zig-zag forwards although occasionally they also hovered backwards for short distances. The flight pattern then became progressively restricted when the beetles were alighting until they were only flying to and fro a few centimetres just before they landed. This hovering flight was shown by all beetles that were about to settle including males that were flying towards females as described above. The beetles were also frequently seen hovering from one clump of vegetation to another or repeatedly hovering up to a plant and between its shoots, then flying rapidly downwind a metre or so and starting to hover towards it again. On the few occasions when there appeared to be no wind the beetles approached plants from any direction and oriented towards the plants as if the wind was blowing from them.

A relatively high density of hovering beetles was always found near the edges of tall plants such as trees and shrubs if these were edible to them (Appendix 1.1)and fewer hovered towards vegetation that was low or never eaten. Usually so many hovered about these tall plants during the large flights in the first two or three weeks of the flight season that they formed swarms extending about 1 m downwind from them but small swarms were only very occasionally observed near low plants when the largest flights occurred. Many of the beetles in these swarms also alighted to mate, rest and this resulted in them being found most abundantly on tall edible plants after dusk. It appeared that the flying beetles were particularly attracted to foliage that was silhouetted against a lighter background or against the sky when viewed from the direction from which the beetles approached. Possibly the irregular outline of the foliage was also important because the beetles were rarely seen hovering towards objects with smooth outlines, such as fence posts, tree trunks, or buildings but they were often seen hovering towards inedible plants although they usually flew away from them after a short time.

Following the observations that beetles were attracted to tall plants some experiments were performed with the sticky traps to find if the effectiveness of an attractant was affected by the position of its source in the field. These experiments were first run in 1971 when all the traps were baited with 50 µl of 6 g/l phenol and then they were repeated in 1972 when the number of traps was increased enabling half to be baited with 6 g/l phenol and the rest to be left as blanks.

When the traps were placed on top, half way up, or near the base of the hedge and on both sides of it, the highest mean catches were always in the highest traps followed by those half way up the hedge, then those on the ground (Figure 4.10a). This effect was most pronounced in the catches of males in phenol baited traps although the numbers of females caught in them also showed it to a lesser extent. Fewer beetles were caught in blank traps than in baited ones and there was a lesser difference between the numbers of males and females caught in blank traps although they also showed the same pattern of higher numbers being caught near the top of the hedge than low down. The catches of the blank traps therefore demonstrated that more beetles settled near the top of the hedge than near its base while those caught in the baited traps also showed that an attractant was much more effective when positioned near the top of the hedge than lower down it. However, there was little difference between the numbers of beetles caught in similarly placed traps on both sides of the hedge in relation to the wind direction, so presumably the beetles could fly between the branches and shoots to reach those traps on the upwind side.

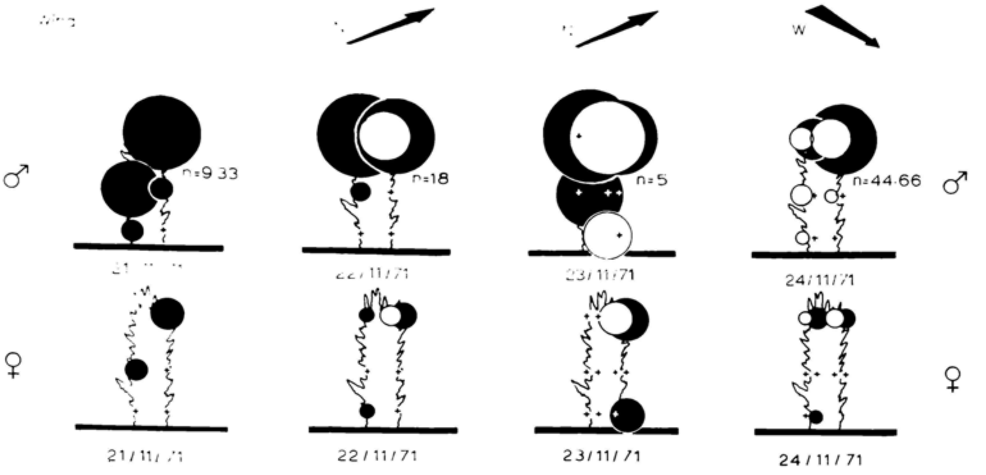
A series of experiments was also performed on different nights to find how the numbers of beetles caught in sticky traps on top of the hedge compared with the numbers caught in sticky traps positioned at different distances up to 20 m from the hedge at grass height or at hedge height. The positions occupied by the traps on each night and their relative catches are shown in Figure 4.10a for the experiments run in 1971 and in Figure 4.10b for those run in 1972 while the means of all the catches are shown in Figure 4.10c. The actual numbers of beetles caught varied considerably from day to day and therefore the catches have been shown in these figures as percentages of the total numbers of male beetles caught on top of the hedge so that catches on different days could be compared. In these experiments the largest numbers of male beetles were again caught in phenol baited traps on top of the hedge followed by those near the ground and at any distance from the hedge. Here the catches were about 35% or less than those on top of the hedge. Traps containing phenol at hedge height caught decreasing numbers of males the further away from the hedge they were placed up to about 6.5 m. Beyond this distance males were caught in approximately equal numbers although the catches were less than 10% of those in traps on top of the hedge. Traps with phenol placed 2 m above the hedge also caught similarly low numbers of males. In blank traps, approximately equal numbers of male beetles were caught at all positions except those 1.5 m or nearer the hedge and here there was a decrease in the numbers caught at ground level and an increase in the numbers caught at hedge height. In addition, fewer male beetles were caught in blank traps than in baited traps at almost all positions except those 6.5 m or further from the hedge at hedge height where approximately equal numbers of males were caught in both sample and blank traps. However, only small numbers of beetles were caught in these positions and it is possible that these similar catches were due to chance.

Proportionally fewer females than males were caught in both types of trap at all positions except at hedge height and 6.5 m or further from the hedge where the numbers of females caught in baited traps were approximately equal to the numbers of males caught in both baited and blank traps. In general the largest numbers of females were caught in baited and blank traps on top of the hedge or within 0.3 m of the top, no females were caught in any traps 2 m above the hedge, approximately equal numbers were caught in phenol baited traps at all other positions, and none or very few were caught in the

Figure 4.10a

Pictorial representation of the mean numbers of male and female *C. zealandica* caught in sticky traps at different heights on a hedge and at different distances from it

The traps were baited with solutions of either 6 or 0 g/l phenol. The area of each spot is proportional to the mean number of beetles caught in all the sticky traps set at the position indicated. For comparative purposes this is expressed as a percentage of the mean number of male beetles caught in the sticky traps baited with 6 g/l phenol on top of the south side of the hedge. This accounts for differences in the numbers of beetles which flew on the different nights. (Data from Appendixes 2.5, 2.6, 2.7, 2.8, 2.9, 2.10)


Wind
W
♂
n=9.33
n=18
n=5
n=44.66
♂
21/11/71
22/11/71
23/11/71
24/11/71
♀
♀
21/11/71
22/11/71
23/11/71
24/11/71

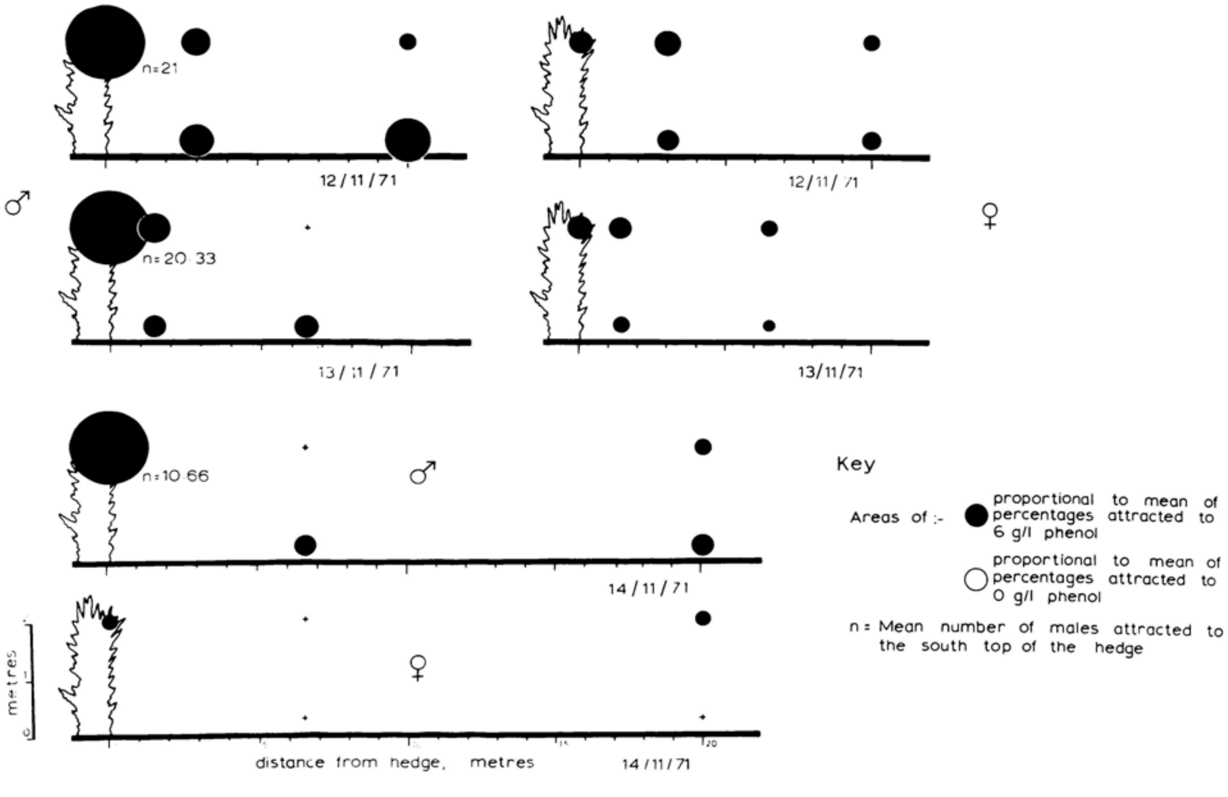

n=21
12/11/71
12/11/71
♂
♀
n=20.33
13/11/71
13/11/71
n=10.66
♂
14/11/71
♀
metres
0
1
2
5
10
15
20
distance from hedge, metres
14/11/71
Key
Areas of :-
● proportional to mean of percentages attracted to 6 g/l phenol
○ proportional to mean of percentages attracted to 0 g/l phenol
n = Mean number of males attracted to the south top of the hedge

Figure 4.10b

Pictorial representation of the mean numbers of male and female *C. zealandica* caught in sticky traps at different heights and distances from a hedge

The explanation of this diagram is the same as that for Figure 4.10 a. (Data from Appendixes 2.8, 2.9, 2.10)

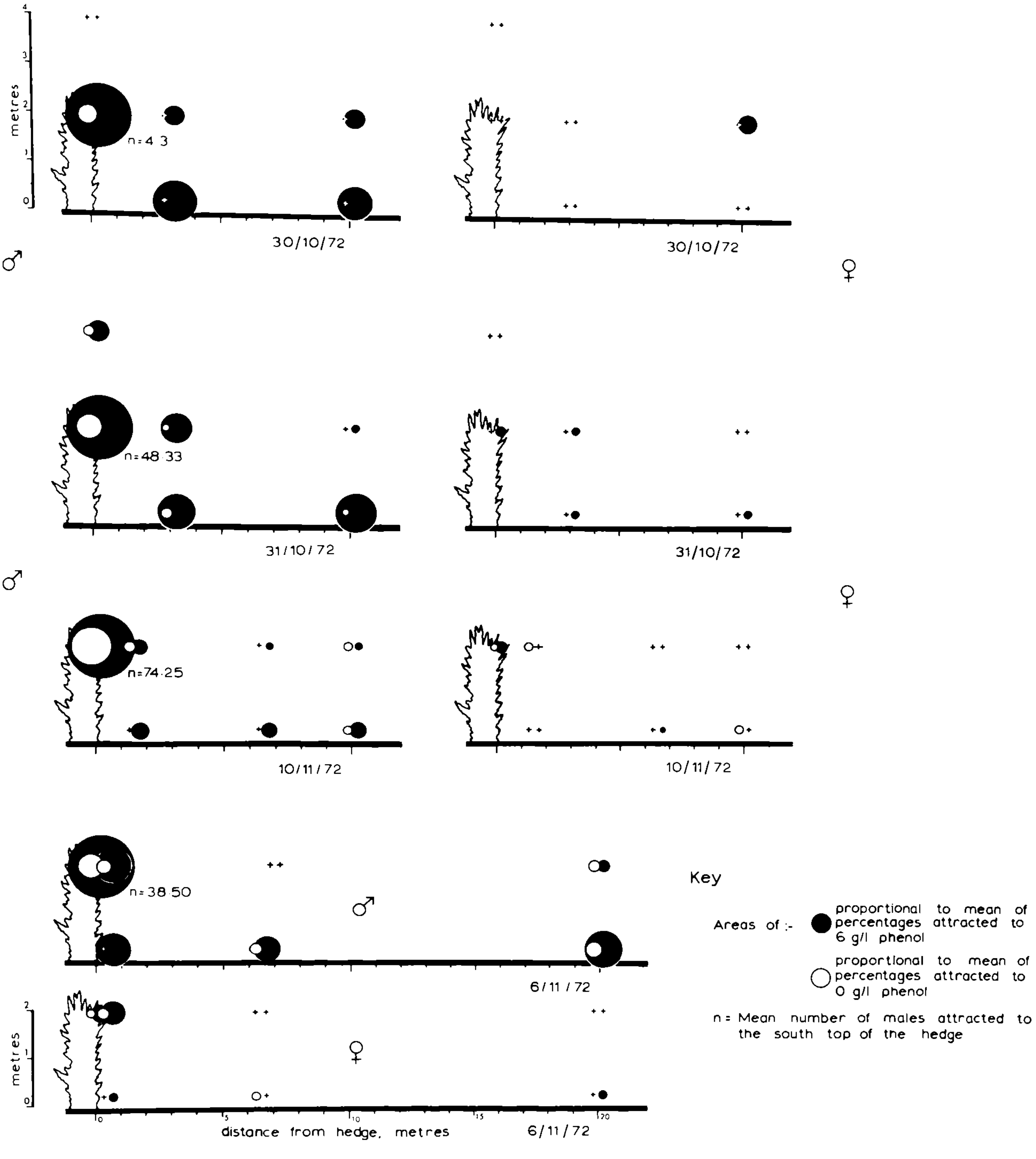
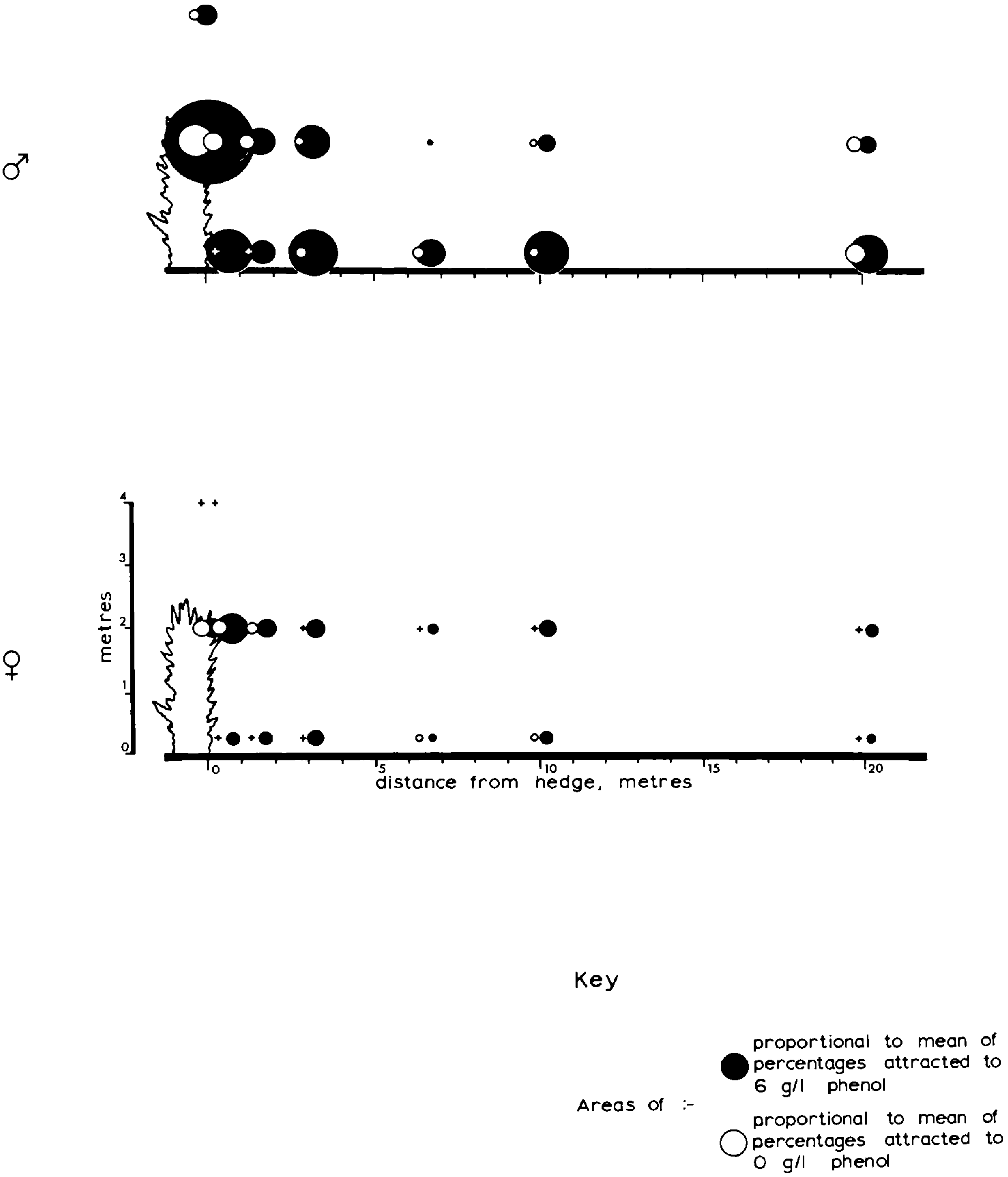

metres
0
1
2
3
4
n=4 3
30/10/72
30/10/72
♂
♀
n=48 33
31/10/72
31/10/72
♂
♀
n=74·25
10/11/72
10/11/72
n=38 50
♂
6/11/72
♀
6/11/72
distance from hedge, metres
0
5
10
15
20
Key
Areas of :-
● proportional to mean of percentages attracted to 6 g/l phenol
○ proportional to mean of percentages attracted to 0 g/l phenol
n = Mean number of males attracted to the south top of the hedge

Figure 4.10c

Pictorial representation of the means of the combined results of the experiments shown in Figures **4.10a and 4.10b**

The explanation of this diagram is the same as that for Figure 4.10a.


♂
♀
metres
0
1
2
3
4
0
5
10
15
20
distance from hedge, metres
Key
Areas of :-
proportional to mean of percentages attracted to 6 g/l phenol
proportional to mean of percentages attracted to 0 g/l phenol

corresponding blanks. This therefore confirmed that phenol was slightly attractive to females. The numbers of beetles caught in blank traps also confirmed the observations that more beetles flew near the top of the hedge than elsewhere in the field while the numbers of males caught in sample traps showed that the most effective position for phenol as an attractant for males was firstly on top of the hedge and then at grass height whereas phenol did not act as an attractant when it was high above the grounds. It thus appears that females are most likely to attract other beetles when they are on the tops of tall vegetation.

The beetles rarely flew at night although occasionally some were observed to fly towards a light. This was probably only a response to being illuminated because they often started flying when they were taken into a lighted room at night. If any had flown when it was too dark for them to have been seen they would have been identified from the continuous buzzing sound made by their wings during flight. Buzzes were, however, frequently heard at night, especially from hedges but these usually lasted much less than a second and were therefore most probably either beetles falling from the hedge or males running about searching for females. On two occasions the beetles were observed to fly at night under normal circumstances. This occurred on the 13th and 14th November, 1973, two and three days after a full moon. On the first of these nights the beetles started flying at irregular intervals after the moon had risen two hours after dark and they were then seen intermittently but with decreasing frequency over the next hour until observation was discontinued. On the second night very few beetles flew and these were only observed over a period of about 15 minutes after the moon had risen three hours after dark. On both occasions the beetles were observed either hovering or flying rapidly on both these occasions in the same way as they did at dusk and probably the main factor contributing to these nocturnal flights was the moonlight because these were the only two nights within 4 days of a full moon when the moon was not obscured by cloud. The other environmental conditions were unexceptional; the temperatures were about 12°C and 14°C on the first and second nights respectively and there was very little wind on either of them.

Some beetles were always found flying at dawn although observations were only made on 5 mornings between the 8th November and 4 December, in 1971 and 1972. On all of these mornings no beetles were seen on pasture plants and they were only observed on hedges.

At dawn many of the beetles on the hedge made brief buzzing sounds when they were observed attempting to fly or when they were observed falling from the hedge but relatively few actually flew. The ones that did fly usually went directly away from the hedge although occasionally they flew back and forth along the hedge a few times first and they were rarely seen to hover towards shoots on the hedge and alight there. However, no beetles were observed hovering towards others on the hedge at dawn and nothing that resembled precopulatory behaviour in any way was ever observed at this time.

Environmental factors affecting mating and flight

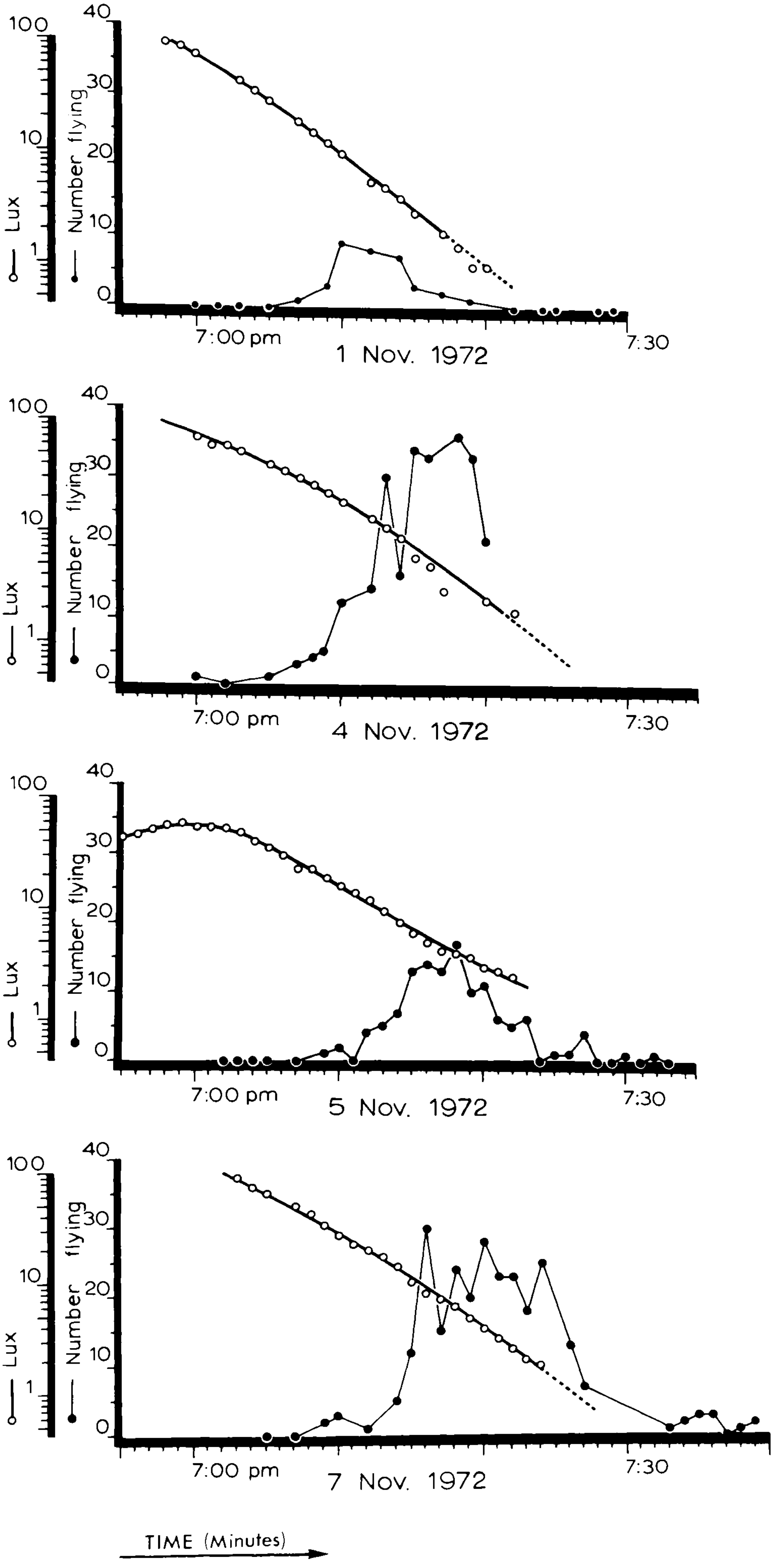
The environmental factors affecting mating and flight in *C. zealandica* will be considered together because most of the beetles mated during the dusk flight periods and because flight plays an important part in their sexual behaviour at dusk.

The environmental factor that appeared to be most important in determining when flight occurred was light intensity. The beetles only flew between a narrow range of light intensities and the relative number of beetles flying at any one time was approximately related to the light intensity at that time. Throughout this study the first flying beetles usually appeared soon after the light intensity had fallen below 100 lux at dusk and only occasionally were any seen flying between 150 lux and 100 lux. By the time the light intensity had fallen to about 50 lux numerous flying beetles were observed and they increased to a maximum between about 10 lux and 1 lux. After this the number flying diminished and the last ones ceased while there was still sufficient light left for the beetles to be seen although the light intensity was below the sensitivity of the lightmeter. These observations were confirmed on four nights in 1972 between the 1st and 7th November when estimates were made of the relative numbers of flying beetles at different times during the dusk flights by photographing the swarm at the top of the hedge. On these occasions the first beetles were usually photographed when the light intensity was about 50 lux, then the numbers in the photographs increased to a maximum between about 10 lux and 1 lux and then decreased again (Figure 4.11). In addition to the photographs, sticky traps baited with phenol solution or

Figure 4.11

Relationship between the light intensity at dusk and the number of *C. zealandica* in flight

The relative numbers of beetles flying were determined from photographs (see Figure 4.2) and the light intensity was determined by placing the sensor of a light meter horizontally and facing upward.


100
10
1
Lux
40
30
20
10
0
Number flying
7:00 pm
7:30
1 Nov. 1972
4 Nov. 1972
5 Nov. 1972
7 Nov. 1972
TIME (Minutes)

with water as blanks were set out on top of the hedge on these 4 nights and the arrival patterns of beetles to them were examined to investigate how the responses of the males to the sex attractant varied at dusk. The results of this are given in Figure 4.12 and showed that the relative numbers of males arriving at phenol baited traps followed approximately the same pattern as the relative numbers of beetles flying although there was a tendency for the numbers of males arriving at the traps to increase and decrease slightly before the numbers of beetles flying. Possibly this effect was due to the beetles finding it easier to settle on the sticky traps when there was more available light early on in the flights.

Few beetles were found on the hedge during the first part of the dusk flights when the light intensity was high and they only started alighting in any numbers after it had dropped below about 10 lux. Males and females settled apparently at the same rate because the ratio of males to females in beatings taken from the hedge at various times during the flight periods on 4 nights in November, 1972 stayed approximately constant (Figure 4.13).

The timing of the dawn flight also appeared to be determined mainly by light intensity and this was confirmed by determining the relative numbers of beetles that flew at different times together with the changes in light intensity on three mornings in 1971 and 1972. The numbers of flying beetles were estimated by periodic counts since it was found that the numbers were too low for them to be estimated photographically. The results are given in Figure 4.14 and show that the beetles flew over the same range of light intensities at dawn as they did at dusk, but that at dawn most flew when the light intensity was between 5 lux and 50 lux whereas at dusk most flew when it was between 1 lux and 10 lux. This discrepancy could be due to the counts being biased towards high light conditions when the chances of observing the beetles in flight would be better.

The numbers of beetles that flew at dusk showed considerable day to day variation although there was a general tendency for them to be largest early in the flight season during the first two to three weeks in November and after this they diminished until few beetles were observed to fly by the end of December. This seasonal variation probably reflected changes in the numbers of suitable adults in the field whereas the short term fluctuations appeared to result from the

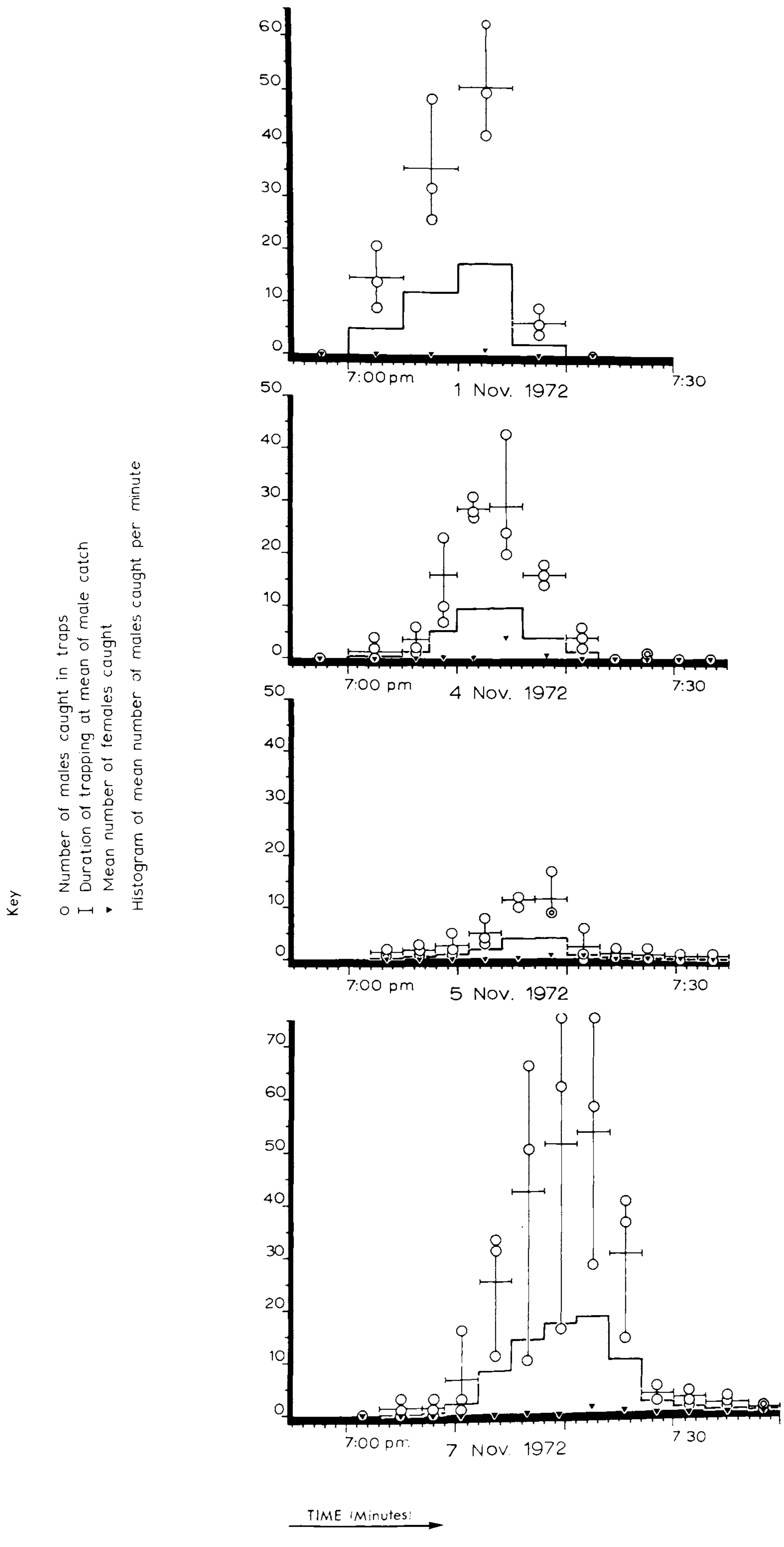
Figure 4.12

The numbers of male and female *C. zealandica* caught in sticky traps baited with 6g/l phenol at different times at dusk

This graph shows the actual numbers of males caught in each trap, the mean numbers of males caught per minute, and the mean numbers of females trapped.

Overlay: Figure 4.11. This shows the relationship between the above catches and the light intensity and relative numbers of *C. zealandica* flying.

Date	Sex of beetles caught	Catches in traps baited with 0 g/l phenol			Total catches in traps baited with 6 g/l phenol		
1/11/72	Male	4	2	4	122	104	99
	Female	0	0	1	0	2	2
4/11/72	Male	3	2	1	85	77	128
	Female	0	1	0	3	4	10
5/11/72	Male	–	–	–	34	47	23
	Female	–	–	–	3	1	1
7/11/72	Male	2	19	4	284	88	283
	Female	0	1	0	3	0	3



Key
○ Number of males caught in traps
I Duration of trapping at mean of male catch
▾ Mean number of females caught
Histogram of mean number of males caught per minute
0
10
20
30
40
50
60
7:00pm
7:30
1 Nov. 1972
7:00 pm
7:30
4 Nov. 1972
7:00 pm
7:30
5 Nov. 1972
70
7:00 pm
7 30
7 Nov. 1972
TIME (Minutes)

<u>Figure 4.13</u>

Relationship between the ratio of male to female *C. zealandica* on the hedge at different times at dusk and the light intensity

The light intensity was measured as for Figure 4.11 while the sex ratio was determined from beetles knocked from a hedge onto a beating tray underneath. The total numbers of beetles collected for each determination are indicated by the small figures next to the symbols.

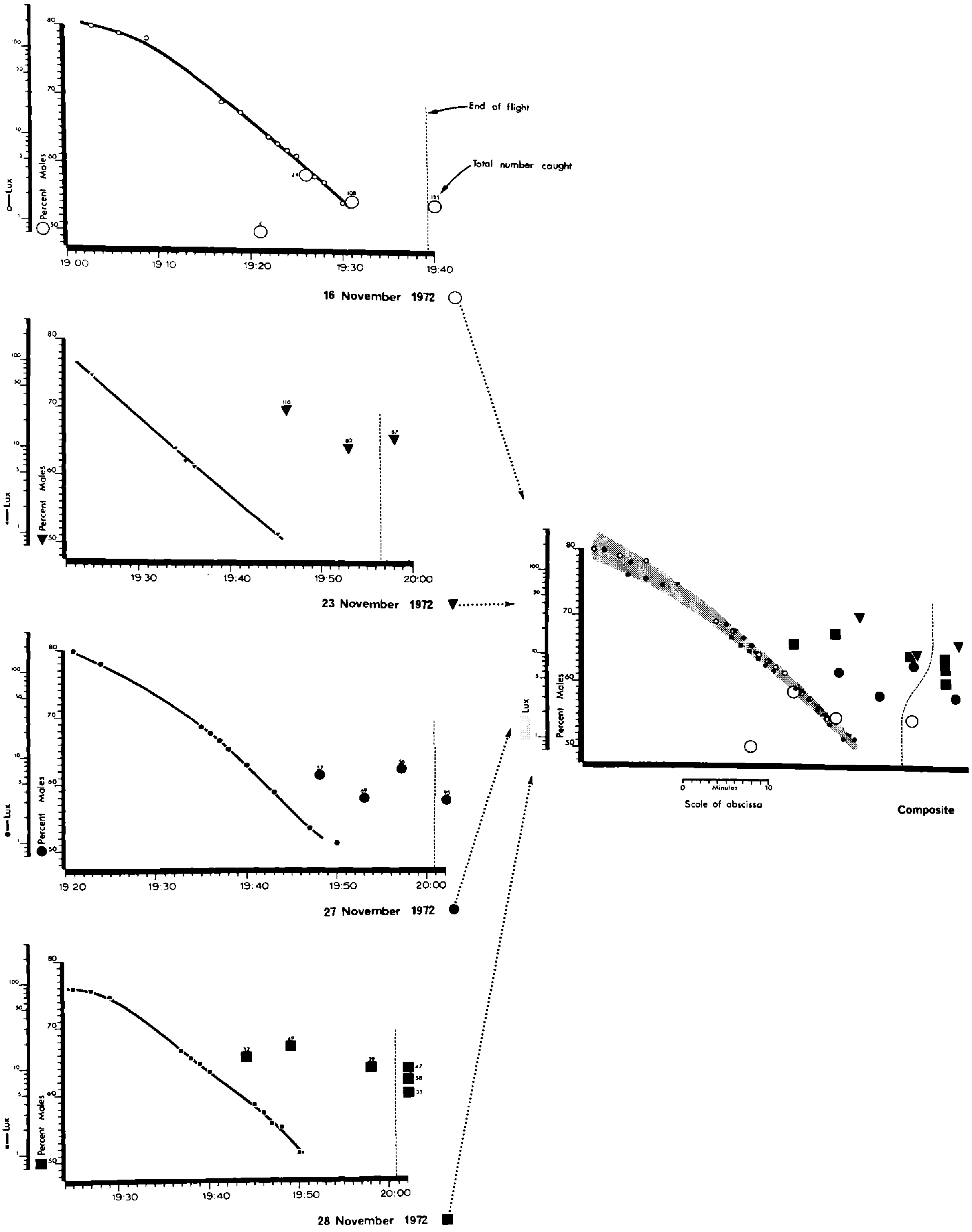
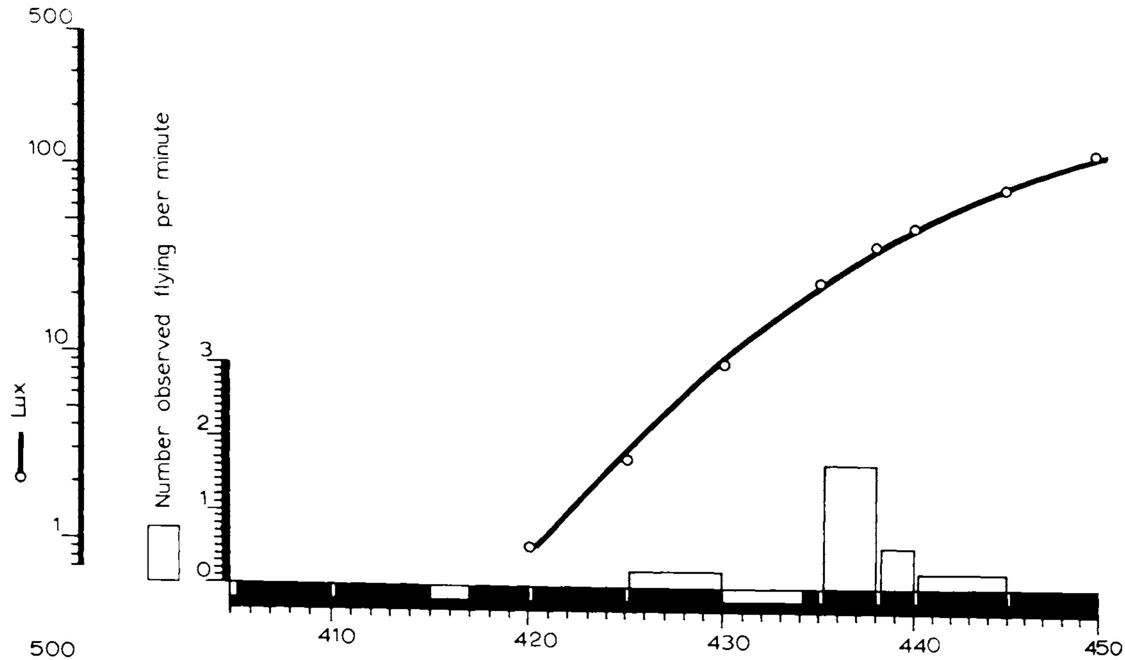

End of flight
Total number caught
Percent Males
Lux
80
70
60
50
100
50
10
5
1
24
108
125
19 00
19 10
19:20
19:30
19:40
16 November 1972
110
82
67
19 30
19:40
19 50
20:00
23 November 1972
19:20
19 30
19:40
19:50
20:00
27 November 1972
19:30
19:40
19:50
20:00
28 November 1972
0
Minutes
10
Scale of abscissa
Composite

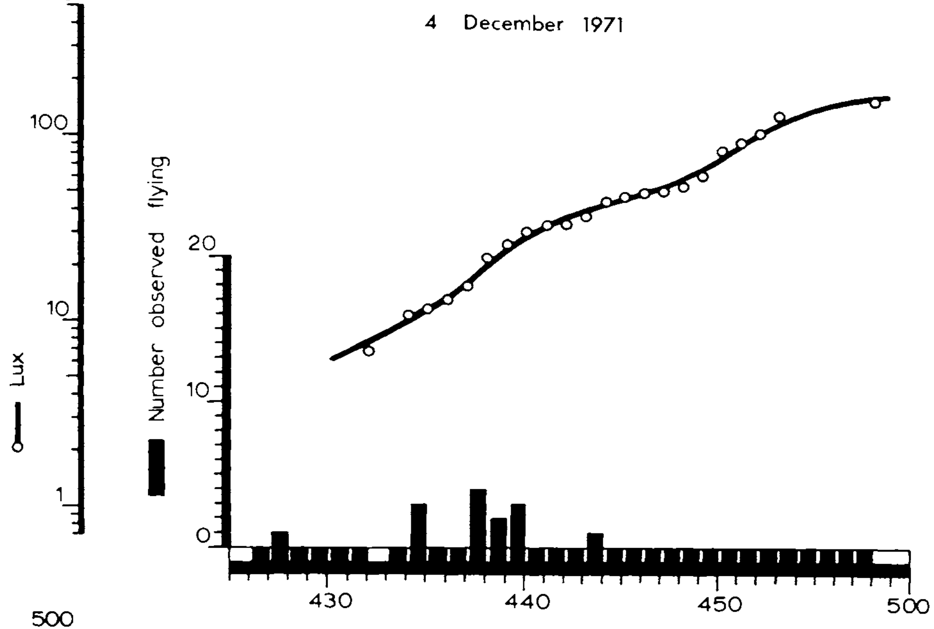
Figure 4.14

Relationship between the light intensity at dawn and the numbers of beetles observed flying

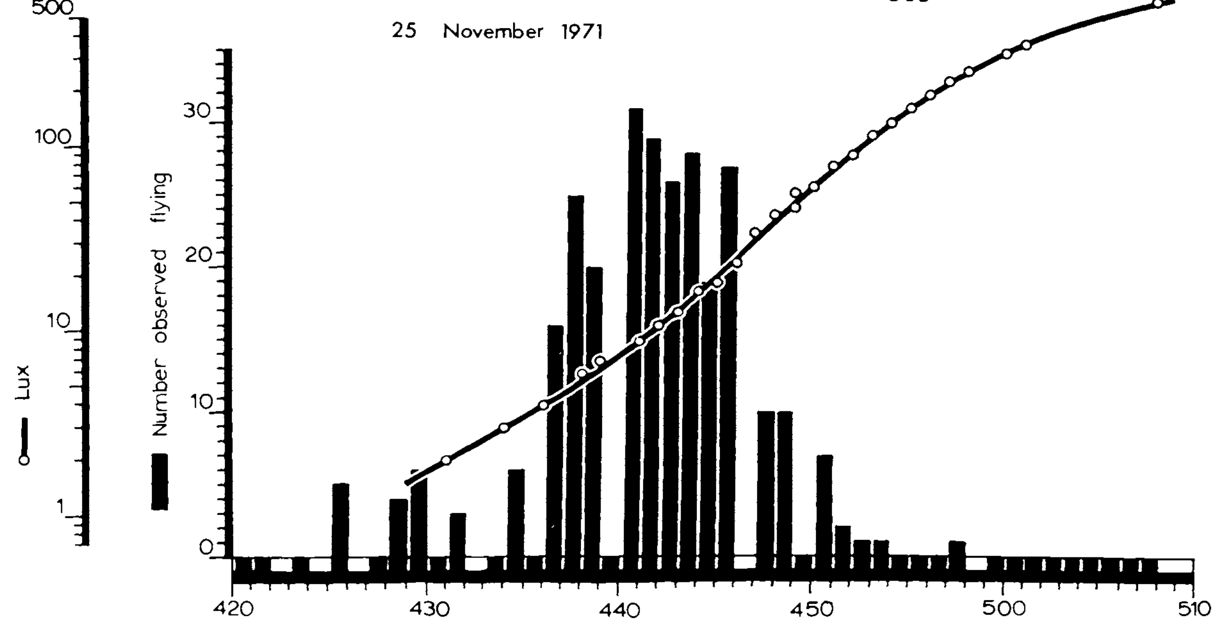
The light intensity was measured as explained for Figure 4.11.



4 December 1971



25 November 1971



9 November 1972

influences of temperature, wind, and rainfall during the dusk flight periods. High temperatures at this time tended to favour large flights while strong winds or heavy rain tended to depress the numbers flying. Often fewer beetles seemed to emerge on those nights when the conditions were unfavourable for large flights but a high proportion of the beetles that were visible after the dusk flights were always found copulating so mating therefore readily occurred under all the environmental conditions experienced during this investigation.

The temperatures at dusk ranged between 9.6°C and 21°C throughout this study (Figures 4.15 and 4.16) but they were never low enough to inhibit flight altogether because some beetles were observed to fly on every night once emergence began. The average wind speeds over the dusk flight periods were recorded in 1972 only, when they varied between 0 m/min and 245 m/min (Figure 4.16) but the beetles usually did not fly in winds of more than about 100 m/min except when they were in sheltered places such as downwind from plants. Rain seemed to depress the numbers of beetles that flew when it was moderate to heavy but light rain appeared to have little effect. It was also evident that surface water could affect the flights but this was only observed once on the 20th November, 1971 when few beetles flew even though the temperature was 14°C and there was no wind or rain. On this occasion a thunderstorm about two hours before dusk had left large areas of the fields covered by water and this had presumably stopped many of the beetles from emerging. The only environmental factor that appeared to have no effect on the numbers of beetles that flew at dusk was the relative humidity. This was only recorded in 1971 and varied between 80% to 100% at dusk, showing a tendency to increase during the evening (Appendix 2.4).

The numbers of beetles caught in the sticky traps reflected the observations on the number that flew each night to some extent. The average numbers of male and female beetles that were caught each night in blank and 6 g/l phenol baited sticky traps are given in Figures 4.15 and 4.16 and these show that generally all the catches declined after the first two to three weeks. It was also apparent that in 1972 a higher ratio of males were caught in phenol baited traps than in the blanks early in the flight season than later on. The same effect was also found in 1971 although it was not as pronounced as in 1972. It therefore appears that the males flew towards phenol more effectively

Figure 4.15

The average numbers of male and female *C. zealandica* caught in sticky traps baited with solutions of 6 or 0 g/l phenol in 1971, together with the temperatures at dusk

The numbers of beetles caught are taken from the results of all the trapping experiments whilst the air temperatures are taken from Appendix 2.4.

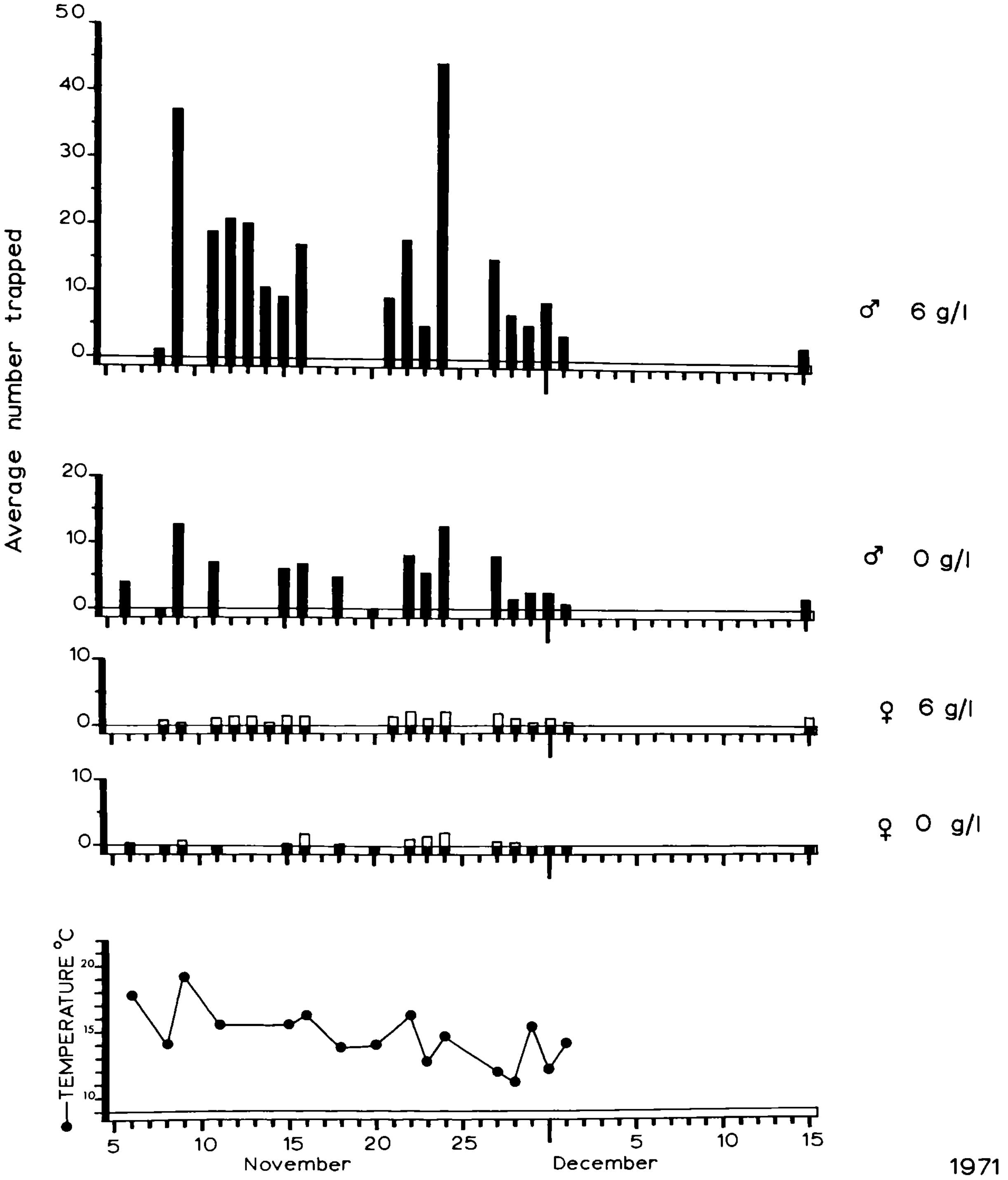
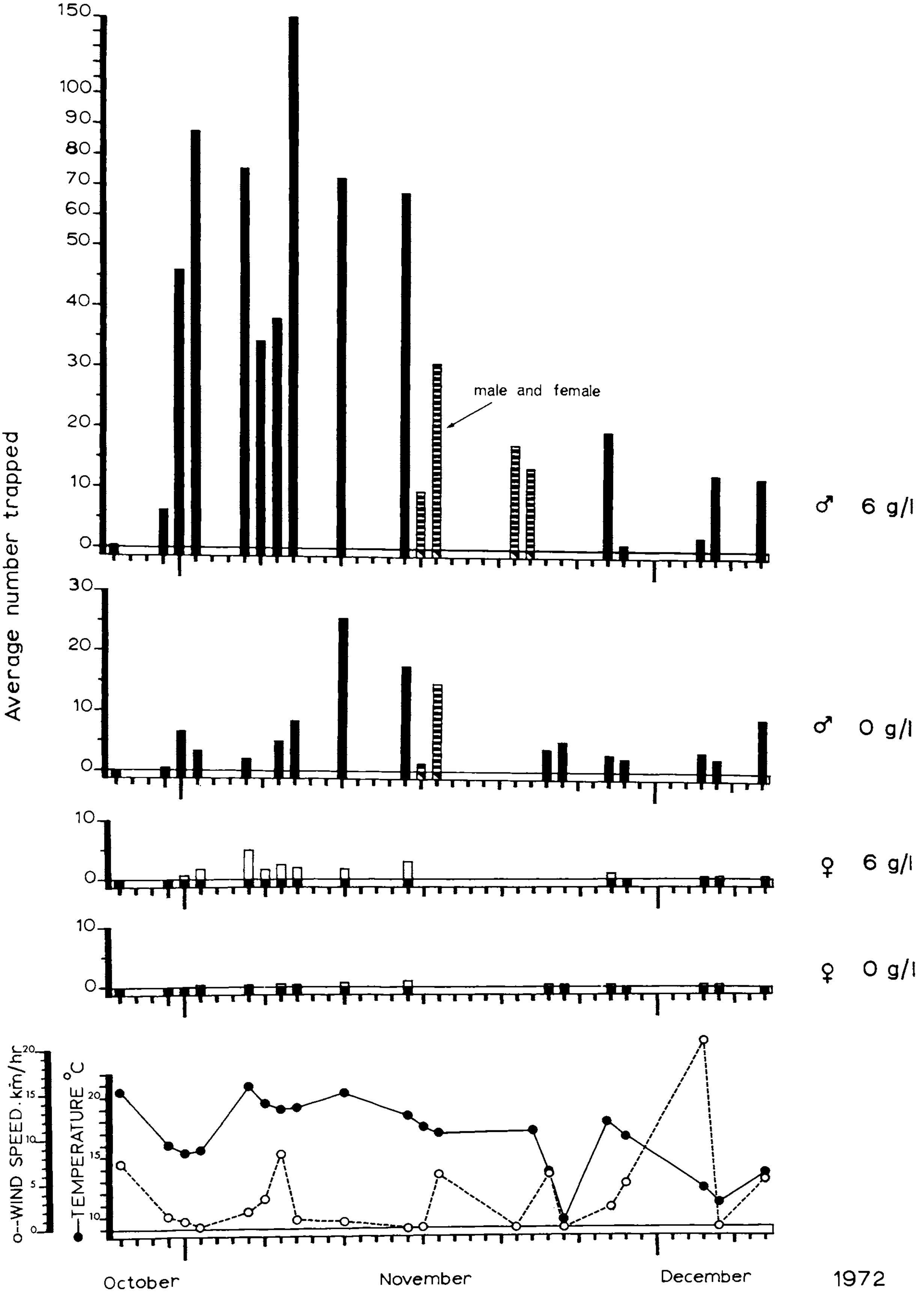

50
40
30
20
10
0
Average number trapped
♂ 6 g/l
20
10
0
♂ 0 g/l
10
0
♀ 6 g/l
10
0
♀ 0 g/l
TEMPERATURE °C
20
15
10
5
10
15
20
25
5
10
15
November
December
1971

Figure 4.16

Relationship between the light intensity
at dawn and the numbers of beetles
observed flying

The light intensity was measured as explained for Figure 4.13.


Average number trapped
150
100
90
80
70
60
50
40
30
20
10
0
male and female
♂ 6 g/l
30
20
10
0
♂ 0 g/l
10
0
♀ 6 g/l
10
0
♀ 0 g/l
o--WIND SPEED. km/hr
20
15
10
5
0
●—TEMPERATURE °C
20
15
10
October
November
December
1972

early in the flight season and therefore more of the males probably also found females at this time.

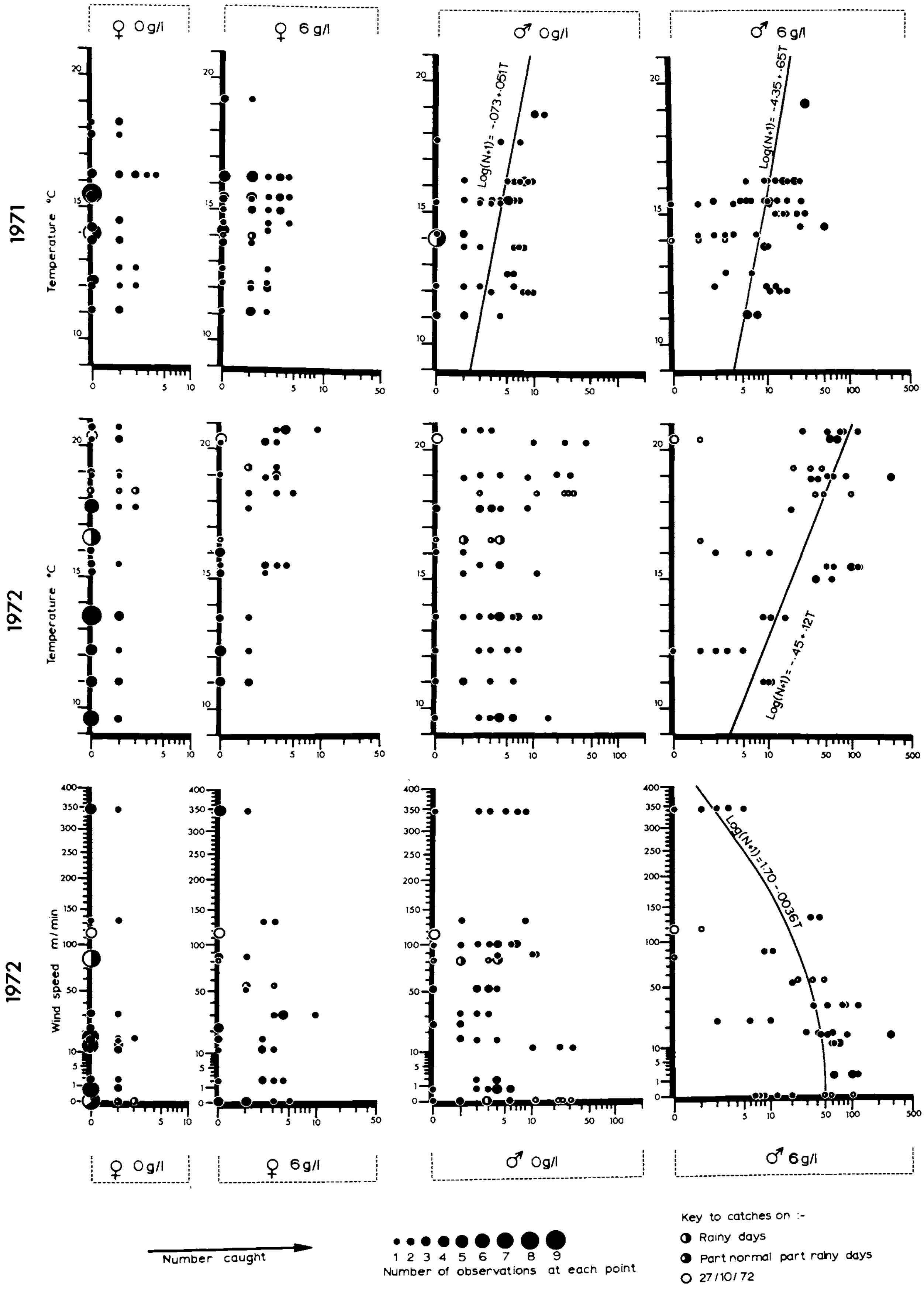
The temperatures and average wind speeds during the dusk flight periods are also given for each night in Figure 4.15 and 4.16 while the numbers of beetles caught in the individual traps at each of these temperatures and wind speeds are given in Figure 4.17. These show that generally the numbers of both males and females in blank and baited traps increased with increasing temperature and decreasing wind speed. When these were analysed statistically, however, only the catches of males in phenol baited traps in 1971 and 1972 and the male catches in blank traps in 1971 were significantly correlated with temperature and wind speed as shown in Figure 4.17. The relationship of these catches with temperature was most significant when the regression was linear while the best relationship with wind speed was logarithmic (Appendix 2.11). The male catches in blank traps were also significantly correlated with temperature in 1972 if the catches on the first night were excluded (Appendix 2.11) and this was justifiable because the beetles had just started to emerge. In addition to these analyses the simultaneous effects of temperature and wind speed on the 1972 catches were also investigated by multiple regression analysis because the windy days tended to be the cold ones and therefore any relationship with one would hold for the other. This analysis showed that wind speed only was significantly correlated with the number of male beetles caught in the baited traps when the catches on all days were considered, though when the catches of the first night were excluded both temperature and wind speed showed significant independent correlations with the numbers trapped (Appendix 2.11). However, the distribution of these catches was not normal, possibly because there were so many zeros, and therefore non-parametric regression methods were used to check the significance of the above findings, which they confirmed (Appendix 2.11). The numbers caught in the blank traps therefore demonstrated that the number of flying male beetles increased with increasing temperature and the baited trap catches demonstrated that the number of male beetles that were attracted to phenol also increased with increasing temperature and decreasing wind speed. The non-significance of the other relationships between the numbers of females caught and temperature or wind speed, and the number of males caught in blank traps and wind speed did not necessarily mean

Figure 4.17

Relationships between the numbers of male and female *C. zealandica* caught in sticky traps baited with solutions of 6 or 0 g/l phenol and the temperature and wind speed at dusk

Wherever the numbers of beetles caught are significantly correlated with an environmental factor ($P<0.05$), the regression line and its equation is also shown.

Note: The numbers of beetles caught, N, are shown on a log scale of $(N+1)$.


♀ 0g/l
♀ 6g/l
♂ 0g/l
♂ 6g/l
1971
Temperature °C
1972
Temperature °C
1972
Wind speed m/min
Log(N+1)= -.073+.051T
Log(N+1)= -4.35+.65T
Log(N+1)= -.45+.12T
Log(N+1)=1.70-.0036T
Number caught
1 2 3 4 5 6 7 8 9
Number of observations at each point
Key to catches on :-
◑ Rainy days
● Part normal part rainy days
○ 27/10/72

that no relationship existed between them but rather that the numbers in the catches were too low for significance to be apparent from the number of samples. All these catches did, however, tend to support the observations that the number of flying beetles increased with increasing temperature and decreased with increasing wind speed (Figure 4.17).

Discussion

Sex attraction

It has been claimed that the female *C. zealandica* uses phenol as a sex attractant for the male (Chapter 1) and it has been demonstrated in the present study that phenol is attractive to male *C. zealandica*. The possibility still exists however, that the female may liberate some synergistic chemicals along with phenol since many sex pheromones have more than one component (e.g. Jacobson, 1972; Roelofs and Carde, 1974), and Osborne and Boyd (1975) have recently found that the resin "Dure$\overset{z}{x}$ 12687" contains an odour synergist for phenol.

It is also interesting that in the present investigation more females were caught in sticky traps baited with phenol than in blanks although the numbers were very low compared with the numbers of males that responded to phenol. In fact, with the exception of one experiment, the female numbers trapped on any night were too small to demonstrate significant attraction. However, when the entire series of trapping experiments was considered, the numbers caught in phenol baited traps was almost always greater than the numbers caught in blank traps (greater on 20 nights, less on 2) and this was significantly different at the $P<0.01$ level using a non-parametric sign test. In addition, the numbers of females trapped also tended to increase with the quantity of phenol used in the traps. Thus a clear indication was obtained that phenol is weakly attractive to female *C. zealandica*. A somewhat similar result was obtained by Ladd (1970) in a field investigation with the rutelinid *Popilla japonica* Newman. Firstly, he found that males were attracted to squashed females, thus demonstrating chemical attraction; then he found that large numbers of males and some females were attracted to traps baited with virgin females, and that the numbers of both sex were greater than the numbers caught in blank traps. Smith and Hadley (1926) also reported that in the field *P. japonica* frequently congregate in "balls" of up to 200 struggling individuals, and Ladd (1970) found that these either consisted of one female with many males, several females with many males, or numerous males by themselves. He concluded that the female releases a powerful sex attractant for the male, and that either an aggregation pheromone is released by the male which attracts both sexes, or a weak pheromone is released by the male which attracts the female. He did not, however, seem to consider the possibility that males might become attractive by previous contact with a female in a ball. This was later resolved by Alder and Jacobson (1971) who obtained an indication from electrophysiological methods that the female *P. japonica* probably produces a sex pheromone for the male and that the male produces a pheromone which attracts both sexes. In addition, aggregation pheromones which are produced by the male have been found in other Coleoptera, particularly amongst the Scolytidae (e.g. Jacobsen, 1972; Borden, 1974), so it is tempting to suggest that a similar acting pheromone is produced by the male of *C. zealandica*. Nevertheless, further research is required to determine whether this is so because, from the present results, it is just as likely that the female *C. zealandica* is weakly attracted by phenol, or that the male produces a weak attractant for the female. In addition, a pheromone may not be involved at all, and the females may be responding for instance to visual or auditory stimuli from the swarm of male beetles flying to the phenol.

A number of female Scarabaeidae are now known to use a sex pheromone which attracts the males. This has been discovered by observations of males being attracted to squashed females, their extracts, or to containers from which females had recently been removed. The species include the melolonthinids *Rhopaea magnicornis* Blackburn (SooHoo and Roberts, 1965), *Phyllophaga decemlineata* (Say) (Lilly and Shorterhouse, 1971), and *Plectris aliena* Chapin (Roberts, 1968); and the rutelinids *Phyllopertha lanceolata* (Say) (Travis, 1939) and *Popilla japonica* Newman (Goonewardene, Zepp, and Grosvenor, 1970). It is also known that the attractant is located in the abdomen of the female and not the head or thorax in *R. magnicornis* (SooHoo and Roberts, 1964), and *P. decemlineata* (Lilly and Shorterhouse, 1971). In addition, Jeannel (1960) also reported that the wingless female *Pachypus cornutus* Oliver (Pachypodinae) emits a pheromone which attracts males, and there are other species with sedentary females and males with greatly developed sensory organs amongst the Melolonthinae, Dynastinae, and Rutelinae (e.g. Britton, 1973) which are likely to use pheromonal communication between the sexes.

At least one melolonthinid might not use a female pheromone: Tashiro and Fleming (1954) obtained negligible attraction of male *Amphimallon majalis* (Razoumowski) to live virgin females or extracts of them in field trapping experiments, and Roelofs, Pulver, Feng, and Gambrell (1967) chromatographed acetone extracts of females and tested the fractions on males in the laboratory without result. The chromatography presumably ruled out the possibility of masking agents being present in the crude extracts, but it may also have separated vital components of the pheromone; or **else** the pheromone may be a highly reactive substance which loses its attractancy.

Mating sites

Ritcher (1958) reports that the choice of mating sites in the Scarabaeidae varies with the subfamily and from genus to genus within the subfamilies. Amongst the Melolonthinae the genera *Serica*, *Diplotaxis*, and *Phyllophaga* mate at night whilst the female continues feeding on foliage (Ritcher, 1958). Females of *Pyronota*, which are active during the daytime, also feed during copulation (Miller, 1925) so that the observation that the female *C. zealandica* also feeds whilst copulating is not unusual for this subfamily. *A. majalis*, a crepuscular species, mostly mates 3 to 6m above the ground on trees (Schwardt and Whitcomb, 1943); *P. decemlineata* also mates on trees at twilight (Lilly and Shorterhouse, 1971); and *Rhopaea verreuxi* Blanchard will mate on the leaves of *Eucalyptus*. *R. magnicornis* and *Rhopaea heterodactyle soror* Blackburn, on the other hand, mate only on the ground (SooHoo and Roberts, 1956). Other melolonthinids mate on flowers in the daytime (Ritcher, 1958); whereas *P. lanceolata* mates in the morning on the ground (Travis, 1939); and *Phyllotocus*, and *Pyronota* presumably also mate in the light (Britton, 1973; Brown, 1966). Copulation during daylight also occurs in the rutelinids *Anomala aenea* Geer (Rittershaus, 1927); *Phyllopertha horticola* (L.) (Rittershaus, 1927; Milne, 1960); and *P. japonica* (Ladd, 1970).

Little is published concerning mating in other Scarabaeidae. *A. tasmaniae* Hope (Aphodiinae) mates at twilight apparently in dung pads (Carne, 1956), and the Dynastinae, which are nocturnal (Britton, 1973), mate either on the surface of the soil, in soil, or beneath the surface of the ground (Ritcher, 1958). The Cetoniinae are nocturnal, and many mate on flowers, whilst the valginid genus *Microvalgus* is only active during daylight (Britton, 1973), and *Valgus* mates within termite galleries in stumps or fallen trees (Ritcher, 1958).

General nocturnal activity

It is interesting to note that Farrell and Wightman (1972) confirmed some of the findings of the present study concerning the nocturnal activities of *C. zealandica*. They reported that mating took place during the first hour after the cessation of flight on the foliage of pasture plants. Furthermore they noted that the numbers of both sexes declined at a similar rate throughout the night: on pasture the time taken for half the beetles to return to the ground mostly varied from 3 to 6 hours after the onset of darkness,whilst the beetles remained on willow trees for longer, half returning after about 6 to 7hours, and the greatest numbers returning at dawn. They detected no flights away from trees during the night, but found that the beetles did fall to the ground from them. They also never observed flight at dawn perhaps because the air temperatures were too low for this (below 10°C), but they observed 8 adults gliding down at an angle from trees. However, flight at dawn has been recorded during the present investigation, and this confirms the observations of Hilgendorf (1902).

Flight and its relationship to mating behaviour

The present study confirmed that males of *C. zealandica* frequently fly to females to mate with them. This had already been indicated from brief comments in the literature and from the results of field trapping studies with phenol published by other research workers (see Chapter 1, and the introduction to this chapter), but the present description of this behaviour is the first detailed one. Flight is also known to occur at the same time as mating in *A. aenea* Geer (Rittershaus, 1927); *A. tasmaniae* Hope (Carne, 1956; Maelzer, 1961); and in *P. horticola* (L.) (Milne, 1960); whereas the male is known to fly to the female in *A. majalis* (Razoumowsky) (Schwardt and Whitcomb, 1943; Gyrisco, Whitcomb, Burrage, Logothetis, and Schwardt, 1954); *P. cornutus* Oliver (Jeannel, 1960); *P. aliena* Chapin (Roberts, 1968); *P. lanceolata* (Say) (Travis, 1939); *P. decemlineata* (Say)

(Lilly and Shorterhouse, 1971); and *R. magnicornis* Blackburn (SooHoo and Roberts, 1956).

Many of the general observations concerning flight in *C. zealandica* made in the present study were later confirmed by Farrell and Wightman (1972) who investigated the flight and feeding activity of this insect in the Nelson province. They reported two types of flight activity: weaving, apparently randomly directed flight at low altitude (<0.5m) and more directed flight at higher altitudes (1-3m). The former was punctuated every few metres (<50m) by the beetle hitting grass stems and falling to the ground, followed by its climbing to the top of a grass stem and taking flight again. The latter flight occurred less often early in the flight season and was predominantly towards the highest silhouettes on the beetles' horizon.

Farrell and Wightman (1972) examined the internal condition of *C. zealandica* adults and found that only 6% of the apparently older beetles feeding on pasture contained eggs, suggesting that some females laid eggs before feeding. They also similarly obtained evidence that most females oviposited before flying to trees thus supporting the evidence already noted in the introduction to this chapter which indicates that the behaviour of female *C. zealandica* changes with age.

The behaviour pattern of *C. zealandica* is suggestive of that of *P. horticola* as reported by Milne (1959, 1960). *P. horticola* flies during the daytime and shows two roughly equal and half-overlapping phases of activity. In phase 1 there is a "feverishly busy concentration" of males alternately weaving in flight close over the ground and alighting to run around. The average height of this swarming flight is about 22cm and it occurs over patches of ground where larvae have developed. The female spends practically her entire life below ground, coming up very briefly now and then to mate, and when she does, she "perambulates" slowly around on the sward and copulation is quickly initiated. In phase 2 swarming shifts to deciduous trees and bushes. The male flight is the same as over the grass. There are, however, more females which fly more often in short hops from one piece of vegetation to another. Some females also fly directly towards tall vegetation, or away from it towards the grass, and this was termed "bee-lining". Male and female outgoing flight is also different: the male outgoers keep low and generally go downward in a curving path to the grass 10 to 20m away, whereas females initially go in mostly straight climbing paths and descend usually 100m or more away. They then either "bee-line" again or dig into the soil. Male outgoers are those that have spent the night on the foliage and are joining the activity on the grass. "Bee-lining" only occurs when the sun is shining, and the beetles go in any direction in calm weather or downwind in a moderate breeze.

Many authors have noted that males of *C. zealandica* start flying earlier in the season than females (Chapter 1) and Farrell and Wightman (1972) confirmed this. They found that males predominated in flight traps from the 2nd to 6th November, whereas females predominated in catches after the 12th November. This also tends to confirm the change in behaviour of females with age. However, in the present study there was only a slight difference between the relative numbers of male and female beetles that landed on hedges during the entire flight season until the numbers of beetles that settled there reached low levels. The beetles also swarmed around the top of the hedge from the very first day of flight onward and this suggested that many of the females flew to the hedges on first emergence or very soon afterwards in the area of study. It is still possible, however, that at this Hamilton location many of the beetles showed similar behavioural changes with age to those at Nelson, and to *P. horticola*, except that the first behavioural phase was very abbreviated.

It appears that different species of Scarabaeidae can be arranged into a series according to their mating behaviour: this ranges from flightless species which mate only near the ground, through species such as *P. horticola* which spend the first half of their lives mating near the ground and the last half mating in trees, and culminates with species which apparently mate only high up in trees, such as *A. majalis* (Razoumowsky) (Evans and Gyrisco, 1959), and *P. decemlineata* (Say) (Lilly and Shorterhouse, 1971). Furthermore, it appears that within this series *C. zealandica* represents an intermediate step between *P. horticola* and those species which mate in trees.

Long range orientation whilst in flight

The behaviour of adult *C. zealandica* when flying towards tall vegetation was found both by observation during the present study, and by Farrell and Wightman (1972) who tracked the flying beetles, to be similar to that described by a number of research workers for *Melolontha melolontha* L. The principal investigations concerning the flight of this latter insect were made by Schneider (1952, 1954) in Switzerland, who facilitated observation of the beetles by tying paper streamers to them, and by Couturier and Robert (1952, 1954) in France, who investigated flight towards silhouettes by positioning lightly coloured smoke screens to obliterate portions of the horizon and subsequently observed the direction of the beetles' flights.

According to these authors, adults of *M. melolontha* fly at dusk. They first describe a spiral flight of increasing radius above the emergence site and then fly towards a specific point. During the preliminary flight the entire horizon is examined optically and a certain section is selected as the primary flight goal. Usually this is the section of the horizon offering the silhouette of maximum height over an arc of 50 to 90° (a hill or woodland), but a near silhouette is as attractive as a distant one of double the apparent height on the opposite horizon. In the absence of a high silhouette, the beetles fly to neighbouring single or grouped trees. If bad weather delays emergence the beetles prefer silhouettes offering the greatest contrast with the sky, and if cloud lowers the contrast of a preferred silhouette, the beetles choose another high contrast silhouette. The beetles fly close to the ground towards these silhouettes and the line of flight is therefore not direct. If the primary flight goal is unsuitable for feeding, a second is selected in renewed spiral flight. Schneider (1952) also notes that *Melolontha hippocastani* Fabr. behaves in a similar way, but prefers trees or similar objects in the immediate vicinity. If such vegetation is lacking, they fly towards the highest silhouette unless this stands out against a very bright sky.

In *C. zealandica* it is not known whether the adults describe a preliminary spiral flight before flying to tall silhouettes, but Farrell and Wightman (1972) reported that beetles from the same area of pasture fly in well defined directions towards a number of different prominent silhouettes, in a manner similar to the flight parting or "flascheide" of *M. melolontha* described by Schneider (1954). Farrell and Wightman (1972) also reported that the average height above the ground at which *C. zealandica* flies when going towards tall silhouettes is 1 to 1.5m over level ground and 0.5 to 1m when ascending a slope. These latter authors confirmed that *C. zealandica* only settles on foliage and not fence posts or tree trunks, and discriminates between different tree species (Appendix 1.1). This, therefore, suggests that long-range orientation toward the silhouette of the target feature is succeeded by a short-range behavioural response involving acceptance or rejection of the site in a similar manner to that reported for *M. melolontha* by Schneider (1954). This was further confirmed in the present study, in that the swarms of *C. zealandica* were densest downwind of plants presenting very irregular or finely broken up silhouettes which had some depth to them in relation to the upwind direction of the beetles' flight paths. It was also noted that *C. zealandica* swarmed around plants of low and medium height as well as tall plants when these were near each other, and that although the swarms of hovering beetles were densest near the top of the vegetation, they also flew downwind from the side edges of isolated plants. This behaviour, therefore, suggests that the highest silhouette is not always the preferred target, and that the flight behaviour of *C. zealandica* may be closer to that of *M. Hippocastani* as reported by Schneider (1952).

Orientation towards silhouettes has also been suggested to occur in the melolonthinids *A. majalis* by Gyrisco *et al.*(1954), *P. decemlineata* by Lilly and Shorterhouse (1971), and in three species of *Pyronota* by Brown (1966). It is also interesting to note that possibly the short-range visual responses of *A. majalis* differ from those of *C. zealandica* and *M. melolontha* because Schwardt and Whitcomb (1943) reported that *A. majalis* flies around telephone poles as well as trees.

The dusk flight behaviour of the aphodiinid *A. tasmaniae* is also of interest in that this scarabaeid apparently does not orientate to distant silhouettes (Maelzer, 1961). According to Carne (1956) the adults of this species all take flight at about the same time and either fly to dung pads where they mate, or fly in broad spirals interupted by short straight flights with changes of height.

Occasionally they also fly in straight lines for longer distances before resuming spiral flight again. Maelzer (1961) also found that *A. tasmaniae* which were not flying towards dung pads flew apparently at random if it was calm, and downwind when there was a wind, irrespective of the wind direction.

The effectiveness of the sex pheromone as influenced by the position of its source in the field

During the present study, the position occupied by the source of the sex attractant of *C. zealandica* in the field was demonstrated to have a very great influence on its effectiveness by the use of sticky traps. By far the largest numbers of beetles were always caught in those traps placed on top of the hedge where the beetles swarmed followed by traps at grass level at some distance from the hedge, whilst traps placed low down, close to the hedge or high in the air were relatively ineffective. The numbers of females caught in blank traps also indicated that most females also alighted on top of the hedge, and furthermore, since the ratio of males to females caught in blank traps on top of the hedge was much higher than the ratio of males to females that eventually settled on the hedge, it was also likely that in these swarms, the females spent less time flying than the males or else the males settled and took off again more frequently than the females. These experiments therefore indicated that aggregation aids the male *C. zealandica* in finding its mate, although there may be other advantages in this behaviour such as bringing the beetles to the growing shoots of plants. However, it is not unreasonable to suggest that the females of *C. zealandica* are more likely to be effective in attracting males, and therefore more likely to be mated, when they are in any situation in the field where swarms form and not only at the top of barberry hedges as investigated here.

Soo Hoo and Roberts (1965) noted that the "adults of many scarab species in Australia are attracted to a common food source, such as *Eucalyptus* trees, where they feed and mate; such species have no need to rely on sex pheromones to bring the sexes together and are not known to produce them". They also noted that the North American *P. lanceolata* feeds on low plants and that its pheromone is presumably of great selective advantage as a mechanism for uniting the sexes.

Farrell and Wightman (1972) have also noted a trend from tree feeding to ground feeding with an accompanying loss of migratory flight. However, as already mentioned, amongst the tree mating Scarabaeidae the female *A. majalis* apparently may not produce a pheromone, whereas the females of *P. japonica* and *C. zealandica* do. Ritcher (1958) also reported that 8 or more different species of *Phyllophaga* find their mates at night on the same trees so different sex pheromones would be of selective advantage at least in this case.

There appears to be two likely explanations to account for the ineffectiveness of phenol baited traps when placed high above the ground (2m), or close to the ground and near the hedge. Firstly, the response of the male may be stronger to the silhouette of the hedge than it is to the phenol at other places; and secondly, the combined pheromone liberated by all the females on top of the hedge may be much greater than that liberated by isolated traps at other places. This would result in the odour from these traps being negligible by comparison so that the beetles would respond to the stronger stimulus from the top of the hedge. Observations of flying beetles suggest that the males fly to the aggregation sites using the visual stimulus of its silhouette as discussed above, and that they then respond to the female pheromone at short distances of perhaps less than 1m. On the other hand, those male beetles flying close to pasture possibly come into contact with the effective region of a female's pheromone more or less by random flight. Furthermore, it also appears that the response of many of the beetles to a high silhouette is not very strong because many fly and are attracted to a phenol source close to the grass and at some distance from the hedge. However, the response to the silhouette becomes increasingly stronger when the angle it subtends above the horizontal increases to more than approximately 30° to 45° (<6.5 - 10m away from a 2m high hedge).

Close range orientation of males flying towards females

In close range orientation visual stimuli from the shoot on which the attractive female is located probably also supplements the influence of the pheromone during the approach of the male because females often describe a few horizontal zigzags before alighting on a shoot of foliage. The zigzag flightpath of a male approaching a female or phenol source is, however, very much more pronounced and this is of interest because it fits the proposed mechanism of Farkas and Shorey (1974) to explain how an insect can orient and fly towards a distant pheromone source. This suggested mechanism is discussed more fully in Chapter 8 in relation to the flight of male *P. operculella* towards its sex attractant. In addition, it also appears likely that in *C. zealandica* the short range visual stimulus is stronger the greater the contrast between the particular piece of foliage (or sticky trap) and its background because the highest density of copulating beetles were found on shoots of foliage above the main mass of vegetation. The contrast would be greatest in the natural situation when the foliage is outlined against the sky, and this would occur more frequently from the beetles' point of view (and depending on its flight path) the further away from other high objects, or the nearer to the top or edges of tall plants the particular piece of foliage is situated. It must be stressed, however, that these explanations of orientation towards both a distant and a near pheromone source in *C. zealandica* are only suggestions and further research is required to confirm them.

Little has been published concerning the flight of males to females in other Scarabaeidae. Travis (1939) reported that numerous males of *P. lanceolata* "were seen flying from various directions and suddenly alighting on the ground around a female". Milne (1960) in describing the behaviour of *P. horticola* noted that in "their random running and flying, males were obviously searching for females. When the male pauses in his running on the grass he not only swings his head from side to side but also spreads open the palmate clubs of his antennae". The female similarly spreads her antennae but Milne (1960) considered that "it is very doubtful if either sex can recognise the other except at very close quarters" and cites only two instances when it appeared possible that flying males could have been responding to females. Males, however, frequently made random changes of direction, and collided with individuals on the grass. The beetles subsequently scrambled over one another and if both were male, they parted immediately to resume searching, whereas a male and female paired immediately. Milne (1960) concluded that pairing in *P. horticola* "depends practically entirely upon chance physical encounters (collisions) between individuals" and added that it is a very efficient method since relatively few unpaired females were visible on the grass. Soo Hoo and Roberts (1965) reported that males of *R. magnicornis* flew at random in search of females if breezes were unsteady or less than 180 m per minute, but in steady breezes of about 134 to 188m per minute the males flew upwind to females. Lilly and Shorterhouse (1971) reported that males of *P. decemlineata* spread their antennae, hovered in flight when near the source of a female extract in 70% ethyl alcohol, and apparently searched excitedly when they landed. This behaviour appeared identical to that exhibited by males when approaching females and indicated to these authors that the males were responding to a chemical sex attractant.

Influence of environmental factors on flight and sex attraction

In the present study light intensity was found to be the most important factor in the initiation of flight both at dawn and at dusk. The best demonstration of this was the two small flights observed during the night when an almost full moon rose and the light intensity increased above the threshold value. It is interesting to note in this respect that Schneider (1952) reported that strong daylight immobilised the adults of *M. melolontha*, that these beetles were activated at dusk, and also that the process of immobilisation and reactivation were both reversible. There is also the likelihood that initiation of flight in *C. zealandica* is under the influence of a circadian rhythm although this was not investigated. Certainly Carne (1956), and Evans and Gyrisco (1958), found that in *A. tasmaniae* and *A. majalis* respectively attempts to induce flight by artificial reduction of light intensity was only successful at times close to that at which flight was normally initiated at dusk. The light intensities at which other scarabaeids fly is "5 to 2ft Lamberts" in *A. tasmaniae* Hope (Carne, 1956); below about 50 lux in *A. majalis* (Razoumowsky) (Evans and Gyrisco, 1958); and below about 10 lux in *P. decemlineata* (Say) (Lilly and Shorterhouse, 1971).

In the present study concerning *C. zealandica* the numbers of males caught in phenol baited sticky traps was shown to be inversely proportional to the logarithm of wind speed and directly proportional to temperature. The lower threshold of temperature was not determined because the air temperature was always too high but it is presumably about 10°C (Kelsey, 1968). In addition, relative humidity apparently had little effect on trap catches, and heavy rain depressed flight whereas light rain apparently had little effect on it. On one occasion low numbers of beetles caught in the traps was apparently due to surface water after a heavy rainstorm.

No research has been published concerning the environmental factors which affect attraction of males to females in other Scarabaeidae although the following has been published concerning flight. Couturier and Robert (1955) found that about 4°C was the minimum temperature at which flights occurred towards forest in *M. melolontha*, but a temperature of about 10°C was about the minimum necessary for oviposition flights away from the forest. Hurpin (1956) reported that for the same species flight of newly emerged beetles was stimulated by a temperature exceeding 12°C at midday, combined with some period of sunshine, and that conditions at dusk had less influence and flights were observed at temperatures as low as 6°C. Ellerton (1956) noted that *Pleocoma oregonensis* Leach, which has peak flights in the morning and in late afternoon, flies either in rain or with an overcast sky and a saturated atmosphere. Carne (1956) reported that *A. tasmaniae* does not fly in winds above 240 to 270m per minute. According to Evans and Gyrisco (1958) strong winds and rain depress flight activity in *A. majalis*. Lilly and Shorterhouse (1971) reported that *P. decemlineata* flew on clear evenings when the temperature was above 15°C. Cloud and strong winds reduced flight, and a heavy rainstorm disrupted flights for two evenings in favourable temperatures. Finally Brown (1966) reported that flight in *Pyronota* appeared to be inhibited below 15 to 20°C, and that flight occurred at temperatures above this despite light drizzle, strong winds, and a partly cloudy sky. This author also pointed out that day flying species (*Pyronota*, *P. horticola*, and *P. japonica*) have a relatively high temperature threshold for flight and that crepuscular species (*A. tasmaniae*, and *C. zealandica*) have a much lower temperature requirement.

Influence of environmental factors on mating

Nothing has been published specifically concerning the environmental factors which affect mating in other Scarabaeidae, although presumably factors which affect flight also affect mating in species where the male flies towards the female. In the present study, the environmental factors under which *C. zealandica* has been observed to mate in the field have been noted, but upper and lower thresholds for this behaviour were not determined. It is interesting to note that this species will mate at the beginning of the night when it is dark so presumably the light requirement for this activity is lower than for flight, and also that strong winds apparently have less effect on mating than on flight.

Mating behaviour

Comparatively little has been published concerning mating and copulation in Scarabaeiodae. Soo Hoo and Roberts (1965) have, however, very briefly described the precopulatory behaviour of the melolonthinid *R. magnicornis*, and this appears to show the closest similarities to the behaviour of *C. zealandica* amongst the published descriptions. These authors reported that the typical sexual response of *R. magnicornis* consisted of "fanning out the lamellae of the antennal club and excited walking, culminating in rapid fluttering of the wings and searching behaviour". It is interesting that *C. zealandica* males also intermittently beat their wings because rapid wing beating is associated with the liberation of male pheromones in Lepidoptera (Chapter 8). Whether a male pheromone is produced by *C. zealandica* which sexually excites or supresses an escape reaction of the female is not known, although, as noted above, there is some evidence from field trapping experiments with phenol that the male might produce a weak attractant for the female.

Conversely, the wing beating may simply represent displacement behaviour or an intention movement for flight, or the sound of the beating wings may have some possible stimulatory influence on the female. Sound is certainly produced by the beetles when they fly and this may act as a cue for swarming although this seems unlikely. The buzzing or humming sounds produced by flying Scarabaeiodea is an obvious feature of their flight and has often been noted. Appropriate organs of hearing are not, however, obvious although the sound could be perceived through vibration of, for example, the elytra or the substrate. The same applies for Scarabaeoidea which stridulate: males of *P. decemlineata* apparently do so with the edges of their wings when they alight near females (Lilly and Shorterhouse, 1971) but whether this has any sexual significance is not known. Other Scarabaeoidea which possess stridulatory organs occur in the Trogidae, Dynastinae, Geotrupidae, and Passalidae: the latter are subsocial and their sound is thought to have some function in keeping the family group intact (Britton, 1973), but the function of the sounds produced by the others is not known.

The spreading of the antennal club noted by Milne (1960) in *P. horticola*, may occur also in *C. zealandica* but this could not be determined by observation during the present investigation although it appeared that it possibly does from photographs of the beetles. This behaviour has been reported in other Scarabaeoidea as noted both above and below, and it seems to suggest some olfactory response.

The report by Henzell *et al.* (1970) that male *C. zealandica* attempted to copulate with squashed females, as well as with dummies containing extract of females or phenol suggests that the sex pheromone alone is all that is required to elicit the full mating behavioural response from the male. However, apart from the possibilities already noted above, visual, gustatory, or tactile stimuli may also play some part both in the mating of *C. zealandica* and in other Scarabaeoidea. This is suggested by the apparent frequency with which males will mount and attempt to copulate with other males both in *C. zealandica*; and in *Lucanus placidus* Say and *Platycercus virescens* (Fabr.) (Lucanidae) (Mathieu, 1969); *P. horticola* L. (Milne, 1960); and in three species of *Pyronota* (Brown, 1966): it is also possible that these males had become attractive by some previous contact with a female.

With respect to mating behaviour, the following has been reported for other Scarabaeidae:

Travis (1939) noted males of *P. lanceolata* often feeding on the same leaf or plant with a female and ignoring her, but when a female was observed to extend her genitalia males within a radius of 15 to 20m immediately flew towards her.

Carne (1956) reports that in *A. tasmaniae* males mount females and perform characteristic precopulatory movements in which the antennae are brought into juxtaposition with those of the female and the hind tarsal claws are used in genital stimulation.

Brown (1966) reported that in three species of *Pyronota*, the male mounts the female by grasping the anterior margin of the pronotum with his fore tarsal claws. The male extends his abdomen downwards and extends and inserts his genitalia (parameres and basal plate) into the female. During copulation the male's body is held at an angle to the back of the female.

Rittershaus (1927), Milne and Loughlin (1956), and Milne (1960) have described the sexual behaviour of *P. horticola*. When a male locates a female he immediately mounts her and clasps the edge of her elytra with his 4 front legs. If the female has not laid most of her eggs she walks downward as vertically as possible and hides under a leaf or some other object where copulation proceeds. At the end of copulation (mean 12 minutes) the female vigorously shakes and shrugs the male off her back by twisting her body and thrusting upward with her upturned legs. When the female has laid all or most of her eggs she no longer conceals herself, but copulation takes the same time and she does not attempt to remove the male afterwards so that he stays on her back for a further 5 to 30 minutes.

With respect to mating in other Scarabaeoidea, the only detailed report known to me is that of Mathieu (1969), for 5 species of Lucanidae. Their precopulatory behaviour, as reported by this author, is more complex than that in the Scarabaeidae discussed above. Initial attraction often appears to be visual, sex recognition is probably by chemotactic stimuli at close range, and the male mounts the dorsum of the female to copulate. Precopulatory behaviour includes the production of sound on the back of the female by the male's mandibles in *Lucanus capreolus* (L.), *L. placidus*, and *P. virescens*; palpitation of the female by the male with the antennae and/or the palpi in *Ceruchus piceus* Walker, *L. placidus*, and *P. virescens*; and jerk-like movements of the male in *L. capreolus*. In addition copulating males of *C. piceus* also make genital thrusts and keep their metathoracic legs in constant motion against the posterior of the female's abdomen. Mathieu (1969) also reports that male lucanids frequently show aggressive displays to one another, followed by biting, grabbing, and head tossing. The frequency of these displays is increased in the presence of a female, but only when a male is mounted on a female does he become vulnerable to side attacks and damage by other males. Aggressive displays have, however, not been reported in Scarabaeidae and have not been observed during the present investigation in *C. zealandica*.

In conclusion, it appears that the mating behaviour of Scarabaeoidea, although poorly known, is generally simple with few different movements being involved, and therefore *C. zealandica* is not exceptional in this respect.

Chapter 5

Investigation into bioassay methods for the sex pheromone of *C. zealandica*

Introduction

Most of the bioassays used by various authors for the sex pheromone of *C. zealandica* have involved field attractancy tests using simple vane traps with bases containing water for snaring beetles which flew into the vanes and then fell (Osborne and Hoyt, 1968, 1969, 1970; Henzell, 1969; Henzell and Lowe, 1970; Hoyt and Osborne, 1971; Osborne and Boyd, 1975). Accounts of two laboratory bioassays have, however, been published. The first was developed by Henzell *et al.* (1969) and consisted of a "Y-choice olfactometer" made of perspex tubing with a flat perspex track for the beetles to walk on. Traps were constructed at the end of each upper arm of the "Y" by placing a wire mesh gate which contained a small hole for the beetles to climb through near the end of the tubing. Air was sucked through the apparatus by an electric hair dryer and expelled from the room. One end of the "Y" was left blank and the other was baited with the test sample. The male beetles (apparently 24 - 51) were then placed in the downwind end of the "Y" where they were confined by removable wire screens. After a 1.5 hour conditioning period they were liberated into the rest of the olfactometer. Responses were measured by the number of beetles caught in each trap after three hours.

The second bioassay was published by Henzell *et al.* (1969) and depended on a visual assessment of the beetles' behaviour. The olfactometer consisted of the top half of a 2.5 litre Winchester bottle (about 15cm in diameter) resting on a plate of glass covered with paper tissue. Ten male beetles were dropped into this and given a conditioning period from 1300 hours until dusk. Two methods were used for presenting the test samples to the beetles: liquids were spotted onto dummies of paraffin wax about the size of an adult *C. zealandica* while frozen sections were applied to filter paper. Each sample was then tested by dropping it amongst the beetles and observing their responses apparently from "dusk ... until about 9.00 p.m." The activity of the samples was then estimated by the percentage of beetles that attempted to copulate with them or with one another.

Both of these bioassays were considered by the present writer to be unsatisfactory, although the "Y-choice olfactometer" came the closest to fitting the requirements of a bioassay design which are considered here to be desirable.(Chapter 1). These included particularly the fact that it depended upon upwind attraction of male beetles to the source of the sex pheromone, and the method of trapping the beetles in order to measure their response. However, the beetles apparently had difficulty in walking on the smooth perspex surface provided because a photograph of the apparatus (figure 1; Henzell *et al.*, 1969) shows that most of the clearly visible beetles are lying on their backs. Possibly this accounts for the long period required for each bioassay. Furthermore, it appeared possible that a roughened surface is more suitable for the beetles to walk on because Henzell *et al.*(1970) provided paper tissue in the second type of olfactometer partly because it "helped the beetles move about".

The second bioassay published by Henzell *et al.*(1969) is subjective and since the test odours are liberated into still air, it can only be considered to give a qualitative assessment of a pheromone. The results obtained by Henzell and Lowe (1970) in fact suggest that the bioassay is not quantitative. They found that 80% of the males responded to each of 0.1, 1.0 and 10.0µg of phenol when spotted onto paraffin dummies. Also, since there is no control of air movement, the results of such a bioassay are likely to be extremely variable (Chapter 1), however the variability of the response obtained by Henzell *et al.* (1970) are not given in detail.

The evident need to develop a better bioassay for the female sex pheromone of *C. zealandica* prompted the present investigation. A bioassay apparatus (described below) was constructed and tested at the start of the flight season in 1970, but it proved to be unsatisfactory. Some experiments were then carried out in the laboratory and field to try to determine why the bioassay did not work, and the results of these indicated an alternative bioassay design. This second bioassay apparatus also described below was subsequently tested during the flight season in 1971 but proved to be unsatisfactory as well. In the interim Hoyt and Osborne (1971) published their identification of the females' pheromone glands (described in Chapter 3) and there was thus no further need of a bioassay for the present investigation, so further research was directed towards investigating the beetles' behaviour in the field. Nevertheless, some field trapping experiments were carried out after the behavioural research had been completed in an attempt to confirm the identification of the pheromone glands but the results were inconclusive because the experiments were carried out late in the season.

Apparatus used for the bioassay experiments

The first apparatus constructed consisted of a large central flight tunnel enclosed within an outer chamber and mounted on a wooden base (Figure 5.1). The flight tunnel had a hardboard floor, and glass walls and roof. A fine bronze screen was also stretched over each end to prevent insects from escaping and to allow air to be drawn through the tunnel. The outer chamber also had glass sides and roof but the ends were closed off by two sheets of glass each with a large central hole. The design of the apparatus allowed the two top plates of glass to be lifted up to gain access to the central flight tunnel. Four holes were drilled in the hardboard floor at equal intervals near one end of the flight tunnel and pitfall traps were fitted into these from underneath. Each trap consisted of two conicle flasks connected by wide diameter glass tubing: one flask formed the trap and had a vertical tube of glass which fitted the hole in the hardboard floor, while the second flask formed the sample chamber and was open to the surrounding air. The wooden frame on which the entire apparatus rested was made airtight, and had a door for access to the traps beneath the flight tunnel.

Air was drawn through the apparatus by a domestic ventilator fan attached to the hole in one of the end pieces of glass by wide diameter plastic tubing. Air was drawn through the flight tunnel at a rate of about 20m per minute (measured by timing smoke) and some air also leaked into the flight tunnel through the traps which were situated at the upwind end of the tunnel. In addition, air passed underneath the flight chamber where the traps were situated preventing any odour from entering the flight tunnel except by way of the traps.

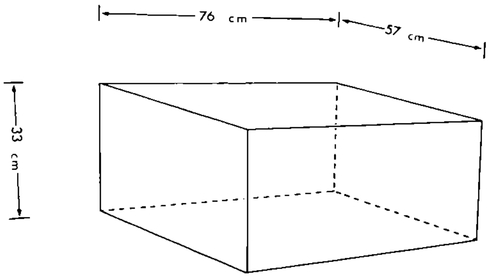
Figure 5.1

The first apparatus used in an attempt to develop a biassay for the sex pheromone of *C. zealandica*

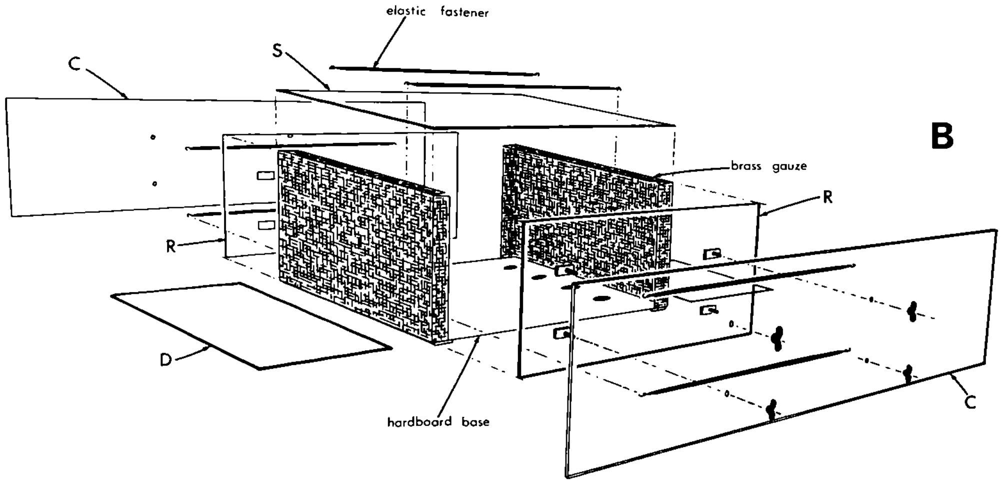
Sizes of glass sheets

A	137cm x 61cm	
B	58.5cm x 59cm	hole 14cm in diameter centred 20cm from lower edge.
C	136cm x 39cm	Each hole 37cm from end and 8cm from edge.
D	59.5cm x 29cm	
S	59.5cm x 76cm	
R	33cm x 76cm	

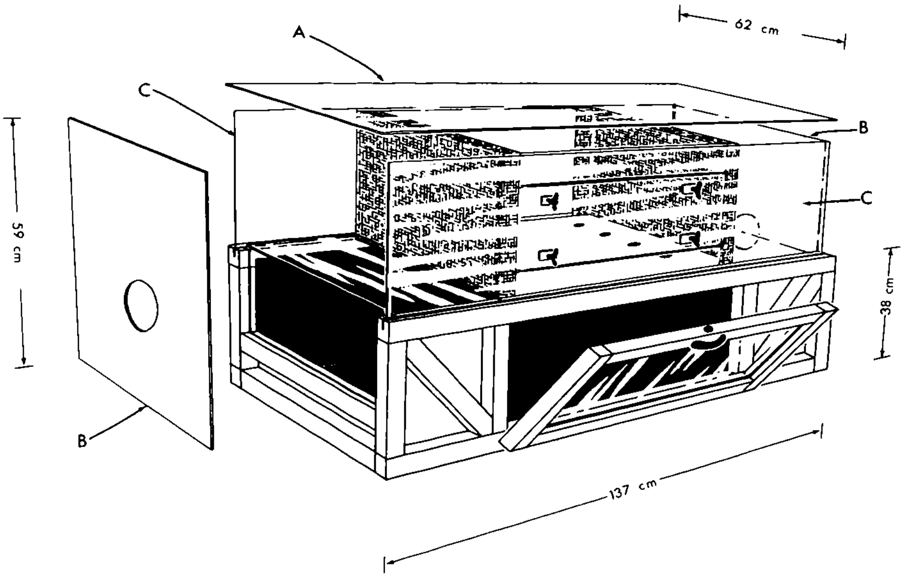
A


76 cm
57 cm
33 cm

B


elastic fastener
S
C
brass gauze
R
R
D
hardboard base
C

C


A
62 cm
C
B
C
59 cm
38 cm
B
137 cm

The live beetles used in each experiment were allowed to dig into a shallow crystalising dish of moist sand and then this was placed at the upwind end of the apparatus. The floor of the flight tunnel was also covered with moist sand to a depth of about 3mm, and this was replaced after each experiment. In both cases the sand was clean boiled river sand. At the conclusion of each experiment, the traps were removed and thoroughly washed using "Decon 75": the flight tunnel on the other hand, was dismantled only after every 4 to 6 experiments and rinsed with "Decon 75".

The second apparatus consisted of a flat chamber supported by a wooden frame (Figure 5.2). The chamber consisted of two large flat glass sheets which were held 1cm apart by means of glass strips along two sides of the chamber. A large diameter hole was drilled centrally near one end of the upper sheet of glass, while 4 small holes were drilled at equal intervals near the opposite end of the lower glass sheet. Organdie netting was stretched over the entire top surface of the lower glass sheet so that it formed the surface on which the beetles walked when they were inside the chamber, and this organdie was also folded over the ends of the chamber to form a screen to prevent the beetles from escaping. In addition, holes were cut in the organdie over the holes in the lower glass sheet so that beetles could fall through into the same pitfall traps as used with the first apparatus. The complete chamber was placed on the wooden frame so that one end fitted into a narrow opening in a perspex box, and this in turn was fitted to a domestic ventilator fan by flexible plastic tubing. The end of the chamber with the traps was placed furthest away from the plastic suction box and air was drawn through the apparatus at a rate of 10 to 15m per minute. Live beetles were dropped into the chamber through the large hole in the upper sheet of glass at the commencement of each experiment, and the hole was then covered by a glass petri dish. Also, each time the apparatus was used, the glass and organdie netting was subsequently thoroughly washed with "Decon 75".

Experimental procedure and results

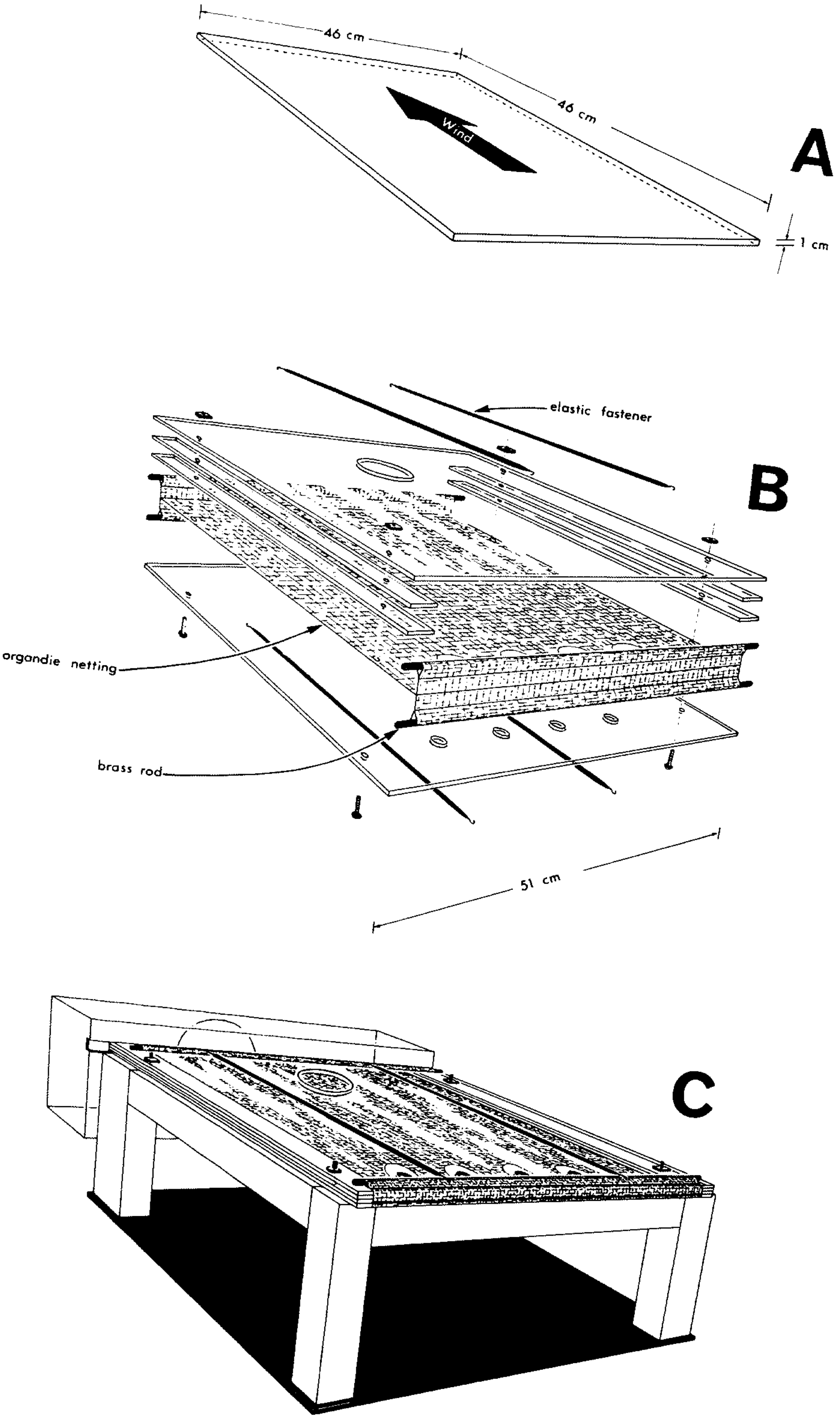
Laboratory experiments with the first apparatus:

Experiments were carried out with the first apparatus every evening between the 2nd November and the 9th November, 1970. The

Figure 5.2

The second apparatus used in an attempt to develop a bioassay for *C. zealandica*

The brass rods placed in seams in the organdie served for hookong the rubber fasteners on and prevented the organdie from wrinkling when it was stretched. The hole in the upper sheet of glass is 4cm from the end of the glass and has a diameter of 5cm, whereas the four holes in the lower sheet of glass have diameters of 2cm and are situated 5cm away from the end of the glass.


46 cm
46 cm
Wind
1 cm
A
elastic fastener
B
organdie netting
brass rod
51 cm
C

temperature on each of these evenings varied between 12.5°C and 16°C and the only light used was that coming from the sky through the window of the laboratory. Each experiment was started at dusk as soon as the first beetle began to dig out of the sand.

Initially the apparatus was set up next to a window in a laboratory so that the air was sucked towards the window and exhausted outside through a long plastic hose. One trap was baited with 5 live females 5 to 12 days old, another trap was baited with two females of the same age which had been squashed onto filter paper, and the other two traps were left blank. Twenty laboratory reared males 5 to 12 days old were used but only 12 emerged from the sand. These all either flew or walked towards the light from the window and climbed about on the wire mesh or walked on the floor of the cage at the base of the mesh. The experiment was concluded after 1 hour of darkness, but no beetles were trapped.

The following day the apparatus was reversed so that the air was sucked from the window and exhausted into the laboratory. The same live male and female beetles were used again and two females 3 to 5 days old were squashed as samples. On this occasion 14 male beetles flew or walked towards the window as in the first experiment and none were observed to show any behaviour which could be regarded as a response to the odours from the female baited traps. No beetles were trapped during the experimental period which concluded after 1 hour of darkness.

On the third day the apparatus was left in the same position and the same male beetles were used again. On this occasion the female beetles in the trap were provided with some shoots of barberry. One of the other traps was baited with 50ml of water with 10ppm phenol, another was baited with 50ml of 100ppm phenol, and the last was left blank. Only 10 of the males came out of the sand and these behaved in the same way as on the two previous nights. The experiment was left to run until 0900 hours the following morning but no beetles were trapped.

The decision was then made to save the laboratory reared beetles until the bioassay had been further investigated using field collected beetles which had been kept without contact with the opposite sex for at least 5 days.

On the 5th November 20 male and 20 female beetles were placed together in the apparatus and the trap holes were corked. At dusk 15 of the males and 10 females came out of the sand and 7 pairs were observed to copulate on the wire mesh screen or floor of the cage by the window. These beetles, however, only appeared to mate after a male had contacted a female whilst walking or climbing about apparently in a random fashion. This experiment therefore demonstrated that *C. zealandica* would mate in the apparatus.

On the following two nights the apparatus was set up in the same position as previously but the traps were each provided with a glass tubular extension which had been dipped in wax blackened with charcoal. These extensions projected upward 10cm into the flight tunnel and it was hoped that it might provide the beetles with an object to fly towards. On both nights the same 40 male beetles were used, and 30 and 28 emerged from the sand. Two of the traps were baited with the same quantities of aqueous phenol as used on the 4th November, another of the traps was baited with 20 field collected females together with barberry, and the last trap was left blank. On 6th November one male was observed hovering in a zizzag pattern for approximately two seconds about 10cm downwind from the tip of the tube with the 100ppm phenol sample but it flew directly to the wire mesh afterwards. On the same evening another beetle flew into the roof of the chamber and fell down the blank trap tube. All the other males behaved in much the same way as those in the first three experiments except that they appeared to fly much more frequently.

It appeared that possibly the directed light from the window interfered with the behaviour of the beetles. The entire apparatus was accordingly transferred to a glasshouse and a small white tent was erected over it. This tent diffused the light from the sky, nearby buildings, and lamp posts and prevented the structure of the glasshouse, nearby houses, trees or hills from being seen from the bioassay apparatus.

Two experiments were carried out in the glasshouse on the 8th and 9th November, but the direction of airflow was reversed on these nights. The same samples as used on the 6th and 7th November were set up again for these experiments and 50 male beetles which had not been used previously were introduced into the flight tunnel. The glasshouse fans were used to draw air past the apparatus and expell it to prevent contamination by the samples. On these occasions 39 and 26 male beetles emerged and their flight appeared to be less directed than previously because they flew all around the cage. However, no beetles were caught in any of the traps although in each case the experiment was left running until the following morning.

Attempts to duplicate the observational bioassay of Henzell et al. *(1970)*

Late in 1970 two attempts were made to duplicate the observational bioassay of Henzell *et al.*(1970) using the remaining laboratory reared beetles (2-3 weeks old) and the actual equipment and constant temperature room used by these authors. This constant temperature room was illuminated by means of two 60w incandescent bulbs shining through Ilford type 900 red filters. The temperature was set at 15°C, the temperature which was used by Henzell *et al.*(1970) for the bioassays rather than the published temperature of 8-10°C (Henzell, *pers. comm.* 1970). In all other respects the experimental arrangement was exactly as already described (see above in the Introduction to this hapter). Three bioassays were set up on both occasions with 5 male beetles in each. The beetles were placed in the apparatus at 13.00 hours to allow them to become accustomed to the conditions and the bioassays were then started at 21.00 hours. On each occasion two of the samples used consisted of a pair of reared females that had been squashed immediately beforehand onto filter paper, while the third sample consisted of a paraffin wax ball about 0.5cm in diameter with 1.0µg of phenol spotted onto it. This quantity of phenol was known to elicit sexual responses from male *C. zealandica* of a similar age in this bioassay (Henzell and Lowe, 1970). On both occasions, however, no sexual responses were observed from any of the beetles during a one hour period.

Field experiments carried out in 1971

It was possible that the traps used in the laboratory experiments did not release the right quantities of attractant or that the beetles did not receive the correct visual cues to aid them in flying to the source of the attractant (Chapter 4). The apparatus was therefore dismantled and the traps, together with the hardboard floor of the flight tunnel, were set up on the 10th November on top of a barberry hedge around which the beetles were known to swarm at Te Awamutu (see Chapter 2 for the location). The same samples were used in the traps as in the previous 4 experiments but this time a peristaltic pump was used to blow air through each of the traps at a rate of approximately 5ml per minute. All noise, fluctuations in pressure, and possible odours were removed from this air by passing it through a large expansion chamber followed by two activated charcoal filters. On this occasion, large numbers of beetles were observed flying near the hedge top, but no beetles were caught in the traps and none were observed hovering within 10cm downwind of them.

Between the 11th to 15th November, and the 17th to 18th November a series of field experiments was carried out at the Te Awamutu location mentioned above. On each of these evenings large numbers of beetles were observed to fly and each of the experiments was run over the entire flight period.

Two types of experiments were carried out: the first consisted of testing various samples in the traps used in the laboratory experiments, and the second type consisted of observing the reactions of beetles towards various samples during the dusk flight periods.

In the first type of experiment three of the traps were used each evening. These were attached to shoots of barberry 1m apart near the top of the hedge, and their air supply was the same as that used on the 10th November as described above. The samples tested were 10 and 20mg "Durez 12687"; 10mg, 1g, and 10g of phenol crystals; 50ml of aqueous phenol in concentrations of 5, 10, 20, 50, 100, and 500ppm, and 0.1 g/l, and 1 g/l; shoots of barberry together with 2, 10, and 100 live female beetles that had been kept in the laboratory; and shoots of barberry together with 1, 2, 3, and 7 females which had been collected the same night at the beginning of the flight period just after they had been found by males but before they had copulated. No beetles were trapped with any of these samples although a female alighted on the exit port containing the 1 g/l phenol sample, and both male and female beetles tended to cluster at an apparently higher density than elsewhere on shoots adjacent to the traps containing samples of phenol in concentrations varying between 10ppm and 1 g/l.

The second type of field experiments consisted of the following procedures. On each of the first two nights three female beetles were caught after they had attracted males, their abdomens were then cut off and the two portions of their bodies squashed onto separate pieces of filter paper. The 6 samples were then placed on the grass in the centre of a field and subsequently observed, but no beetles were attracted to any of them. The following night the same experiment was repeated except that the filter papers with the samples were attached to the top of a hedge by paperclips. Again no beetles were observed to be attracted to any of these samples.

On the 14th November 4 attractive females were caught and tied to wooden spatulas by copper wire. The wings of two of the females were removed and then they were all attached to the hedge by paperclips. Again no male beetles were observed to be attracted to any of these females, although it was likely that the females could control their attractiveness and that they were too disturbed to release their pheromone since they struggled continuously.

The next evening 5 glass rods were tied to the hedge. Each rod had a small ball of black wax with a diameter of about 7mm attached to its top and samples were spotted onto this wax. Two of the rods had 10μg and 50μg of phenol applied to them in a solution of diethyl ether, whilst the other three had extract containing the equivalent of 5 females spotted onto them. This extract was made by grinding up 15 female beetles with about 5ml of diethyl ether, filtering it, and subsequently allowing the ether to evaporate to about 0.5ml. On this occasion one male was observed to hover towards and alight on the wax ball with 10μg of phenol. The male then walked over the wax for about 3 seconds before flying off again. It also appeared that other males occasionally may have hovered towards the 10 and 50μg phenol samples, but no response was observed towards any of the extract samples.

On the 17th November, a series of filter papers was attached to the hedge by paperclips and phenol in quantities of 0, 20, 40, 60, and 80μg were spotted onto them in a diethyl ether solution at the beginning of the flight period. However, the wind changed direction so that it blew towards the side of the hedge with the samples and few beetles were observed flying on that side. The experiment was then repeated on the 18th November and some beetles were observed to hover towards or settle on each of the shoots of barberry with phenol samples attached, whereas no beetles were observed behaving in a similar fashion to the shoots with the blank filter paper.

Finally, in 1970, a series of field experiments was carried out late in the flight season. In each of these experiments folded

Erratum

Line 3:
"... the first were ..." *should read* "... the first beetles were ..."

filter papers were fastened onto the collected and counted at the end of the flight period. These results are given in Table 5.1 together with the quantities of phenol used. No significant attraction was demonstrated by this method although it was noticed that most of the beetles which alighted on the shoots flew away again and therefore the numbers obtained at the end of the flight period did not give a good representation of the attractiveness of the samples. These experiments, however, provided the idea for developing the sticky traps used for the field attraction experiments in 1971 described both below and in Chapter 4.

Observations of the behaviour of the beetles both in the laboratory and in the field showed that the beetles would mate if a male had not first flown to the female he copulated with immediately beforehand, and that even when a male did fly towards a female he usually landed a short distance away and then ran about searching for her (Chapter 4). In addition, the various experiments suggested that too many unknown sensory requirements had to be provided before the males would fly to the females. The most likely of these appeared to be diffused lighting and a large irregular silhouette such as that of a tree or hedge and both of these would be difficult to provide in the laboratory situation. It seemed likely, however, that the males would walk towards the source of the odour from an attractive female if they were prevented from flying. A second bioassay apparatus which satisfied this requirement was therefore constructed (as described above) and tested in 1970.

Laboratory experiments with the second bioassay apparatus

The second apparatus was set up in the same room as was used for bioassaying the female pheromone of *P. operculella* (see Chapter 9). The suction fan was connected to the ventilator outlet of the room and the room was illuminated by incandescent bulbs which could be controlled by variable resistors. One of these lights was set up permanently under the table on which the bioassay apparatus was placed so that it was directed towards the wall opposite the downwind end of filter papers were fastened onto the hedge with paperclips and then various quantities of phenol in diethyl ether were spotted onto them as soon as the first were observed to fly. The numbers of beetles which rested on each shoot with an attached sample were subsequently collected and counted at the end of the flight period. These results are given in Table 5.1 together with the quantities of phenol used. No significant attraction was demonstrated by this method although it was noticed that most of the beetles which alighted on the shoots flew away again and therefore the numbers obtained at the end of the flight period did not give a good representation of the attractiveness of the samples. These experiments, however, provided the idea for developing the sticky traps used for the field attraction experiments in 1971 described both below and in Chapter 4.

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Table 5.1

The numbers of beetles that settled on shoots of a hedge with samples of phenol on filter papers.

		male	female	male	female	male	female	male	female	male	female	No. of replicates	Temperature at end of flight
30/11/70	Amount of phenol ug	0		5		10		20				3	17°C
	Average No. of beetles caught	0.33	0.67	1.0	0.67	0.67	0.67	3.00	3.33				
3/12/70	Amount of phenol ug	0		20		20		30				5	18°C
	Average No. of beetles caught	0	0.40	0.40	0.60	1.20	1.20	0.80	0.50				
5/12/70	Amount of phenol ug	0		10		20		30		40		10	16°C
	Average No. of beetles caught	1.00	0.40	1.50	0.80	0.40	0.30	0.50	0.50	1.00	1.00		
6/12/70	Amount of phenol ug	0		20		40		60		80		10	15°C
	Average No. of beetles caught	0.60	0.20	1.30	0.60	0.90	0.60	0.40	0.50	0.70	0.60		

the apparatus and illuminated the apparatus with approximately 0.1 lux. The other lights were similarly placed under the table and pointed in all directions. These were used for providing an artificial dusk when the variable resistance which controlled their output was manipulated so that the light intensity taken vertically from the table top was reduced linnearly from 100 lux to 0.1 lux over a period of 25 minutes prior to the start of the bioassay.

The apparatus was first tested on the 16th November with beetles which had been previously collected in the field and kept in the laboratory separated from the opposite sex (see Chapter 2). The trap holes were corked and 15 males and 15 females were dropped in at 2035 hours. At the end of the dusk period at 2135 hours 6 copulating pairs were observed, but it was noted that the beetles still attempted to fly even though the low roof of the chamber restrained them from doing so. For all subsequent experiments, therefore, the container in which the beetles were kept was taken into the bioassay room, its lid was replaced with a glass sheet, and the beetles were allowed to emerge from the soil and fly during the dusk period. At the end of that time only beetles on the surface of the soil or on the willow branches in the container were used for the bioassay experiments. In addition, the beetles used were replaced in the container after the experiments.

Between the 17th and 24th November a number of experiments were carried out using the bioassay apparatus and field collected beetles. The numbers of beetles trapped are given in Table 5.2 together with the details of the various samples used. For each experiment the position of the sample amongst the blanks was randomised and the ordering of the results in Table 5.2 is purely one of convenience. It is apparent, however, that none of the samples showed attraction in this experimental situation.

The conclusion reached was that it was pointless attempting to use this apparatus for bioassaying the female sex pheromone of *C. zealandica* so no further experimentation was carried out with it.

Field experiments using sticky traps in 1972

Two attempts were made in 1972 using the sticky traps described in Chapter 4 in field experiments to try to verify that the accessory glands of the female contained the attractant as reported by

Table 5.2

Numbers of male beetles trapped in experiments with a variety of samples in the second bioassay apparatus.

Description of samples	Numbers of beetles trapped SAMPLE	 BLANK	 BLANK	 BLANK	Number of male beetles used
11 live field collected females	4	8	6	5	30
10 live field collected females	5	9	5	2	30
10 live field collected females and some twisted willow	11	11	7	6	50
10 live field collected females and some twisted willow	2	2	1	0	28
2 squashed attractive females collected the same night	5	5	4	3	25
10 squashed attractive females collected the same night	4	6	5	5	30
50µl of 20g/l aqueous phenol on a disc of seed test paper	4	4	3	0	30
50µl of 20g/l aqueous phenol on a disc of seed test paper	8	6	5	4	30

Hoyt and Osborne (1971). For each of these experiments, 5 attractive females were caught in the field just after males had found them, and they were taken back to the laboratory where their abdomens were separated from the rest of their bodies and their guts and accessory glands were excised. Separate chloroform extracts were then made of these parts according to the method described in Chapter 4. In addition, the traps were prepared and set out in the same manner as detailed in Chapter 4.

The results of these experiments are given in Table 5.3, and it is apparent that they were inconclusive, probably because of the lateness in the flight season when the experiments were carried out.

Discussion

There appear to be a number of likely explanations to account for the failure of the present attempts to develop a laboratory bioassay for the female sex pheromone of *C. zealandica*. One possibility is that the male requires some essential factor or factors before responding to the pheromone, and that these were not present in the laboratory situations. In support of this Roelofs *et al.* (1967) reported that such requirements are often very precise. In the field, however, all of the necessary factors must be present when the beetles mate so that experiments carried out in these situations must provide reliable results.

The field experiments demonstrated that many of the samples used were not attractive and this was shown to be so for squashed female *C. zealandica*. The possibility exists, however, that the pheromone was covered by some other portion of the squashed beetles but this seems unlikely since a large number of such samples were used altogether. Another possibility is that these crude preparations contained a masking agent and it is also probable that some substances are present in the body of the female which react with phenol since it is a reactive chemical. Such explanations would also account for the lack of attraction shown by extracts of females in the field experiments. It is, however, apparent that this masking or reaction with phenol is not complete because female extracts were shown to be attractive in the experiments with sticky traps (Chapter 4), and Henzell *et al.* (1969, 1970) found that both squashed females and their extracts elicited

Table 5.3

Numbers of beetles caught in traps containing either filter paper blanks, 50μl samples of 6 g/1 phenol or dried chloroform extracts of parts of beetles.

(* Significantly different from the mean of the blank samples at 0.05 level in a t-test)

	SAMPLE		BLANK	6 g/1 PHENOL	EQUIVALENT OF 5 MALES/TRAP GUT	 REMAINDER	EQUIVALENT OF 5 FEMALES/TRAP GUT	 ACCESS. GLNDS	 REMAINDER
3-12-72	TRAP POSITION		A H O W Y	G N U X Z	B I P	C J Q	D K E	F M T	E L S
	Male catch		5 2 7 0 3	5 1 3 2 0	3 7 5	5 3 4	2 3 7	1 8 5	0 1 4
	Female catch		0 0 1 0 0	0 0 1 0 0	1 0 0	1 0 0	2 0 1	0 0 0	0 0 0
	Mean S.D.	Male	3.40 2.70	2.20 1.92	5.00 2.00	4.00 1.00	4.00 2.65	4.67 3.51	1.67 2.08
	Mean S.D.	Female	0.20 0.45	0.20 0.45	0.33 0.58	0.33 0.58	1.00 1.00	0.33 0.58	0.00 0.00
	SAMPLE		BLANK	6 g/1 PHENOL	EQUIVALENT OF 5 MALES/TRAP GUT	 REMAINDER	EQUIVALENT OF 5 FEMALES/TRAP GUT	 ACCESS. GLNDS	 REMAINDER
7-12-72	TRAP POSITION		B I P	A H O	C J Q	W D K R Y	E L S I	F M T	G N U Z
	Male catch		11 12 4	9 11 17	6 3 18	2 2 1 6 1	2 2 7 2	3 11 1	8 6 4 5
	Female catch		0 0 0	0 1 0	0 0 1	0 0 0 0 0	0 0 1 0	0 0 1	1 0 0 1
	Mean S.D.	Male	9.00 4.36	12.33 4.16	7.25 7.37	2.50* 2.38	3.25 2.50	5.75 1.71	5.00 5.29
	Mean S.D.	Female	0.00 0.00	0.33 0.58	0.25 0.50	0.00 0.00	0.25 0.50	0.33 0.58	0.50 0.58

attraction and sexual responses from males in the laboratory.

The field experiments in the present investigation also indicated that the traps may not have been effective. This could explain why the live females were apparently not attractive when in the traps, although, on the other hand, this could be due to them not releasing their attractant since it is quite possible that they have precise requirements for doing so. One well known example, which serves to illustrate the diversity of such conditions, occurs in the moth *Antheraea polyphemus* (Cramer). Riddiford (1967), and Riddiford and Williams (1967a, 1967b) reported that the females of this species must perceive the chemical *trans*-2-hexanal which is released from the leaves of red-oak, *Quercus rubra* L. sec., before they will release their sex pheromone. Many other plant species also liberate this chemical although they apparently give out other odours as well which stop the females from releasing their attractant. However, the inclusion of food plants with the females of *C. zealandica* in the present study presumably discounts this as a possible reason for the lack of attraction. Concerning the attractiveness of live female *C. zealandica*, it is interesting that Kelsey (1967) obtained an indication that laboratory reared females, when caged in 2.54cm^3 gauze-sided cells and placed on the ground surface in an uninfested area, could attract males upwind towards them, although apparently no controls were run. In addition, in the present study (Chapter 4), the results of experiments with sticky traps using live females did not exclude the possibility that captive females could attract males in the field; but they did indicate that if this occurred there would be marked differences in attractiveness between individual females, and the same conclusion was also reached by Kelsey (1967).

It appeared possible that the traps used in the present study may have released the attractant in the wrong manner, or in the case of phenol, in the wrong concentrations. It can be noted that large simple vane traps have been used successfully by other research workers for studying attraction in *C. zealandica* (Chapter 1) but these possibly allowed the beetles to be caught by blundering into them whilst at an early stage of orientation to the phenol, whereas small traps have to provide other more specific cues in addition to the odour source. The effectiveness of the sticky traps developed at a later time in this study (Chapter 4) suggests that a visual stimulus comprising an irregular silhouette contributes greatly to close range orientation of pheromone-stimulated male *C. zealandica*. It is obvious, therefore, that a trap for use in a bioassay would have to be developed primarily on the basis of field experimentation.

One aspect of this present investigation which is difficult to understand, is that Henzell and his research associates managed to obtain both attraction and sexual responses from male *C. zealandica* in the laboratory. In the case of experiments with the "Y-choice olfactometer" (Henzell *et al.*, 1969) this was even obtained after the male beetles had been exposed to the pheromone odour for 1.5 hour (during the preconditioning period). This is of interest because many insects are known to habituate to their sex pheromones (e.g. Jacobson, 1972; Shorey, 1974). Certainly the conditions under which the present laboratory experiments were carried out appeared to the present writer to be closer to those at dusk than those under which Henzell *et al.* (1969, 1970) carried out their experiments. These authors used a constant temperature room which was "illuminated by means of two 60 watt incandescent bulbs shining through two Ilford type 900 red filters having a transmittance above 6,200 A " and they adjusted the intensity of this light by means of a variable resistance until it "approximated conditions at dusk when mating is known to occur". The lighting conditions in the field at dusk, however, were never observed to be as red as those used by these authors. Possibly the failure to duplicate the second bioassay of Henzell *et al.* (1970) using their actual equipment could have been due to chance because only 6 replicates were run. This failure also suggests that perhaps there was some detrimental influence affecting the beetles reared in the present study although the rearing method was very similar to that used by Henzell *et al.* (1970) (see Chapter 2).

It is interesting to note that male scarabaeid beetles vary in their responses to caged live females, to crushed females, and to extracts of females. Travis (1939) found that in *Phyllophaga lanceolata* (Say) "exposure of a crushed female initiated flights of males, but produced no visible responses from the females" and that "(crushed) males produced no apparent response from either sex". Soo Hoo and Roberts (1965) reported that male *Rhopaea magnicornis* Blackburn were attracted to caged females, cups which had housed 4 to 15 day old virgin females, and abdomens dissected from females. Ladd (1970) found that males of *Popilla japonica* Newman were attracted to tethered and squashed females, and attempted to copulate with females held in the hand. However, they found that although males were attracted to glass jars which had recently held virgin females, extracts of females in ethyl acetate, acetone, benzene, methylene chloride, and ethyl alcohol were not attractive. In contrast, Roelofs *et al.* (1967) could find no evidence of a sex pheromone in *Amphimallon majalis* (Razoumowsky) even though they tested caged virgin females aged between 1 and 8 days, crude ethyl alcohol extracts of them, and separate acetone extracts of males and females after elution on Florisil columns with varying proportions of diethyl ether and petroleum ether. They also tested these chromatographed fractions in the laboratory but obtained negative results. The chromatography was carried out in order to remove possible masking agents from the crude extract but chromatography may not always do this, as for example, when the masking agent is an optical isomer of the pheromone. In addition, chromatography may also separate components of the pheromone which act together in eliciting a response. These experiments are therefore inconclusive in demonstrating that *A. majalis* does not have a sex pheromone. It can be further noted that since *C. zealandica* uses the reactive chemical phenol as its sex pheromone, and that both this insect and *A. majalis* are in the Melolonthinae, *A. majalis* may also use a reactive chemical for this purpose which is rapidly deactivated during extraction. Nevertheless, the conclusion reached by Roelofs *et al.*(1967) was that *A.majalis* probably does not use a sex pheromone, and the results presented in this chapter, when considered by themselves certainly suggest a similar conclusion with respect to *C. zealandica*, and yet it is known that the female of this insect does produce a sex pheromone.

CHAPTER 6

Materials and rearing methods for *P. operculella*

Included in this chapter is a section on the methods employed for transferring live adults from one container to another, and a section on the investigation into rearing methods. The latter incorporates estimates of the mortality and the duration of the various stages in the life cycle of *P. operculella* reared in the laboratory.

Materials

The original culture of *P. operculella* was started from specimens supplied by Mrs M. Foote, Plant Diseases Division, Department of Scientific and Industrial Research, Mt. Albert, Auckland. After this larvae were collected two or three times a year from potato plants growing at Tamahere Gardens, Cambridge Road, Tamahere, Hamilton, and new cultures were started from them. This ensured that laboratory bred moths were genetically similar to wild moths even if the rearing procedure imposed some selective pressures on them.

The possible importance of starting new cultures is demonstrated by the various selective effects shown to have occurred in other insects after they had been reared in the laboratory for some generations. Fye and Labrecque (1966) observed differences in mating behaviour of laboratory reared male *Musca domestica* L. in competition with wild ones. Alley and Hightower (1960) noted differences in mating frequency of the screw-worm fly, *Cochliomyia hominivorax* (Coquerel) which were attributable to their laboratory colonization, and Spates and Hightower (1967) found that laboratory rearing also affected the sexual aggressiveness of males of the same flies. Guthrie and Carter (1972) observed that the moths *Ostrinia nubilalis* (Hübner) lost their ability to survive on their natural host after extended laboratory colonization. However, by backcrossing the laboratory strain to a wild one, they were able to recover the survival abilities of this insect on its natural host. Recently, Raulston (1975) reported that in the laboratory, females of the laboratory adapted strain of the moth *Heliothis virescens* Fabr. had a higher percentage of matings than the wild strain and that this effect was noticeable within three generations. This data indicated that the selection pressure and conditioning produced by artificial laboratory systems altered the mating and oviposition behaviour of the moth.

Rearing of *P. operculella*

All moths were reared by the method described below because this produced a homogeneous population and therefore helped to ensure that experimental results were as repeatable as possible. This method was based upon the ones used by Finney *et al.* (1947), and Platner and Oatman (1968) with minor modifications that were adopted after being examined for adverse effects on the moths.

The moths were kept and reared in a darkroom where the temperature was usually between 19°C and 22°C but varied on rare occasions between extremes of 15°C and 26°C, and the relative humidity ranged between 46% and 85%. Fluorescent ceiling lights were used for light periods being controlled by a time switch to provide a constant photoperiod of 14 hours 45 minutes of light to 9 hours 15 minutes of darkness. During the day the insects received about 150 lux, and in the dark periods they were indirectly illuminated with about 0.1 lux or less from a 40 W bulb supplied through a variable resistance. In order to reduce accidental interference with experiments performed at the beginning of the dark periods, the lights were timed to switch off each day at 2100 hours.

Moth larvae were reared in continuous cultures on potatoes loosely packed with crumpled paper towelling in transparent plastic 1 litre jars or 10 litre aquaria covered with organdie netting. Potatoes of any variety and size as sold by a greengrocer were used and their skins were punctured using the points of a bed of nails set vertically into a lead base (flower holder) because the larvae bore into the potatoes to feed and these holes provided easier access for newly hatched ones. The potatoes were replaced with fresh ones as they dried out or went rotten.

Pupae were collected once a week by completely unpacking the cultures. They were found in cocoons which the mature larvae had spun amongst the potatoes and paper towelling after emerging from the potatoes. Many of the cocoons were ripped open while being unpacked thus exposing the pupae and those that remained intact were carefully opened with fine forceps.

Virgin moths were always obtained from pupae that had been previously segregated by sex. Male and female adults, therefore, never came into contact with each other until they were put together for the experiments. The sexes of the pupae were clearly distinguished under a dissecting microscope by the relative positions occupied by the genital scars on their last abdominal segments as shown in Figure 6.1 . Male pupae also tended to be more tapered, slimmer, and lighter than females but these latter characteristics were not reliable for accurate sexing.

The pupae were always sexed twice because errors of up to 3% occurred the first time. The process of sorting the pupae was made considerably easier by placing two plastic trays next to the microscope stage, one for males and one for females, and learning their positions so that the operator could drop pupae into them without having to look away from the microscope. This reduced eye fatigue and therefore allowed the sexing of large numbers of pupae.

Pupae were kept in *Drosophila* culture jars in a small amount of damp sand or vermiculite. This prevented the adults from drying out while they were emerging and thereby gave high yields of undamaged insects suitable for experimental purposes. Newly emerged adults were transferred to 1.2 litre wide mouthed glass containers (2 pint "Agee" jars) with organdie netting fastened over the openings by screw bands. Each container had a 12 mm hole drilled through its side to facilitate the introduction of moths when using an aspirator (described in the following section) and to enable dead moths to be removed easily. When not in use the holes were corked. Filter papers were placed on the organdie and dampened initially with 10% sucrose solution, and then with water on every subsequent day to provide food and moisture. The moths were kept together with the opposite sex after they had been used for experimental purposes and then the females readily laid their eggs through the organdie onto the filter papers. Filter papers with eggs were removed every few days and left in the potato cultures for the larvae to hatch.

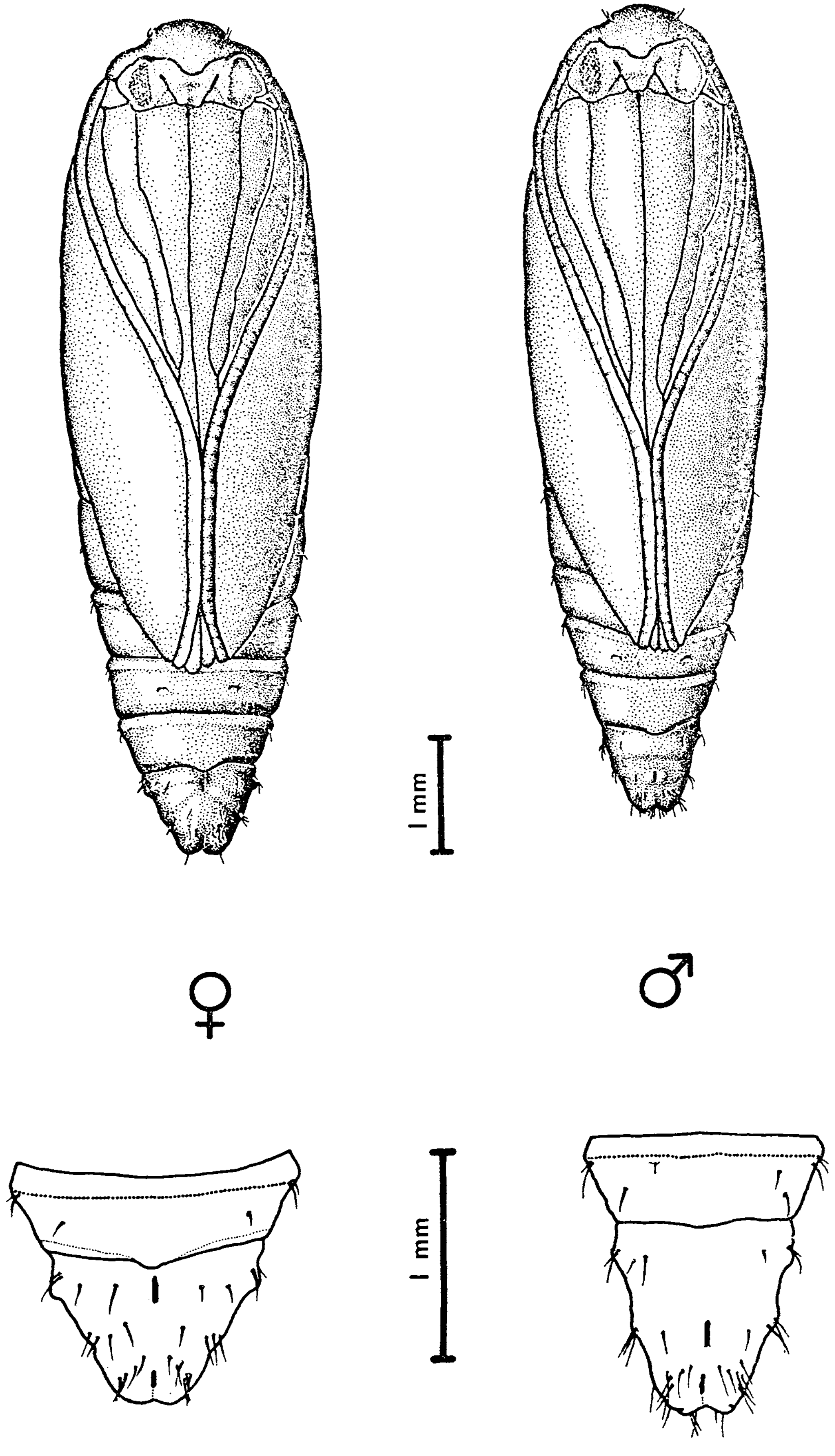
Transfer of Live Moths

Live moths were transferred with the aspirator described below, and when using this advantage was also taken of their behaviour towards light. The moths' initial reactions when disturbed by movement

Figure 6.1

Sexual dimorphism in the pupa of *P. operculella*

Note the differences in the positions occupied by the genital scars of male and female.


1 mm
♀
♂
1 mm

or by jarring were to fly towards light and then after a few minutes to seek crevices or darker areas to rest. Agee jar containers were therefore opened without losing the moths by holding their bases towards the light and periodically tapping them. Alternatively, the containers were opened near a window or sheet of glass with a light shining from the other side, and the moths aspirated from the glass when they flew towards it. This method was also used for removing moths from complex pieces of apparatus.

The aspirator was based on a common design shown in Figure 6.2 using two rubber bungs each with a central hole to plug a glass tube. The end of a flexible clear P.V.C. tube was inserted through one of the bungs as an inlet tube while the hole in the other bung was countersunk on its inner surface and covered with grade XXX bolting silk to seive most of the moth scales from the air. Those scales that remained were removed before the air was inhaled by a dust filter consisting of a glass expansion chamber that tapered at one end to an inlet tube fitting into the bung on the aspirator. This inlet tube was extended into the expansion chamber and bent so that air impinged against the wall as it was sucked through, and the scales adhered to a coating of "Stickem Special" on the walls and on crumpled filter paper loosely packed inside the expansion chamber. The end of the dust filter was closed by another rubber bung with the end of the P.V.C. mouthpiece tubing fixed through it. This also provided easy access for cleaning the filter.

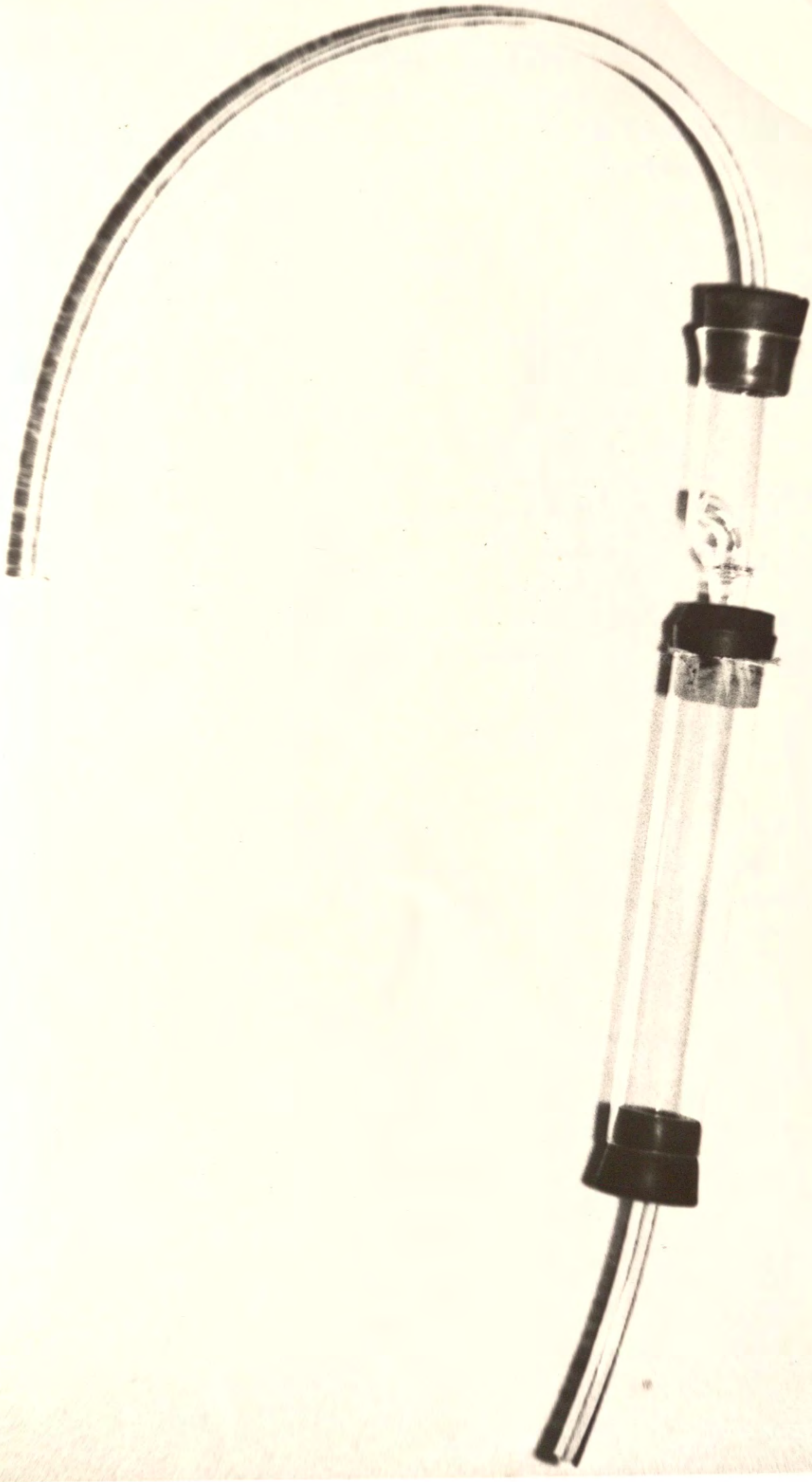
Moths were gently sucked into the aspirator which was large enough internally for them to slow down and remain undamaged. Once inside they had no difficulty clinging or walking on the glass. They could then be counted by sucking air through the aspirator moderately fast because this caused them to remain in one place. The moths were transferred from the aspirator by holding its inlet tube downward then simultaneously tapping and blowing so that the moths fell toward the inlet opening and were blown out.

This method of transferring the adults proved very quick and convenient. It also avoided damaging the moths by direct handling or the possible harmful and variable effects of chilling or using an anaesthetic such as carbon dioxide.

Figure 6.2

An aspirator for use with adult *P. operculella*

Note the filter chamber with inlet tube bent so as to direct air onto the side of the chamber.
(x $\frac{1}{2}$)



Investigation into the rearing methods for *P. operculella*

This section deals with the investigation of modifications of the mass rearing methods of Finney *et al.* (1947), and Platner and Oatman (1968). These modifications were principally concerned with obtaining virgin moths; however, other modifications were made to the rearing containers, the method for obtaining pupae, and the conditions under which the insects were reared. These changes required that the durations of the stages in the moth's life cycle were determined together with the mortality so that the rearing procedure could be managed efficiently. In addition, an attempt was made to find whether or not sorting the pupae adversely affected them. This was done by determining the total duration and mortality of the insects that had been collected as larvae when they emerged from the potatoes and sorted when they pupated, and comparing these against insects that had been left undisturbed. An experiment was also carried out to determine whether the larvae were affected adversely by the degree of crowding experienced under the rearing procedure.

Both Finney *et al.* (1947), and Platner and Oatman (1968) collected pupae that had been infested by parasites by allowing mature larvae to drop from the potatoes into trays of sand where they spun cocoons. Sodium hypochlorite solution was then used to dissolve the cocoons liberating the moth and parasite pupae. These authors did not determine the yield of viable moth pupae. In the present study, the preferred method was to obtain pupae directly from their cocoons by opening them with forceps. Thus, any harmed by this method were obvious and could be discarded, whereas possible chemical damage might be difficult to detect. However, attempts were made to facilitate collecting the pupae by providing the larvae with artificial sites in which they could spin cocoons, these sites being so arranged that they could be easily opened up later to expose the pupae.

It was clearly advantageous to keep mortality to a minimum after the tedious procedure involved in sorting the pupae and therefore the effects of keeping pupae in empty containers or with damp vermiculite was also investigated to find which treatment produced the highest proportion of adults. Finally, the availability of food and water on the life expectancies of virgin adults was investigated to ensure they were sustained in good condition.

Methods

The conditions and methods used for rearing the moths were identical to those described in the preceding section on the rearing method, except for the following instances required for the purposes of this investigation:- The insects were examined daily. Incubation times were found by placing the filter papers with eggs in clear plastic pots, 4 cm high by 5 cm in diameter, with snap-on plastic lids. The eggs and newly hatched larvae used in each experiment were all either laid or hatched on the same day. When the duration of the larval stage was being determined the potato cultures were unpacked daily for examination and only those larvae that had started to spin cocoons were removed and counted. This was necessary because immature larvae often came out of the potatoes and later burrowed back into them.

The effects of crowding on the larvae was investigated by placing different numbers of newly hatched larvae on known quantities of potato in separate containers. The densities of larvae used ranged from 1 to 4 per gram of potato and they were originally placed on the potatoes using a camel hair brush. All the containers were subsequently left alone for 30 days, then they were opened and the numbers of larvae, pupae, and adults amongst the potatoes were counted. In all other respects the rearing method was exactly the same as described in the previous section concerned with this.

Corrugated cardboard and holes drilled into wood were tried as alternative pupation sites. The corrugated cardboard was cut into 15 strips, 3 cm wide by 20 cm long, at right angles to the corrugations. They were placed at random in a newly prepared larval culture where they were left for 30 days before being examined. Holes drilled into wood were provided in three special containers consisting of opaque plastic tubes, 10 cm in diameter and 20 cm long, closed at one end by organdie netting and at the other by removable circular plugs each cut from two flat pieces of wood 12 mm thick and bolted together. One hundred holes 4 mm in diameter were drilled through the inner boards and 3 mm into the outer ones so that when they were unbolted and pulled apart any cocoons inside the holes were also opened. Freshly prepared potatoes with larvae that had just hatched were placed in these containers and they were then left lying on their sides for 30 days before being examined.

The number of days taken from eclosion to hatching by the adults was determined by two methods:- In the first, the potatoes were unpacked daily and mature larvae that had just begun to spin pupation chambers were collected and kept individually in the same type of plastic pots used for the eggs. They spun their pupation chambers in these in the angles between the walls and the floor where they could be examined through the clear plastic. In the second method the potato cultures were left undisturbed except for opening the containers each day and firmly tapping them to make the adults fly out so that they could be collected and counted.

Adults were also kept in the same type of plastic pots used for the eggs. However, the centres of their lids were removed and the rims were then used to fasten organdie netting over the pots. Filter papers were placed on the organdie netting and either left dry, wet with water daily, or initially wet with 10% sucrose solution and subsequently remoistened with water every day. The filter papers were replaced if mould appeared on them and the procedure was restarted. Measured quantities of liquids were not used for this experiment because this was felt unnecessary for rearing and the experiment was designed to simulate rearing methods.

Virgin moths were kept in the plastic pots in groups of up to 5 individuals of the same sex provided they had all emerged on the same day, whereas moths that were allowed to mate were kept as one male and female of the same age per pot.

Results

The eggs hatched on the average about 7 days after being laid with a range of 6 days to 11 days, and up to 60% of them died (Table 6.1). It was also noted that most of these eggs, including those that hatched, shrivelled a small amount after being laid.

The larvae spent a mean time of 20 days feeding in the potatoes with a range of 15 to 38 days (Table 6.3). They then left the potatoes and began to construct silken cocoons. At this stage they were collected and placed in individual containers where they spun their cocoons and pupated after about 2 to 5 days. There was no significant difference between the duration of the larval stage of males and females. On the average 16% of the larvae died before they were collected and a further 22% died after being handled (Table 6.3).

TABLE 6.1

Incubation period for the egg of *P. operculella* (in days)

TRIAL NUMBER	MEAN	S.D.	RANGE	NUMBER OF EGGS HATCHED	% NOT HATCHED
1	7.30	0.48	7-9	139	NR[1]
2	7.25	0.45	7-9	172	50.6
3	6.93	0.28	6-8	143	58.7
4	6.75	0.51	6-9	182	36.5
5	8.42	0.78	7-11	125	NR[1]
TOTAL	7.27	0.75	6-11	761	(47.89[2])

[1] NR = number not recorded

[2] Total mortality from known samples only

TABLE 6.2

Interval between the larvae leaving their potatoes and pupating (in days).

SEX	MEAN	S.D.	RANGE	NUMBER	% THAT DIED
Male	4.20	1.11	2-6	43	13.95
Female	4.23	1.50	2-7	43	

TABLE 6.3

Duration of larval stage. Number of days after eclosion taken by larvae to emerge from potatoes and then to pupate. Data from three trials.

	EMERGENCE FROM POTATOES					PUPATION OF MALES				PUPATION OF FEMALES				Total % Mortality to pupa
	Mean	S.D.	Range	Number Emerging	% Mortality	Mean	S.D.	Range	Number Pupating	Mean	S.D.	Range	Number Pupating	
	20.44	4.63	15-36	109	-	24.81	4.07	19-37	40	25.57	3.52	19-39	46	-
	20.01	1.68	16-25	77	17.2	22.83	1.61	20-25	23	23.71	1.24	21-26	28	45.2
	18.23	3.48	15-38	67	14.1	20.89	1.57	19-24	28	20.73	1.22	19-23	26	30.8
TOTAL	19.79	3.78	15-38	253	15.79 [1]	23.06	3.38	19-37	92	24.03	3.36	19-39	88	38.6 [1]

[1] Total mortalities from known samples only.

In another experiment where larvae were collected after emerging from their potatoes and kept in separate containers, they took 2 to 7 days with a mean of 4.2 days to pupate (Table 6.2). The mortality after this treatment was 14%.

Yields of insects varying from 45% to 78% were obtained when larvae were reared at densities between 1 per gm and 4 per gm of potato (Table 6.4). However, within the scope of the experiment, no increase in mortality was detectable at the high larval densities.

The effect of rearing larvae at higher densities than this was not investigated because the intention was always to use excess potato for rearing the insects for this study.

No advantages were conferred by providing the larvae with alternative pupation sites. When strips of corrugated cardboard were made available few larvae spun their cocoons inside them and these cocoons were still difficult to open. In addition, when given wood with drilled holes usually more than 88% of the larvae pupated elsewhere (Table 6.5). Thus this technique was not considered worthwhile.

Male pupae required significantly longer to mature than females and hatched after 10 to 14 days with a mean of 12.5 days, while females hatched after 9 to 13 days with a mean of 11.2 days (Table 6.7). More than 77% of the pupae kept in damp vermiculite hatched successfully but less than 56% did so when kept in empty containers because many of these moths were unable to detach themselves from their pupal exuviae. However, dry conditions affected males more than females with respective losses of 54.8% and 44.4% whereas the respective losses in damp vermiculite were 22.9% and 20.4%.

A total mortality of almost 60% occurred during the larval and pupal stages when the pupae were collected, sexed under the microscope, and kept in damp vermiculite until they hatched, whereas no mortality was observed in undisturbed cultures when the adults were collected as they emerged (Table 6.6). However, there was no significant difference between the two treatments for the time taken from eclosion until the adults emerged from their pupae.

The life expectancies of virgin moths depended on whether they were starved, given water, or given 10% sucrose solution (Table 6.8). Their life expectancies increased with significant

TABLE 6.4

Results of rearing *Phthorimaea operculella* on different quantities of potatoes

INITIAL WEIGHT OF POTATO (gm)	AVERAGE WEIGHT OF POTATOES (gm)	% REDUCTION IN WEIGHT OF POTATO AFTER 30 DAYS	NUMBER OF NEWLY HATCHED LARVAE PLACED ON POTATOES	WEIGHT OF POTATO PER LARVA (gm)	% ADULTS THAT HATCHED WITHIN 30 DAYS
200	66.7	42.5	200	1	66.0
476	68.0	27.3	239	2	58.7
371	61.8	24.3	185	2	45.4
496	70.9	20.2	198	2.5	54.5
394	65.7	23.6	134	3	66.4
503	83.8	20.3	168	3	52.4
695	77.2	50.4	235	3	51.1
331	82.8	17.2	110	3	68.2
402	80.4	20.4	134	3	67.9
407	81.4	15.0	123	3.3	49.6
434	86.8	21.7	109	4	78.0
697	174.3	15.4	175	4	60.0
469	117.3	17.3	117	4	74.4

TABLE 6.5

Data from experiments in which larvae of *P. operculella* had access to holes for pupation

NUMBER OF NEWLY HATCHED LARVAE	TOTAL YIELD OF PUPAE AND EXUVIAE (%)	PROPORTION PUPATING INSIDE HOLES (% OF YIELD)	WEIGHT OF POTATC TO EACH LARVA (gm)
239	58.6	19.29	1.99
198	54.5	5.56	2.51
200	74.0	8.78	2.97

TABLE 6.6

Interval between eclosion and the emergence of the adult.

TREATMENT	SEX	MEAN	S.D.	RANGE	n	% NOT HATCHING
Collected as larvae and sexed after pupation	Male	32.37	1.99	28-37	30	57.14
	Female	31.55	1.66	30-35	28	
Moths collected after emerging	Male	32.04	2.16	29-38	23	0
	Female	31.05	2.19	29-38	17	

TABLE 6.7

Number of days taken for pupae to hatch, when kept with or without damp vermiculite.

TREATMENT	SEX	MEAN	S.D.	RANGE	NUMBER	% NOT HATCHING
Kept in empty containers	Male	12.45	0.89	10-14	31	54.8
	Female	11.26	0.86	9-13	27	44.4
Kept with damp vermiculite	Male	12.66	0.80	11-14	35	22.9
	Female	11.11	0.65	10-12	49	20.4

TABLE 6.8

Relationship between length of adult life and diet in virgin *P. operculella*

DIET	SEX	LIFESPAN IN DAYS				SIGNIFICANCE LEVEL BETWEEN MALE & FEMALE LIFESPANS (t-test)
		MEAN	S.D.	n	RANGE	
starved	Male	9.75	2.12	20	5-14	8.40***
	Female	22.05	6.21	21	8-35	
water	Male	17.45	5.97	31	3-35	5.69***
	Female	26.48	6.52	31	8-34	
10% sucrose in water	Male	33.61	13.32	21	15-67	0.084
	Female	33.41	14.78	23	5-74	

TABLE 6.9

Length of adult life of mated *P. operculella* when fed 10% sucrose in water

SEX	LIFESPAN IN DAYS				SIGNIFICANCE LEVEL BETWEEN MALE & FEMALE LIFESPANS (t-test)
	MEAN	S.D.	n	RANGE	
Male	12.70	5.47	47	2-23	3.78***
Female	16.39	4.02	49	7-26	

differences between each treatment in the order of those starved, those given water, and those given sucrose solution. When males and females were provided with sugar and water their lifespans were not significantly different with mean lengths of 33.6 days and 33.4 days respectively. However, females lived significantly longer than males when either given water or starved, and their respective mean lifespans were 17.5 and 26.5 days with water and 9.8 and 22.1 days when starved.

In contrast to virgin moths, both male and female moths that were allowed to mate had very much reduced lifespans even though all were provided with sugar and water. The mean lifespan of mated moths was 12.7 days and this was significantly shorter than that of the mated females which was 16.4 days (Table 6.9).

Discussion

The known host plants of *P. operculella* larvae are listed by Cunningham (1969). All belong to the Solanaceae and are closely related: they comprise 15 species of *Solanum* including *S. tuberosum* L. (potato) and *S. melongena* L. (egg plant), 12 species of *Nicotiana* including *N. tabacum* L. (tobacco), *Capsicum frutescens* L. (chili), *Lycopersicon esculentum* Mill. (tomato), 4 species of *Physalis* including *P. peruviana* L. (Cape gooseberry), 3 species of *Datura*, and 5 other species from different genera. The larvae are mostly leaf miners in these plants but they also attack the fruit of *L. esculentum*; the stems of *N. tabacum*, *Solanum nigrum* L. and *Physalis*; and the tubers of *S. tuberosum* (Cunningham, 1969). Finney *et al.* (1947) noted that the favoured food plants were potato and tobacco, while potato tubers have been widely used for rearing *P. operculella* in the laboratory.

According to Finney *et al.* (1947) there appears to be little advantage in using one variety of potato in preference to another for rearing *P. operculella* although they reported that the larvae matured on the average a day earlier when reared on the Russet variety, a mealy potato, than on White Rose, a non-mealy one. In addition, it has also been noted by Platner and Oatman (1968) that the Russet variety of potatoes are more fully consumed by larvae than White Rose. Both Finney *et al.* (1947), and Graf (1917) also reported that the development of the larvae seemed to be affected by the quality of the food rather than the amount, and that larvae in leaves and stems of potato develop more rapidly than in tubers. However, all these effects were only very minor and therefore any potatoes supplied by a greengrocer were used in the present study.

Finney *et al.* (1947) noted that small sized potatoes must be used when rearing *P. operculella* because they have the greatest surface area for the larvae to infest. This was confirmed by Platner and Oatman (1968) who found that the most desirable potato size was between "2.25 and 4 inches" in length and that each potato can support an average of 75 and as many as 90 to 100 larvae. Both Finney *et al.* (1947) and Platner and Oatman (1968) were concerned with rearing parasites of *P. operculella* as economically as possible, whereas in the present study size of potato was considered to be of no consequence since the larvae were always reared on an excess. The majority of potatoes used, however, fell within the recommended size range of Platner and Oatman (1968). Furthermore, in the present study the skins of the potatoes were punctured to allow the larvae ample opportunity to gain entrance because this has been the normal practice of many authors (for example Finney *et al.*1947; Platner and Oatman, 1968, 1972; Broodryk 1971).

Larvae were reared at low densities in potatoes during the present study to ensure they were as healthy as possible. Foote (*pers.comm.*, 1970) advised that rearing at low densities tended to prevent excessive mortality due to disease, and Broodryk (1971) concluded that crowding and consequent malnutrition apparently increased susceptibility to mortality factors such as disease. The latter author reported that when the larvae were crowded above 1 per 5 g of potato the percentage that survived to pupate decreased, and the mean weight of the resulting pupae also decreased. He found, for example, that the percentage that survived and their mean pupal weight were 97% and 11.4 mg respectively at a density of 1 larva per 5 g of potato, 75% and 10.7 mg at 1 per 3 g 56% and 10.3 mg at 1 per 2 g, 43% and 9.7 g at 1 per 1 g and 23% and 8.2 mg respectively at 1 per 5 g of potato. The percentages that survived at densities between 1 per 1 g and 1 per 3 g of potato differed from the results of the present study although this is probably due to chance variation since Broodryk (1971) only used 39 to 157 larvae at each density between 1 per 2 g and 1 per 5 g of potato, and replicates in the present study each with from 109 to 235 larvae showed variations of up to 18% (Table 6.4). Broodryk (1971), however, used 618 to 2552 larvae at each density between 1 per 1 g to 5 per 1 g of potato so possibly his results are correspondingly closer to mean values. When the results of both this and the present study are considered together they clearly indicate that the larvae are more healthy when reared at low densities.

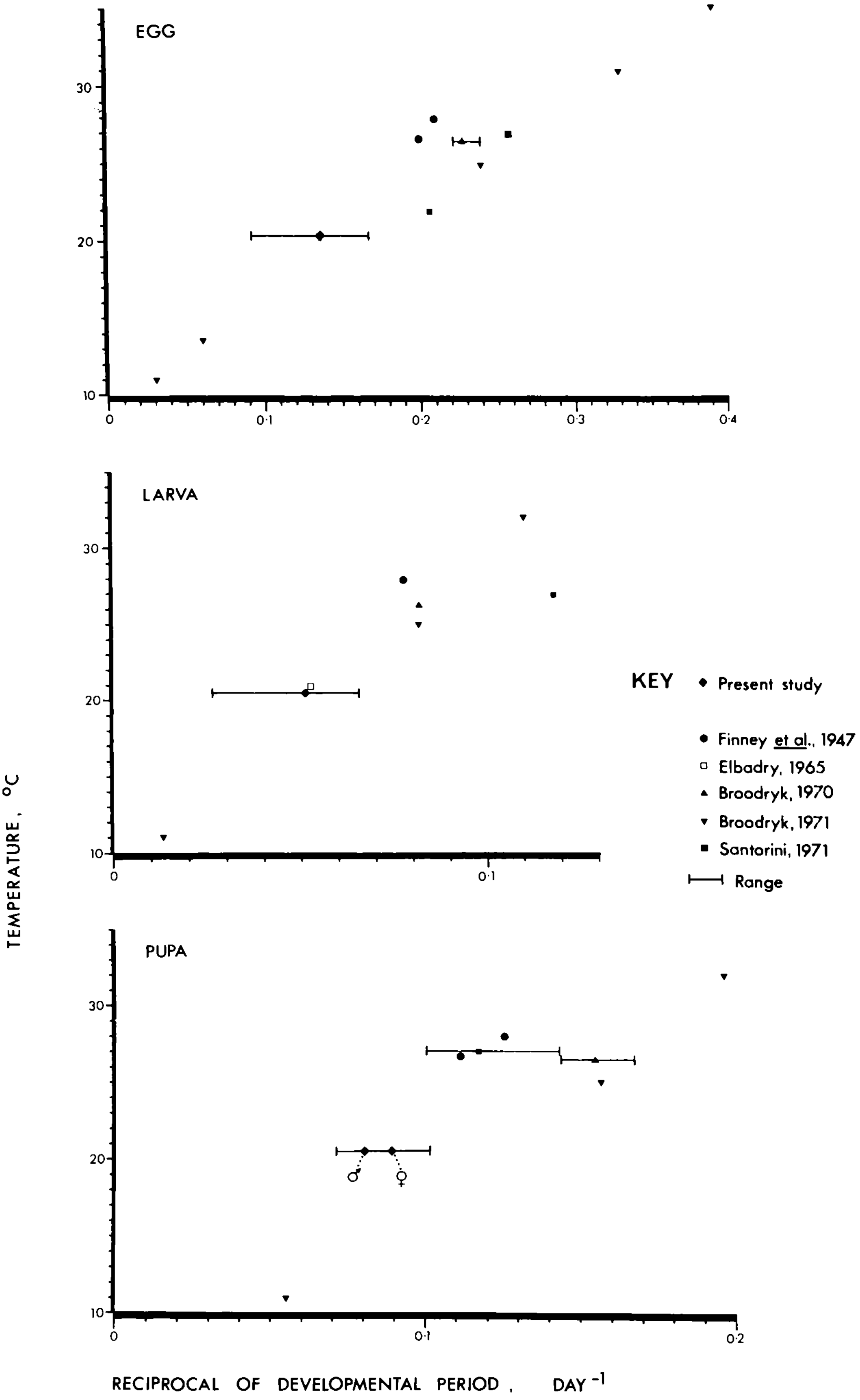
It appears probable that the optimum temperature for rearing *P. operculella* is near room temperature if it is not necessary to breed them quickly. Broodryk (1971) reported that male and female adults fed honey produced twice the numbers of eggs (an average of 71.5, range 0-232) when they were kept at 21°C than they did at any of the temperatures 11, 16, 25, 29 and 39°C. However, this author noted that the eggs, larvae, and pupae all develop apparently normally at temperatures between 11°C and 32°C but they all die if kept at 39°C. Finney *et al.* (1947), however, stated that outbreaks of disease could result from rearing the larvae at temperatures above 30.6°C, but noted also that a suitable temperature was 28°C and that the moths attained their greatest size at temperatures lower than this.

The mean times spent by *P. operculella* in the egg, larva, and pupa in the present study were compared with the times determined by other workers at different temperatures by plotting the reciprocal values of developmental time against temperature as shown in Figure 6.3 This makes the relationship between time and temperature linear (Uvarov, 1931), and therefore makes comparisons easier. Figure 6.3 demonstrates that the mean developmental periods in the present study generally compared well with those of Finney *et al.* (1947), Elbadry (1965), Broodryk (1970, 1971), and Santorini (1971) although the developmental period of the larva according to Santorini (1971), and the pupa according to Broodryk (1970, 1971) were markedly shorter than those determined in the present study and by other workers. Possibly these differences are due to physiological adaptation to the general temperature characteristics of the area in which the population of moths has existed for some time. Broodryk (1971) has also suggested this to explain why the threshold of development of South African tuber moth eggs (9.5°C) is 4.25C° below that of Egyptian ones. A further point of difference noted between the pupae observed in the present study and those examined by Santorini (1971) was that the males took significantly longer to develop than females in the present study whereas Santorini stated that they both took the same time (mean of 8.5 days) at 27°C. Other workers, however, did not examine males and females separately.

Figure 6.3

Comparisons between the mean durations of egg larval and pupal stages of *P. operculella* determined in the present study with the values published by other research workers

Since the other research workers used different temperatures the comparisons have been facilitated by plotting the reciprocals of development time against temperature which makes the relationship linear.


EGG
LARVA
PUPA
30
20
10
0
0·1
0·2
0·3
0·4
TEMPERATURE , °C
RECIPROCAL OF DEVELOPMENTAL PERIOD , DAY $^{-1}$
♂
♀
KEY
◆ Present study
● Finney *et al.*, 1947
□ Elbadry, 1965
▲ Broodryk, 1970
▼ Broodryk, 1971
■ Santorini, 1971
├─┤ Range

It was noted that the proportion of eggs that failed to hatch in the present study (Table 6.1) appeared to be rather high when compared with those of Broodryk (1971) which ranged from 7% to 13%. Desiccation was probably not the cause of this even though the eggs shrivelled a certain amount because Broodryk (1971) found that the percentage of eggs that hatched at 26.5°C was approximately the same over a relative humidity range from 11.1% to 100%. Large numbers of eggs were, however, always available during the present study and therefore the cause of this high loss was not investigated.

During the present study a higher proportion of normal moths was obtained from pupae that were kept in dampened sand when compared to those kept in dry sand. However, it appears from the data of Broodryk (1971) that relative humidity alone has no effect on the proportion of pupae that die or become deformed adults. This author kept pupae at 5 different relative humidities between 11.1% and 100% at 26.5°C and reported losses ranging from 14.0% to 22%. These losses were close to those obtained in the present study when pupae were kept in damp sand and therefore it appears likely that the detrimental effect of dry sand was due to excessive water loss resulting from abrasion and that this was minimised by damp conditions.

Santorini (1971) reported that the lifespans of adult male and female *P. operculella* depended on whether they received food and water. He gives the mean number of days spent by male and female moths as 8.4 and 8.8 days respectively when provided with no food or water, 8.5 and 9.0 days with water, 9.5 and 11.8 days with 8% honey, and 11.6 and 11.0 days respectively when provided with 8% "Biopol", a food used in apiculture. These moths were, however, kept at 27°C and allowed to mate so the results cannot be compared directly with those of the present study, but they do show the same trends. The only other published data found on the lifespan of adult *P. operculella* was that of Brookryk (1970) who reported that the average for males and females was 13 days when fed honey and kept at 26.5°C. It is, however, not clear whether this author means that the lifespan is 13 days for both male and female moths or whether this is for a mixed population, and he does not specify their mated state.

Overall, the data obtained in the present study on the development of *P. operculella* compared well with those of other workers, and therefore it was unlikely that the present rearing procedure was detrimental to the insects in any way. It was, however, particularly important that the adult moths were kept in a healthy state because they were the experimental subjects in the present study. Most adults were used when they were aged less than 5 days and their longevity under the rearing conditions indicated that they were very healthy. Additional confirmation of this was also obtained for the females from the investigation of oviposition (Appendix 1.2), since the numbers of eggs they laid compared favourably with the numbers determined by other workers.

CHAPTER 7

The reproductive organs of *P. operculella*

Introduction

The internal reproductive organs of *P. operculella* have not been described elsewhere although the structure of the male and part of the female external genitalia have recently been reported by Povolný (1964) for taxonomic purposes. Part of this study was therefore a detailed description of the morphology and histology of the internal reproductive organs of the adult to provide background information both for any future work on the reproduction of this moth and also for those aspects dealt with here in the following chapters. A full investigation of the testes and ovaries was not done although general observations made on them during the course of this study are presented here. Also included here, is an investigation into sexual dimorphism of the imago and complete descriptions of the external genitalia because these constitute essential basic information for studies of the sexual behaviour of these moths.

Methods

The methods include all those used during the investigation of the reproductive organs of *C. zealandica* which are described in Chapter 3. However, there were two minor differences:- Firstly, the moths were used when aged between 1 and 3 days old unless otherwise stated. Secondly, the copulating moths were quick frozen by first allowing them to copulate in small glass tubes covered with organdie netting over each end, then, after one of the pieces of organdie was removed, they were dropped into liquid nitrogen.

The fixatives listed for *C. zealandica* generally worked well with *P. operculella*. However, they gave poor results for the ovaries, spermathecal glands and accessory glands in the female and therefore Altmann's fixative with chrome alum (Humason, 1967) was used for them. These organs were fixed in this for 2 hours, then transferred directly to 70% alcohol followed by the normal dehydration, clearing and embedding procedure as used for *C. zealandica*. Good fixation of ova complete with chorions was not achieved and they also shrank badly. This could have been solved by puncturing the eggs as is normal procedure when preparing them for sectioning (Humason, 1967), but this was not considered necessary for the present study since the structure of the egg was not investigated.

The moths' genitalia were extended, in order to be examined under the scanning electron microscope, by placing the moths between strips of plasticene sandwiched between glass microscope slides and gently squeezing the slides together. When the genitalia were visible the moths, together with plasticene and slides, were dropped into liquid nitrogen and subsequently allowed to warm up to -35°C. The moths were then removed from between the slides and prepared for examination as described in the methods section of chapter 3.

Sexual dimorphism in the adult

Adult male and female moths are best distinguished by their external genitalia which are described in detail below under appropriate section headings.

Both sexes are very similar in general shape and colour, but they can be easily distinguished with the unaided eye by the shape of the abdomen. When viewed ventrally, that of the female is relatively broad and tapers to a point (Figure 8.8B), whereas that of the male is narrower and of a more uniform thickness along most of its length except the tip which is broad and spatulate shaped (Figure 8.8A). This thickening is caused both by elongated scales which arise from the 8th sternite and by tufts of elongated scales that arise from the posterior lateral edges of the 7th segment. The latter are termed coremata and normally lie laterally alongside the other terminal structures. All the scales at the end of the male's abdomen are coloured a slightly yellow to orange grey compared with the rest of the moth which is brownish grey. Povolný (1964) also notes that, in the male, the colour of the end of the abdomen and the macroscopically visible coremata sharply define the genus *Phthorimaea* from the other genera of the tribe Gnorimoschemini.

The other sexually dimorphic structures are the hair pencils consisting of long thin scales which arise dorsally on each hindwing near the anterior edge and close to the base. Povolný (1964) also notes these and terms them "osmeteriches". In the present study (Chapter 8) they were found to be used during mating behaviour and they constitute part of the male sex pheromone organs. They are described in detail in Chapter 10.

No other sexually dimorphic structures were found in the present study although a small scale morphometric investigation was made during the search for them. The dimensions of the parts examined are given in Table 7.1 and show that most measurements did not differ between male and female. The male, however, did have a significantly longer total body length, prothoracic leg length from femur to tarsus, and metathoracic tarsus length, while the eyes were significantly further apart and of larger diameter (Table 7.1).

The male internal reproductive system

The morphology and histology of the male internal reproductive system of *P. operculella* has not previously been described. However, there is a fairly extensive literature on the internal reproductive systems of other Lepidoptera. This includes morphological descriptions for various microlepidoptera (Stitz, 1901, Lasiocampidae (Williams, 1940), Tineoidea (Williams, 1947), and Noctuidae (Callahan, 1958, 1960; Callahan and Chapin, 1960). Detailed descriptions are also available for Zygaena (Hewer, 1932); *Plodia,* and *Anagastia kuhniella* (Zeller) (Norris, 1932); *Galleria mellonella* (L.) (Khalifa, 1950); *Colias philodice eurytheme* (Boisduval) (Stern and Smith, 1960); *Rhyacionia buoliana* (Schiffermuller) (Shen and Berryman, 1967); *Laspeyresia caryana* (Fitch) (Tedders and Calcote, 1967); and *Diatraea grandiosella* (Dyar) (Davis, 1968). Morphological descriptions together with some histology are available for some Saturniidae (Ruckes, 1919), *Leucinodes orbonalis* Guen. (Srivastava, 1960b), and *Heliothis zea* (Boddie) (Callahan and Cascio, 1963), while detailed histology is given for A. *kuhniella* (Zeller) (Musgrave, 1937) and *Choristoneura fumiferana* (Clemens)(Outram, 1970).

The internal reproductive system of the male *P. operculella* is basically the same as that found in all the higher Lepidoptera Imms, (1960). The terminology used here follows that of Callahan (1958).

The internal reproductive system (Figure 7.1) consists of a pair of testes fused into a single structure, two vasa deferentia and their seminal vesicales, a pair of accessory glands, a ductus ejaculatorius duplex and a ductus ejaculatorius simplex. The latter joins the endophallus which leads to the exterior through the penis or

TABLE 7.1

Lengths of various external parts of *Phthorimaea operculella* taken from 26 male and 20 female moths.
All measurements in mm.

	MALE		FEMALE		Student-t value
	MEAN	S.D.	MEAN	S.D.	
Total body length (head to abdomen	6.571	0.236	6.200	0.639	2.651*
Labial palp 2nd segment length	0.776	0.051	0.771	0.069	0.284
3rd segment length	0.718	0.051	0.692	0.075	1.312
Eye max. diameter	0.502	0.020	0.458	0.029	6.112***
Min. distance between eyes	0.646	0.027	0.618	0.040	2.867**
Antenna length	4.954	0.333	5.025	0.377	0.679
Forewing length	6.312	0.232	6.321	0.505	0.085
Hindwing length	5.085	0.187	5.068	0.365	0.194
Prothoracic leg femur length	1.103	0.053	1.056	0.095	2.135*
tibia length	0.933	0.075	0.858	0.100	2.847**
tarsus length	1.353	0.059	1.290	0.088	2.819**
Mesothoracic leg femur length	1.401	0.059	1.379	0.049	1.248
tibia length	1.426	0.078	1.391	0.116	1.134
tarsus length	1.625	0.066	1.628	0.102	0.125
Apical spur length	0.510	0.038	0.525	0.077	0.888
Metathoracic leg femur length	1.498	0.070	1.489	0.118	0.324
tibia length	2.276	0.120	2.247	0.158	0.718
tarsus length	2.409	0.087	2.309	0.163	2.640*
medial spur length	0.911	0.056	0.913	0.062	0.082
Apical spur length	0.695	0.032	0.701	0.053	0.464
Valva length	1.198	0.084			
Penis length	1.270	0.044			
width (midway)	0.072	0.005			
Distance from posterior edge of 8th sternite to anterior end of scleratised ductus bursae			0.717	0.060	
Extended ovipositor length			0.982	0.209	
Width along posterior edge of 8th sternum			0.442	0.037	
Apophysis posterior length			1.272	0.066	

Figure 7.1

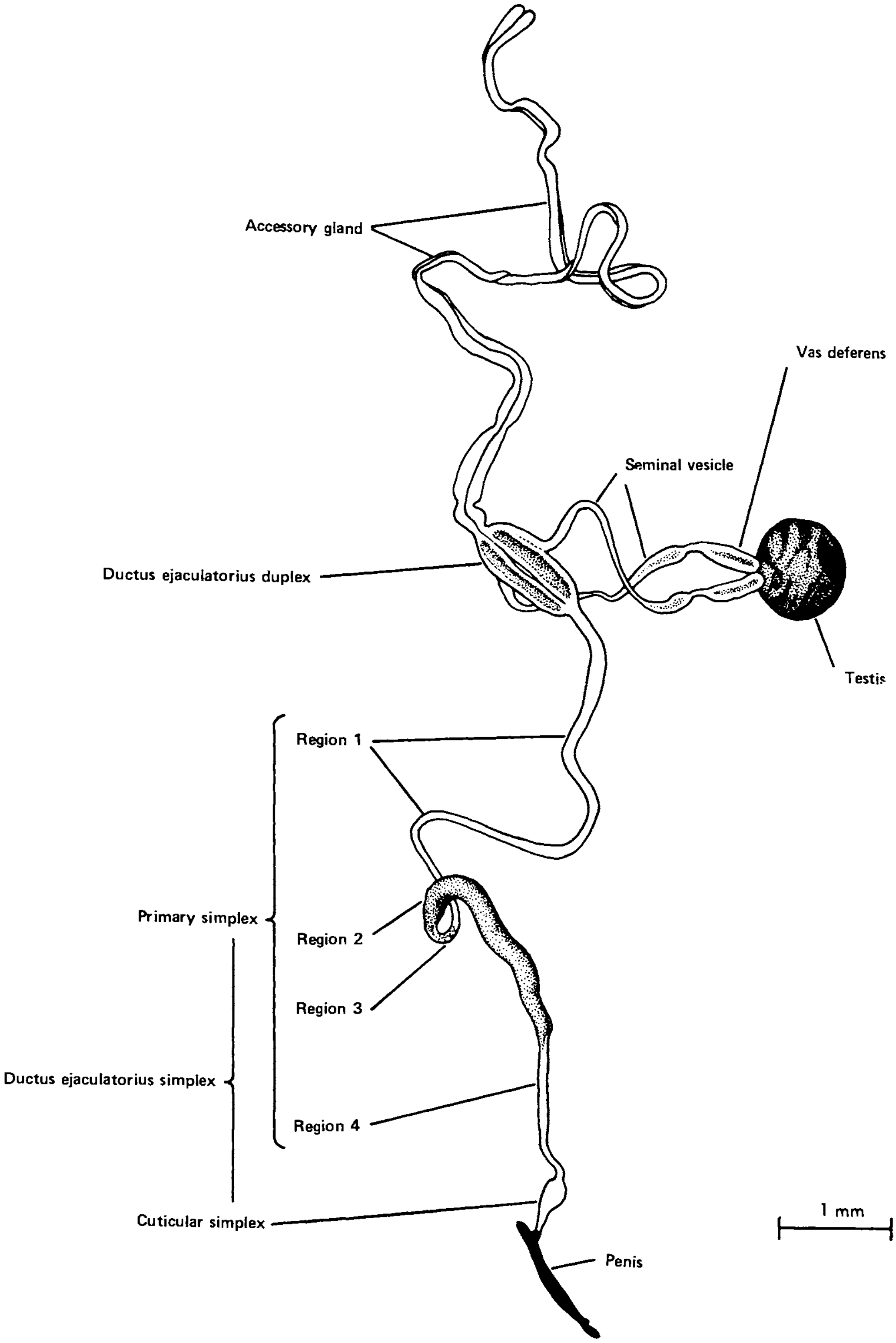
The internal reproductive organs of the male *P. operculella*

Errata

On the diagram:

"Region 2" *should be labelled* "Region 3"

"Region 3" *should be labelled* "Region 2"


Accessory gland
Vas deferens
Seminal vesicle
Ductus ejaculatorius duplex
Testis
Region 1
Primary simplex
Region 2
Region 3
Ductus ejaculatorius simplex
Region 4
Cuticular simplex
1 mm
Penis

phallus. Some of the dimensions of the above parts are given in Table 7.2.

The testes

The fused testis is a conspicuous approximately spherical organ which is coloured a mottled deep reddish brown. Its maximum diameter varies between 0.50 mm and 0.88 mm (Table 7.2) and it lies dorsally in the 2nd to 3rd abdominal segments just beneath the pericardium.

Each testis consists of 4 follicles enclosed within their own connective tissue sheaths (Figure 7.2) which have an inner layer of pigmented cells. Around the outside these sheaths are fused into a common sheath or scrotum which encloses and binds together both testes. The scrotum consists of a tough layer of connective tissue fibres and muscle about 2 μ thick with an inner layer of pigmented cells.

The follicles are mostly filled with sperm bundles but there is a relatively small germarium situated dorsally followed by a region containing cysts of cells at various stages of spermatogenesis (Figure 7.2). The sperm bundles consist of closely packed parallel spermatozoa enclosed within their cysts along approximately their entire lengths. Each sperm bundle is between 10 to 13 μ wide by about 550 μ long and it is coiled into a left-handed spiral of 9 to 10 turns with an outer radius of about 30 μ.

The vasa deferentia

The paired vasa deferentia are transparent to slightly translucent white tapering tubes which arise next to each other on the ventral surface of the testes and then run in a ventral direction in the abdomen. Where they arise from the testes they have an average width of 0.14 mm and this reduces to an average of 0.075 mm where they join the seminal vesicles. The left vas deferens is also usually longer than the right one with respective mean lengths of 0.71 mm and 0.69 mm but occasionally in individuals they are the same length (Table 7.2).

Each vas deferens is composed of a single layer of columnar epithelial cells ~~cells~~ which rest on an outer basement membrane and are surrounded by a sparse network of of muscle fibres.

TABLE 7.2

Dimensions of the male internal reproductive organs of *Phthorimaea operculella*.
Measurements taken from 10 males.
All measurements in mm.

				RANGE	
		MEAN	S.D.	max.	min.
Testis	max. diameter	0.720	0.132	0.875	0.500
Vas deferens	max. width	0.138	0.029	0.175	0.080
	min. width	0.075	0.024	0.100	0.030
	length of left	0.708	0.116	0.875	0.500
	length of right	0.689	0.115	0.875	0.500
Seminal vesicles					
	max. width	0.140	0.035	0.200	0.088
	min. width	0.039	0.016	0.063	0.020
	length of left	2.288	0.543	3.250	1.625
	length of right	1.805	0.698	2.550	0.625
Accessory glands	length	9.90	1.63	12.3	7.8
	width	0.048	0.018	0.075	0.025
Ductus ejaculatorius duplex					
	length	1.040	0.276	1.750	0.750
	width	0.165	0.029	0.225	0.125
Ductus ejaculatorius simplex					
region 1 width by ductus ejaculatorius duplex		0.125	0.018	0.125	0.100
regions 1 and 2	length	5.076	0.636	6.30	4.00
	average width	0.060	0.013	0.075	0.038
region 3	length	2.328	0.681	3.20	1.25
	max. width	0.174	0.059	0.225	0.100
region 4	length	0.577	0.093	0.75	0.50
	width	0.095	0.027	0.13	0.06
regions 5 and 6	length	0.421	0.042	0.58	0.38
	max. width	0.206	0.033	0.23	0.15

Figure 7.2

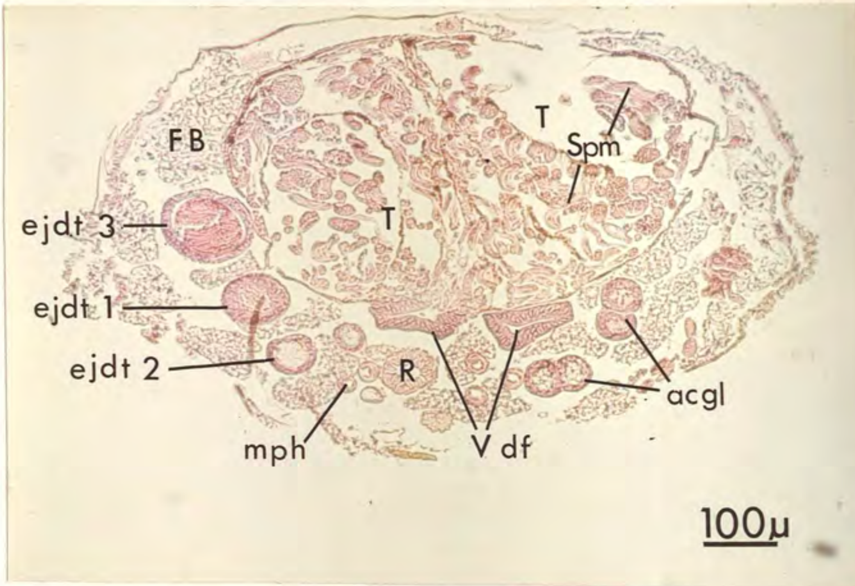
Histological micrographs of the male internal reproductive organs of *P. operculella*

Upper Cross section through the abdomen in the region of the testis. Note the sperm bundles.

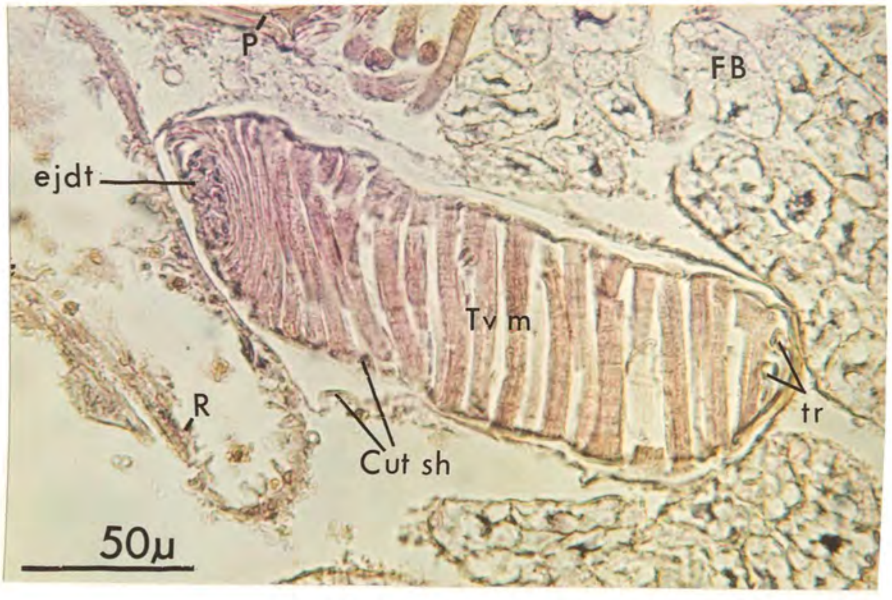
Middle An oblique section through the anterior of the 5th region of the ductus ejaculatorius simples (cuticular simplex) showing the greatly developed circular musculature.

Lower An oblique section trhough the middle of the 5th region of the ductus ejaculatorius simplex showing the transverse muscles and the cuticular sheaths.

acgl	accessory gland
Cm	circular muscle
Cut sh	cuticular sheath
ejdt	ductus ejaculatorius simplex
ejdt 1 ejdt 2 ejdt 3	regions 1 to 3 of the ductus ejaculatorius simplex
FB	fat body
mph	Malpighian tubules
P	penis
R	rectum
Spm	spermatozoa
T	testis
tr	tracheae
Tv m	transverse muscle
V df	vas deferens


FB
T
T
Spm
ejdt 3
ejdt 1
ejdt 2
R
mph
V df
acgl
100µ


FB
Cm
ejdt
R
50 µ


P
FB
ejdt
Tv m
R
Cut sh
tr
50µ

The epithelial cells vary slightly over the length of the vas deferens: Those of the thinner region (Figure 7.3 A) are between 21 µ and 24 µ tall, their nuclei are rouned with diameters of 8 to 9 µ and situated basally while their cytoplasm is densely basophilic in their basal regions fading to lightly basophilic apically where some eosin staining is visible. The intercellular membranes of these cells are distinct apically but are more difficult to distinguish basally amongst the basophilic cytoplasm. The epithelial cells of the thicker region of the vas deferens are generally between 14 to 16 µ tall, their rounded to oval nuclei are also basally situated but slightly smaller with maximum diameters of 5 to 8 µ; their cytoplasm is densely and evenly basophilic. These cells therefore resemble those of the thinner region except that they lack the apical lighter staining region.

The epithelial cells grow together at the junction with the testis to apparently close off the vas deferens (Figure 7.2 A) but there are probably distendable spaces between them which are normally occluded because sperm bundles can often be observed passing between the cells. At the other end of the vas deferens the epithelial cells are enlarged to form a plug which occludes the lumen. However, these cells probably are not joined apically, thus allowing the sperm bundles to pass through.

Ruckes (1919) reported that the epithelial cells of the vas deferens in some Saturniidae were crowded and grouped into ridges or clusters which he termed pseudonidi, and that the cells were distorted as a result of this. However, this epithelium is uniform and composed of columnar cells in *A. kuhniella*, *L. orbonalis*, *H. zea*, and *C. fumiferana* according to Musgrave (1937), Srivastava (1960b), Callahan and Cascio (1963), and Outram (1970) respectively. In *A. kuhniella* their intercellular membranes are figured by Musgrave (1937) as only being distinct apically, and they appear to be entirely indistinct in *C. fumiferana* from the diagram by Outram (1970). These cells have also been reported to have a distinct brush boarder in *L. orbonalis* by Srivastava (1960b), and to have one only when close to the testes in *A. kuhniella* according to Musgrave (1937). Ruckes (1919) described the cytoplasm of the cells in the lower portions of the vas deferens in Saturniidae as appearing fibrous with prominent apical vacuoles. Similarly, the free ends of the cells of the upper vas deferens in *C. fumiferana* are densely vacuolated and form an irregular boarder with the lumen where large "gobbets" of secretion are budded off

Figure 7.3

Histology of the male internal reproductive organs of *P. operculella*

(All transverse sections)

A. Vas deferens

B. Vesicula seminalis

C. Ductus ejaculatorius duplex

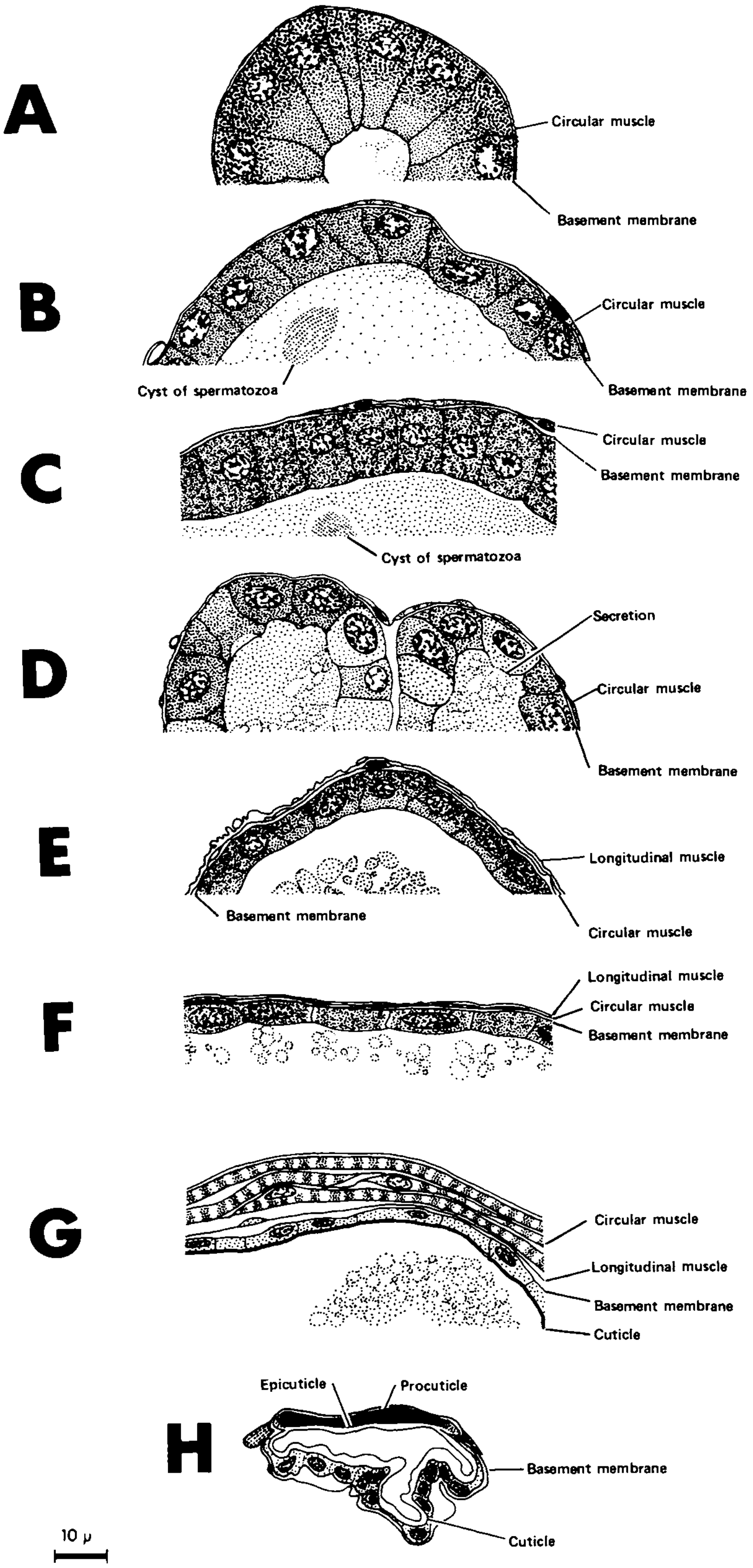
D. Accessory glands

E. First region of the ductus ejaculatorius simplex (primary simplex)

F. Third region of the ductus ejaculatorius simplex (primary simplex)

G. Anterior of the 5th region of the ductus ejaculatorius simplex (cuticular simplex)

H. Endophallus


A
Circular muscle
Basement membrane
B
Circular muscle
Cyst of spermatozoa
Basement membrane
C
Circular muscle
Basement membrane
Cyst of spermatozoa
D
Secretion
Circular muscle
Basement membrane
E
Longitudinal muscle
Basement membrane
Circular muscle
F
Longitudinal muscle
Circular muscle
Basement membrane
G
Circular muscle
Longitudinal muscle
Basement membrane
Cuticle
H
Epicuticle
Procuticle
Basement membrane
Cuticle
10 μ

(Outram 1970). However, Strivastava (1960b) did not observe any vacuoles in these epithelial cells in *L. orbonalis*.

The muscular layer surrounding the vas deferens may be absent or thin in Lepidoptera: Stitz (1901) states that the majority of male organs are entirely devoid of musculature and Musgrave (1937) tentatively referred to the thin layers of doubtful origin surrounding most of the reproductive organs in *A. kuhniella* as basement membrane. In addition, Srivastava (1960b) makes no mention of muscle in *L. orbonalis* except around the cuticular simplex of the ejaculatory duct. Norris (1932), however, suggested that Stitz (1901) was incorrect in his findings. Ruckes (1919) found one muscle layer surrounding the vas deferens of some Saturniidae, Callahan and Cascio (1963) observed a single layer of circular muscle in this position in *H. zea*, and Outram (1970) reported a well defined but thin circular muscle layer there in *C. fumiferana*.

Vesiculae siminales

The seminal vesicles of *P. operculella* are long thin tubes of varying diameter. Near their junction with the vasa deferentia they are relatively wide, averaging 0.14 mm in diameter but they taper to an average diameter of 0.039 mm (Table 7.2) after approximately 0.5 mm to 1 mm. After this they continue as thin tubes until they come to within about 0.5 mm of the ductus ejaculatorius duplex when they gradually expand slightly again. They become attached to their respective branch of the ejaculatorius duplex one quarter to one third along its length from its posterior end and run alongside them to enter posteriorly. Most authors consider the upper dilated portion of the seminal vesicles to be part of the vas deferens while Callahan and Cascio (1963), and Outram (1970) considered it to be a first seminal vesicle in *H. zea* and *C. fumiferana* respectively. These authors called the dilation near the ejaculatorius duplex the second seminal vesicle because it differed histologically from the first. In *P. operculella*, however, there is no significant histological difference between these two regions (see below) and therefore the entire duct is simply called the seminal vesicle.

As with the vasa deferentia, the left and right seminal vesicles differ in length, the left having a mean length of 2.29 mm and the right one of 1.81 mm (Table 7.2). The left seminal vesicle also traverses a more complex path through the abdominal organs than does the right but these paths vary in different individuals. Generally, the first swollen region of the left seminal vesicle continues in a ventral direction from its vas deferens to the left of the folded ejaculatory ducts, it then bends under the rectum and turns dorsad again to the right of the ejaculatorius duplex and dorsally over it to attach to its left-hand side. The ductus ejaculatorius duplex is itself usually placed ventral and to the left of the testis but it is rotated and twisted around so that its originally ventral surface is dorsal and its originally anterior end now faces posteriorly: this torsion accounts for the left seminal vesicle running dorsally over the ejaculatorius duplex to attach to its left branch. The right-hand seminal vesicle simply runs first in a ventral direction from its vas deferens and then folds back on itself to attach to the right side of the ejaculatorius duplex.

Each seminal vesicle is histologically similar throughout its length. It consists of a layer of cuboidal to flattened cells which rests on an outer basement membrane surrounded by a well developed layer of circular muscle (Figure 7.3 B). The cytoplasm of these epithelial cells is densely basophilic, their intercellular walls are fairly distinct and their rounded to oval nuclei are centrally or slightly basally situated and have a maximum diameter of about 5 to 7 µ. For the most part these cells are approximately cuboidal and have heights of between 7µ and 11µ depending on how stretched out they are. Near the ductus ejaculatorius, however, these epithelial cells become flattened and their heights range between 5 µ and 7 µ.

The contents of the seminal vesicles consist of sperm bundles and a few free spermatozoa floating in either a non-staining liquid or one containing small eosinophilic globules of less than 0.5 µ in diameter. No evidence was found as to the origin of this secretion but possibly the non-staining liquid is secreted by the epithelial cells of the vasa deferentia or seminal vesicles while the eosinophilic globules possibly originate from the accessory glands because they resemble their secretion. In addition, when the seminal vesicles were observed during dissection they often appeared clear or slightly translucent near the vasa deferentia but contained increasingly larger areas of a slightly milky liquid near the ductus ejaculatorius and this closely resembled that filling the accessory glands and ductus ejaculatorius duplex. In addition the sperm bundles could also be seen within the clear regions of the seminal vesicles.

The epithelial cells of the seminal vesicles of some Saturniidae are columnar and are not grouped into pseudonidi according to Ruckes (1919). In *A. kuhniella*, those of the seminal vesicles are irregularly shaped and frequently separated basally but fuse apically. In this insect the cells of the lower vas deferens are also irregularly shaped and their intercellular walls are only sometimes visible (Musgrave, 1937). Low columnar to cuboidal cells constitute the seminal vesicle epithelium of *H. zea* according to Callahan and Cascio (1963). In *C. fumiferana* as described by Outram (1970), the epithelial cells of the first seminal vesicle are columnar and their secretion is liberated into the lumen in fine droplets while the cells of the second seminal vesicle are low to cuboidal. None of the above authors except Outram (1970) noted vacuoles in the seminal vesicle cells or those of the lower vas deferens and Srivastava (1960b) states that none occur in *L. orbonalis*. Finally, a thin layer of circular muscle surrounding the seminal vesicles has been reported in some Saturniidae, *H. zea*, and *C. fumiferana* by Ruckes (1919), Callahan and Cascio (1963), and Outram (1970) respectively.

The ductus ejaculatorius duplex

The ductus ejaculatorius duplex of *P. operculella* consists of two swollen sausage shaped tubes which lie adjacent to each other (Figure 7.1). Anteriorly each of these drains a seminal vesicle and an accessory gland and posteriorly both join the end of the ductus ejaculatorius simplex. The ejaculatorius duplex forms a reservoir for both the accessory glands and seminal vesicles and it therefore appears a translucent pale white colour because of their secretions. In size the ejaculatorius duplex varies in length between 0.75 and 1.75 mm and the individual branches vary in maximum width from 0.13 to 0.23 mm (Table 7.2).

Each branch of the ejaculatorius duplex is composed of a single layer of cells similar to those of the seminal vesicles, which rest on an outer basement membrane surrounded by a well developed layer of circular muscle (Figure 7.3 C). Possibly the circular muscle overlies a sparse layer of longitudinal muscle but this cannot be detected at all in the sections.

The epithelial cells of the ductus ejaculatorius duplex have been reported by Ruckes (1919) to have prominent apical vacuoles in some Saturniidae, but no vacuoles were reported in those of *L. orbonalis* by Srivastave (1960b). In *H. zea* the cytoplasm of these cells contained granules and clear globules which move apically and bud off according to Callahan and Cascio (1963). Outram (1970) noted that in *C. fumiferana* this cytoplasm was dense but contained small apical vacuoles and he also observed that the cells were in a very active secretory state and no distinct inner boarder was visible. Both Callahan and Cascio (1963), and Outram (1970) reported that these epithelial cells degenerate during the life of the adult in *H. zea*, and *C. fumiferana* respectively, until at old age only the basement membrane and muscle is left. However, no evidence of degeneration in these cells was found in *P. operculella* during the present study.

In some Saturniidae the ductus ejaculatorius duplex was reported by Ruckes (1919) to be surrounded only by a layer of circular muscle but in *H. zea* and *C. fumiferana* it is enclosed by inner longitudinal muscles and an outer layer of circular muscle according to Callahan and Cascio (1963), and Outram (1970) respectively.

Anteriorly the walls of the ejaculatorius duplex of *P. operculella* merge imperceptably into those of the accessory glands and seminal vesicles, and the lumens of all are continuous, but posteriorly these cells enlarge where the two branches come together and they almost occlude the lumen.

In *A. kuhniella* the lumen of each branch of the ductus ejaculatorius duplex is separated, at least partially, from that of the accessory gland by a partition according to Musgrave (1937) and he presumes that this is broken down during copulation. However, this is the only specific description of what happens at the ends of these organs in other Lepidoptera and therefore their lumens are probably continuous with those of the adjoining structures.

The accessory glands

The paired accessory glands are closely bound together along their whole length. They are long thin blind ending tubes which vary in length between 7.8 mm and 12.3 mm and in width between 0.13 mm and 0.23 mm (Table 7.2). They arise from the originally anterior end of the ejaculatorius duplex which is twisted to face posteriorly and run repeatedly back and forth at least three times within the 2nd to 7th abdominal segments, coiling as they do so.

Histologically they are composed of an inner epithelial layer of approximately cuboidal cells resting on a basement membrane and bound together by a common connective tissue sheath with a network of muscles in it (Figure 7.3 D).

The epithelial layer consists of two types of cells which intergrade with one another. Both types vary in height from 3 to 9 μ, have more or less distinct intercellular walls and have rounded to slightly oval nuclei with maximum diameters of 6 to 8 μ. The first type of cells are indistinguishable from the epithelial cells of the ejaculatorius duplex, except that they are usually smaller, while the second type have eosinophilic cytoplasm and are actively secretory. Dense clusters of globules of non-staining secretion can be seen to arise from the middle of their apical surfaces where the cell walls are indistinct. Vacuoles, however, occur singly and rarely in these cells. All intermediate stages occur between these two cell types although secreted globules are only found arising from cells with non-basophilic cytoplasm or with cytoplasm containing only a few small scattered basophilic granules. It is therefore possible that these cell types represent the same cells at different stages of a secretory cycle.

The secretion surrounding the non-staining globules in the accessory glands is a pale homogenous liquid which stains lightly eosinophilic but as it moves proximally it gradually changes to a dense mass of deep staining eosinophilic droplets with diameters of up to 0.25 μ surrounded by clear space or non-staining liquid. These droplets probably condense from the homogenous liquid although they could possibly also be artifact.

Musgrave (1937) recognized 4 regions in the accessory glands of *A. kuhniella* on the basis of their secretions and Outram (1970) similarly recognized three regions in those of *C. fumiferana*. However, according to these authors the accessory glands of these insects are histologically similar throughout their lengths. Callahan and Cascio (1963) also found this epithelium was histologically uniform in *H. zea* and suggested that the apparent differences between the various regions are due to physiological changes in the secretion.

Ruckes (1919) describes the cytoplasm of the accessory gland cells in some Saturniidae as spongy and not obviously vacuolated, and no vacuoles were reported in these cells in *A. kuhniella* by Musgrave (1937), or in *L. orbonalis* by Srivastava (1960b). However, Callahan and Cascio (1963) describe these epithelial cells in *H. zea* as low columnar with central nuclei, and their secretion accumulates in apical vacuoles which rupture liberating the secretion into the lumen. After this the cell walls reform leaving an even border. Outram (1970) reports that in *C. fumiferana* these cells are also low columnar and their cytoplasm contains many vacuoles which are periodically discharged as fine droplets into the lumen of the gland in a similar process to that described by Callahan and Cascio (1963) for *H. zea*.

Ruckes (1919) reported that the accessory glands of some Saturniidae were surrounded by a single layer of longitudinal muscle. Callahan and Cascio (1963) made no mention of any muscle surrounding these organs in *H. zea*. Outram (1970), however, described an inner longitudinal layer of muscle and an outer circular layer investing each accessory gland in *C. fumiferana*.

The ductus ejaculatorius simplex

In *P. operculella* the ductus ejaculatorius simplex arises from the originally posterior end of the ductus ejaculatorius duplex, and forms a long thin tube which runs back and forth longitudinally three times within the 2nd to 7th abdominal segments and finally disappears into the dorsal side of the base of the penis.

Externally, it is divided into 5 regions by its width and by the appearance of its contents; the first 4 of these can be grouped together as the primary segment since they are not lined with cuticle while the last one has an intima and is therefore termed the cuticular segment.

The first region is usually a transparent or occasionally slightly translucent white duct with a uniform or slightly tapering diameter of 0.075 to 0.038 mm along most of its length although this broadens to a mean diameter of 0.13 mm immediately before it joins the ejaculatorius duplex. Posteriorly it runs into the second region without any change in diameter but this is marked by the secretion inside taking on the appearance of a number of closely packed clear globules. The total length of the combined first and second regions varies from 4.0 to 6.3 mm (Table 7.2) but the second region is only about 0.2 mm or less in length. The third region forms an expanded storage organ for a liquid containing small dense white droplets. It varies in length from 1.25 to 3.2 mm and in maximum diameter from 0.06 to 0.13 mm. The fourth region is a clear uniform tube with a mean width of 0.095 mm which varies in length from 0.50 to 0.75 mm.

All these 4 regions of the primary segment of the ejaculatorius simplex are histologically similar. They consist of an inner single layer of low columnar to cuboidal cells resembling those of the ejaculatorius duplex (Figures 7.3 E, F). These cells rest on an outer basement membrane surrounded by an inner layer of longitudinal muscles and an outer layer of circular muscles. The cells of all 4 regions vary in height from 4 to 9 µ and contain large rounded to oval nuclei with maximum diameters of between 5 and 8 µ. The intercellular walls are fairly distinct and the cytoplasm is moderately basophilic, although that of the cells in the first two regions stains slightly deeper with haemotoxylin than the cytoplasm of the third and fourth regions. Furthermore, in the third region the cytoplasm by the intercellular walls sometimes stains lighter than in the rest of the cells (Figure 7.3 F). In sections the secretion of the first region appears finely granular or sometimes contains eosinophilic globules similar to those found in the third region described below. In the second region the secretion appears as closely packed large clear refractive blocks with irregular sides and with widths of about 5 µ. The secretion in the third region consists of densely packed spherical globules which are moderately eosinophilic and vary in diameter from 1 to 12 µ although most lie in the 2 to 4 µ size range. In the fourth region the secretion appears to be largely clear but it sometimes contains a few individual spermatozoa scattered irregularly through it.

The primary simplex is generally considered to be the area in which the spermatophore precursors are produced (Outram, 1970). The epithelial cells of the 4 regions of the primary simplex in *P. operculella* are probably glandular although no vacuoles can be observed in their cytoplasm. Probably their secretion is liberated as extremely small droplets and it is therefore difficult to determine how many different glandular regions there are. On the basis of secretion alone there would be a maximum of 4. However, Callahan and Cascio (1963) concluded that there were only two glandular regions in the primary simplex of *H. zea* instead of the original 4 suggested by Callahan and Chapin (1960). What had been previously observed was the secretion undergoing physiological changes during the reproductive life of the moth and therefore a similar physiological change could also occur in the secretion in *P. operculella.* Among other Lepidoptera there is also doubt as to whether morphological divisions of the primary simplex represent discrete glandular regions. Stitz (1901) divided the whole duct into three glands with a varying number of intermediate portions, termed "Schaltstucke". Norris (1932) considered that there were 4 secretory areas and no intermediate ones in *A. kuhniella,* based on the different types of secretion, whereas Musgrave (1937) considered that there were 4 secretory areas plus 4 intermediate ones in the same insect. Khalifa (1950) concluded, from the secretions, that there were only two discrete secretory regions in the primary simplex of *G. mellonella* even though it was composed morphologically of 6 sections. Srivastava (1960b) describes three distinct morphological regions in *L. orbonalis.* Outram (1970) found 7 morphologically distinct regions in *C. fumiferana* which were also distinguishable by the secretions they contained.

The appearance of the secretions of the primary simplex in histological sections have been described for *A. kuhniella, H. zea,* and *C. fumiferana* by Musgrave (1937), Callahan and Cascio (1963), and Outram (1970) respectively. They vary considerably from a homogenous material, sometimes with vacuoles or globules in it, to one that was composed solely of granules or globules. In both *A. kuhniella* and *C. fumiferana,* there was also a region filled with angular blocks of a homogenous secretion similar to those found in the present study, in the second region of *P. operculella.*

Ruckes (1919) noted that the epithelium of the primary simplex of some Saturniidae was columnar, and Srivastave (1960b) observed no vacuoles in any of these cells in *L. orbonalis.* Musgrave (1937) reported that the epithelial cells of the various regions of the primary simplex of *A. kuhniella* were columnar or irregular in shape, and that sometimes those of the fourth intermediate region had a faint striated border next to the lumen. Callahan and Cascio (1963) found that the cells of the second (anterior) secretory region in *H. zea* were low columnar to cuboidal with smooth apical surfaces, but they degenerated completely in old moths. These authors also describe the cells of the first (posterior) secretory region as being columnar and producing secretion by "budding". Outram (1970) reported that the cells of all regions of the primary simplex of *C. fumiferana* were histologically similar, being columnar with even borders next to the lumen, and with granular cytoplasm which stained darkly with haematoxylin. In old moths all these cells degenerated considerably, but not completely.

Ruckes (1919) noted that the primary simplex of some Saturniidae was invested by longitudinal muscle only. Callahan and Cascio (1963) reported that the "second" (anterior) secretory region of *H. zea* is surrounded only by a layer of circular muscles whereas the "first" (posterior) secretory region has an inner longitudinal layer surrounded by circular muscle. In *C. fumiferana* the entire primary simplex is surrounded by a layer of longitudinal muscle fibres and a thin outer layer of circular muscle according to Outram (1970).

In *P. operculella* the cuticular segment of the ductus ejaculatorius simplex varies in total length from 0.38 to 0.58 mm and it is translucent white in colour. The fifth region forms a ventral swelling of the duct which is laterally compressed and reaches a maximum width between 0.15 and 0.23 mm. Usually this region is bent into a "C" with the widest part of the swelling occurring on the middle and inner part of the "C". Anteriorly and posteriorly the swelling tapers and gradually assumes a rounded cross-sectional shape. Posteriorly this becomes produced into a rounded duct to the penis.

The epithelial cells of the entire cuticular segment are the same as those of the endophallus (Figures 7.3 G, H). They are flattened cells 1 to 3 μ in height with eosinophilic cytoplasm and with deeply staining oval nuclei 3 to 5 u in maximum diameter. Their intercellular walls are indistinct but apically they secrete a thin intima about 1 u in thickness which lines the lumen of the duct and they rest basally on an outer basement membrane. This epithelial lining is usually folded into longitudinal ridges inside its outer muscular layers.

A thin sparse inner layer of longitudinal muscle surrounds the epithelial cells and this is enclosed within a well developed layer of circular muscle. At the anterior end of the 5th region (Figure 7.2 B, 7.3 G) the circular muscle layer is between 18 to 25 μ thick and is enclosed within a thin connective tissue sheath. The muscle is thickest posteriorly and forms a sphincter which controls the flow of fluid out of the primary segment of the ejaculatorius simplex. Posterior to this sphincter the rest of the cuticular segment is completely encircled by a tubular epidermal sac with thin flexible walls which arises from the edges of the foramen at the base of the sclerotised penis through which the ejaculatory duct enters. The anterior end of this epidermal sac joins the connective sheath which surrounds the anterior sphincter. Most of the length of the cuticular segment is therefore covered by a thin walled epidermal sheath.

The **circular** muscles show basically the same arrangement in the rest of the cuticular segment. However, they become reorganized to run ventrally under the epithelial lining of the duct from insertions on either side of the epidermal sheath (Figure 7.2 C). In the region of the swelling this muscle layer becomes enormously developed so that it is about 100 μ thick and 80 μ wide but it tapers posteriorly until it is only about 10 to 20 μ thick just before the duct enters the penis.

The cuticular simplexes of most Lepidoptera, whose internal reproductive organs have been described in detail, resemble each other, except for some Tineoidea described by Williams (1947). This author reported that *Tischeria tinctoriella* (Chambers) had a sac-like structure which joined the ejaculatory duct just before it entered the penis, while that of *Tischeria badiiella* (Chambers) was similarly joined by a long coiled structure. Histologically, the cuticular simplex of *P. operculella* is very similar to that found in *A. kuhniella* and *C. fumiferana* according to the descriptions of Musgrave (1937), and Outram (1970) respectively, and it is similar to that of *H. zea* as described by Callahan and Cascio (1963) except that this moth has a small diverticulum which branches from the ejaculatory duct in the anterior of the bulbous muscular part. In *A. kuhniella*, the cuticular simplex is enclosed by a double cuticular wall (Musgrave, 1937), whereas Callahan and Cascio (1963) only mention a single outer cuticular sheath in *H. zea* and Outram (1970) described a single outer cuticular sheath in *C. fumiferana* surrounded by a double connective tissue sheath. Callahan and Cascio (1963) suggested that the ejaculatory duct which lies entirely free within the surrounding muscles and cuticular sheath, is thereby allowed to stretch and follow the eversion of the endophallus during copulation. However, no evidence was found in the present study that the endophallus of *P. operculella* everts during copulation.

The process of spermatophore production has been followed by other workers in a number of moths. According to Norris (1934), in *Plodia* it is partly found within the bursa copulatrix of the female and partly within the ejaculatory duct of the male. The secretion from the posterior segment of the primary simplex flows into the bursa and hardens into a gelatinous mass whereas the secretion from the middle segment flows into the cuticular simplex where it is moulded to form the neck and horns of the spermatophore. The outer layer of this secretion hardens and the secretion from the anterior segment is then forced down, followed by the sperm and accessory gland secretion, pushing the soft core of the neck into the bursa thereby distending the first gelatinous secretion into a sac. Finally, the neck and horns are expelled from the cuticular simplex into the bursa to complete spermatophore transfer.

Spermatophore formation in *H. zea* has been followed in detail by Callahan (1958), and Callahan and Cascio (1963). At the onset of copulation the endophallus is locked into the bursa by evertion of the endophallic caecae and the secretion from the first secretory region of the primary simplex is discharged into the bursa copulatrix. Simultaneously the sperm bundles in the duplex begin to break down and the sperm mix with the secretions there. A gel produced by the second secretory region of the simplex is then moved posteriorly by contractile movements. It becomes progressively more resilient and forms on its outer surface a rod-like spermatophore precursor in the muscular region of the cuticular simplex. This is moved on by waves of contraction from the transverse muscles. The mass of sperm trails the precursor and mixes with some of the internal core of the precursor in the cuticular simplex. As the spermatophore coils down around the endophallus the inner core moves towards the tip of the spermatophore and the sperm follow the retreating core. As the tip reaches the corpus bursae the core draws progressively into the tip and expands it into a bulb-like corpus. At the completion of copulation, the core comes up to the top of the spermatophore corpus and hardens to a plastic-like consistency. Beneath this and down into the neck of the spermatophore is the sperm mixed with accessory gland and duplex secretions.

A similar but somewhat simpler process of spermatophore production has been described for *Laspyresia pomonella* L. by Ferro and

Akre (1975). In this moth the primary simplex secretes the spermatophore precursor and this forms as a resilient tube in the cuticular simplex. Within 15 minutes of copulation it is pushed into the bursa copulatrix via the endophallus and once inside, it expands with the addition of a clear liquid under pressure. After 40 minutes the male ejaculates the seminal fluid which is firstly a milky fluid containing free sperm and later a compact substance with sperm bundles. Sperm transfer is finally completed 50 to 60 minutes after the initiation of mating.

Further study is required to determine how the spermatophore of *P. operculella* is formed but it could be similar to any of those described above. Any spermatophore precursor would, however, have to be shaped into a thin rod to allow it to pass through the narrow tubular penis (described in the following section). In addition, the structural simplicity of the spermatophore of *P. operculella*, as described in the section on the female internal reproductive organs, could indicate that it is entirely discharged as a liquid or plastic gel into the bursa copulatrix where it hardens after discharge of the sperm. If this occurs, then the resulting structure could no longer be strictly termed a spermatophore since it is not formed within the male and passed to the female but it is generally accepted, that in some insects the spermatophore only assumes a definite form within the body of the female (Imms, 1960; Chapman, 1975). If the male secretions harden after being introduced as a liquid into the female then this would represent a method of sperm transfer intermediate between direct sperm transfer and insemination by means of a spermatophore.

When considered overall, the internal reproductive organs of the male *P. operculella* have no unusual histological features compared with other Lepidoptera; almost every detail having its counterpart in another species. A particularly close histological similarity exists, however, between the primary simplex of *C. fumiferana* and that of *P. operculella*, while, to a lesser extent, the cuticular simplex of *C. fumiferana*, *A. kuhniella* and *P. operculella* although complex structures, are also similar. The vas deferens, seminal vesicles and ejaculatorius duplex of *P. operculella* show the closest resemblance to those of *L. orbonalis*. Of particular note is the lack of direct histological evidence of secretory activity in the epithelial cells of these organs in *P. operculella*. It seems unlikely that this was due to the technique used in preparing the sections because sections of the entire abdomen show, for example, vacuoles in the cells of the mid-gut, and a brush border in the Malpighian tubules. The possibility therefore exists that the accessory glands produce all of the secretion and the differences in different regions are due to physiological changes that occur with time. The explanation favoured here, however, is that the epithelial cells of these regions are secretory and that the secretion is liberated as very fine droplets from the surfaces of these cells, but the use of the electron microscope is required to confirm this.

The male external genitalia

Although the male external genitalia have been figured and briefly described by Povolný (1964) a more detailed morphological investigation was made of them in the present study. This included the use of the scanning electron microscope to show the three dimensional relationships between the various parts. The terminology used here is that of Povolný (1964), and Klots (1970).

The 7th abdominal segment of the male is the last one with a typical sternum and tergum. It differs only from the more anterior ones in possessing a conspicuous tuft of elongated scales, or corematis, on either side (Figure 7.4 B). These arise from the extreme posterio-lateral edges of the segment and reach approximately 1 mm in length. Normally they lie laterally alongside the other posterior abdominal structures but they become extended laterally during precopulatory behaviour when a male approaches a female. (Chapter 8).

The remaining tergite and sternite of segment 8 are produced into hood-like flaps (Figure 7.4 A, B) which normally project caudally and enclose the external genitalia dorsally and ventrally. They articulate with each other only by narrow lateral extensions, and posteriorly they taper into thin flat plates with curved cross-sections. A row of slightly elongated scales arises from the posterio-lateral edges of the 8th tergite while the 8th sternite is clothed ventrally by elongated scales. The largest of these reach approximately 0.4 mm in length and arise from the anterior region, while those posteriorly become progressively shorter (Figure 7.4 B). Both tergite and sternite are moved through approximately 30° to 45° dorsally and ventrally respectively during precopulatory behaviour when a male approaches a female. Together with the coremata they then form a splayed ring of scales surrounding the central genitalia.

Figure 7.4

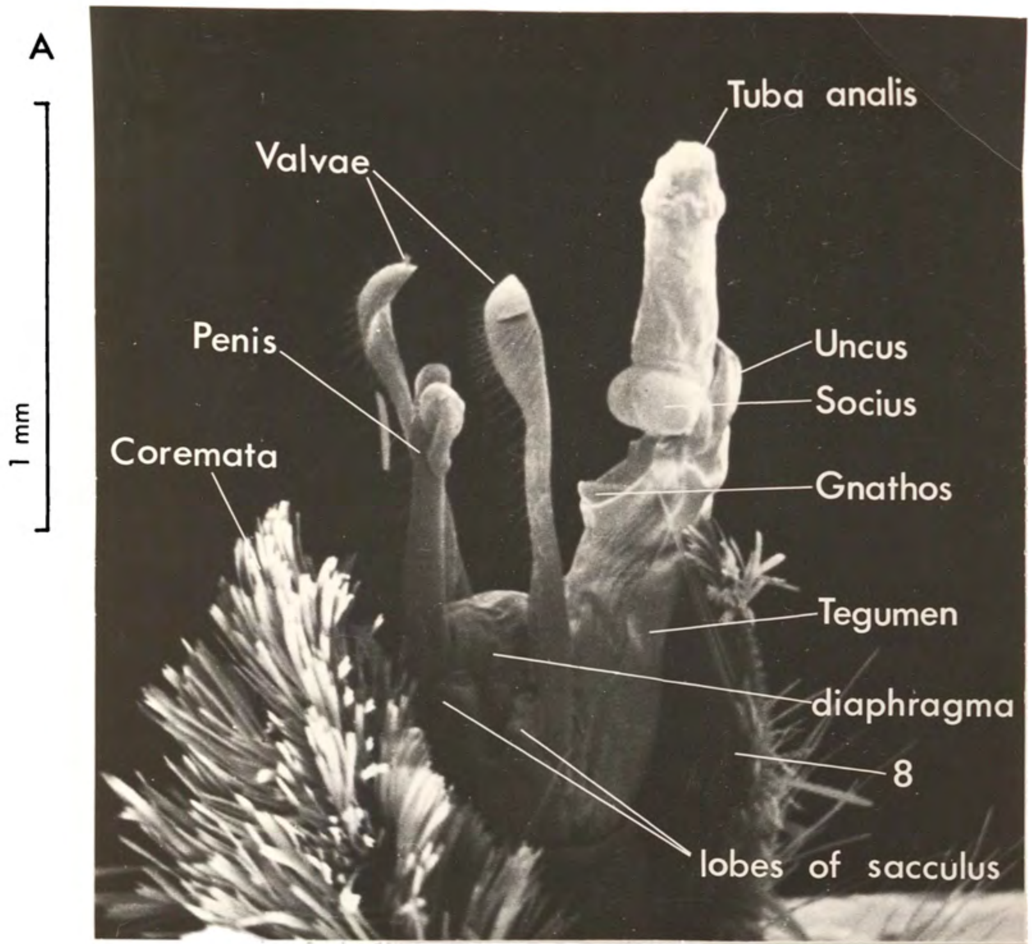
Scanning electron micrographs of the male external genitalia of *P. operculella*

The sterna are indicated by roman numerals and the terga by arabic numerals.

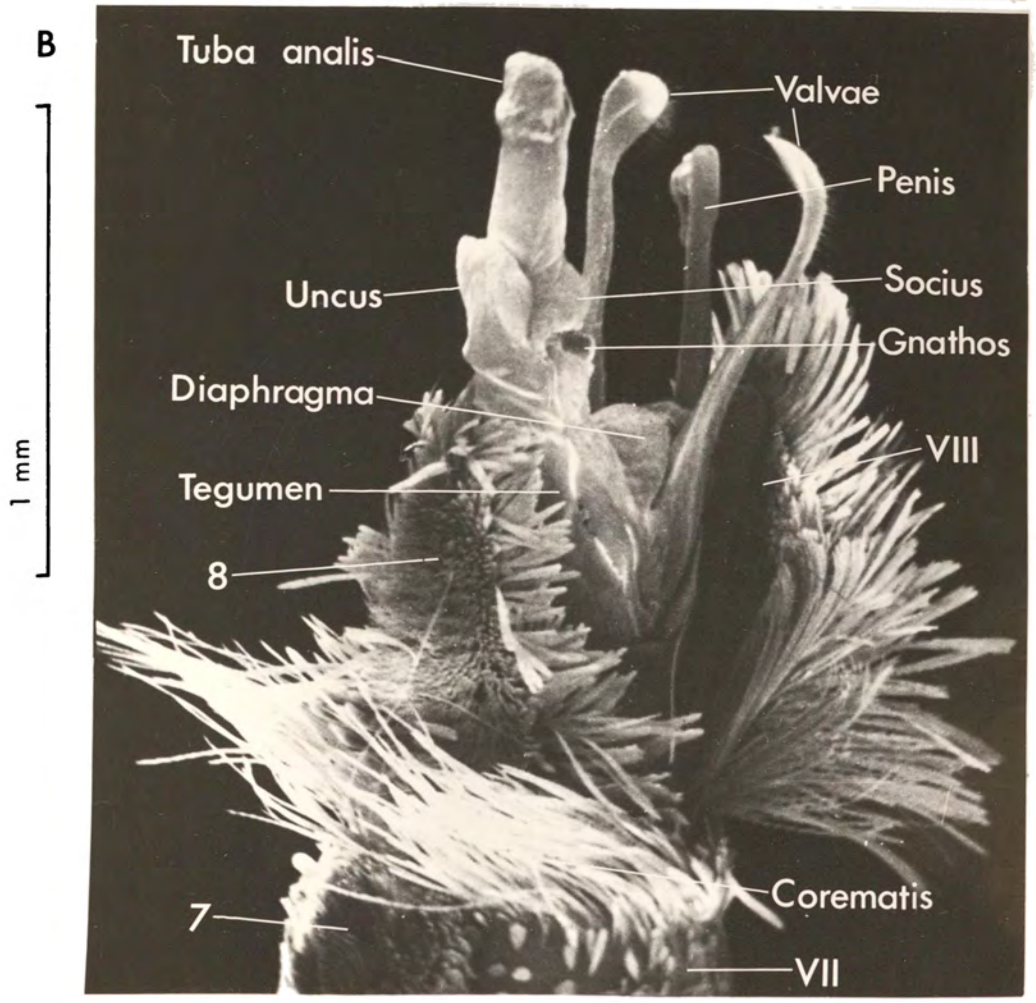
A. Ventro-lateral view of the genitalia

B. Dorso-lateral view of the genitalia.

A


1 mm
Tuba analis
Valvae
Penis
Coremata
Uncus
Socius
Gnathos
Tegumen
diaphragma
8
lobes of sacculus

B


1 mm
Tuba analis
Valvae
Penis
Uncus
Socius
Gnathos
Diaphragma
VIII
Tegumen
8
Corematis
7
VII

The parts of the external genitalia immediately posterior to the 8th segment are the dorsal tegumen and the ventral vinculum (Figures 7.4 A, B; 7.5 A, B). These articulate laterally and form a transverse ring around the abdomen within the bases of the 8th tergite and sternite. The tegumen forms a tube which is elongated caudally and open ventrally. At its ventral posterior edges it articulates with the anterior lateral edges of the gnathos, an oval dished area of sclerotised cuticle with numerous short bristles. Normally the gnathos lies rotated so that its anterior end projects ventrally but when the structures posterior to the tegumen are extended, it comes to lie within the posterior part of the tegumen, with its anterior end cephalad. Dorsally the tegumen ends bluntly and articulates with the flattened triangular uncus. Also arising from the caudal margin of the tegumen but ventral to the uncus are a pair of soft rounded socii densely covered with short bristles. These join together ventrally and the tuba anus opens posteriorly between them and the uncus. The uncus is normally flexed ventrally to lie parallel with the gnathos but it can be extended to project posteriorly from the tegumen.

Externally, the vinculum is a thin sclerotised band which runs from one side of the anterio-lateral end of the tegumen to the other in a "V". Internally, it is produced into a wide flattened invagination, the saccus, which has the same "V" cross section as the rest of the vinculum but runs cephalad and ends bluntly. The vinculum is joined posteriorly by the valvae along its entire edge. These fuse ventrally but are divided into two dorsal arms, one on either side, which form the functional claspers. The dorsal arms have mean lengths of 1.20 mm (Table 7.1) and they articulate with the base of the valvae and the anterio-ventral tips of the tegumen before extending caudad as long thin tubular arms. They expand at their posterior tips and turn medially and slightly dorsally towards the uncus.

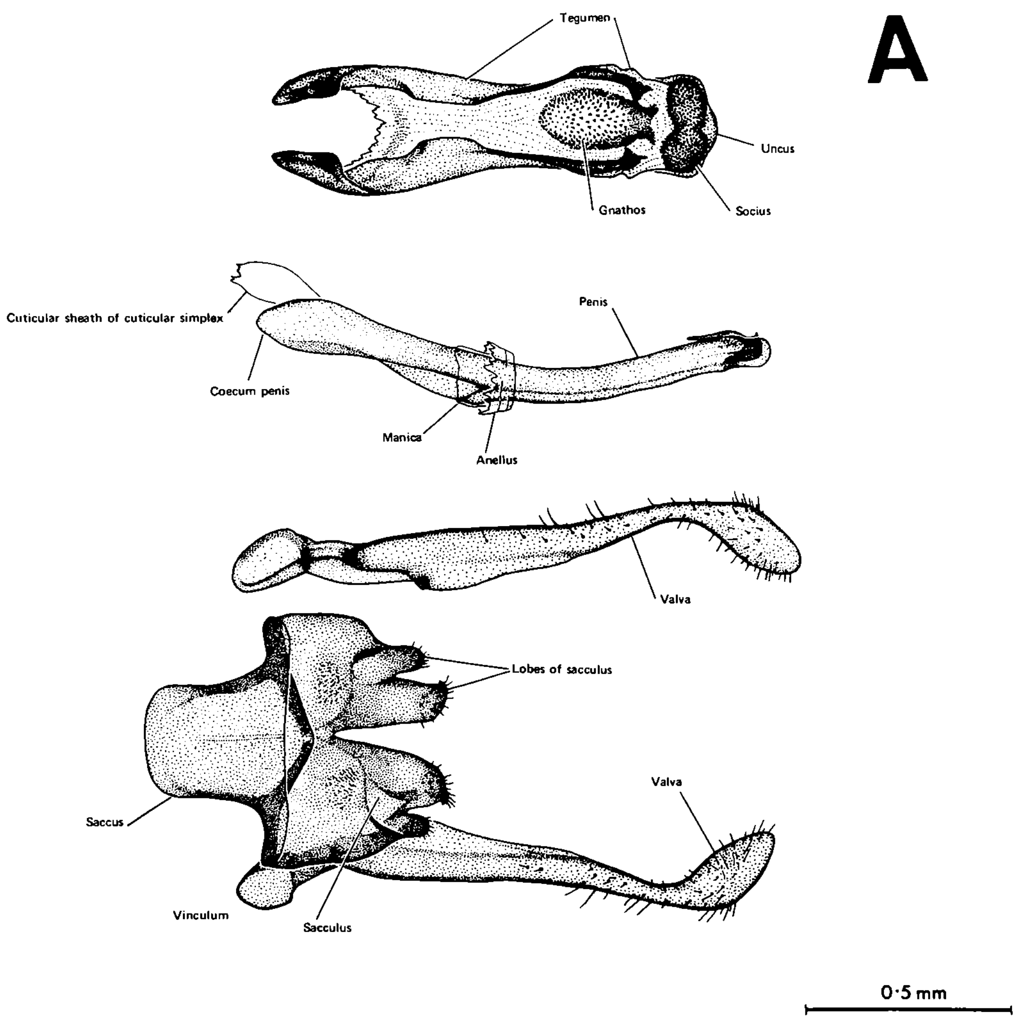
The base of each valva is represented only by the sacculus, all the other regions having been lost (Figure 7.5 A, B). Each sacculus is, however, divided posteriorly into dorsal and ventral lobes. These are provided with numerous short bristles and their posterior edges curve medially and are serrated into a series of short wide teeth. Ventrally, the sacculi are almost separated by a deep cleft between them but they fuse anteriorly to this.

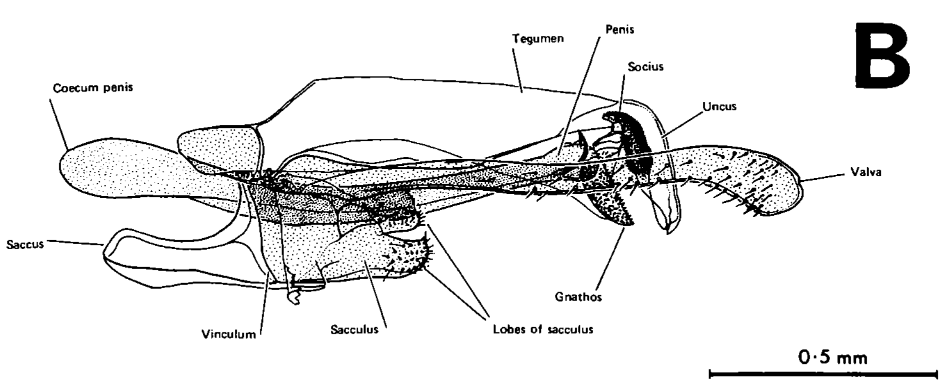
Figure 7.5

The external genitalia of the male
P. operculella

A. The separate components.

B. A whole mount after maceration.


A
Tegumen
Uncus
Gnathos
Socius
Cuticular sheath of cuticular simplex
Penis
Coecum penis
Manica
Anellus
Valva
Lobes of sacculus
Valva
Saccus
Vinculum
Sacculus
0·5 mm


B
Tegumen
Penis
Socius
Coecum penis
Uncus
Valva
Saccus
Gnathos
Vinculum
Sacculus
Lobes of sacculus
0·5 mm

The end of the abdomen, between the tegumen and the valvae, is sealed off by the diaphragma formed of thin cuticle (Figure 7.4 A, B). This also extends dorsally between the ventral edge of the tegumen to the anterior edges of the gnathos. The penis projects posteriorly through the centre of the diaphragma, between the sacculi: it is a long thin tubular sclerite with a mean length of 1.27 mm and a width of 0.072 mm (Table 7.1). Anteriorly it projects into the abdomen and ends with a slight swelling, the caecum penis. Posteriorly, it ends with a rounded flap on the left side and a heavily sclerotised short spine which curves dorsally on the right side. The penis is also curved so both ends are dorsal to the middle region. It is joined about a third of its length from the anterior end by the diaphragma which runs posteriorly a short distance close to the penis as the manica and then folds back on itself as a cone-shaped anellus before joining the rest of the diaphragma (Figure 7.5 A). This allows the penis to slide anterio-posteriorly to some extent.

The cuticular simplex of the ejaculatory duct enters the penis through a dorsal foramen on the caecum penis. However, before it enters, it is surrounded for a short distance by a double walled tubular sleeve of thin cuticle arising from the edges of the foramen as already described in the previous section. Within the penis, the cuticular simplex joins the endophallus or vesica which runs as a straight tube of thin cuticle and opens at the posterior end of the penis. Sections of the penis (Figure 7.3 H) reveal that a sclerotised bar runs anterio-posteriorly within the posterior wall of the endophallus and this serves for the attachment of numerous muscle fibres that run anteriorly from it and insert on the internal surface of the caecum penis. This suggests that the endophallus may become everted out of the penis to some extent and that these muscles then withdraw it again, but no evidence of this was found by examining serial sections of copulating moths.

The male external genitalia are of great taxonomic importance; the detailed shape of the parts serving for identifying the species and the general shape of some of them serving to distinguish the genus. The latter aspect has been dealt with by Povolný (1964) who states that the macroscopically visible coremata alone sharply define the genus *Phthorimaea* from the remaining Gnorimoschemini while the oval shaped gnathos also makes it distinct. This author also notes that the long pouch formed by the 8th tergite and sternite is more strongly developed than in the other genera, while the shape of the aedeagus, and the lobed processes of the sacculus clearly demonstrate its inclusion in the Gnorimoschemini.

The female internal reproductive organs

The female internal reproductive system of *P. operculella* has not been described elsewhere, although those of one other gelechiid, *Sitotroga cerealella* Oliver, have (Joubert 1964a, 1964b). There is, however, a fairly extensive literature on these organs in other Lepidoptera. Williams (1947) described and compared those of a number of Tineoidea, while Eidmann (1929), and Weidner (1934) treated a variety of species from a total of 9 families of Lepidoptera. Detailed descriptions also include those of *Plodia* and *Anagastia kuhniella* (Zeller) (Norris, 1932), *Zygaena* (Hewer 1932), *Heliothis zea* (Boddie) (Callahan, 1958), *Pseudaletia unipuncta* (Haw.) and *Peridroma margaritosa* (Haw.) (Callahan and Chapin, 1960), *Colias philodice eurythene* (Boisduval) (Stern and Smith, 1960), *Leucinodes orbonalis* Guen. (Srivastava, 1960a), *Pectinophora gossypiella* Saunders (Wellso and Adkisson, 1962), and *Choristoneura fumiferana* (Clemens) (Outram, 1971). Some histology is available for the internal reproductive organs of the female of *Porthetria* ***dispar*** L. (Behrenz, 1952), *L. orbonalis* (Srivastava, 1960a), and *H. zea* (Callahan and Cascio, 1963), while good histological descriptions have been given for those of *A. kuhniella* (Musgrave, 1937) and *C. fumiferana* (Outram, 1971a).

The female internal reproductive system is typically ditrysian (Imms, 1960, Klots, 1970; Common, 1973) having two separate openings, the ostium oviductus situated on the ovipositor and the ostium bursa situated ventrally on segment 8. These serve for egg laying and for copulation respectively.

The internal reproductive organs of *P. operculella* (Figure 7.6A,B) consist of paired ovaries, paired lateral oviducts, median oviduct, vagina, spermatheca and associated gland, two accessory glands each with a reservoir, bursa copulatrix, and a ductus seminalis with a bulla seminalis.

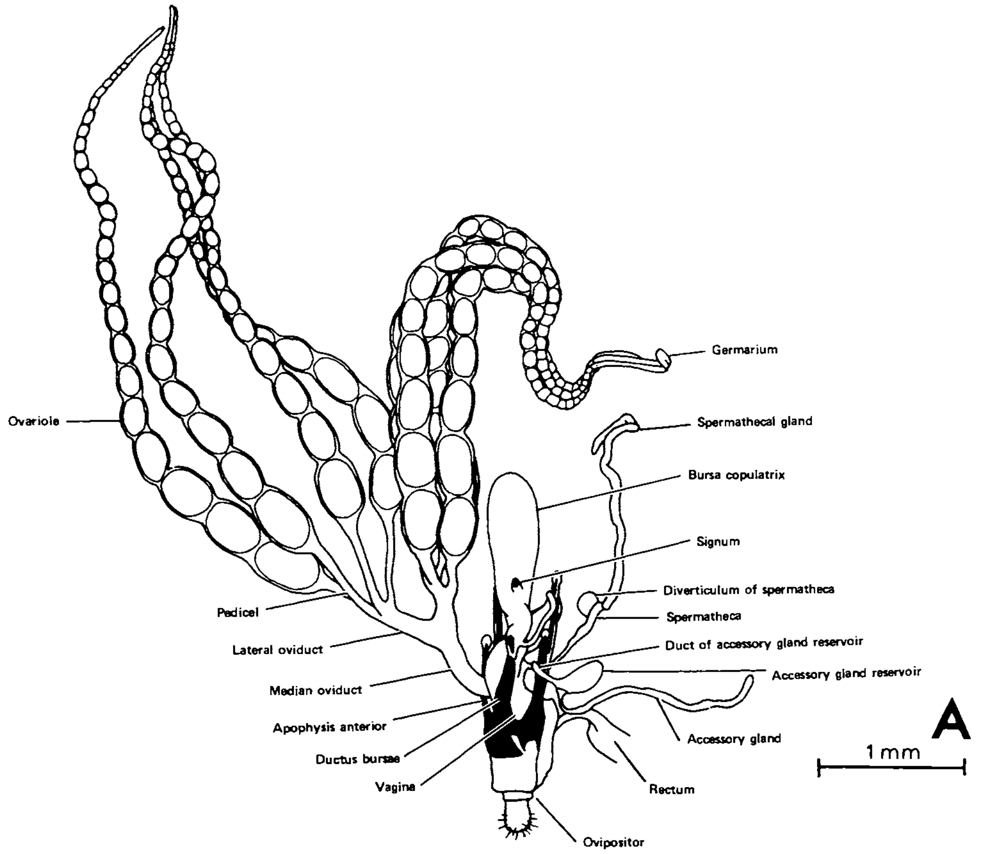
The ovaries lie within the 2nd to 6th obdominal segments. They are folded back on themselves dorsally about midway to two thirds along their lengths so that their distal ends run anteriorly. Occasionally the ends are folded a second time to run posteriorly. Each ovary consists

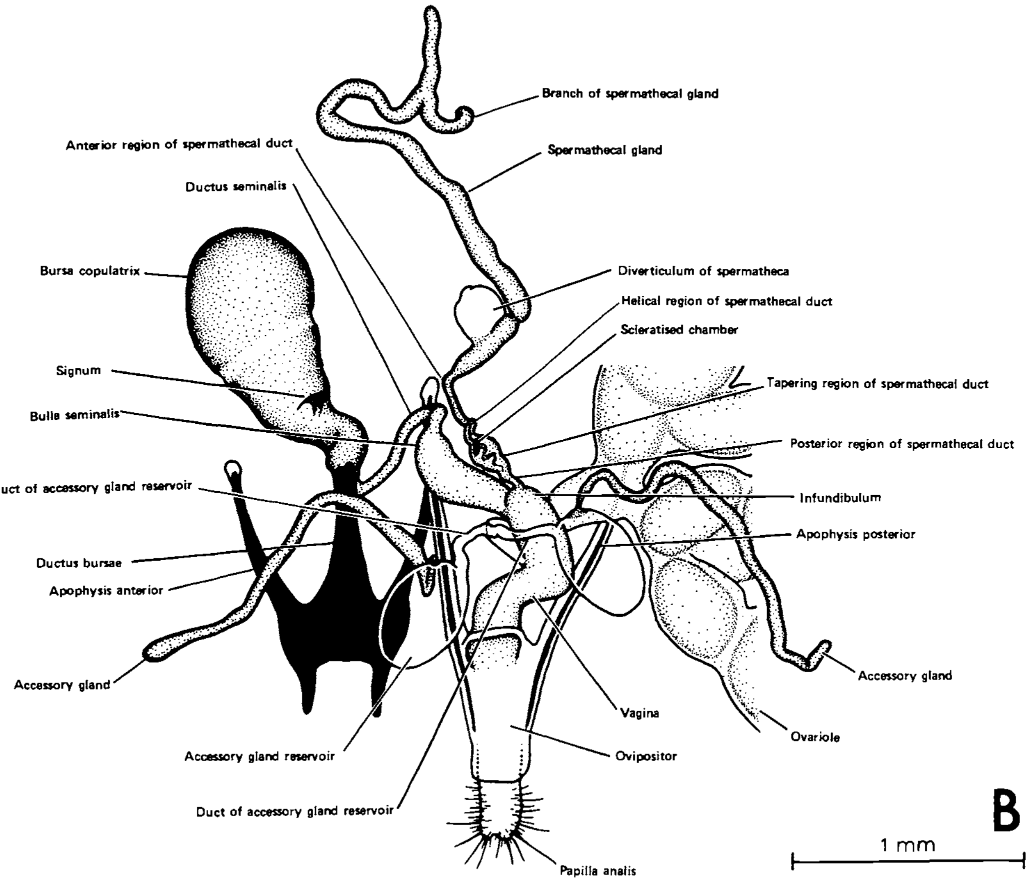
Figure 7.6

The internal reproductive organs of the female *P. operculella*

A. Ventral view after exusion from the female.

B. The organs after complete dissection.


Germarium
Ovariole
Spermathecal gland
Bursa copulatrix
Signum
Diverticulum of spermatheca
Pedicel
Spermatheca
Lateral oviduct
Duct of accessory gland reservoir
Accessory gland reservoir
Median oviduct
Apophysis anterior
Accessory gland
Ductus bursae
Vagina
Rectum
Oviposito
1 mm
A


Branch of spermathecal gland
Anterior region of spermathecal duct
Spermathecal gland
Ductus seminalis
Bursa copulatrix
Diverticulum of spermatheca
Helical region of spermathecal duct
Scleratised chamber
Signum
Tapering region of spermathecal duct
Bulla seminalis
Posterior region of spermathecal duct
Duct of accessory gland reservoir
Infundibulum
Apophysis posterior
Ductus bursae
Apophysis anterior
Accessory gland
Accessory gland
Vagina
Ovariole
Accessory gland reservoir
Ovipositor
Duct of accessory gland reservoir
B
1 mm
Papilla analis

of 4 polytrophic ovarioles which are for the most part separated from each other although they are bound together by a common connective tissue sheath at their distal ends. There is no terminal filament, the ovarioles simply ending at their germaria which lie free in the abdominal cavity. Generally the ovarioles of each ovary do not end at precisely the same point but some terminate slightly before the others so that their ends become somewhat staggered.

Most of the remainder of each ovariole constitutes the vitellarium which connects by a short pedicel to the lateral oviduct. Within a vitellarium the developing ova can be seen gradually increasing in size as they move down the ovariole. The most distal are not at first apparent but by about a quarter of the way down they can just be discerned as slightly denser white regions within the translucent white of the ovarioles. The more mature ova acquire an opaque white colour and cause the walls of the ovarioles to bulge outward. It is possible to see from 5 to 20 ova and mature eggs in each ovariole but when an ovariole is full with eggs, the pedicel also becomes filled with them and then it is impossible to distinguish visually where the pedicel ends and the vitellarium begins. Typically, though, the pedicel is a short thin translucent white tube (Figure 7.6 A).

The ovarioles range from 2.5 to 7.2 mm in total length. At their distal terminations the ovary has a width of between 0.03 mm and 0.08 mm but the maximum diameter reached by any one ovariole ranges from 0.25 to 0.38 mm depending on the degree of maturity of the last ovum within it (Table 7.3). The pedicel varies in width from 0.075 to 0.113 mm and the largest of these dimensions is only attained when it is filled with mature eggs which have become orientated with their long axes at right angles to the walls of the pedicel (Figure 7.6 B).

The germarium of each ovariole is a small tube-like sac consisting of a thin wall of connective tissue which encloses loosely packed undifferentiated oogonia and occasional small interstitial cells (Figure 7.7 A). These interstitial cells have relatively small nuclei that stain darker than those of the oogonia and which probably become the follicular epithelial cells (Bonhag, 1958). Both types of cell pass down the ovariole and begin to differentiate and form groups when they are about 0.01 mm from the distal end of the ovariole. Each group consists of an oocyte with eosinophilic cytoplasm, 5 nutritive cells or trophocytes with basophilic cytoplasm and, surrounding

TABLE 7.3

Dimensions of the female internal reproductive organs of *Phthorimaea operculella*.
Measurements taken from 10 females.
All measurements in mm.

		MEAN	S.D.	RANGE	
				max.	min.
Ovary					
Terminal filament	width	0.047	0.016	0.075	0.030
Ovariole complete	length	4.430	1.653	7.2	2.5
	max. width	0.350	0.050	0.375	0.225
Number of eggs in ovary (average of both)		9.30	4.18	20	5
Pedicel	width	0.088	0.014	0.113	0.075
Lateral oviduct	length	0.400	0.142	0.625	0.125
	width	0.239	0.081	0.375	0.175
Median oviduct (to junction with ductus seminis)	length	0.972	0.133	1.188	0.813
	width	0.109	0.028	0.175	0.075
Vagina (from junction with ductus seminis to ostium oviductus)	length	2.044	0.433	2.813	1.500
	width	0.128	0.028	0.175	0.075
Accessory gland	length	1.738	0.312	2.25	1.19
	width	0.053	0.010	0.063	0.045
Accessory gland reservoir	length	0.475	0.125	0.775	0.325
	width	0.179	0.079	0.325	0.100
Accessory gland duct	length	0.478	0.135	0.750	0.250
	width	0.036	0.013	0.050	0.020
Spermathecal gland (to constriction)	length	1.728	0.425	2.41	0.94
	width	0.072	0.018	0.100	0.050
Spermatheca	length from constriction	0.288	0.078	0.450	0.200
	max. width	0.163	0.040	0.225	0.100
Spermatheca duct from spermatheca to scleratised part of duct	length	1.125	0.311	1.75	0.88
	width	0.038	0.010	0.063	0.025
Scleratised part of spermathecal duct to vagina	length	0.553	0.083	0.700	0.475
	max. width	0.099	0.010	0.113	0.075
	min. width	0.027	0.006	0.038	0.020
Ductus seminalis	length	1.125	0.195	0.38	0.75
	min. width	0.038	0.009	0.050	0.025
	max. width (bulla seminalis)	0.108	0.029	0.179	0.075
Corpus bursa	max. width	0.488	0.077	0.563	0.344
length to scleratised part of ductus bursa		1.203	0.209	1.56	0.94

Figure 7.7

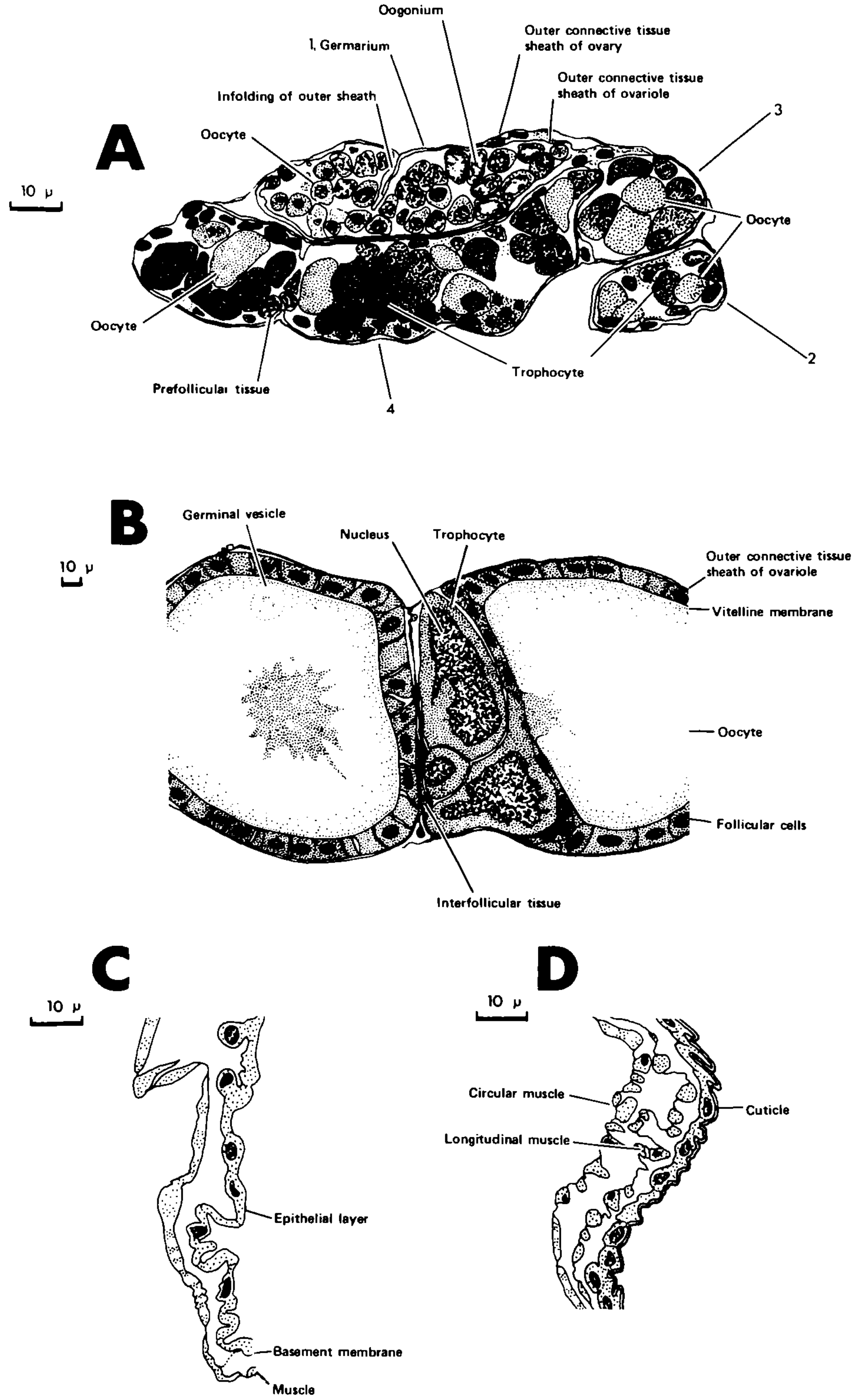
Histology of the ovaries and oviducts of *P. operculella*

A. Oblique section through the germarium passing through different regions of the four ovarioles. These are indicated in order from anterior to posterior by the numerals 1 to 4.

B. Longitudinal section through the vitellarium. Note the material passing from the trophocytes into the ovum to the right.

C. Lateral oviduct. (T.S.)

D. Median oviduct. (L.S.)


A
10 μ
Oogonium
1, Germarium
Outer connective tissue sheath of ovary
Infolding of outer sheath
Outer connective tissue sheath of ovariole
3
Oocyte
Oocyte
Oocyte
Prefolliculai tissue
4
Trophocyte
2
B
10 μ
Germinal vesicle
Nucleus
Trophocyte
Outer connective tissue sheath of ovariole
Vitelline membrane
Oocyte
Follicular cells
Interfollicular tissue
C
10 μ
Epithelial layer
Basement membrane
Muscle
D
10 μ
Circular muscle
Cuticle
Longitudinal muscle

all of these, prefollicular cells still with small darker staining nuclei and eosinophilic cytoplasm. The connective tissue walls of the germarium also fold inwards to act as a supporting membrane for the prefollicular cells (Figure 7.7 A).

The groups of cells grow in size and pass into the vitellarium sheath which contains a few circular muscle fibres and is continuous with that enclosing the germarium. However, the outer connective tissue sheath which surrounds the distal end of the ovary is lost

Within the vitellarium (Figure 7.7 B) the trophocytes and their oocytes continue to grow in size. The trophocytes become irregularly shaped, their nuclei enlarge and also become irregular in shape but their cytoplasm retains its basophilic nature and stains evenly. The oocyte stays ovoid in shape, yolk droplets become visible within it, a vitelline membrane forms as a thin uniformly eosinophilic layer next to the follicular epithelium, and its nucleus enlarges and contains relatively large nucleolus-like bodies. The nucleus is now termed a germinal vesicle (Bonhag, 1958) and is situated close by the vitelline membrane about halfway along the ova. The follicular cells multiply, grow around the group of trophocytes and the oocyte, and acquire a distinct basement membrane. They also grow as a flat plate between the oocyte and its trophocytes but a central hole is left for communication between the two. Through this hole an evenly basophilic substance appears to flow from the trophocytes into the ovum where it eventually breaks up into small granules and looses its basophilic property (Figure 7.7 B).

The cytoplasm of the follicular cells becomes basophilic in the vitellarium and these cells at first both multiply and grow to enclose the expanding cells beneath them. However, they only reach a maximum size of about 12.5 μ high and 17.5 μ wide. When the oocyte reaches approximately 200 μ in diameter the follicular cells apparently stop dividing and become gradually flattened out as the oocyte continues to grow. By this stage the trophocytes which have previously started degenerating are represented by a small basophilic mass at the anterior end of each ovum. These eventually degenerate completely, the follicular epithelium secretes a structureless chorion around the oocytes and then becomes continuous with the walls of the pedicel. Here the epithelium is mostly about 3 μ in thickness reaching up to 7.5 μ where nuclei bulge outward. The cytoplasm of these cells is still densely basophilic.

Surrounding the epithelium of the pedicel is a connective tissue sheath with occasional circularly orientated muscles which is continuous with the sheath around the vitellarium.

The lateral oviducts

The lateral oviducts are usually somewhat flattened tubes of a translucent white colour, but they can contain eggs when the ovaries are full. They vary in length from 0.13 to 0.63 mm, and in width from 0.18 to 0.38 mm (Table 7.3). The maximum width occurs only when they are distended with eggs.

The lateral oviducts consist of a single layer of epithelial cells resting on an indistinct basement membrane (Figure 7.7 C). This epithelium is usually complexly folded but longitudinal folds predominate. The intercellular membranes are also indistinct, the cytoplasm eosinophilic and the nuclei rounded or distorted where the epithelium is folded. Where the cells are flat they vary from 2 to 7 µ in thickness.

Surrounding the epithelial layer is a well developed layer of circular or oblique muscle. Longitudinal muscle is not apparent in *P. operculella*, but it occurs sparsely in *C. fumiferana* (Clemens) (Outram, 1971a), and *A. kuhniella* (Zeller) (Musgrave, 1937).

Outram (1971a) describes the epithelium of the lateral oviducts of *C. fumiferana* as being composed of low columnar cells with indistinct walls and no lining of cuticle. Musgrave (1937) describes these cells in *A. kuhniella* as columnar but he is uncertain as to whether or not there is a very thin lining of cuticle. Srivastava (1960a), however, noted an indistinct lining of cuticle in these organs in *L. orbonalis*.

The median oviduct and vagina

In *P. operculella*, the median oviduct and vagina form a continuous tube which resembles the lateral oviducts in appearance, being flattened and translucent white. The median oviduct merges imperceptibly with the vagina and there is no infundibulum separating them as occurs in some other Lepidoptera (Klots, 1970). Therefore, the term median oviduct is used here to refer to that part of the duct anterior to the junction with the ductus seminis and the term vagina to the posterior part. Klots (1970) notes, however, that some taxonomists refer to the entire duct as the oviduct or oviductus. The median oviduct varies in length from 0.81 to 1.19 mm, and the vagina from 1.50 to 2.81 mm (Table 7.3). Both vary in width from 0.08 to 0.18 mm although on the average the vagina is the slightly wider (Table 7.3). Anteriorly the vagina also bulges slightly both dorsally and ventrally where it is joined by the spermatheca and accessory glands respectively.

The median oviduct and vagina are very similar histologically (Figure 7.7 D). Both are lined internally by a thin layer of cuticle less than 0.25 μ thick and the epithelial layer lying on this is similar in all respects to that of the median oviduct. Surrounding both ducts is a layer of longitudinal muscle fibres enclosed within a layer of circular muscle.

The arrangement of common oviduct and vagina in *P. operculella* is similar to that described in *L. orbonalis* and *C. fumiferana* by Srivastava (1960a), and Outram (1971a) respectively. In these insects the rectum is also separate from the vagina and both this and the common oviduct are lined with cuticle. In *L. orbonalis*, the common oviduct has an inner layer of longitudinal muscle and an outer layer of circular muscle. These also extend over the vagina but here they are surrounded by an additional outer layer of longitudinal muscle (Srivastave, 1960a). In *C. fumiferana* the columnar epithelial cells lining these organs have indistinct intercellular walls and are surrounded by a layer of circular muscle interspersed with a few longitudinal fibres. These organs are histologically similar to the lateral oviducts except that the muscle layer is thicker (Outram, 1971a). In contrast, *A. kuhniella* has a vestibulum and its vagina fuses with the rectum which run together as a common tube to the end of the ovipositor (Musgrave, 1937). This arrangement is, however, often found in Lepidoptera (Klots, 1970). Musgrave (1937) is also uncertain whether the common oviduct of *A. kuhniella* has a cuticular lining, but he notes that it is "straggly" in the vestibulum and well developed in the vagina. He describes occasional intercellular boundaries in the epithelial cells of the common oviduct and vestibulum, an indistinct basement membrane in the vestibulum, and a definite and regular hypodermis in the posterior region of the vagina. The common oviduct is figured by him as being surrounded by a layer of circular muscles with occasional longitudinal fibres running through it. He also describes the vestibulum and upper part of the vagina as being surrounded by a thick layer of circular and longitudinal musculature, which gradually disappear posteriorly.

The accessory glands

The accessory glands of *P. operculella* are long thin translucent white organs which coil about within the 7th and 8th abdominal segments. They range in length from 1.29 to 2.25 mm and have a mean diameter of about 0.05 mm (Table 7.3). Posteriorly, each narrows slightly and empties into a reservoir which in turn communicates with the vagina by a short thin duct. However, both reservoir ducts fuse immediately before joining the vagina so they share a common opening. The reservoirs and their ducts are completely transparent and are difficult to see except for small circular areas surrounding the openings of the accessory glands. These have a similar colour to the accessory glands centrally but fade to transparency around their edges. The reservoirs vary in size depending largely on the amount of clear secretion they contain. Their maximum length ranges between 0.3 and 0.8 mm, while their maximum width ranges between 0.1 and 0.3 mm. The reservoir ducts have an average length of 0.5 mm and an average width of 0.04 mm (Table 7.3).

The accessory glands of *P. operculella*, together with their reservoirs and ducts resemble those of most Lepidoptera whose internal reproductive systems have been described in detail. They differ, however, from those of *S. cerealella* as reported by Joubert (1964b). This author describes a single long gland or "flagellum" which is drained by a narrow duct from which two reservoirs or "sacculi" arise as diverticulae, one at the end of the gland and the other halfway along the duct. In addition, the accessory gland reservoir and duct system of *C. fumiferana* differs from that of *P. operculella* according to Outram (1971a). He describes two long paired accessory glands which empty into a single bilobed reservoir, and this in turn is drained by a single short duct.

Each accessory gland of *P. operculella* and the white area surrounding its opening on the reservoir differs histologically from the remainder of the reservoir and its duct.

The accessory glands (Figure 7.8 C) are lined by an intima approximately 0.25 u thick. This is surrounded firstly by a single epithelial layer which is in turn surrounded by a layer of secretory

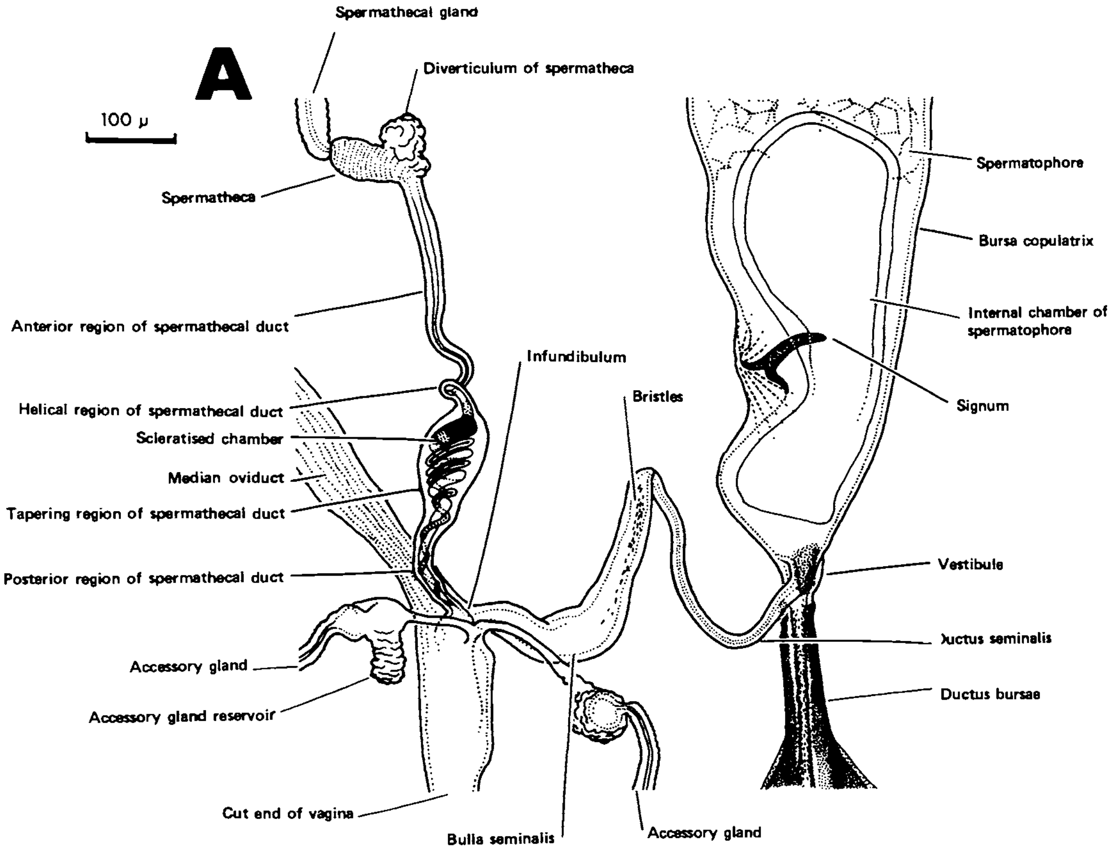
Figure 7.8

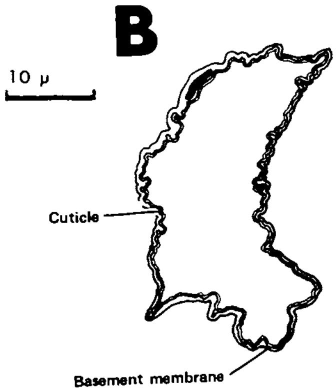
Structure of some of the female internal reproductive organs and histology of the accessory glands of *P. operculella*

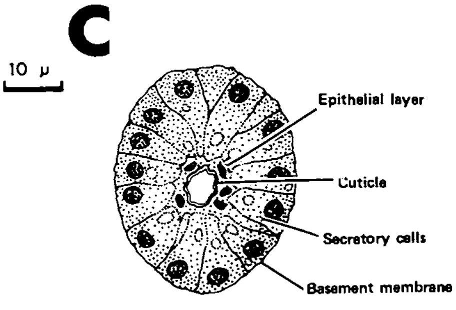
A. A whole mount of some of the internal reproductive organs.

B. Accessory gland reservoir. (T.S.)

C. Accessory gland. (T.S.)


A
100 μ
Spermathecal gland
Diverticulum of spermatheca
Spermatheca
Anterior region of spermathecal duct
Infundibulum
Helical region of spermathecal duct
Scleratised chamber
Median oviduct
Tapering region of spermathecal duct
Posterior region of spermathecal duct
Accessory gland
Accessory gland reservoir
Cut end of vagina
Bulla seminalis
Bristles
Accessory gland
Spermatophore
Bursa copulatrix
Internal chamber of spermatophore
Signum
Vestibule
Ductus seminalis
Ductus bursae


B
10 μ
Cuticle
Basement membrane


C
10 μ
Epithelial layer
Cuticle
Secretory cells
Basement membrane

cells resting on a basement membrane. No muscle layer is visible and none occurs in *A. kuhniella*, *H. zea* and *C. fumiferana* according to Musgrave (1937), Callahan and Cascio (1963), and Outram (1971a) respectively.

The epithelial layer of the accessory glands in *P. operculella* consists of poorly defined cells which vary in thickness from 1 to 4 µ. Their cytoplasm is eosinophilic and they contain flattened or irregularly shaped nuclei which stain deeply and have a maximum diameter of 2 to 6 µ. The secretory cells surrounding these have better defined walls and vary in thickness from 7 to 11 µ. Their cytoplasm is also eosinophilic but contains apical vacuoles with diameters of usually 3 to 5 µ. The nuclei are rounded, about 5 µ in diameter, and are situated close to the basement membrane.

Histologically, the accessory glands of *P. operculella* closely resemble those of *C. fumiferana* as described by Outram (1971a). This author observed an inner poorly defined layer of epithelial cells with irregular deeply staining nuclei surrounded by a layer of tall glandular cells resting on a distinct basement membrane. These glandular cells had large round nuclei and large secretion filled vacuoles which were apically situated. A similar histology of the accessory glands was also reported for P. *dispar* by Behrenz (1952) but this author in addition observed fine canals, the "Sekretkanalche", which pass through the inner epithelial cells or "Kanalzelle" and drain apical vacuoles or "Binnenblaschen" in the secretory cells or "Drusenzelle".

The arrangement of two layer of cells in all these insects and particularly in P. *dispar* suggests an organisation similar to that described for the spermatheca of *Periplaneta americana* L. by Smith (1968), and Gupta and Smith (1969). This is treated in detail below when discussing the spermathecal gland of *P. operculella*.

A cuticle lining of the accessory glands has also been noted in *A. kuhniella* by Musgrave (1937) and in P. *dispar* by Behrenz (1952), but it was not mentioned by Srivastave (1960a) in *L. orbonalis*, or by Callahan and Cascio (1963) in *H. zea*. The accessory glands are also composed only of a single layer of cells in the other Lepidoptera investigated. In *L. orbonalis*, these cells have basal vacuoles (Srivastava, 1960a), and in *H. zea* they are cuboidal with highly vacuolated cytoplasm and medial nuclei (Callahan and Cascio, 1963), while these cells have large apical vacuoles in *A. kuhniella*

(Musgrave, 1937).

In *P. operculella* the areas immediately surrounding the openings of the accessory glands are similar, histologically, to them. However, the remainder of the reservoirs and their ducts (Figure 7.8 B) are lined by an intima less than 1 µ thick. This is overlain by a single layer of thin flat epithelial cells mostly between 0.5 µ and 1 µ thick, which are continuous with the epithelial layer of the accessory glands and rest on a basement membrane. In some parts of the reservoirs and more frequently in the ducts no epithelial cells are visible at all and it appears as if the intima forms the complete wall. It is, however, possible that in these regions the cells are so thin that they cannot be resolved at the magnification available. Where epithelial cells can be seen their intercellular membranes are indistinct, their cytoplasm is eosinophilic, and their nuclei are flattened.

A sparse muscle layer surrounds the reservoirs and its duct but muscle fibres are only occasionally visible in the sections.

No differences were observed between the epithelial cells of the accessory gland reservoirs and their ducts in newly emerged and old moths corresponding to the differences observed by Outram (1970a) in *C. fumiferana*. In this insect the epithelium is thick and actively secretory in young moths but the cells degenerate rapidly within 2 to 3 days of emergence and become reduced to a thin syncitium-like sheet of tissue with a few scattered vacuoles and nuclei. Possibly a similar change may occur in the pupa of *P. operculella* but this was not investigated. Degeneration of the cells may also explain their apparent non-existence in some regions of the reservoirs and their ducts of *P. operculella*.

The epithelium of the accessory gland reservoirs in *L. orbonalis* is also formed by very flat cells which are not clearly distinguishable, but the walls of the ducts are composed of prominent cells surrounded by inner and outer longitudinal muscle with a circular layer between them (Srivastava, 1960a). In *H. zea* some of the cells of the reservoirs resemble those of the accessory glands with highly vacuolated cytoplasm but they become low and disappear altogether in the centre portion of the reservoirs. They then consist of a cuticular "pectinate" membrane surrounded by a single layer of circular muscle (Callahan and Cascio, 1963). In *A. kuhniella*, Musgrave (1937) describes the wall of each reservoir and its duct as "extremely difficult to distinguish" and it "apparently consists of a basement membrane upon which lie scattered epithelial cells".

The spermatheca

The spermatheca of *P. operculella*, its distal gland, and proximal duct form a relatively complex structure as shown in Figure 7.8 A.

The spermathecal gland is generally a simple straight tube but sometimes it branches once near its distal end as shown in Figure 7.6 B. Detailed records of the proportion of moths with branching spermathecal glands were not kept because of the difficulty involved in dissecting out the organ to check if it was straight or not. However, 3 of the 10 females carefully dissected to obtain the measurements given in Table 7.3 had branched glands.

The spermathecal gland coils around within the 7th and 8th abdominal segments usually towards the left side of the female. It is translucent white in colour, being similar to most of the other organs, and it varies in length from 0.9 to 2.4 mm and in width from 0.05 to 0.10 mm (Table 7.3). However, immediately before it joins the spermatheca it constricts.

In most Lepidoptera the spermathecal gland is usually a blind tube with approximately uniform sides which entwines among the other organs, although in *Tischeria malifoliella* (Chambers) and *T. badiiella* (Chambers) it is helically coiled (Williams, 1947). *C. philodice eurytheme*, however, has a complexly branching spermathecal gland (Stern and Smith, 1960).

The histology of the spermathecal gland in *P. operculella* resembles that of the accessory glands. It is lined by a thin intima, less than 0.25 µ thick, surrounded by two concentric layers of cells, the outer ones of which are secretory and rest on a basement membrane. No muscle layer surrounds the gland (fig. 7.9A).

The cytoplasm of both layers of cells is amongst the deepest staining in the abdomen. It is strongly eosinophilic and also contains numerous basophilic granules, but these are slightly more closely packed in the basal regions of the outer cells than elsewhere.

The inner epithelial cells have poorly defined intercellular membranes and are from 1 to 4µ thick. Their nuclei stain deeply and have slightly irregular shapes with maximum diameters of between 3 and 5 µ. The outer secretory cells are larger than these, being

Figure 7.9

Histology of the spermatheca of *P. operculella*

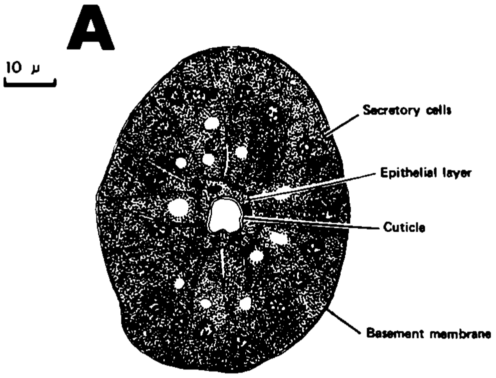
A. Spermathecal gland. (T.S.)

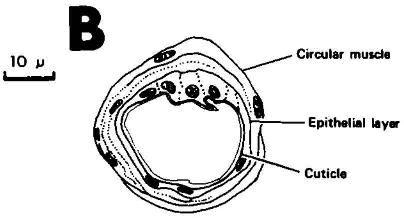
B. Duct of spermathecal gland. (T.S.)

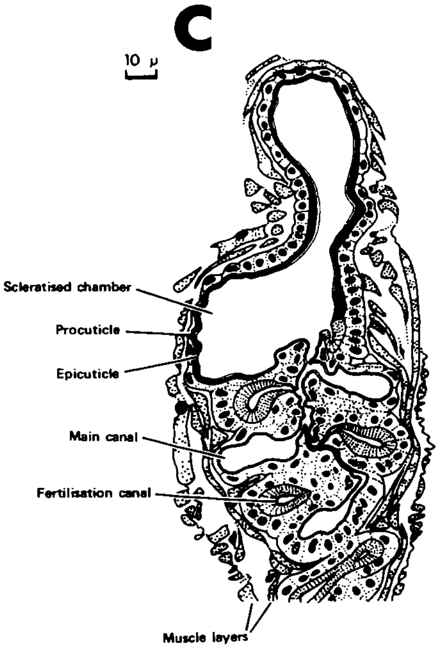
C. Anterior region of the spermathecal duct showing the sclerotised chamber and the alternately arranged main and fertilization canals. (L.S.)

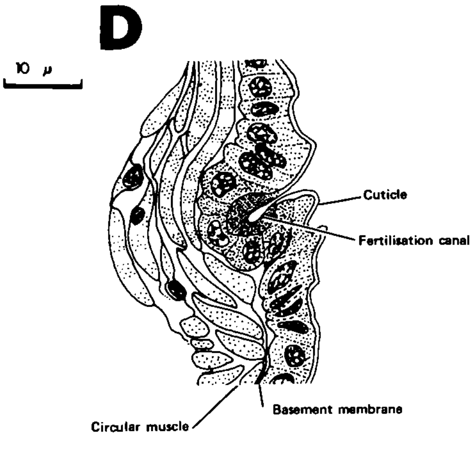
D. Posterior region of the spermathecal duct showing a transverse section through the fertilization canal. The lumen of the main canal is to the right. (L.S.)

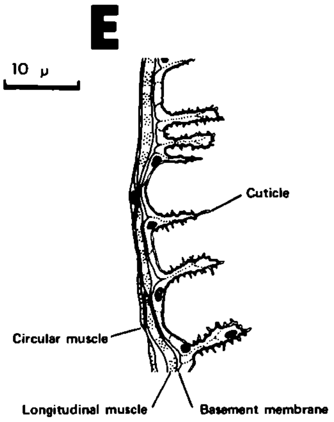
E. Spermathecal diverticulum. (L.S.)


A
10 μ
Secretory cells
Epithelial layer
Cuticle
Basement membrane


B
10 μ
Circular muscle
Epithelial layer
Cuticle


C
10 μ
Scleratised chamber
Procuticle
Epicuticle
Main canal
Fertilisation canal
Muscle layers


D
10 μ
Cuticle
Fertilisation canal
Circular muscle
Basement membrane


E
10 μ
Cuticle
Circular muscle
Longitudinal muscle
Basement membrane

generally about 16 μ thick although they can vary from 5 to 26 μ. Their intercellular walls are better defined than those of the epithelial cells although they are still somewhat obscured by the dense staining cytoplasm. Often gaps are also apparent between these cells which are probably artifactual. The nuclei of the secretory cells are rounded with diameters of 3 to 7 μ and they are situated near the centre of the cells or slightly basally. Their chromatin is also granular and evenly dispersed compared to other nuclei, and this makes the nuclei harder to see in the cytoplasm. Apically, each of these cells contains a large single vacuole with a diameter ranging from 2 to 8 μ and occasionally there are other smaller vacuoles elsewhere in the cells.

The function of the spermathecal gland is most likely to provide an exogenous nutrient for the spermatozoa (Davey, 1965) while the general arrangement of the cells of the spermathecal gland and especially the fact that the glandular cells are separated from the gland lumen by another inner layer of cells suggest that they may have a similar organisation to those described in the spermathecal bulb of *P. americana* by Smith (1969) and Gupta and Smith (1969). These authors showed by electron microscopy that the spermathecal bulb is lined with cuticle surrounded by a layer of epidermal cells and each of these cells is flanked on its basal surface by an elongated secretory cell. Each pair of cells works as a unit in which the products of the gland cell are afforded a channel allowing them to pass through the intima of the spermatheca. Each gland cell is furnished with an extracellular duct which traverses the intima and is made up of two distinct parts; a distal one inserted into the secretory cell and a proximal one encircled by the underlying epithelial cell.

An histological structure of the spermathecal gland very similar to that described here for *P. operculella* has been reported for *A. kuhniella* by Musgrave (1937), *P. dispar* by Behrenz (1952), and *C. fumiferana* by Outram (1971a). In *P. dispar* the histological structure shows additional similarities with that of *P. americana* described above since Behrenz (1952) also reports that a fine secretion canal is visible penetrating each epithelial cell or "Kanalzelle" and ends in a terminal vesicle or "Endblaschen" surrounded by secretion in the apical region of the secretory cells. However, the spermathecal gland of *L. orbonalis* and *H. zea* is composed of only a single epithelial layer (Srivastava, 1960a; Callahan and Cascio, 1963). All the above authors do not mention any muscle surrounding the spermathecal gland.

The spermatheca of *P. operculella* consists of a short tube with an expanded sac-like diverticulum arising from one side near its posterior end. The diverticulum varies in shape from a broad longitudinal swelling along the tube to a rounded sac with a relatively narrow opening. Posterior to this the spermatheca generally tapers slightly and merges into the spermathecal duct but this junction is often not apparent.

The spermatheca of Lepidoptera is frequently a simple tube which expands somewhat. However, a sac-like diverticulum was observed to arise from it in *Dendrolimus pini* L. and *Panolis piniperda* Panz by Eidmann (1929) who termed it the lagena receptaculi. A similar structure was reported in *Bombyx mori* L. by Weidner (1934) and in *H. zea* by Callahan and Cascio (1963).

The spermatheca in *P. operculella* is translucent white in colour except sometimes for its diverticulum which may be transparent when distended with fluid and sperm. The morphological appearance of the diverticulum also varies with the degree of distention: when empty its walls are folded and puckered but when full they are smooth. The total length of the spermatheca was difficult to measure because its posterior limit was often obscure. However, its length to the posterior end of the diverticulum varied from 0.20 to 0.45 mm. Its width through the widest point by the diverticulum was 0.23 to 0.10 mm and this largely depended on how much fluid and sperm it contained (Table 7.3).

The epithelial wall of the spermathecal diverticulum is flat or folded into gentle ridges but in the main tubular region it is thrown internally into numerous narrow transverse folds which project 10 to 15 μ into the lumen (Figure 7.9 E). The entire spermatheca is lined by cuticle less than 1 μ thick and this is thrown into small secondary folds on the transverse folds. These secondary ones run at right angles to the transverse folds, and are concentric with the outer surface of the spermatheca. The epithelial cells, themselves, lie on a basement membrane and are usually about 1 to 2 μ tall in the non-folded regions. However, they become very distorted and stretched inside the transverse folds since the basement membrane follows the outer surface of the organ and does not enter the folds. The cytoplasm of these cells is eosinophilic, their intercellular walls are indistinct, and their nuclei generally occupy positions near the bases of the transverse folds. The spermatheca is surrounded by an inner layer of longitudinal muscle fibres and an outer layer of circular ones. The epithelial lining of the spermatheca is smooth in *H. zea* (Boddie)(Callahan and Cascio, 1963), and *S. cerealella* (Joubert, 1964b). It is slightly folded into transverse ridges in *L. orbonalis* (Guen.)(Srivastava, 1960a), formed into numerous villi in *C. fumiferana* (Clemens)(Outram, 1971a) and into longitudinal folds in *A. kuhniella* (Zeller)(Musgrave, 1937). Possibly the spermathecae of *D. pini*, *P. piniperda*, *Plodia* , and *B. mori* are also similarly folded since they are carefully figured with transverse lines which make them distinct from the other organs by Eidmann (1929), Norris (1932), and Weidner (1934) respectively.

The muscular layers around the spermatheca in *C. fumiferana* as reported by Outram (1971a) are similar to those described here for *P. operculella*. They differ from those of *A. kuhniella* which are complex; fibres in both layers running circularly and turning longitudinally (Musgrave, 1937); and in *H. zea* where there is one loosely connected layer of circular and longitudinal muscle (Callahan and Cascio, 1963). No muscle was found in *L. orbonalis* by Srivastava (1960a).

The spermathecal duct is morphologically divided into a number of regions (Figures 7.6 B, 7.8 A). Anteriorly, it is a straight tube which becomes bent into approximately one whirl of a helix and then joins a thickened region. This tapers posteriorly and becomes a relatively straight tube which is twisted into a slight spiral before joining a swelling on the vagina, termed the infundibulum by Musgrave (1937). Internally, at the anterior end of the thickened region is a sclerotised spheroidal chamber which is compressed somewhat anterio-posteriorly. The helical region enters this anteriorly and to one side, but just before it does so its lining becomes progressively sclerotised. Within the tapering region posterior to the sclerotised chamber the lumen is just visible coiled into a helix but this internal structure will be described below under the histology.

The spermathecal duct is mostly coloured the same translucent white as the other organs, except for the sclerotised region which is brown. The first region, from the posterior end of the spermathecal diverticulum to the sclerotised chamber, varies from 0.88 to 1.75 mm long and from 0.025 to 0.063 mm wide. The remainder of the duct to its junction with the infundibulum on the vagina is from 0.48 to 0.70 mm long, 0.075 to 0.113 mm in diameter at its widest part, and 0.020 to 0.038 mm in diameter at the narrow posterior tube (Table 7.3).

In the posterior region of the duct the lumen becomes distincly divided into two canals which intercommunicate along their entire lengths (Figure 7.9 C, D). One side of the duct, termed the subsiduary fertilization canal is lined with thick cuticle, reaching 5 µ at its widest place. Fine eosinophilic strands also traverse the cuticle here. Overlying it is an epithelium of cuboidal cells 5 to 11 µ thick, with large rounded nuclei and slightly basophilic cytoplasm. The other side of the duct is termed the main canal. Its cellular lining is less well developed, being 0.25 to 0.5 µ thick, and apparently homogeneous. It becomes folded gently into one to three shallow longitudinal ridges. The epithelial cells overlying it, in contrast to the fertilization canal, are more irregular, and vary from 5 to 11 µ thick, have less distinct intercellular membranes and a cytoplasm that is eosinophilic only. The cells of both canals rest on a common basement membrane.

In the thickened tapering region of the spermathecal duct, posterior to the sclerotised chamber, the main and fertilization canals are arranged into helical spirals which alternate with each other (Figure 7.8 C, D). A longitudinal section through the centre of the entire region, therefore, shows alternating cross sections through these canals along one side and diagonally opposite ones along the other side (Figure 7.9 C). The two ducts are packed close together near the sclerotised chamber but the path of each lengthens towards the narrow region and the main canal eventually straightens out. The fertilization canal, however, retains its helical path and continues in a lengthened spiral around the main canal to the infundibulum. Its lumen narrows considerably before it enters the vagina (Figure 7.9 D) but it retains a thick "horseshoe" lining of cuticle along its entire length overlain by a more or less regular cuboidal epithelium. The main canal remains as a wide canal with slightly folded internal walls lined with a thin intima until it reaches the infundibulum.

Surrounding the entire structure in the thick tapering region is an inner layer of circular muscles and an outer layer of longitudinal ones. These longitudinal ones become reorganised in the posterior region and run circularly, combining with the inner layer to form a thick complex circular sheath 5 to 10 μ thick.

In the infundibulum the fertilization canal becomes smaller and opens out before finally losing its distinction with the other cells. The lumen of the main canal, however, continues undiminished into that of the vagina. All the cells become very thin and tall and the whole epithelial layer varies from 7 to 35 u thick. As in the vagina and spermathecal duct their apical surfaces are covered by intima and they rest on a basement membrane. Their cytoplasm is eosinophilic and their nuclei are apically situated. The epithelium is surrounded by a layer of circular muscle which joins those of the vagina and gradually becomes reorganised into their orientations.

The spermathecal duct of *P. operculella* resembles fairly closely that of *Operophthera brumata* L. reported by Weidner (1934), and *S. cerealella* described by Joubert (1964b). Other Lepidoptera in which it is helically coiled, but not in the same manner as *P. operculella*, include *A. kuhniella* (Zeller) (Musgrave 1937), *T. badiiella*, *T. malifoliella* and *Lithocolletis fitchella* Clemens (Williams, 1947), *L. orbonalis* (Guen) (Srivastava, 1960a), *C. philodice eurythene* (Boisduval) (Stern and Smith, 1960), and *H. zea* (Boddie) (Callahan and Cascio, 1963). Histologically, the anterior part of the spermathecal duct of *P. operculella* is very similar to that of *C. fumiferaba* as described by Outram (1971a), while the remainder of it with spirally intertwined main and fertilization canals resembles those of *A. kuhniella*, *H. zea*, and *C. fumiferana* as described by Musgrave (1937), Callahan and Cascio (1963), and Outram (1971a) respectively. A sclerotised chamber also exists in A. *kuhniella*, but is absent in the other Lepidoptera mentioned above.

The ductus seminalis and Bulla seminalis

The seminal duct connects the vestibulum of the bursa copulatrix to the vagina, and like most of the other organs it is translucent white in colour. It varies in total length from 0.75 to 1.38 mm and shows a consistent variation in width along its length. The first half of it, from the bursa copulatrix is narrow, ranging between 0.025 mm and 0.05 mm in diameter, then it expands into a broad tube reaching between 0.075 mm and 0.179 mm in maximum diameter (Table 7.3) to form a bulla seminalis. This constricts again before it joins the vagina.

The entire seminal duct is lined by a thin cuticular intima less than 0.25 μ in thickness. This is smooth except for a narrow band where it is produced into fine sclerotised bristles. These run along one side in the middle of the duct extending over about a quarter of its total length and running part way into the bulla seminalis (Figure 7.8 A). The bristles vary from 7.5 to 14 μ in length and lie close to the walls of the duct, pointing towards the opening to the vagina.

The epithelium of the narrow region is thrown into large longitudinal folds (Figure 7.10 B). It consists of a single layer of poorly defined cells between 1 μ and 3 μ thick, with slightly irregularly shaped nuclei and eosinophilic cytoplasm. Surrounding this is a thick layer of closely packed muscle fibres which make observation of the basement membrane on which the epithelial cells rest difficult. Individual fibres also are difficult to distinguish but they appear to be circularly orientated.

The epithelium of the bulla seminalis resembles that in the remainder of the seminal duct (Figure 7.10 A). When empty it is also thrown into longitudinal folds, but when distended the cells are between 1 μ and 5μ thick. Their intercellular walls are more distinct than those of the narrow region

The ductus seminalis and bulla seminalis of *P. operculella* are very similar to those of *O. brumata* as reported by Weidner (1934), and *H. zea* as described by Callahan (1958). Frequently, however, in Lepidoptera the bulla seminalis arises as a diverticulum or sac-like structure from the ductus seminalis and the cuticular lining of both organs are often provided with various sclerotised spines or plates (Klots, 1970). In *H. zea* the epithelium of the ductus seminalis is degenerate and 4 or 5 bands of circular muscle surround it (Callahan and Cascio, 1963) while circular muscle only is described by Srivastava (1960a) as surrounding that of *L. orbonalis*. Musgrave (1937), however, reports that its musculature in *A. kuhniella* is complex. Where it is attached to the bursa copulatrix it is composed at first of scattered longitudinal fibres but these entirely disappear and circular muscles reappear just before the duct moves from the bursa copulatrix. This then gradually assumes the form of the layer surrounding the bulla seminalis, being composed of both circular and longitudinal fibres.

Occasionally, in the present study, an egg was observed in the bulla seminalis of *P. operculella*. This has also been recorded as

Figure 7.10

Histology of the bursa copulatrix and ductus seminalis of *P. operculella*

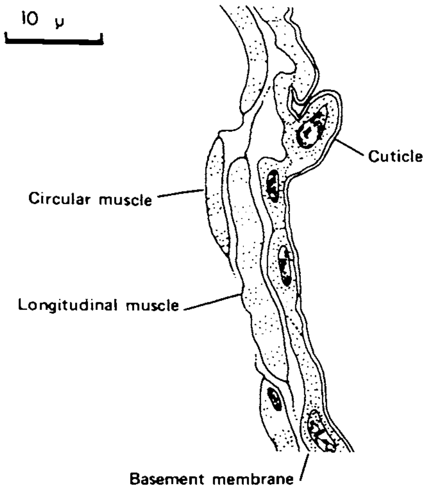
A. Bulla seminalis.(Oblique section.)

B. Narrow region of ductus seminalis.(T.S.)

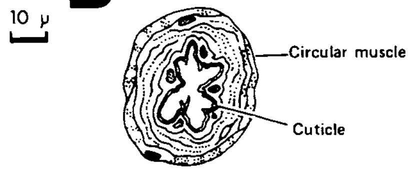
C. Anterior region of bursa copulatrix.(Oblique section.)

D. A longitudinal section of the bursa copulatrix in the region of the signum bursae.

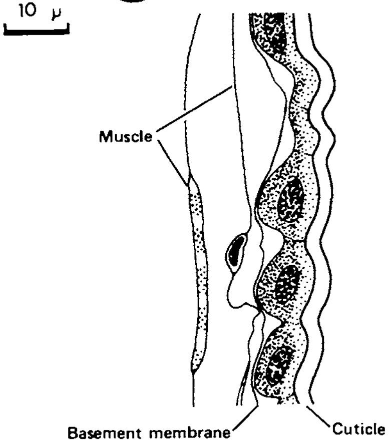
A


10 µ
Cuticle
Circular muscle
Longitudinal muscle
Basement membrane

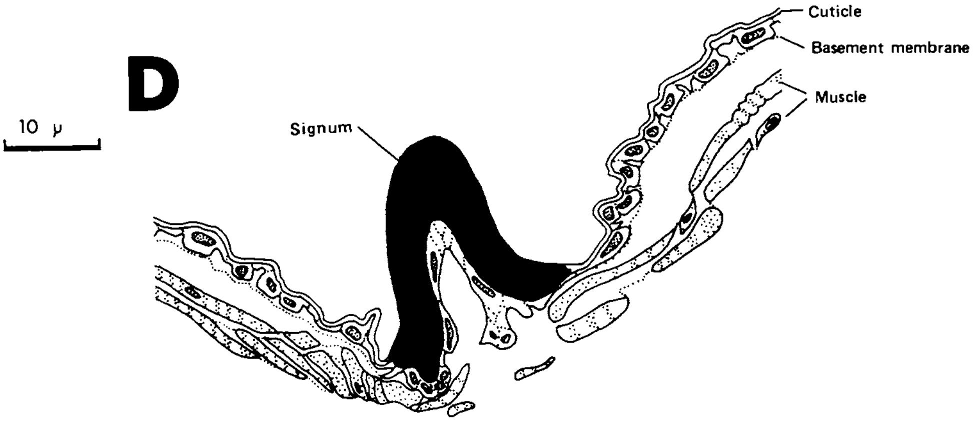
B


10 µ
Circular muscle
Cuticle

C


10 µ
Muscle
Basement membrane
Cuticle

D


10 µ
Signum
Cuticle
Basement membrane
Muscle

occurring in *Hydrocompa nymphaeata* L. by Stitz (1902). This author only observed it in one specimen and considered it accidental. Probably this is also the case in *P. operculella*. Norris (1932) observed several cases of one or two eggs being present in the bulla seminalis of both *Plodia* and *Ephestia*, but also found an egg in the vestibulum of most specimens killed during oviposition. Their cephalic poles were pressed against the entrance of the ductus seminalis and he therefore considered that fertilization took place there. Joubert (1964b, 1969) usually found at least one egg and sometimes up to 4 or 5 in the bulla seminalis of *S. cerealella*, *Cadra cautella* Walker, *Plodia interpunctella* (Hübner) and A. *kuhniella*. He also reported that sperm was only exceptionally found in the spermathecae of these insects and concluded that the bulla seminalis of each plays a primary role in the process of fertilization.

The bursa copulatrix

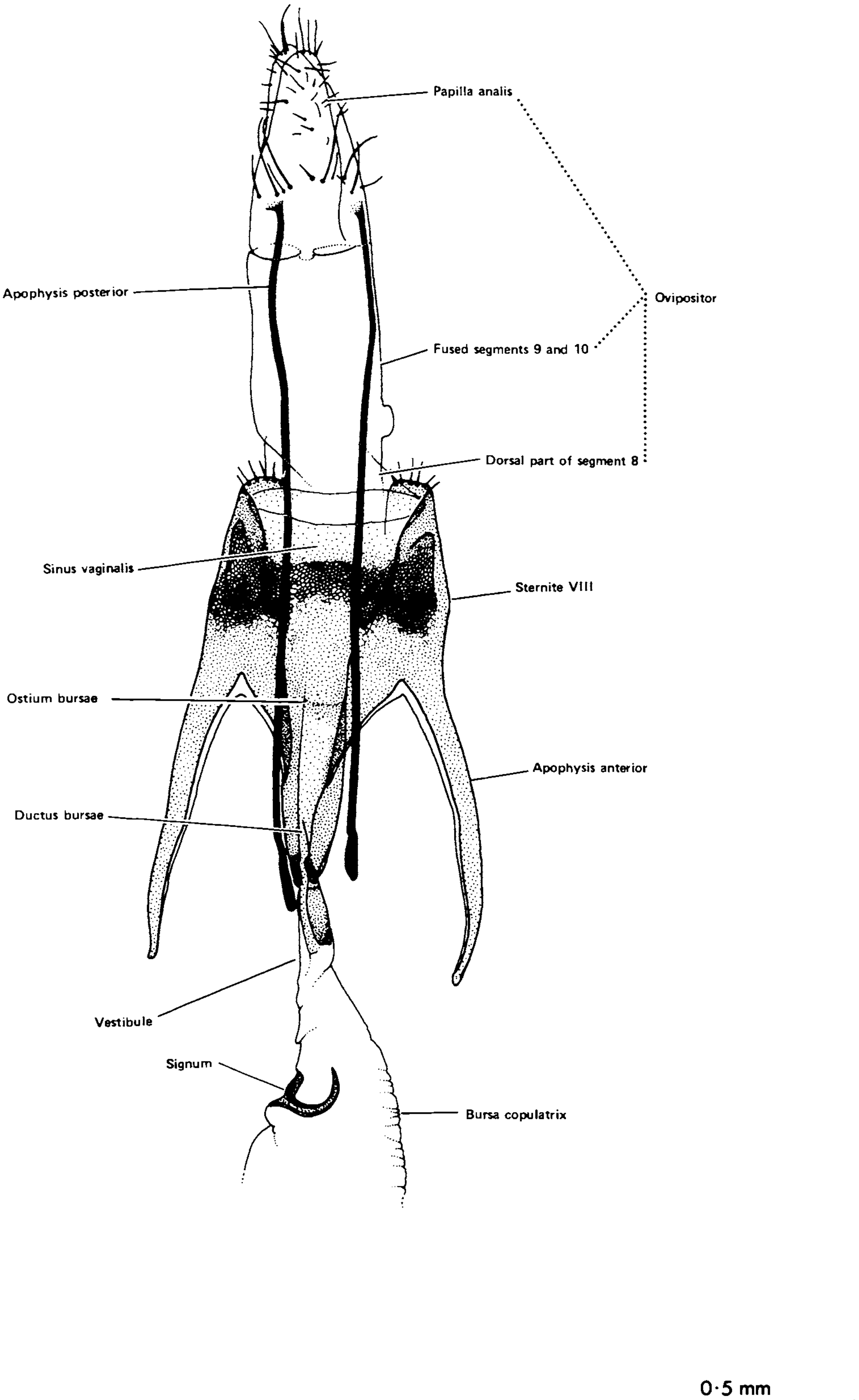
The bursa copulatrix or corpus bursae of P. *operculella* Figures 7.6 A,B; 7.8 A) is a large sac-like and pear shaped structure which tapers posteriorly to a vestibulum and then joins the anterior end of the ductus bursae. Its size varies largely on the number of spermatophores it contains but its length, from the end of the ductus bursae, ranges from 0.94 to 1.56 mm and its maximum width from 0.34 to 0.56 mm (Table 7.3). Ventrally the wall of the vestibulum is sclerotised into a shield shaped plate and the ductus seminalis enters the vestibulum dorsally and often towards the left hand side. About one third of the way along the bursa copulatrix from the ductus bursae a tooth-shaped signum or signum bursae arises as a sclerotised invagination of its wall (Figures 7.6 A, B; 7.8 A; 7.11). This projects inwards and caudad and has a circular cross section. It varies in length from 80 to 100 µ and in diameter from 18 to 25 µ. Two short sclerotised arms run from opposite sides out of the base of the signum in the wall of the bursa copulatrix and curve outward at their tips.

The bursa copulatrix is usually a translucent to transparent white colour except for the brown sclerotised regions, but it can contain slightly yellow coloured spermatophores on occasion. It often has a slightly lighter band running transversely around it about midway and any spermatophores within it can be easily seen through its walls.

Figure 7.11

The external genitalia of the female
P. operculella

A whole mount after maceration. The cuticular lining to some of the internal reproductive organs have been omitted for clarity.


Papilla analis
Oviposito
Apophysis posterior
Fused segments 9 and 10
Dorsal part of segment 8
Sinus vaginalis
Sternite VIII
Ostium bursae
Apophysis anterior
Ductus bursae
Vestibule
Signum
Bursa copulatrix
0·5 mm

Where the cuticular lining of the bursa copulatrix is unsclerotised it is smooth and between 1.5 µ and 2 µ in thickness (Figure 7.10 C, D). The epithelial cells which secrete it have rounded basal surfaces and lie on a well developed basement membrane. However, they only attach to the basement membrane centrally so there are gaps between it and the regions of their intercellular contacts (Figure 7.10 C). These gaps become pronounced when the epithelium is compressed because the basement membrane often folds outward from the cells. The epithelial cells themselves usually vary from about 1 µ at their edges when stretched and flattened to about 20 µ when compressed but they are generally between 6 to 11 µ in maximum thickness. Their intercellular membranes are fairly distinct and their nuclei are rounded with diameters of 10 to 12 µ unless the cells are flattened. They occupy basal positions surrounded by basophilic cytoplasm which stains deeply in newly emerged moths and less so in moths older than about 7 to 11 days. Apically, about midway along the cells the cytoplasm changes sharply to eosinophilic and then often appears to consist of strands running at right angles from the cuticle (Figure 7.10 C). This transitional region is always distinct in both young and old moths.

Surrounding the epithelial layer posteriorly are two layers of muscle which run diagonally around the bursa copulatrix but at an angle to each other and they both attach to the arms which arise from the signum. However, anteriorly, to the lighter transverse band, only one thin connective tissue sheath can be discerned surrounding the bursa copulatrix and this has only occasional muscle fibres running over it.

Although the bursa copulatrix may be absent in Lepidoptera it is commonly enlarged and sac-like; and possesses sclerotised signa varying from inward projecting teeth to scobinate patches (Klots, 1970). Paired signa in the form of teeth occur in some Gelechiidae, as for example in *S. cerealella* Oliver (Joubert, 1964a, 1964b), and *Stegasta bosquella* (Chambers) (Klots, 1970). The functions different authors have ascribed to the signa have been reviewed by Callahan and Cascio (1963) and include being excitatory or stimulatory, serving to tear up or saw open the spermatophore, and holding the smooth body of the spermatophore in the bursa. These authors consider the latter is correct for *H. zea* and it probably also applies for *P. operculella*, serving to position the spermatophore opening near the ductus seminalis. However, the sclerotised and narrow ductus bursae in *P. operculella* must also prevent the hardened spermatophore from moving very much.

Histologically, the bursa copulatrix of both *H. zea* and *C. fumiferana* is uniform. That of *H. zea* is surrounded by 4 to 8 layers of muscle and the basement membrane of the epithelial layer is indistinct (Callahan and Cascio, 1963). In *C. fumiferana* it has a heavy coat of muscle which is particularly thick by the signum, and its cuticular lining has a distinct epicuticle and procuticle (Outram, 1971b). In contrast, the bursa copulatrix of *A. kuhniella* and *L. orbonalis* is divided histologically into three regions, showing similarities to that of *P. operculella*. The anterior part in *A. kuhniella* has no muscle, and the epithelium is composed of cuboidal cells which are partially separated by cuticular infoldings and have apically striated cytoplasm. In the region of the signum the cuticle is thicker, the epithelial cells are irregularly shaped and are surrounded by inner longitudinal and outer circular muscle layers. Posterior to this, the epithelial cells are no longer separated by cuticle and their intercellular walls become indistinct. In addition, only a layer of circular muscle is present (Musgrave, 1937). In *L. orbonalis*, the anterior region of the bursa copulatrix also has no musculature, the middle one has thin inner longitudinal and outer circular layers of muscle, while both layers become thickened in the posterior region and the intercellular membranes of the epithelial cells become indistinct (Srivastava, 1960a).

The spermatophore

The spermatophore of *P. operculella* takes the shape of the bursa copulatrix it is formed within. It consists of two regions, an outer hard and generally transparent region which encloses an inner posterior sac. The hard transparent part can sometimes be slightly cloudy white or yellow, while air bubbles often become incorporated into it and small cracks form within it running in different directions giving it an irregularly crystalline appearance. The inner sac is almost always visible, inside the spermatophore. It opens posteriorly, is opaque white in colour, and sections of it reveal that it alone contains the spermatozoa which are both free swimming and bound into cysts.

When more than one spermatophore was observed within a bursa copulatrix the last one had displaced the older ones anteriorly so that it occupied the posterior space with its inner sac opening near the ductus seminalis. The ultimate fate of spermatophores in the bursae copulatrices were not investigated here, so it was not determined if they eventually became absorbed or not. Possibly they do not or this only occurs very slowly since female moths copulate infrequently after their initial mating (Chapter 8) and this may be due to their bursae copulatrices being already distended.

The spermatophores of Lepidoptera frequently take the shape of the bursae copulatrices they are deposited in and sometimes they also extend into the ducts leading from them (Williams, 1939,1941). In *S. cerealella*, however, the spermatophore is quite unlike that of *P. operculella:* it is long and thin, and is coiled into the bursa copulatrix, thereby distending it because of its resiliency (Joubert, 1964b). It also appears that the spermatophores of Lepidoptera are often permanent in the bursae copulatrices of the females once deposited there. Williams (1939) states that the "presence of a spermatophore in the bursa copulatrix indicates that the female has mated once; and when more than one is present each one indicates a different mating if the mating behaviour is the same as in *Ephestia kuehniella* Zell." Callahan and Cascio (1963) believed that it was highly unlikely that any secretion, or conversely material within the bursae, could pass through the thick epicuticle lining of the bursa copulatrix of *H. zea*. Landa (1960), however, maintained that the digested spermatophore of the scarabaeid, *Melolontha melolontha* L. penetrates the chitinous lining of the bursa copulatrix. Possibly this occurs in *G. mellonella*, since Khalifa (1950) reports that its spermatophore completely disappears 10 days after copulation. It is also possible that, if the spermatophore is digested, the products could pass along the ductus seminalis and be absorbed elsewhere.

In conclusion, the morphology and histology of the internal reproductive organs of the female *P. operculella* show no unusual structures when compared with those described for other Lepidoptera. However, when they are considered in detail as a whole they show some differences with all other moths, although when each organ is considered individually it has its counterpart somewhere. The bulla seminalis in, *P. operculella* is, however, an exception but this is most probably only because this organ arises as a diverticulum from the ductus seminalis in each of the other moths in which it has been examined histologically. As regards the other organs, remarkable similarities occur, if size alone is ignored. Thus the oviducts, vagina, accessory glands, spermathecal gland and spermatheca of *C. fumiferana*, the spermathecal gland and spermatheca of *A. kuhniella*, and the bursa copulatrix of *L. orbonalis* closely resemble the corresponding organs in *P. operculella*. In addition, the accessory gland reservoirs and duct, and the spermathecal duct of *A. kuhniella* also bear close resemblances histologically to these organs in *P. operculella*.

The female external genitalia

The female external genitalia have only been incompletely described before by Povolný (1964) who figured and briefly described the sclerotised structure consisting of the fused apophyses anteriores, 8th sternite and ductus bursae. The terminology used in the present description is that of Povolný (1964), and Klots (1970).

The 7th abdominal segment in the female is the last with a typical sternum and tergum but both these sclerites possess a row of slightly elongated scales that arise from their posterior edges (Figure 10.1 A). Together, these scales and the posterior region of the 7th segment form a chamber which encloses the other terminal abdominal structures when they are withdrawn.

Posterior to the 7th segment is a thin walled ovipositor which, when extended, reaches a mean length of 0.98 mm (Table 7.1). This is formed from the dorsal part of segment 8, together with segments 9 and 10. The posterior boundaries of these segments are probably marked by transverse rings which become visible around the ovipositor when it is not quite fully extended. The tergal and sternal areas are not clearly delimited since each segment forms a complete tube of thin cuticle. Between the dorsal parts of segments 9 and 10 is a large eversible fold of thin cuticle which normally lies withdrawn anteriorly under the dorsal surface of segment 9 where it forms a pocket or invagination (Figure 10.5 A, B). This is the location of the female sex pheromone gland which is described in detail in Chapter 10.

Caudally from the 10th segment arise a pair of soft broad and thick lateral lobes, the papillae anales, which are covered in long bristles (Figures 7.11). Anteriorly, they fuse together where they join the 10th tergum at a constriction and posteriorly they enclose each side of a genital chamber into which the dorsal anus and ventral vagina open.

A pair of apophyses posteriores arise laterally from the constriction at the posterior end of the 10th segment and run anteriorly within the ovipositor into the abdomen (Figure 7.11). These are long thin rods of heavy cuticle with a mean length of 1.27 mm. They are joined anteriorly by numerous muscle fibres which are responsible, at least in part, for extending the ovipositor by pulling caudally from their insertions on the apophyses anteriores.

The above interpretation of which areas of the ovipositor correspond to which abdominal segment is open to question. A study of the musculature could perhaps resolve this, but in its absence the interpretation of Klots (1970) has been followed here. This differs, however, from that of Adeesan *et al.* (1969) who named the regions here termed the 10th and 9th segments as the 9th and 8th segments respectively.

Ventrally, at the base of the ovipositor, a sclerotised sternite of the 8th segment curves transversely around underneath the ovipositor (Figure 7.11). This tapers and fuses medially with the sclerotised tube of the ductus bursae and fuses laterally with the apophyses anteriores. These latter also project posteriorly from the edge of the 8th sternite as short flat blades with rounded ends (Figure 10.3B). Ventrally and dorsally, the 8th sternite is ornamented by closely packed pits with broad rims which have depths of about 7 µ and widths of 4 to 10 µ. They each also frequently have a short bristle projecting from their posterior rim (Figure 10.3B).

The 8th sternite, at the point where it is joined by the apophyses anteriores has a mean breadth of 0.44 mm, while the total distance from the anterior end of the ductus bursae to the posterior edge of the 8th sternite is a mean of 0.72 mm (Table 7.1).

Immediately dorsal to the 8th sternite is a broad invaginated sinus vaginalis. Ventrally and laterally it is bounded by the sclerotised 8th sternite and apophyses posteriores but dorsally it consists of thin cuticle except for a pair of posterio-lateral oblong sclerotised plates which run anterio-posteriorly. These plates together with the thin walled posterio-dorsal cuticle of the sinus vaginalis are ornamented with pits and spines similar to those on the 8th sternite. The sinus vaginalis tapers anteriorly and joins the posterior end of the sclerotised ductus bursae at the ostium bursae. Internally, all these parts are armed with short spines between 10 µ and 14 µ in length which project posteriorly.

Povolný (1964) notes that the female genitalia of all genera in the tribe Gnorimoschemini are relatively uniform.

CHAPTER 8

The sexual behaviour of P. *operculella* and the environmental factors that affect it.

Introduction

Very little has been published concerning mating in P. *operculella*. Adeesan *et al.* (1969) reported that the female moths attracted the males by means of a pheromone and they described the behaviour of the males when they were caged with newly emerged females as becoming "highly excited, exhibiting sexual responses such as clasper-extension, fluttering of wings, and spinning flight".

Before commencing work on a behavioural bioassay a detailed investigation of the sexual behaviour of P. *operculella* was made. Initially the moth's diurnal pattern of copulatory activity was investigated in the laboratory and related to mating to determine when the moth was most sexually active. This knowledge was essential for all subsequent experiments on mating behaviour. Two experiments were next carried out in constant illumination to investigate the relevance of photoperiod to sexual behaviour. The effect of temperature was also examined to obtain some indication as to how the moths might behave in the field. When these laboratory experiments had been completed the pattern of arrival of male moths to females in the field was determined using sticky traps baited with live moths or their extracts. These field experiments were carried out to check that the results of the laboratory studies accounted for the behaviour of this insect under natural conditions, but they indicated that certain temperatures have an important influence on the timing of sexual behaviour. The effects of temperature changes were therefore further investigated in the laboratory with the result that they enabled a method to be devised to induce the moths to mate in daylight so that this behaviour could be observed and described.

Methods

All observations and photographs of mating behaviour taken in the laboratory were made when the moths were enclosed in a flat observation chamber shown in Figure 8.1. This had narrow sides, an organdie top and a removable perspex lid which was used for removing the moths and cleaning out the chamber after an experiment. Drilled through one side of the observation chamber was a 12 mm diameter hole fitted with a cork and this was used for inserting the aspirator when the moths were introduced. Observations and photographs were taken through the perspex lid; the distance between the top and the lid of the observation chamber was small (2 cm) to minimise the depth of field required when taking photographs.

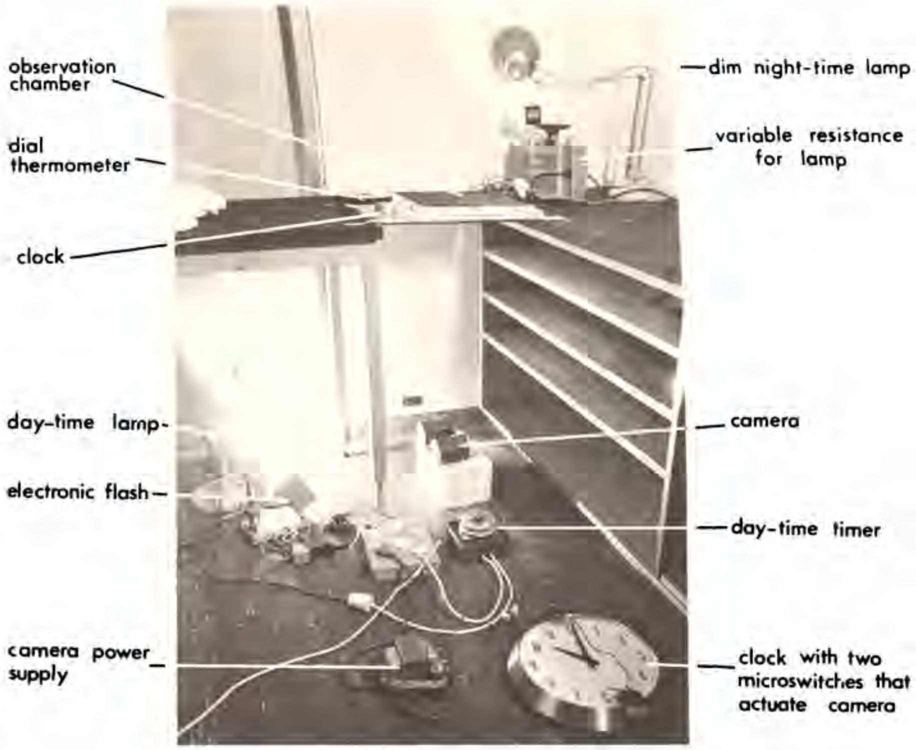
The diurnal mating activity in the laboratory and the effects of temperature on this were studied by placing the moths together in the observation chamber and photographing them at intervals throughout the night. The observation chamber was placed on a sheet of glass supported between two tables and an automatic camera with an electronic flash was mounted underneath on the floor (Figure 8.1). A clock and a wall thermometer were placed next to the observation chamber so that the time and temperature were recorded on each photograph. Sheets of black paper (not shown in Figure 8.1) were used to shield the observation chamber from the sides and the top, partly as a photographic background and also to screen the moths from the rest of the room because they were easily disturbed by the sight of moving objects. During the day illumination was provided by an incandescent lamp placed under the table and controlled by a timeswitch. Variations in the intensity of illumination were made by using different wattage bulbs and placing the lamp at different distances from the moths. During the night the moths were also illuminated indirectly with less than 0.1 lux from a lamp operating through a variable resistance. The photographs were taken initially by hand operating the camera but eventually an automatic triggering device was constructed to allow photographs to be taken every half hour or hour over long periods of time. However, if additional photographs were required these were taken by manually activating the camera. The aperture of the camera and the output of the electronic flash were adjusted so that the flash provided most of the light for exposing the film and therefore the photographs taken in the daytime differed very little from those taken at night.

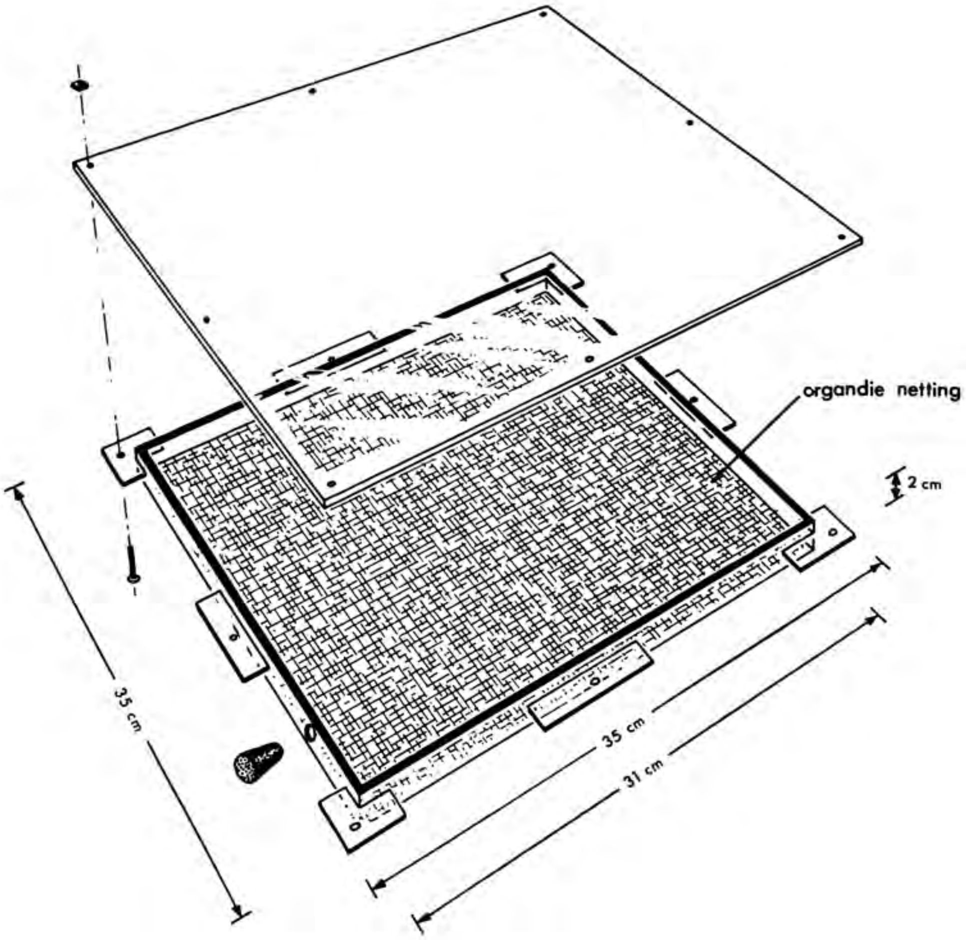
<u>Figure 8.1</u>

Equipment for determining when
P. operculella copulates

Upper Experimental arrangement for automatically photographing *P. operculella* every hour or half-hour. The observation chamber was placed lid downward on a sheet of glass next to a dial thermometer and a clock. The automatic camera was positioned beneath so as to photograph all three. Black cardboard was used to shield the observation chamber from the top and front but was removed for this picture.

Lower Construction details of the observation chamber. The lid and sides were made from clear perspex, and the moths were observed or photographed through it. Also organdie netting was dyed black to contrast with the light grey of the moths.


observation chamber
dial thermometer
clock
day-time lamp
electronic flash
camera power supply
dim night-time lamp
variable resistance for lamp
camera
day-time timer
clock with two microswitches that actuate camera


organdie netting
2 cm
35 cm
35 cm
31 cm

Observations on the moths' diurnal copulatory activity at room temperature were all made in the darkroom used for rearing the moths in, but the room lights were switched off and the moth cultures were illuminated by a lamp controlled by the same timeswitch that operated the experimental lighting because this avoided any possible errors in synchronising both sets of lights. A constant temperature room and a refrigerated storeroom were used when temperatures down to 10°C and 0°C respectively were required and both of these rooms were large enough for the entire apparatus to be set up inside them. However, when the moths were placed in the refrigerated storeroom it was also necessary to enclose the observation chamber from the top and sides with a polystyrene foam box to eliminate cyclical fluctuations in temperature that were large enough to start some of the moths mating. Temperature changes during the experiments were usually effected by moving the entire apparatus to the appropriate room. When a temperature change was made during darkness the observation chamber was kept enclosed inside a lightproof photographic bag until the equipment had been moved.

When the observations were made on mating behaviour the observation chamber was inverted so that the moths could be observed from above, and the results of the experiments described here on their diurnal copulatory activity patterns made it possible to make these observations during daylight. This was accomplished by keeping the male and female moths in separate containers overnight in a domestic refrigerator at approximately 5°C, then placing them together in the observation chamber at room temperature at dawn when they mated. Photographs were taken of these moths by mounting a Nikon F camera and a Rollei computer electronic flash unit onto a frame which could be freely moved over the perspex lid of the observation chamber. The camera was therefore kept at a fixed distance from the moths so that the focusing and flash outputs could be preset and photographs could be quickly taken by positioning the camera over the subject.

Sticky traps were used in the field to study the arrival patterns of moths to virgin females and their extracts. Each trap was constructed from two clear plastic 1 litre jars bolted together base to base with a wide central hole drilled through from one to the other (Figure 8.2). The baits were placed in a glass tube pushed into the central hole and organdie netting was fastened over both ends of the tube by rubber bands when live females were used as bait. These females

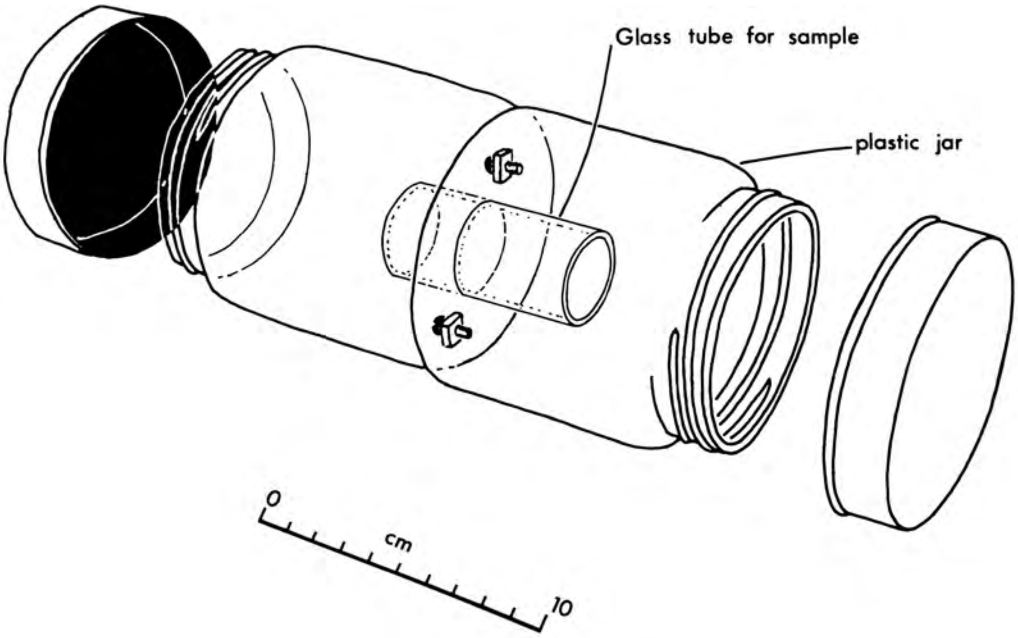
Figure 8.2

A sticky trap used for *P. operculella* and its positioning in a potato field

A. A sticky trap.
Two clear plastic jars were bolted together by their bases. A glass tube in which the sample was placed was inserted through a hole drilled in the bases. "Stickem special" was applied to the insides of the jars. During transport the lids were kept on the jars.

B. Positioning of sticky traps in the field. Each sticky trap was placed on top of a mound and shielded by a potato plant.

A



B

were also only used in groups because this counteracted individual variability and helped to ensure that the traps were attractive. The extracts consisted of the equivalent of 30 females in 100µl of chloroform prepared in the same manner as the extracts for *C. zealandica*, described in Chapter 4. These were sealed into flat bags of 0.002mm thick polythene, 6cm wide by 14.7cm long together with 1gm of perlite which kept the sides of the bags apart and soaked up the extract. "Stickem Special" was applied to all the inner surfaces of the traps except the glass tubes; the screw top lids of the plastic jars were used for closing the traps during transit.

All the field work was done at the Tamahere location given in Chapter 6. The potato patches consisted of 15 or more parallel mounds of earth approximately 0.5m apart and over 150m long in which the potatoes were planted, and the traps were placed on the central mounds at least 10m apart under large potato plants where they were protected from the weather to some extend (Figure 8.2). During the day it was also necessary to place half cylinders of aluminium foil over the traps to prevent the sun from overheating and killing any live moths used as bait. The configuration in which the traps were positioned in the patches varied depending on the direction of the wind: when this was greater than about 45° to the direction of the mounds the traps were arranged down the middle mound but when the wind direction was less than 45° to the direction of the mounds the traps were distributed equally between the central mound and the 4th mound on either side of it in a regular sequence of three diagonal rows of three traps. The traps were therefore arranged across the wind, or so that there was at least 30m between one trap and the next one downwind.

Experimental procedure and results:

Initial laboratory experiments

The diurnal copulatory pattern of the moths was determined firstly by photographing groups of them at regular intervals over one or more days and then counting the number copulating in the photographs. The copulating moths were readily distinguishable from the single ones because they were attached to each other by the tips of their abdomens and faced in opposite directions (Figure 8.7 C, D).

Initially the diurnal copulatory pattern was determined with the moths under natural light conditions. Fifteen pairs of virgin moths between 0 and 11 days old were placed together in the flat observation chamber in a room with floor to ceiling windows forming its northern wall and photographs were taken of them over a period of 31 hours. All these moths were found to remain single until night time where the light intensity had dropped well below 1 lux (the minimum reading of the Lux meter) except for those pairs that began copulating soon after they were placed together at 1200 hours and separated again within 4 hours (Figure 8.3 A). Once it was dark, however, the proportion copulating reached a maximum within an hour when 73% of the moths became paired. This proportion remained copulating for a further two hours and then reduced during the rest of the night except for occasional small increases due either to more moths mating, or to errors in counting caused by pairs becoming temporarily obscured. By the time dawn arrived only one pair of moths was copulating and these remained together for less than an hour. After this no other moths mated until it became dark again on the second night when one pair copulated for less than an hour. This experiment, therefore, showed that the majority of moths copulated at the beginning of the dark period and only a small proportion copulated later in the night and in the morning.

Five similar experiments were then performed to determine what time the moths copulated when they were kept in the rearing room where the light intensity changed abruptly from 150 lux to approx. 0.1 lux from day to night. Equal numbers of virgin male and female moths between 1 and 3 days old were again placed together in the observation chamber for each experiment and photographed at intervals over 1 to 4 days. The electronic flash was, however, covered by a Kodak 1A red filter during the first two experiments and left uncovered during the last three to determine if the colour of the light affected copulation. However, there were no apparent differences in the copulatory patterns between these two treatments (Figure 8.3 B, C, D, E, F). In each experiment few or none of the moths copulated when they were first placed together during the day and those that did separated again within two hours. After this all the moths remained unpaired until the lights went out. The proportion copulating then reached a maximum during the first one to two hours of darkness when between 45% to 85% of the moths became paired. Following this the proportion then decreased until less

Figure 8.3

Time of day when *P. operculella* copulates

Each histogram represents the percentage of females photographed copulating at 20°C.

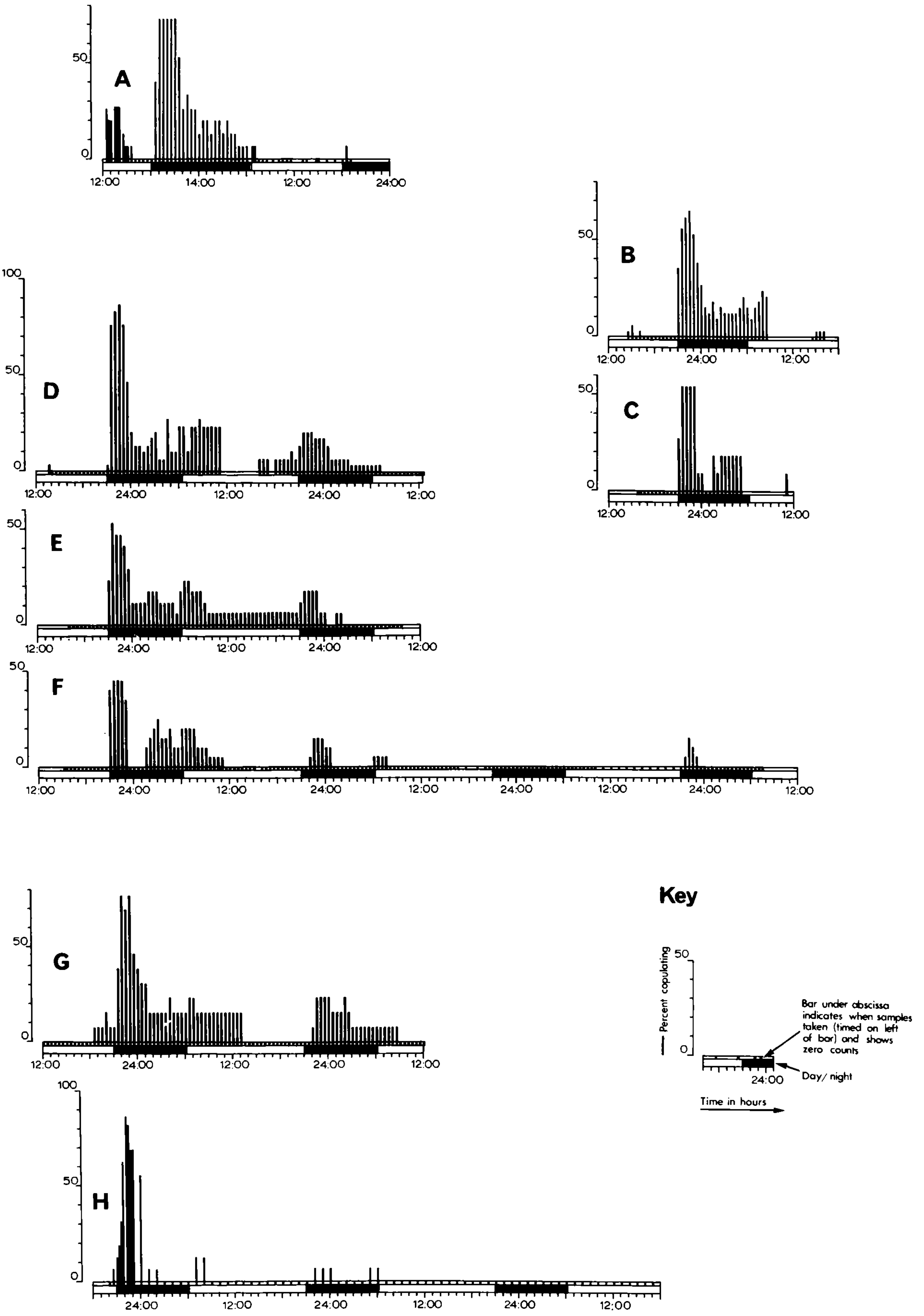
A in natural light.

B to H in artificial light; H also in total darkness at night.

B & C: Kodak 1A filter over electronic flash.

Number of moths used in experiments:

	Male	Female
A	15	15
B	34	34
C	11	11
D	30	30
E	17	17
F	20	20
G	39	13
H	32	16


A
50
0
12:00
14:00
12:00
24:00
B
50
0
12:00
24:00
12:00
C
50
0
12:00
24:00
12:00
D
100
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0
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12:00
G
50
0
12:00
24:00
12:00
24:00
12:00
H
100
50
0
24:00
12:00
24:00
12:00
24:00
12:00
Key
Percent copulating
50
0
24:00
Bar under abscissa indicates when samples taken (timed on left of bar) and shows zero counts
Day/ night
Time in hours

than 20% were paired after three to four hours of darkness and then it remained at a low level for the rest of the night. The proportion of copulating moths did, however, show a tendency to increase slightly during the last half of the night and decrease again before the lights turned on. In the morning the proportion copulating increased once again although usually less than 20% of the moths became paired and most of these separated within 4 to 5 hours. A few of the moths continued to copulate until the second night when there was another small increase in copulation during the first two to three hours of darkness but this involved less than 20% of the moths. The proportion then decreased again until few copulating pairs were found by the end of the second night. Some moths also copulated at the beginning of the third day but these usually separated within two hours (Figures 8.3 D, F). Photographs of the moths were only taken over 4 days on one occasion (Figure 8.3 F) and then no moths were found to copulate between the morning of the third day and the start of the 4th night. Those that copulated on the 4th night all separated again within 2.5 hours.

These 5 experiments showed that most of the moths copulated during the first night after they were placed together and that the largest proportion copulated soon after the lights were turned off on any night, although some copulated at other times later in the night and when the lights were turned on in the morning. This copulatory pattern was close to that of moths kept under natural lighting conditions so that abrupt changes in light intensity between day and night did not appear to affect the moth's copulatory behaviour except for the possibility that in artificial light a larger proportion copulated when the lights turned on when compared with the number that copulated at dawn under natural light. This latter observation could, however, also have been a chance occurrence since only one experiment was carried out in natural light.

The similarities between the copulatory patterns of moths photographed with red and white light suggested that the light from the electronic flash did not affect their copulatory behaviour. However, it was possible that the moths could be equally sensitive to both colours and therefore it was necessary to establish whether or not the light flashes affected them. This was investigated by keeping three groups of 20 pairs of moths in undisturbed darkness for different periods of time and then comparing the proportions found copulating with those in the previous experiments. Each of these three groups of moths consisted of 15 virgin males and 15 virgin females placed in a 1 litre jar covered with organdie netting. All three jars were kept in the rearing room until the lights were turned off; they were then removed in turn after 1.5, 3, and 7 hours of darkness. The proportions of moths found copulating after these times were 80%, 10%, and 20% respectively and since these were within the range of proportions found copulating at the same times when they were photographed, it appears that the electronic flashes did not significantly affect their copulatory behaviour.

All the experiments performed so far were only concerned with copulating moths and therefore no estimates of the diurnal mating pattern could be made until it was known how often they copulated in a night. This was therefore determined by placing 40 male and 20 female virgin moths, 1 to 3 days old, in the observation chamber, and keeping them in the rearing room until all the moths had separated the following day. The females were then dissected so that the number of spermatophores in the bursa copulatrix of each could be counted. More males than females were used on this occasion to increase the chances of multiple copulations being detected should they occur, but even so only 18 females each had one spermatophore inside them and two females had none. This demonstrated that the females mated once during this period and when related to the previous experiments, it also showed that most of the females mated soon after the light was turned off at night and only a few mated during the remainder of the night or in the morning. The relatively slow changes in the proportions of moths copulating in these experiments also indicated that the moths copulated for long periods of time ranging from possibly less than an hour to over 13 hours. This was confirmed by many photographic sequences in which copulating pairs occupied identical or very similar positions.

The diurnal copulatory pattern of *P. operculella* was then investigated when there were more males than females to check that this did not affect the copulatory behaviour of the females. Initially 32 male and 16 female virgin moths between 1 and 3 days old were placed in the observation chamber and the proportion that copulated at different times over three days was determined using the same photographic method as used above. In addition, it was later found that the lamp producing the night time illumination had been accidently removed by another student near the start of the experiment and therefore the moths received no light during the dark periods. The copulatory pattern of the moths, however, was still similar to those previously observed (Figure 8.3 H) and this therefore demonstrated that the moths could mate in total darkness and indicated that an excess of males did not affect the copulatory behaviour of the females.

This experiment was repeated again for completeness using the usual night-time illumination and the results were similar to those of the previous experiment (Figure 8.3 G). This, therefore, confirmed that the copulatory behaviour of the females was unaffected when there were more males than females.

Following this, a similar experiment was performed using 10 males and 30 females to find how often the males copulated. On the first night this was similar to the previously observed patterns but the initial pattern was repeated again on each of the following two nights although the maximum number of males that copulated at the beginning of each night decreased slightly each time (Figure 8.4 G). This, therefore, indicated that the males only mated once in 24 hours and that they mated again on subsequent days whereas the females usually only mated once and subsequently did not mate for at least three days.

All of the above experiments were performed at 20°C but it was likely that the moths usually experienced lower temperatures than this in the field if they mated at night. It was therefore decided to determine their diurnal copulatory activity patterns when they were kept at 15°C, 10°C, and 5°C. Virgin moths between 1 and 3 days old were again used in the observation chamber in each experiment but more males were used than females and the observation chamber was kept in a constant temperature room. The moths were then photographed over a period of three days except for the experiment at 10°C which only ran for two days before the refrigeration unit iced up and the temperature rose to 15°C. However, the experiment was continued at this temperature for another two days. The results of these experiments (Figure 8.4 B, C, D) showed that the proportion of moths that copulated after the lights went off became progressively smaller the lower the temperature, whereas approximately the same proportion copulated at dawn at all temperatures, and therefore at 10°C and 5°C relatively more moths copulated at dawn than at the beginning of the night. The moths also showed a tendency to

Figure 8.4

The influences of different temperatures and constant light on the time of day when *P. operculella* copulates

Each histogram represents the percentage of males photographed copulating. Artificial light was used in each experiment.

A. The copulatory pattern of 10 males at 20°C, when provided with 30 females 0 to 1 day old.

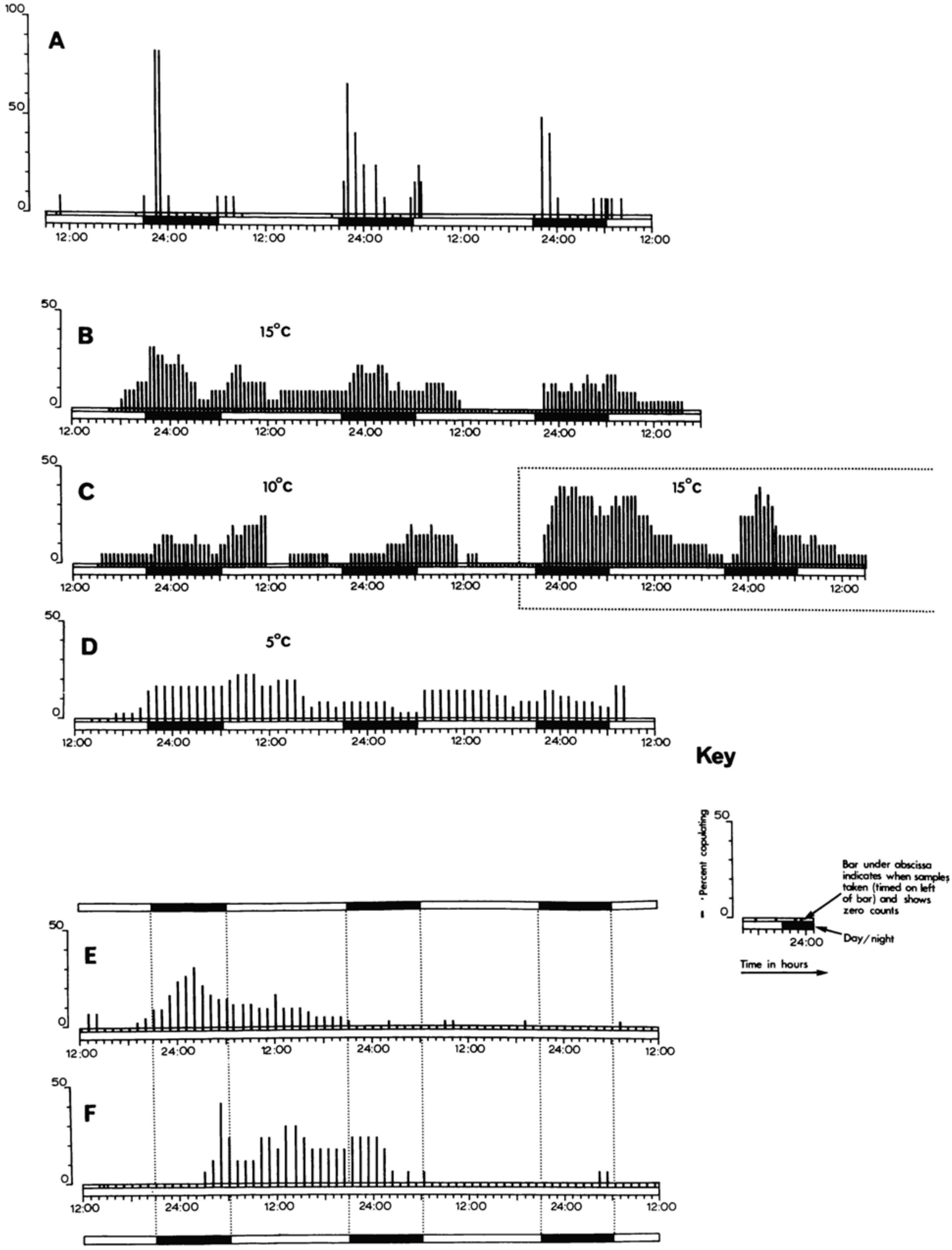
B. The copulatory pattern of 22 males and 40 females at 15°C.

C. The copulatory pattern of 20 males and 40 females at 10°C for two days followed by 15°C for a further two days.

D. The copulatory pattern of 30 pairs of moths at 5°C.

E. The copulatory pattern of 42 pairs of moths 1 to 4 days old in a constant light of 150 lux.

F. The copulatory pattern of 17 pairs of moths 1 to 4 days old, in a constant light of 250 lux.


A
100
50
0
12:00
24:00
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12:00
24:00
12:00
B
15°C
50
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C
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12:00
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24:00
12:00
24:00
12:00
D
5°C
50
0
12:00
24:00
12:00
24:00
12:00
24:00
12:00
Key
Percent copulating
50
0
Bar under abscissa indicates when samples taken (timed on left of bar) and shows zero counts
24:00
Day/night
Time in hours
E
50
0
12:00
24:00
12:00
24:00
12:00
24:00
12:00
F
50
0
12:00
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12:00

begin mating three or more hours before the lights turned off on the first night and there was a noticable time lag before they started mating after the light turned off on subsequent nights. At the lower temperatures they also tended to spend increasingly longer periods in copulation than at 20°C.

The possibility that the mating activity of the moths was influenced by an endogenous circadian rhythm was breifly investigated by keeping equal numbers of males and females, 1 to 3 days old, in constant light and photographing them over a period of three days. Two separate experiments were performed under light intensities of 150 lux and 250 lux. In the first experiment the moths began copulating just before the normal dark period (Figure 8.4 E) after which their numbers increased steadily until they reached a maximum at 0200 hours the next day and then gradually declined again over the next 20 hours. Following this they only copulated occasionally at irregular times. During the second experiment the moths did not begin copulating until 0300 hours on the second day when the proportion copulating increased to a maximum after two hours and then fluctuated irregularly over the next 24 hours or so (Figure 8.3 F). Following this no moths copulated until 0300 hours on the third day when one pair were found to do so for about an hour. The first experiment, performed under the same light intensity as that in which the moths were reared, showed that the moths' mating activity was possibly influenced by a circadian rhythm, while the higher than normal light intensity during the second experiment probably inhibited mating and caused the observed delay. However, no further investigations were carried out on endogenous rhythms although these experiments did demonstrate that it was essential to keep the moths under constant photoperiod conditions for behavioural experiments.

Field trapping experiments

When the laboratory experiments described above had been completed a study of the diurnal sexual activity of the moths in the field was undertaken to investigate whether their behaviour under natural conditions was similar to that observed in the laboratory. It was, however, difficult to find many moths in the field and also difficult to observe their sexual behaviour and therefore an indication of their diurnal sexual activity was obtained by studying their arrival patterns to sticky traps baited with live virgin moths or their extracts.

Initially, three sticky traps were constructed and tested in the field to find if they were effective. Two of these traps were each baited with 10 live females and the other was baited with 10 females crushed between two pieces of filter paper. All three traps were then placed in a potato patch on the evening of 13th April, 1972 and collected the following day. The two sticky traps beited with live females were found to contain 20 and 25 male moths respectively and no female moths, while the trap baited with crushed females caught no moths at all (Table 8.1). The sticky traps were therefore shown to snare moths and so a further 6 were constructed.

The next day all 9 sticky traps were used to check that female moths only attracted males in the field and that females were not attracted by males or females. Three of the sticky traps were therefore each baited with 7 live males between 1 and 2 days old, three were each baited with 7 live females of a similar age and the remaining three were left blank. The traps were then set in the potato patch before dusk and examined periodically during the night.

The results of this experiment (Table 8.2) showed that no moths were caught in the male baited traps and no females were caught in any of the others whereas a total of 48 males were caught in the three female baited traps and two males were caught in the blank traps. In addition, it also appeared that most of these males were caught soon after dark although some were also caught later in the night or in the morning. On analysis the female baited traps were found to be significantly more attractive than either the male baited traps or the blanks ($P<0.05$) whereas the catches of male baited traps and blanks were not significantly different from each other. This therefore confirmed the laboratory studies in Chapter 9 which showed that female moths were only attractive to males and that males were not attractive. It was, however, difficult and time consuming identifying the sex of every moth snared in the sticky traps because they became entangled in the adhesive. Therefore, in subsequent experiments no attempt was made to do this and all the trapped moths were considered to be males.

The sticky traps were next used on 4 separate occasions to determine the arrival patterns of male moths to live virgin females. In each of these experiments three of the traps were always left blank.

TABLE 8.1

Numbers of male and female moths caught in 3 sticky traps containing samples of 10 live or 10 crushed females.

The traps were set in the field at 0635 hours on 13/4/72 and collected at 0845 hours on 14/4/72.

Sample in trap	10 live females	10 live females	10 crushed females
No. of males caught	20	25	0
No. of females caught	0	0	0

TABLE 8.2

Numbers of male moths caught in sticky traps during different times over a two day period.
The sticky traps contained either 7 live males, or 7 live females, or were left empty as blanks.

The traps were set out at 0630 hours on 15/4/72.

Time of inspection	Temperature (C) at inspection time	Order of traps and samples in them	7 males	7 females	blank	7 females	7 males	blank	7 males	7 females	blank
15/4/72		Numbers of male moths trapped									
2000	16		0	1	0	10	0	0	0	14	1
2100	14		0	0	0	1	0	1	0	0	0
2330	3		0	0	0	0	0	0	0	0	0
16/4/72											
0920	12		0	8	0	7	0	0	0	3	0
1430	22		0	0	0	1	0	0	0	0	0

However, 7 females between 1 and 3 days old were used to bait each of the other 6 traps in the first two experiments, and three females of a similar age were used to bait each of these traps in the last two experiments. The traps were placed in the potato patch and examined at intervals over one or more days.

The individual trap catches for these 4 experiments are given in Appendix 2.14 and the individual catches per hour of both blank and baited traps are given in Figure 8.5.

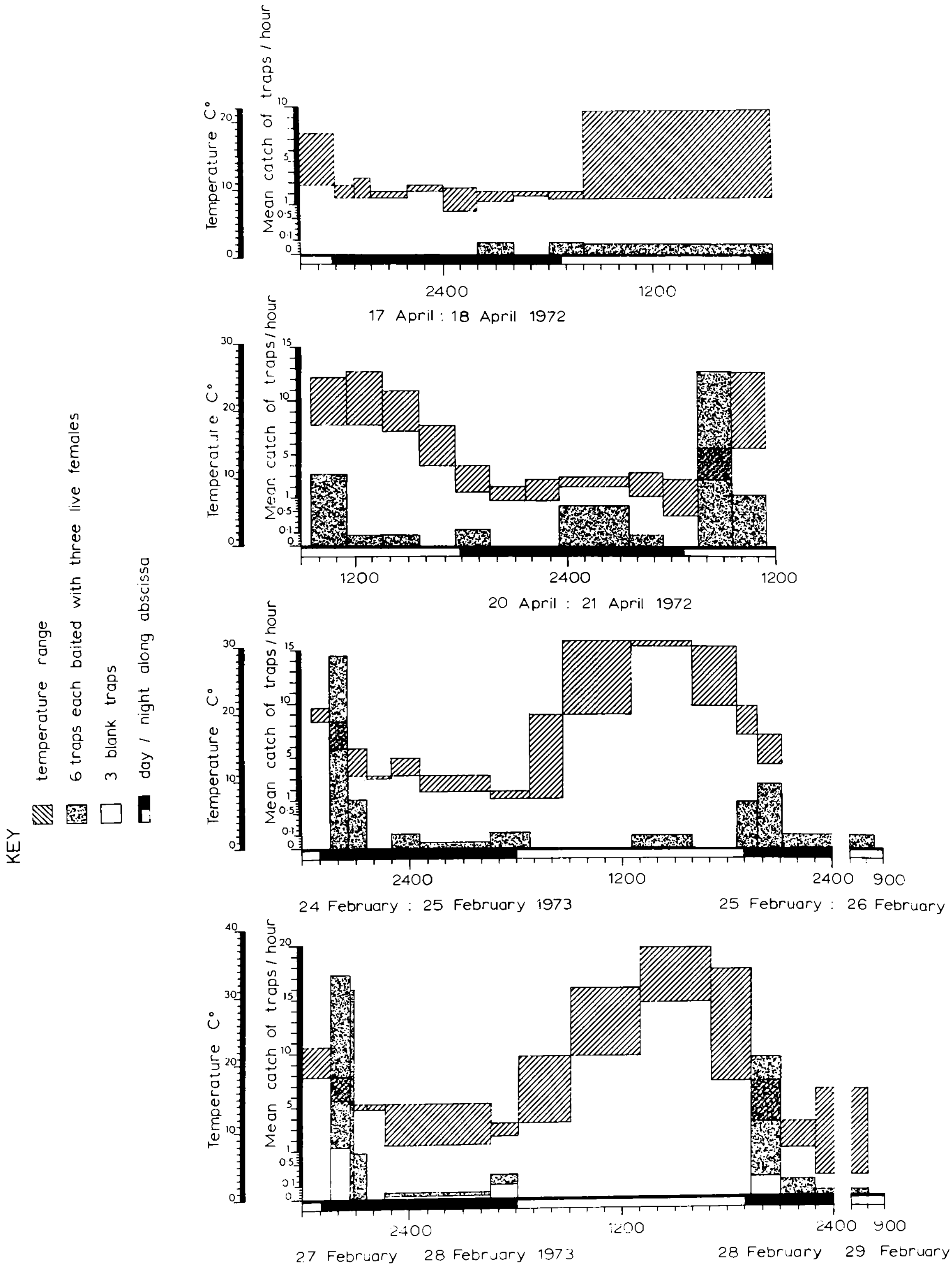
In the first experiment on 17th and 18th April, 1972 no moths were caught in any of the blank traps and only two were caught in the baited traps during the first night; one between 0200 hours and 0400 hours, and one between 0600 hours and 0800 hours. However, the temperature was mostly below 10°C during the entire night and this possibly accounted for the low catches. The traps were then left until 1900 hours the following night when 5 more moths were found in the female baited traps and it was possible that these moths had been caught during the daytime when the temperature was high.

In the second experiment on 20th and 21st April, 1972 most of the moths were caught in the mornings when the temperature was above 10°C and only two moths were caught during the two hour period over dusk when the temperature fell below 10°C. However, 19 were caught later in the night when the temperature rose above 10°C. This experiment therefore confirmed that attraction of males to females was inhibited at temperatures lower than about 10°C, and the results also suggested that possibly attraction was stimulated again when the temperature rose above 10°C.

On the last two occasions when the sticky traps were set out on 24th to 26th February and 27th to 29th February, 1973 the temperature stayed above 10°C except for short periods at the ends of the nights. Most of the moths were also caught in the female baited traps during the first two hours or so of darkness while few were caught at other times during the nights. The numbers caught did, however, increase slightly at the end of the nights or during the dawns but the increases when the temperatures rose above 10°C were only slight compared with those observed previously. It therefore appeared likely that big catches at dawn probably only occurred if the moths had been inhibited from being attracted to females at the beginning of the night. In addition, this

Figure 8.5

The arrival pattern of *P. operculella* to sticky traps baited with live virgin females


KEY
▨ temperature range
6 traps each baited with three live females
☐ 3 blank traps
day / night along abscissa
Temperature C°
Mean catch of traps / hour
2400
1200
17 April : 18 April 1972
20 April : 21 April 1972
24 February : 25 February 1973
25 February : 26 February
900
27 February 28 February 1973
28 February 29 February

increase in the numbers caught on 25th February occurred between temperatures of 8°C and 10°C so that inhibition probably occurred just below 10°C. Apart from the moths caught at dawn only two others were caught in baited traps during the daytime between 1230 hours and 1600 hours on 25th February and no moths were caught in any blank traps between 24th to 26th February, while a total of 9 were caught in them between 27th to 29th February. However, 8 of these were caught during the 2 hour period at the beginnings of the nights and one was caught at dawn indicating that the moths were also most active at these times.

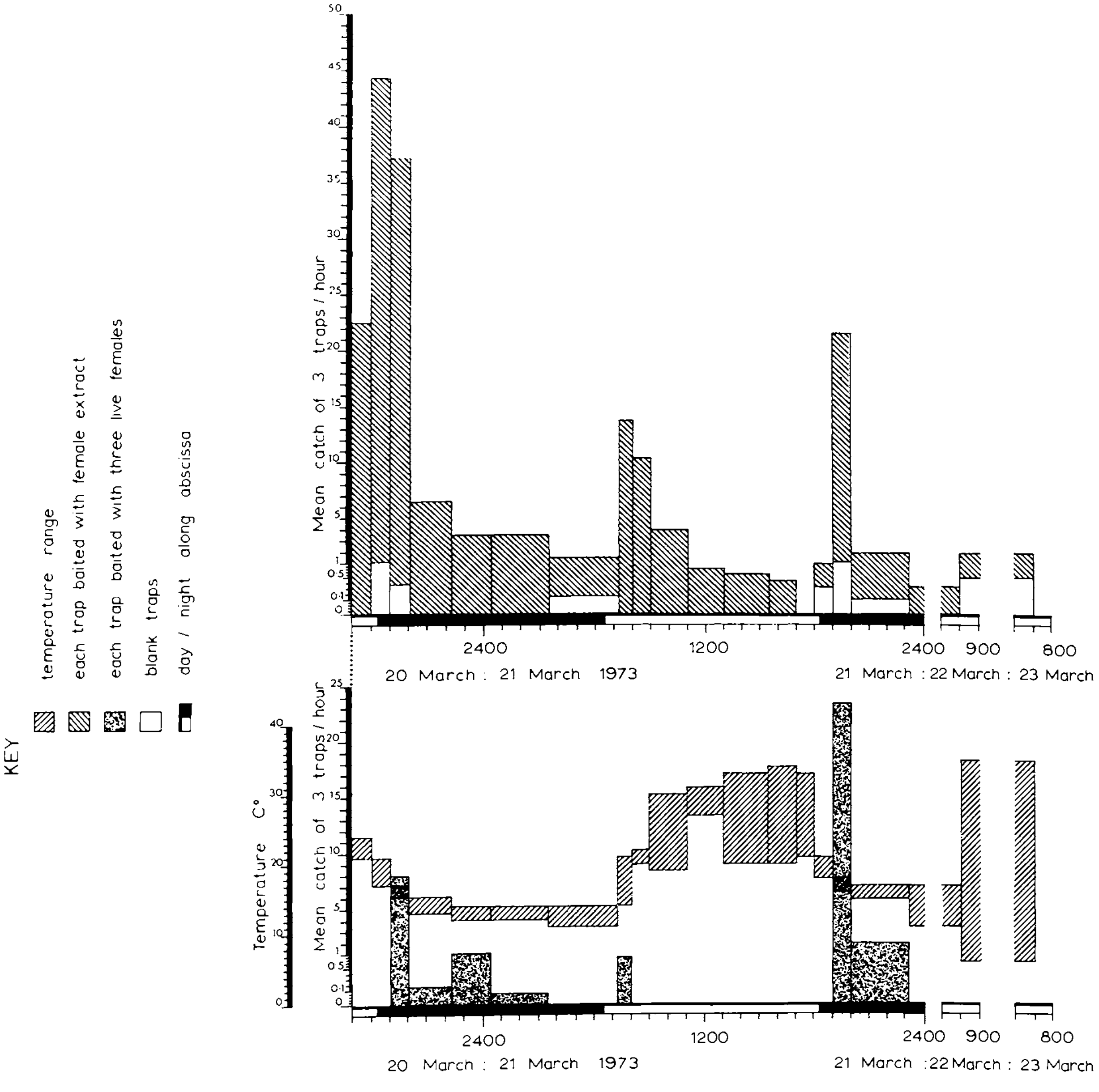
These experiments confirmed the findings of the previous laboratory experiments when the temperature was above 8°C to 10°C, and showed that at temperatures below this attraction was inhibited. However, the laboratory experiments showed that mating still occurred at low temperatures and therefore this inhibitory effect was probably the result of the moths not flying below 8°C to 10°C.

It was possible that the arrival patterns of moths in the previous experiments were influenced by two behavioural factors since live females were used as the baits in the traps. The females might have definite periods when they are attractive and the males might likewise respond to the females at definite times. If this is the case then the observed behaviour occurs when these periods overlaped. One last experiment was therefore performed on 20th to 23rd March, 1973 using extracts of virgin females as bait in the sticky traps to determine when the males were sexually responsive. In this experiment three traps were each baited with 100 µl of chloroform extract containing the equivalent of 10 females, three more traps were each baited with three live virgin female moths between 1 and 3 days old, and the remaining traps were left blank. The traps were then placed in the potato patch at 1700 hours on 20th March and examined at intervals afterwards. Also, the live female baited traps were removed at 2315 hours on 21st March but the extract baited traps and the blanks were left for a further 31 hours to check whether the extract lost its attractancy over this period.

The temperature stayed above 11°C during the period when the female baited traps were set in the field and the arrival pattern of moths to them was similar to those in the previous two experiments with maximum catches occurring soon after dark and with smaller numbers being caught at other times during the night and in the morning (Figure 8.6).

Figure 8.6

The arrival pattern of P. *operculella* to sticky traps baited with live virgin females and chloroform extracts of them


KEY
▨ temperature range
▧ each trap baited with female extract
▩ each trap baited with three live females
☐ blank traps
day / night along abscissa
Mean catch of 3 traps / hour
50
45
40
35
30
25
20
15
10
5
1
0·5
0·1
0
2400
1200
2400
900
800
20 March : 21 March 1973
21 March : 22 March : 23 March
Temperature C°
40
30
20
10
0
25

The arrival pattern of moths to the extract baited traps, however, differed from these; the maximum numbers of moths were still caught at approximately the same times as they were caught in the female baited traps, i.e. at the beginning of the night and in the morning (Figure 8.6 B) but they started being caught in the extract baited traps one to two hours before they were caught in the female baited traps at night and they were also caught during most of the daylight hours. It was also apparent that the extract baited traps became progressively less attractive during the trapping period, probably because the attractant was gradually lost. During the first 13 hour trapping period, between 1700 hours and 0800 hours, a mean of 12.7 moths were trapped per trap per hour while a mean of only 2.5 moths per trap per hour were caught during the same period the following night. In addition, the mean catches per trap per hour during the 24 hour periods from 0800 hours to 0800 hours were 6.88, 2.28, and 2.01 respectively over the three days not counting the moths that would have been caught during the 9 hours before the traps were set. It was also possible that the large numbers of moths caught during this experiment significantly lowered the number of moths left in the potato field so that catches later on were lower than if the population had been undisturbed, and therefore the numbers of moths caught during this experiment are considered to give only an approximate indication of the relative responsiveness of the males to the female attractant.

The results of this experiment, therefore, demonstrated that males reach their peak responsiveness to females at about the same times as live females release their attractant. However, the males were capable of being attracted to females at any time during the day and since this did not occur when live females were used the conclusion was reached that females control the release of their attractant and are responsible for non-attraction occurring during most of the daylight hours.

In this last experiment, fewer moths were snared in the blank traps than in any of the other baited traps (Figure 8.6) and most of these were caught at the beginning of the nights whereas only a few were caught during the rest of the nights or during the dawns. These results, therefore, confirmed those of the previous experiment and demonstrated that the moths flew mostly at the beginning of the night when the temperature was above about 10°C although some also flew at other times during the night, and that there was a small increase in flight activity at the end of the night or at dawn.

Laboratory experiments involving temperature changes

The results of two of these field trapping experiments indicated that the moths would mate when the temperature rose if they were prevented from mating at their normal times by low temperatures and therefore this was investigated further in the laboratory. It was hoped that this investigation would provide a method for allowing the moths to be observed mating during the daytime. A series of experiments was therefore performed in which equal numbers of virgin male and female moths, 1 to 3 days old, were photographed at intervals when placed together in the observation chamber and kept at between 0°C and 1°C in a refrigerated room for various lengths of time. They were then transferred to another room with a temperature between 20°C and 22°C. The times when the temperature change was made in each experiment are given in Figure 8.7 together with the resulting copulatory patterns of the moths.

Few of the moths were found to copulate when the temperature was below 1°C except in the last experiment when up to 20% of the moths copulated (Figure 8.7 K). In this case it was likely that the insulating polystyrene foam box had not been correctly positioned over the observation chamber and this had allowed the temperature inside to fluctuate enough for some of the moths to copulate. When a transfer was made, it usually took approximately 15 minutes for the temperature inside the observation chamber to change and soon after this there was always an increase in the proportion of moths that copulated. A maximum of between 37% and 45% of the moths were found to copulate within 1 to 2 hours after the temperature change if this occurred 3.5 hours before or after dark on the first or second nights, and when the lights turned on after the first night or an hour after this (Figures 8.7 A, C, D, E, G, I). However a maximum of only about 20% of the moths were found to copulate during the same period of time if the temperature change occurred when the lights turned out on the first and second nights or when the lights turned on after the second night or 4 hours after this (Figures 8.7 B, H, J). In addition, less than 10% of the moths copulated when the temperature change occurred three hours after the lights turned on after the first

Figure 8.7

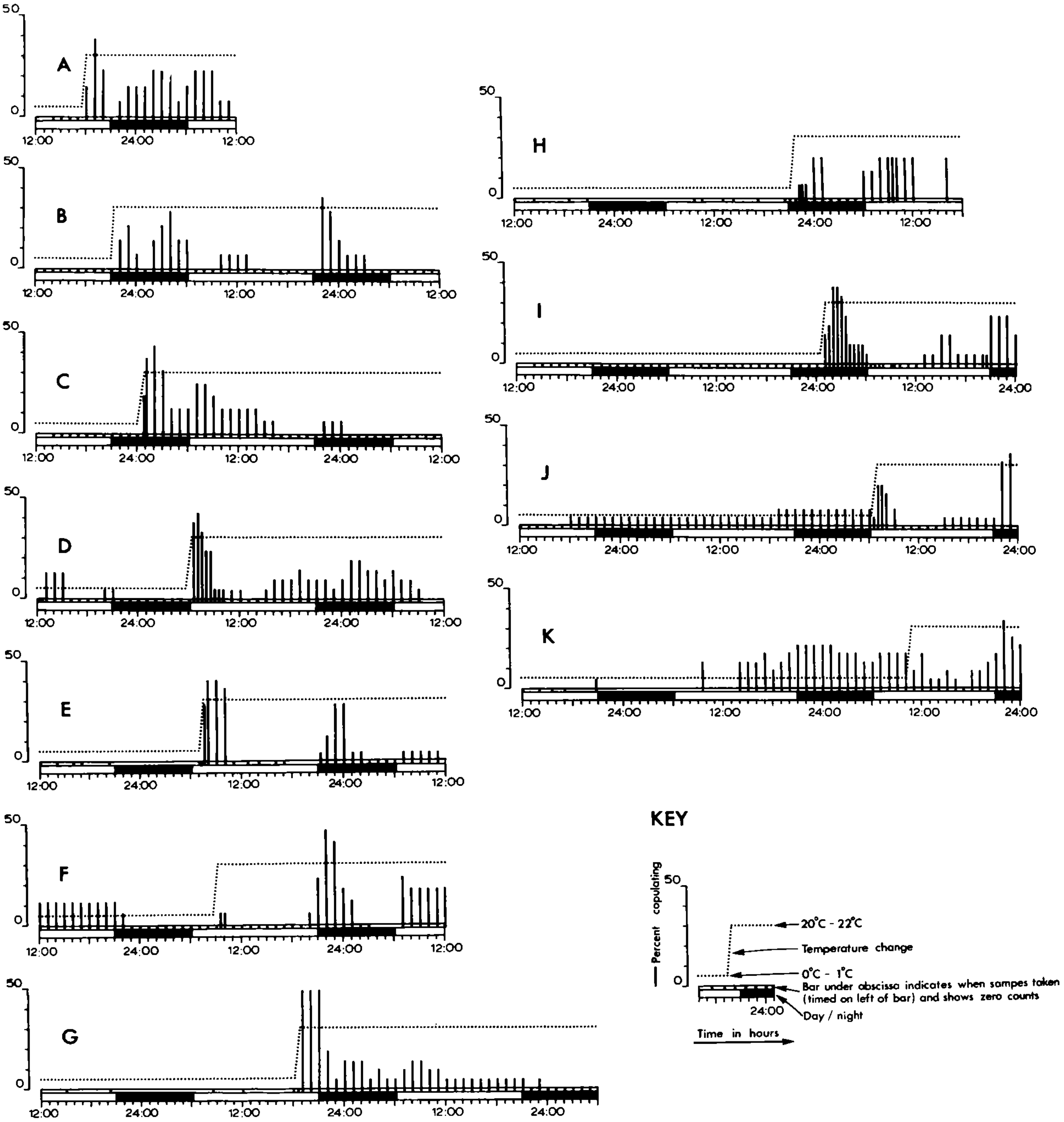
The effects on the copulatory behaviour of *P. operculella* of a rise in temperature above a previously inhibitory level at different times of the day

Numbers of pairs of moths used:

A	13
B	14
C	16
D	21
E	25
F	17
G	21
H	15
I	21
J	25
K	23

erratum: in key

"... when sampes taken ..." should read
"... when samples taken ..."


A
B
C
D
E
F
G
H
I
J
K
50
0
12:00
24:00
KEY
Percent copulating
20°C - 22°C
Temperature change
0°C - 1°C
Bar under abscissa indicates when sampes taken (timed on left of bar) and shows zero counts
Day / night
Time in hours

night (Figure 8.7 K). The moths also showed similar copulatory patterns when the temperature changes occurred at the same times on the first and second days, showing that it was unlikely that these observations were chance extremes and that once the moths had been cooled their subsequent behaviour was not affected much by the periods of time they were kept cold. Since the results of the last experiment were questionable, it therefore follows that the results of the 6th experiment (Figure 8.7 F), in which the temperature change occurred three hours after the lights turned on were not confirmed. Nevertheless, such a low percentage of moths copulated when the temperature changed three hours after the lights turned on that it was unlikely many would ever do so at this time. This investigation therefore demonstrated that if the moths had been prevented from mating by low temperatures many of them would copulate when the temperatures rose at most times during the day, with the exception that few would copulate if the temperature change occurred at the start of the night or in the middle of the day.

Nine of the above laboratory experiments were continued into the night following the temperature rise and these showed that most of the moths which did not copulate in the period immediately following the temperature change did so at the normal times during the following night and morning.

Mating behaviour

Female moths seldom move just before copulation and they appeared to be attractive to the males who searched for them and performed characteristic sexual displays before copulating. Occasionally a male was also observed to copulate with a female which ran or walked close by whilst he was searching for another female, but this only occurred if the female stopped after the male had intercepted her.

Before copulation attractive females are normally found in a resting posture with legs and wings close to the body and with antennae directed backwards. Only occasionally are they seen in an alert posture with legs splayed out and with their antennae held anterio-laterally. In this latter position the antennae are waved dorso-ventrally at a rate of 5 to 10 beats per second; the antennae on either side of the head being moved in opposite directions with short pauses at the top and bottom of each stroke. However, both these postures are also adopted by males and non-attractive female moths, and the only characteristic which distinguishes attractive females is that their ovipositors are usually extended so that up to half their lengths project from the tips of the abdomens (Figures 8.8 B, C). The ovipositors are not visible when the moths are examined dorsally and they can only be seen if the females are standing on glass or perspex and are examined from underneath. Each attractive female also fully extends her ovipositor at intervals and expands its mid region before partially withdrawing it again. Sometimes the female also bends her ovipositor ventrally at the junction of its 9th and 10th abdominal segments before withdrawing it, and in this position it is usually possible to see that the dorsal intersegmental membrane between the 9th and 10th segments is dilated dorsally into a pad with an approximately hemispherical shape. Each complete extension and withdrawal movement of the ovipositor is usually accomplished within one second, and most females perform these movements up to about 4 times within a period of 10 seconds. Some of the females which eventually copulated were, however, never observed to move their ovipositors but only left them half extended.

Male moths usually approach attractive females by either flying to within about 0.25m of them and running the rest of the way, or, if they are already within about 0.5m or so of them, by running only. Occasionally a male was observed to begin flying or running towards a female from a resting position and then he either started immediately from the resting posture or else he first took up the alert posture as described above for a female and waved his antennae about. However, because of the rapidity of the antennal movements it was not possible to decide from observation whether a male in the alert posture beat his antennae any faster than normally when about to move towards a female.

When males were observed to fly towards females in the laboratory each followed a more or less direct flight path which gently zigzagged from one side to the other on an approximately horizontal plane. Usually the males also alighted close by the females without flying about near them. In the field, however, the only males observed flying towards females were those seen approaching females in sticky traps in the mornings. They flew slowly upwind towards traps, constantly facing into the wind, and going alternately from one side to the other over a total distance of up to 10cm. These males also

Figure 8.8

Photographs of mating in *P. operculella*

A. A male waving its antennae in the alert posture

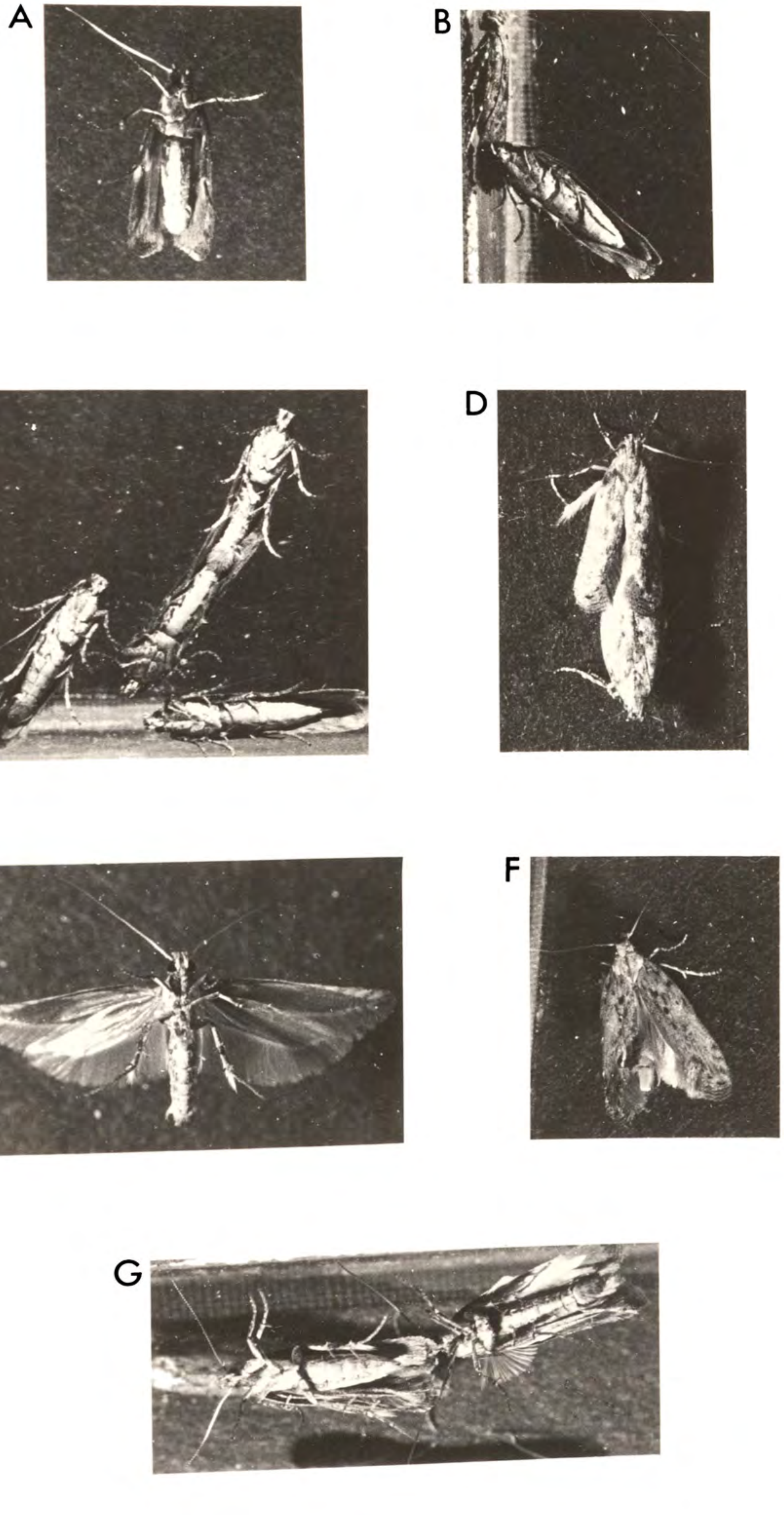
B. A female in the resting posture with its ovipositor extended.

C. A pair of copulating moths together with a male and a female in the resting posture. Note also that the resting female has its ovipositor extended.

D. A copulating pair.

E and F. A pheromone stimulated male beating its wings whilst running about with its abdomen curved dorsally, and its coremata and genitalia splayed out.

G. A male with hairpencil extended approaching an attractive female.


A
B
C
D
E
F
G

frequently alighted on vegetation in their flight path and rested or ran about at intervals for a few seconds before continuing to fly. As they approached within about 0.25m or so of the sticky traps the amplitude of their side to side flight movements tended to decrease progressively and they usually alighted on or very close to the sticky traps before running into them and becoming snared.

The running behaviour of a male approaching a female changes as he comes nearer to her: initially he runs in more or less straight lines with few changes in direction but the frequency of his turning movements increases the nearer he gets to the female until when he is within 5cm or so of her he almost continuously turns in curves and spirals with radii of about 0.5 to 2cm. He then begins to display the first distinctive behaviour pattern of mating referred to here as the "fluttering dance". Up until the performance of this it is impossible to determine with certainty if the male is responding to a female or not, because males perform all the other behavioural patterns when females are not present. However, the behaviour described below was only observed when a male was close to a female. During the fluttering dance the male continues to run about in curves and spirals as he does beforehand but he beats his wings rapidly as if he were flying, moving them over a wide arc so that they almost meet dorsally. At the same time he curves the end of his abdomen dorsally and opens out the large scales of the coremata (Figures 8.9 E, F). Sometimes the male alternates short periods of running with periods of the fluttering dance, but usually he performs the fluttering dance continuously until his circling movements bring him within one or two centimetres of the female. At this distance he folds his forewings slightly posteriorly over his hindwings, while still continuing to beat them, and opens out the two tufts of long thin scent scales from their anterio-dorsal pockets on his hindwings (described in Chapter 10). When extended the scales of each hairpencil radiate out from their attachments to the wing and form a hemispherical brush (Figure 8.9 A, B). Eventually, when the male contacts the female, he usually moves sideways or turns until he is posterior to and slightly to one side of her. He then twists his abdomen to the side nearest the female so that its tip is directed laterally (Figure 8.9 E) and often slightly anteriorly. From this position the male moves forward alongside the female so that his abdomen passes under her wings and he usually

Figure 8.9

Photographs of mating in *P. operculella*

A. A male approaching an attractive female.
The male has temporarily folded his wings but his hairpencils are still extended. He has also bent his abdomen towards the right where it is hidden by the wings (c.f. Figure 8.9C).

B. A male approaching an attractive female.
The male has bent his abdomen to the right as in Figure 8.9A, but he is still beating his wings. His hindwings have been slid back over his forewings and his hairpencils are extended.

C. A pheromone stimulated male approaching two females.
The male's hairpencils are extended and he is bending his abdomen to the left and also beginning to extend his left wings in preparation to adopting the posture shown in Figure 8.9D.

D. A male attempting to copulate with a copulating pair.
Note that his left wings are extended over the back of one of the copulating moths and that his hairpencils are still extended.

E. A ventral view of a male approaching an attractive female.
Note that his abdomen is curved towards the side nearest the female, and that his hairpencils are extended.

F. Two pairs of copulating moths, one of which has just started copulating. The male of the latter pair has hairpencils extended and abdomen bent into a "C" prior to thrusting it toward the female.

A

B

C

D

E

F

moves it about, sometimes by moving his whole body, until his genitalia contact the end of the female's abdomen. While the male is moving alongside the female, he also stops beating his wings and partially folds them, but he still usually leaves his hairpencils extended (Figures 8.8 G; 8.9 A). In addition, when he has positioned himself alongside the female he extends the pair of wings nearest to her so that they go over her back and touch it (Figure 8.9 D). As soon as the male's genitalia contact the end of the female's abdomen he fastens them on and then he usually executes one or two quick thrusting movements by bending his abdomen into a "C" (Figure 8.9 F) and subsequently pushing it towards the female. Once the male is copulating firmly he straightens out his body so that he is facing in the opposite direction to the female (Figure 8.8 C, D) and at the same time he also usually shuffles his wings to fold them properly. Sometimes, when a male is unsuccessful in his first attempt to copulate, he either backs to his former position posterio-laterally to the female and moves towards her once more, or else he moves away and begins his fluttering dance again.

Males responding to attractive females also try to copulate with any other moths they contact including other males, but only once were two males ever found copulating with each other. These were discovered at the end of a bioassay (Chapter 9) after both had entered a trap in response to the odour from two squashed females. However, they never succeeded in separating; one died after two days and was then carried around for a further two days until the other also died. Males were also frequently observed trying to copulate with females which were already copulating (Figure 8.9 D) but they were never observed to be successful. However, they displayed all the normal mating behaviour and even usually moved their abdomens about in the vicinity of the females' abdomens once they had closed with them, rather than near the heads of the copulating moths (Figure 8.9 D).

The mating behaviour of the male moths described above is performed fairly rapidly and usually takes between 2 and 5 seconds from the time they begin their fluttering dance to the time they copulate. However, on occasions this can be completed within one second or take up to about 30 seconds. In addition, male moths also often try to copulate with paired moths for periods of up to about 5 minutes although this is done intermittently, each attempt seldom lasting more than about 15 to 30 seconds.

The role of the hairpencil organs and the antennae during mating

At attempt was made to demonstrate the role of the male hairpencil organs in courtship by removing them and subsequently observing what effect this had on their mating behaviour. Nineteen pairs of male moths aged between 1 and 3 days were used for this experiment. Each pair was anaesthetised with carbon dioxide, and one moth was then kept as a control while the hindwings were dissected off the other. However, dissection without damaging the rest of the moth proved difficult because the insects were small and their scales made them slippery. These difficulties also resulted in the operations often taking 5 or more minutes during which the effects of the anaesthetic frequently wore off. When this occurred both the control moth and the one being dissected were anaesthetised again. Overall, only three amputations were considered to be satisfactory and the moths which were successfully operated on were kept together with the controls until the following night when they were placed in a refrigerator at 4^{o}C. The next morning they were transferred to an observation chamber at room temperature and 12 females, 1 to 3 days old, which had also been held in the refrigerator overnight, were added. These females began extending their ovipositors as they warmed up, and the males began reacting to them. The three control male moths copulated within 2, 3 and 5 minutes of the females being introduced, while the ones without hindwings performed all the usual precopulatory behaviour patterns, including fluttering and partially folding their forewings. However, whenever one touched a female or placed his wing on her back, the female usually moved away a few paces or ran away so that the male was unable to copulate. These males frequently attempted copulation until they lost the female and then they often rested for a period before starting to search for others. All three amputated males did eventually copulate after 15, 16 and 25 minutes respectively, and this was significantly longer ($P < 0.005$) than normal males took. This experiment therefore indicated that the hindwings were involved in subduing the escape reaction of the females during mating and this was attributed to a pheromone dispensed by the hairpencils.

Another experiment was performed to find if the removal of antennae from females had the same effect as removing the hindwings of males. The experimental procedure was the same as that used in the previous experiment, and the moths were again aged from 1 to 3 days old.

Dissections were performed until three moths were satisfactorily operated on and then these moths, together with their respective controls, were kept overnight in a refrigerator at 4°C and introduced into the observation cage together with 12 males in the morning. On this occasion the three control females copulated within 3, 4, and 4 minutes of being placed with the males while those without antennae showed similar escape behaviour to females being courted by males lacking hindwings. They copulated after 2, 8 and 15 minutes but these times were not significantly different from those of normal moths. However, the sample was small and the behaviour of these females supported the hypothesis that amputation of the males' hindwings deprived males of a pheromone organ. It also indicated that some receptors for this pheromone were located on the females' antennae.

Lastly, an experiment was performed to determine the effect of depriving males' of their antennae. Again, the same experimental procedure was followed with moths of 1 to 3 days old. Three males whose antennae had been removed were used with three controls and 12 females. This time the males lacking antennae were not observed to show any mating behaviour within one hour of being placed with the females, whereas the control males copulated after 1, 3 and 4 minutes. This indicated that in the male, the only receptors for the female sex pheromone are located on the antennae.

Discussion

Mating in moths, as in all insects, may be influenced by a number of factors including the physiological variables of age of the individual, mating history, previous exposure to the sex pheromone, time of day, and circadian rhythmicity; together with environmental variables such as light intensity, temperature, wind speed, and host substrate (e.g. Jacobson, 1972; Birch, 1974). The importance of some of these factors in relation to mating in *P. operculella* has now been determined, together with the conditions under which this species mates.

The time of day when mating occurs

The time of day when *P. operculella* is sexually active has been investigated in the present study both in the field, by determining the periodicity of male attraction to virgin females in sticky traps;

and in the laboratory, by determining the diurnal pattern of copulation. The conclusions as to when mating occurs are in close accord, and therefore the diurnal periodicity of sexual activity in *P. operculella* can now be considered as fairly well established.

Sexual activity in *P. operculella* is greatly influenced by temperature, but since most of the adults of this species occur in the field in New Zealand from late spring, through summer and into mid autumn, they will usually experience evening temperatures higher than about 10° to 15°C. Under these conditions most mating activity occurs during the first hour of darkness and drops rapidly during the second hour to a very low level for the rest of the night. At dawn there is a small increase in mating activity for one or possibly two hours but after this the moths rarely mate again until the next evening. Mating also occurs at definite times of the day in other moths but varies depending on the species. For example it occurs in the late afternoon or early evening in *Choristoneura fumiferana* (Clemens) (Sanders, 1971; Sanders and Lucuik, 1972), *Laspeyresia pomonella* (L.) (Wong, Cleveland, and Ralson, 1971), *Diatraea saccharalis* (Fabr.) (Walker, 1965), and *Platynota stultana* Walsingham (AliNiazee and Stafford, 1971); in the middle of the night in *Desmia funeralis* (Hübner) (AliNiazee and Stafford, 1973); in the latter part of the night in *Dioryctria abietella* (Denis & Schiffermüller) (Fatzinger and Ascher, 1971), *Heliothis virescens* Fabr. (Gentry, Lawson, and Hoffman, 1964), and *Manduca sexta* (Johannson) (Allen and Hodge, 1955); or in the early morning in *Hyphantria cunea* Drury (Hidaka, 1972).

The timing of mating behaviour in Lepidoptera is also frequently reflected in the timing of male flight. Many moths apparently exhibit differences in the timing of nocturnal flight activity amongst the sexes (Williams, 1935, 1939; Beck, 1968) and the peak flights of males of a number of species occur near the time when mating is known to occur (Graham, Glick, and Martin, 1964; Saario, Shorey, and Gaston, 1970). Edwards (1962), however, has also demonstrated that characteristic peaks of male activity in certain Lepidoptera may occur both in the presence and absence of females. However, nothing has been published on the flight activity of male and female *P. operculella* except for Rothschild (1972 - 1973) who noted that males predominated in the catches of suction traps and gave no other details. Recently Mr S.L. Goldson

(*Pers. comm.*, 1975) has investigated the flight activity in this species for an M.Sc. degree (Lincoln College) and found that mostly males were caught in his suction traps, and that the majority of these were caught just after the onset of darkness. These results therefore support the findings of the present investigation on the timing of mating in *P. operculella*.

Variation in mating time amongst different moths is a possible means of aiding reproductive isolation between closely related sympatric species which use the same female sex pheromone. In some cases this can be the major isolating mechanism as reported for the saturniids *Callosamia promethea* Drury and *Samia cynthia* (Drury) by Wilson and Bossert (1963) because the first mates in the afternoon from about 4 to 6 p.m., and the second does so at night between about 10 p.m. and 2 a.m. The time of day when mating occurs has also been suggested as being at least partially responsible for preventing cross matings in some species of the *Holomelina* complex (Arctiidae) which use the same female pheromone (Roelofs and Cardé, 1971), and in three species of Plusiinae which also share a common female pheromone (Kaae, Shorey, McFarland, and Gaston, 1973). In addition, the sympatric crambinids *Agriphila plumbifimbriella* Dyar and *Crambus harpipterus* Dyar, which mate in the early and late part of the night respectively, may be temporally isolated but it is not known if they use the same sex pheromone (Krehoff, 1974).

In the present study the field experiment with sticky traps containing live virgin female moths and crude extracts of female pheromone clearly indicated that in *P. operculella* the time of day when mating occurs is determined primarily by the female releasing her sex pheromone. The males on the other hand have a very broad time period over which they will react to the pheromone but even so their responses reach maxima just after dusk and dawn, and these therefore coincide with the times when the females become attractive.

Control of the time of mating by the female is perhaps to be expected since the males of most moths have a very broad period of time when they will respond to the sex attractant and the female usually has a narrow and distinct period when she "calls" (Cardé and Roelofs, 1973). There are exceptions, however, and some moths show the reverse with the female calling for long periods as for example reported for three species of *Hemerocampa* by Grant *et al.* (1975) and for *Porthetria dispar* (L.) by Carde, Doane, and Roelofs (1974). In these cases the time of mating may be controlled by a periodicity in the sexual activity of the male rather than the female. Another exception has been reported for *Plodia interpunctella* (Hübner) by Brady and Smithwick (1968). This species mates at any time of day, and no rhythmicity could be found in either female calling behaviour or male response to the sex pheromone.

It also appears probable that mating in *P. operculella* is partly influenced by an endogenous circadian rhythm because in one experiment virgin moths, when kept on a normal photoperiod and then placed together in constant light, mated approximately 24 hours after the start of the previous scotophase. Further experimentation is, however, required to demonstrate whether or not mating does follow a circadian periodicity.

Considering that almost every aspect of the life of an insect may be influenced by a circadian rhythm (Beck, 1968), then the suggestion that mating behaviour in *P. operculella* is also under the influence of one seems quite reasonable. Furthermore, such an influence on mating has been demonstrated in every moth where this has been investigated, except for *P. interpunctella* (Hübner) (Brady and Smithwick, 1968) which mates at any time of the day or night. Calling behaviour of the female *Anagasta kuhniella* (Zeller) has been reported to follow a circadian rhythm by Traynier (1970), and strongly photoperiod entrained circadian rhythms of pheromone release have been reported for *Holomelina immaculata* (Reakirt) by Cardé and Roelofs (1973), *Trichoplusia ni* (Hübner) by Sower, Shorey, and Gaston (1970, 1971), and for *D. abietella* by Fatzinger (1973). The male *P. operculella* may, however, also show a circadian periodicity of response to the female sex pheromone because this has been demonstrated in the male *A. kuhniella* by Traynier (1970), and in *Autographa californica* (Speyer), *H. virescens*, *Spodoptera exigua* (Hübner) and *T. ni* by Shorey and Gaston 1965.)

The effects of light and temperature on mating activity

The female periodicity in *T. ni* is entrained by a lights-on stimulus (Sower *et al.*, 1971), and this may also be the important factor in entraining the female rhythm in *C. fumiferana* (Sanders and Lucuik, 1972).

In contrast, Cardé (1974) reports that the critical cues for initiating calling behaviour in *H. immaculata* is lights-off and a decrease in temperature. The latter signal also overrides the apparent inhibitory effect of constant light.

In the present study light and temperature have also both been shown to be important factors in determining when mating occurs in *P. operculella.* Sexual activity in this species takes place in the field in the evening when it is quite dark, and this is supported by the results of a laboratory bioassay experiment which indicated that male attraction to the pheromone is inhibited by a light intensity between 1.0 and 0.1 lux (Chapter 9). The moths, however, also mate after dawn in the field or after the lights turn on in the laboratory so light is not altogether inhibitory. Possibly, in this species, a change in light intensity from either day to night, or night to day is the stimulus which triggers mating behaviour although the onset of darkness is the most effective of the two. Temperature, however, has a very important modulating effect on light change:- In the laboratory under the usual photoperiod at which the moths were reared, progressively lower constant temperatures decrease the proportion of moths that mate. This is due to a reduction in the number that mate at the beginning of the scotophase and not to those that do so at the start of the light period, because this proportion stays approximately constant from 15°C to 5°C. Additional effects of low temperatures are that some of the moths begin mating before the onset of darkness at temperatures of 15°C and below, and that the period spent in copulation is increased. In the field, on the other hand, male attraction to females in sticky traps ceases somewhere between 8 to 10°C apparently because flight is inhibited at temperatures below this. This is supported by Mr S.L. Goldson (*pers. comm.*, 1975) who found that the lower threshold for flight occurred at 10°C. In addition, Rothschild (1972-1973) also reported that the catches of *P. operculella* in suction traps and traps baited with virgin females were both influenced by temperature but he gave no details, whereas Langford and Cory (1932) reported that the adults of this species are active flyers between 14.4°C and 15.6°C but that they only crawl at 11.1°C. These latter authors also noted that mating occurs between 17.8°C and 35°C while Graf (1917) reported that it occurs between 15°C and 18.3°C. In the present study the moths were found to be capable of mating at all temperatures between 1 to 2°C and 20 to 22°C, although they rarely did so at the lowest of these.

The field trapping experiments also revealed that a rise in temperature stimulated sexual activity in *P. operculella*, if the temperature fell below about 10°C before dusk and rose above this in the morning. No tendency was discovered for the moths to mate while the temperature was falling prior to nightfall so that the low level of mating activity observed at low temperatures in the laboratory, was possibly a result of the female moths being only very slightly attractive at these times, and that this was accentuated by the confined cages in which the moths were held. Confirmation that an increase in temperature stimulated mating activity in *P. operculella* was obtained from the laboratory experiments. However, for these experiments the temperature had to be lowered to about 1 to 2°C before **mating** was sufficiently inhibited for the purposes of the investigations and this was presumably the temperature which inhibited the males from walking to the females. These low temperatures probably did not adversely affect the moths because Langford and Cory (1932) reported that *P. operculella* could be exposed to temperatures as low as -8.3°C for several hours without this affecting their ability to lay eggs.

The laboratory investigations showed that mating activity was triggered by a temperature rise if this occurred about three hours before the onset of darkness, during the dark period, and soon after the lights switched on, but that it had relatively little effect if it occurred in the middle of the light period or if it coincided with the onset of darkness. In addition, if the temperature rise occurred during the dark period then some of the moths also mated later during the following light period. Possibly this accounts for those male moths which were occasionally attracted to females during the daytime in the field.

In the natural situation, the temperature probably drops to an inhibitory level at some time during the late afternoon or night and the temperature rises again the next morning. The field trapping experiments, however, indicated that such a temperature rise is only effective if the moths were prevented from mating by low temperatures during the previous evening, because when the temperature drop occurred after the mating period then very little attraction was observed when it rose again in the morning. Presumably the adaptive significance of this behavioural response is that it allows the moths to mate during the cooler months of the year when the temperature only rises above 10°C in the daytime.

In other species of nocturnal moths high light intensities are generally considered to be inhibitory to sexual behaviour although it will occur in high light intensities in some circumstances. For example Shorey (1966) reported that *T. ni* will sometimes mate in the laboratory above 0.3 lux, the normal inhibitory threshold value for this species. However, only a relatively small proportion will do so, and they show an inverse correlation with light intensity up to about 300 lux. Generally, the males of moths also have a broad time period when they are responsive to the female pheromone, as already mentioned, and this period frequently extends well into the light period. For example Bartell and Shorey (1969a) reported that male *Epiphyas postvittana* (Walker) entrained to a 14L:10D photoperiod reached their maximum responsiveness from 2 hours before until 6 hours after the onset of darkness. Other examples are cited by Jacobson (1972), but no reports were found of other moths with similar mating behaviour patterns to *P. operculella* with respect to light. Perhaps the closest was that of Fatzinger (1973) who investigated the effect of a diel light cycle on the precopulatory behaviour of the phycitinid *D. abietella*. The male is responsive to the female pheromone mainly during the last 8 hours of the scotophase when entrained to a 12L:12D photoperiod, but also shows another minor peak in responsiveness during the first 6 hours of the light period. In the female calling mostly takes place during the last 6 or so hours of darkness but continues with diminishing frequency into the first hour of light. Both of these observations suggest that a small amount of mating behaviour may occur in this species after dawn. It is of further interest in this respect, that Fatzinger (1973) found that the calling periods of females entrained to a 6L:6D photoperiod were approximately 24 hours apart but occurred only in the light periods, so that light is not altogether inhibitory to mating activity in this species.

Relatively few studies have been carried out on the effects of temperature on mating behaviour, and many of these are concerned only with the lower threshold for sexual activity. Shorey (1966) obtained a significant correlation between temperature, within the range of 10 to

15°C and the percentage of *T. ni* that mated. He also found that inhibition of wing vibration and flight of males exposed to the female sex pheromone increased within a similar temperature range and suggested that the inhibitory effect of low temperature on mating may be attributable in part to a reduction in male pheromone responsiveness as the temperature decreases from 15 to 9°C. Male responsiveness to virgin females in *P. stultana* is also influenced very much by prevailing temperatures according to AliNiazee and Stafford (1972), with the threshold for stimulation being close to 9.2°C. These latter authors also suggested that possibly this temperature was the one at which male flight capacity becomes very limited. For *C. fumiferana*, Sanders and Lucuik (1972) reported that the optimum temperature for mating is 18°C, although these moths will mate readily between 15 and 24°C. Furthermore, they noted that even at the low temperature of 9°C some individuals mated but copulation times increased, being two times as long at 9°C than at 28°C.

Some influences of changing temperatures on the sexual activity of moths have also been reported. Sparks (1963) noted that 100% mating could be attained with *Ostrinia nubilalis* (Hübner) in the laboratory if the temperature was varied from 31.1°C to 18.9°C from day to night, and suggested that a decrease in temperature may enhance male responsiveness to the sex pheromone. Klun (1968) found that, in this species, the male responsiveness to the pheromone in a bioassay is greater at 20 to 23°C than at 27°C. In addition, it is interesting to note that Klun (1968) reported that male *O. nubilalis* were inactivated by cooling to 4°C and did not respond to the sex pheromone when warmed up, although they responded if kept at 27°C for a further 24 hours. This appears to be quite different from the situation in *P. operculella* which responds almost immediately it is warmed up.

A number of authors have reported shifts in mating time due to temperature. Sower, *et al.* (1971) found that the mean time of calling by *T. ni* females occurred 4 hours earlier at 18°C as compared with 24°C, although much fewer moths called at any given time at 18°C. In other cases the temperature effect can shift sexual activity into the daytime. Comeau (1971) reported that attraction by male *Argyrotaenia velutinana* (Walker) to live females or synthetic pheromone took place in mid-afternoon at temperatures lower than 15°C, whilst it occurred after sunset at temperatures above 20°C. He also found that the females commenced calling at approximately sunset at 20°C, and at 2, 4, and 8 hours prior to sunset at 17°C, 15°C, and 12°C respectively. Sanders and Lucuik (1972) found that in *C. fumiferana*, the time at which 50% of the females called when compared with the results at 20°C occurred 1 hour earlier at 18°C, 2 hours earlier at 12.5°C, and 4 hours earlier at 11°C. Cardé *et al.* (1975) found that in *P.dispar* the response of male attraction to synthetic female pheromone in the field occurs approximately from sunset to 4 hours after sunset on a warm day and night (17-30°C), but commenced two hours prior to sunset on a cool day and night. Moreover, these shifts took place even if the temperature was decreased one hour prior to normal lights-off, indicating that long term temperature conditioning is not necessary for temperature-induced changes in the calling rhythm. These latter authors also found that at constant 15°C initiation of calling by the female occurred approximately two hours earlier than at 24°C in either a 16L:8D, or 12L:12D photoperiod regime. A temperature decrease, however, can modify to some extent the initiation of calling within the diel cycle for this species and can induce calling during a photophase, apparently acting in a manner similar to the lights-off cue. A similar effect was reported to occur in *Cadra cautella* Walker by Hagstrum and Tomblin (1973) although in this instance it was oviposition that was stimulated by the fall in temperature and acting like the lights-off cue. They concluded that it is "not unreasonable to suppose that an insect should respond to both temperature and light, since in many natural situations the midpoint in the falling portion of the temperature cycle typically occurs at about the same times as onset of darkness". Generally, however, temperature has been found to have little effect on the periodicity of rhythms although it has been found to influence activity, eclosion, oviposition, pupation, and diapause in at least 15 species of insects. Of these phase-setting influences of temperature on endogenous rhythms have been reviewed by Beck (1968), and the publications concerning the influence of thermoperiod on the timing of a variety of behavioural patterns are listed by Hagstram and Tomblin (1973).

The effect of humidity on mating

The effect of humidity on mating in *P. operculella* was not investigated in the present study although mating did not appear to be impaired at the relative humidities experienced by the moths in this study (from approximately 40% to 100%). Possibly, it may not have any effect because the moths will oviposit at all relative humidities between 3% and 100% (Langford and Cory, 1932). However, the effect of relative humidity on sexual activity in other moths has been investigated very little but it may or may not affect it. Snow and Callahan (1962) reported that successful mating of *Heliothis zea* (Boddie) in captivity was never achieved when the relative humidity fell below 80%, whereas Shorey (1964) found that the low humidity within a glasshouse (30% to 50%) did not appear to affect copulatory efficiency in *T. ni*, and he later could find no correlation between night-time relative humidities and mating frequency in *T. ni* (Shorey, 1966).

The relationship between age and mating behaviour

The ages at which male and female *P. operculella* are most sexually active was not investigated in the present study although it appears from observations of their sexual behaviour and egg laying (Appendix 1.2) that most normally mate during their first and second days after emergence. It is also evident from the investigation with the bioassay (Chapter 9) that virgin females contain pheromone at all ages and that males are not very responsive to the female pheromone during their first day after emergence but that they become strongly attracted by it when aged from two days to about two weeks.

In other species of moths the female can be most attractive at any age from immediately after emergence to 3 or 4 days old, but attractiveness usually declines after this. Females which are at their most attractive within their first day after emergence include *C. fumiferana* (Clemens) (Outram, 1968), *Gypsonoma haimbachiana* (Kearfott) (Payne and Stewart, 1971), and *Synanthedon pictipes* (Grote and Robinson) (Wong *et al.*, 1969); whereas the female of *Crambus trisectus* (Walker) is most attractive 2 to 3 hours after emergence and remains attractive for 2 to 3 days (Banerjee and Decker,1966): and the females of *D. funeralis* and *D.saccharalis* are most attractive during their entire first

1 to 2 days of life (AliNiazee and Stafford, 1973; Perez and Long, 1964, respectively). In other moths attractiveness of the female increases initially with age, and this occurs for example in *Adoxophyes orana* Fischer von Roslerstamm which reaches a plateau of attraction from two days onward (Minks and Noordink, 1971); in *Orgyia leucostigma* J.E. Smith which is most attractive when 2 to 3 days old (Percy, Gardiner, and Weatherston, 1971), and in *L. pomonella* which reaches peak attraction at 3 days of age (Butt and Hathaway, 1966). Dickens (1936) also reported that attractiveness of females increases with age in many of the Phycitinae.

Variations in the quantity of pheromone with age of the female have also been determined for some moths. Examples of this include 7 species of Noctuidae, in which Shorey, McFarland, and Gaston (1968a) found a rapid increase in the quantity of sex pheromone within 0.5 days after emergence, and the amount present subsequently stayed at the same level or declined slightly 5 days or so after emergence; *Vitula edmandsae* (Packard) (Phycitinae) in which Weatherston and Percy (1968) found that pheromone production reached a maximum at two days and then declined; *O. leucostigma* (Lymantridae) in which Grant (1975) found that the pheromone concentration is highest in 1 to 2 day old moths; and *Mamestra configurata* (Walker) (Noctuidae) in which Struble, Jacobson, Green, and Warthen (1975) found the greatest amount of pheromone was present in 7 to 9 day old females. Mating may also cause a decline in attractiveness of the female or pheromone production, as it does in *C. fumiferana* (Clemens) (Outram,1968), *D. saccharalis* (Fabr.) (Perez and Long, 1964), and *O. leucostigma* J.E. Smith (Grant, 1975) but in other moths, such as many noctuidae (Shorey *et al.*, 1968a), mating has very little effect.

The male's response to the female pheromone usually increases to a peak within a few days of emergence and often stays at this level until death. This occurs for example in a number of Noctuidae (Shorey, Morin, and Gaston, 1968b); and *E. postvittana* (Walker) (Bartell and Shorey, 1969a); and in *M. configurata* (Walker) (Jacobsen, Green, and Warthen, 1972). In other species responsiveness increases to a peak and then declines again, as it does for example in *A. velutinana* (Walker) (Roelofs and Feng, 1967), and *C. fumiferana* (Clemens) (Outram, 1968).

Nothing else is published about the other factors which may affect the sexual behaviour of *P. operculella* although Goldson *(pers.*

comm., 1975) found that wind speed had very little effect on the catches of this species in the field and this may indicate that wind speed also has little effect on sexual behaviour. However, it was found in the present study that the moths could be more or less immobilised in an aspirator by sucking air through it sufficiently fast (Chapter 6), but *P. operculella* may possibly mate amongst low foliage in the natural situation where the wind is drastically reduced.

Other environmental and physiological factors may of course influence mating in *P. operculella* but further research is necessary to establish the importance of these. However, those factors which have been found to influence sexual behaviour in other Lepidoptera have been reviewed by Shorey (1974).

Mating behaviour

Moths show a considerable amount of diversity between different species in their mating behaviour. Typically, this usually involves a female sex pheromone which attracts the male and elicits courting behaviour. When close to the female the male also usually produces an "aprodisiac pheromone" (Birch, 1974) which either stimulates the female or subdues her escape reactions thus allowing the male to effect copulation. There are, however, a few exceptions: the male of *Achroia grisella* (Fabr.) produces a sex attractant for the female whilst the female apparently does not produce a pheromone although she is also guided by sound perceived through abdominal tympanic organs (Dahm, Finn, and Roller, 1970). In some species, the male may also produce an attractant which is effective at short distances whilst the long distance sex attractant is produced by the female. This occurs for example in *Galleria mellonella* (L.) (Roller, Biemann, Bjerke, Norgard, and McShan, 1966), and in *Phlogophora meticulosa* (L.) (Ford, 1955). A slightly different method of sexual communication again is employed by *Hepialus humuli* L. according to Warren (1969); the male of this species attracts the female visually, and the female then emits a pheromone which is effective at close range.

Precopulatory behaviour of the female

The female of most species of moth, when receptive or when releasing sex attractant, adopts a characteristic posture which is usually referred to as "calling". In *P. operculella* this is very difficult to observe because the female takes the normal resting position and alternately fully extends and partially withdraws the ovipositor from the tip of the abdomen. A similar calling behaviour has been reported for *Prionoxystus robiniae* (Peck) by Solomon and Neel (1973), and for *Tineolabiselliella* (Hummel) by Roth and Willis (1952). The latter insect will, however, also occasionally bend the abdomen dorsally so that the tip protrudes slightly between the wings and then vibrate its ovipositor, and it will raise the abdomen and protrude its ovipositor if it is stimulated by a male and then separated from it (Roth and Willis, 1952). Possibly *Hofmannophila pseudospretella* (Staint.) shows a similar behaviour to *P. operculella* because Woodroffe (1951) reports that the female has no calling attitude.

Usually, the calling position is quite distinct from the resting position in moths. Frequently the calling posture adopted is with wings parted but resting along the dorsal side and with the tips often touching the substrate, with the abdomen bent dorsally into a "C" so that it protrudes between the wings, and with the ovipositor extended. The behaviour is shown, for example, in *Podosesia syringae syringae* (Harris), and *Podosesia syringae froxini* (Lugger) (Nielsen and Balderston, 1973); and in *V. edmandsae* (Packard) (Weatherston and Percy, 1968). A similar posture is also adopted by *T. ni*, but the wings are vibrated at intervals (Shorey, Andres, and Hale, 1962; Shorey, 1964; Shorey and Gaston, 1970), and in *D. saccharalis* the antennae are vigorously moved between bursts of wing movement (Walker, 1965). Other calling females may hold their wings vertically over their bodies as does *Lambdina fiscellaria lugobrosa* (Hulst.) according to Ostaff (1974), and *H. cunea* according to Hidaka (1972). In addition, the female *H. zea* extends the wings out at about 45° to the body and vibrates them with the abdomen exposed, whilst the wingless female of *O. leucostigma* raises the last few terminal abdominal segments (Percy, *et al.*, 1971).

Rhythmic extension and retraction movements of the ovipositor apparently occurs commonly in calling female moths. It has been reported in the Lymantridae *Dasychira fascelina* (L.), *Dasychira seleutica* (L.) and *Hypogymna morio* (L.) by Gotz (1951), and *P. dispar* by Doane (1968); in the phycitinid genera *Ephestia, Anagastia*, and *Plodia* by Richards and Thompson (1932), Dickens (1936), Gotz (1951) and Brady and

Smithwick (1968); in *O. leucostigma* by Percy *et al.*, (1971); in *P. robiniae* by Solomon and Neel (1973) and in species in the "aurantiaca complex" by Roelofs and Carde (1971). Birch (1970a) also reported that caged *P. meticulosa* repeatedly curled the abdomen ventrally to touch the gland to the surface of the cage. However, no such ovipositor movements were observed in *C. fumiferana* by Sanders (1969) or Weatherston and Percy (1970a); or in *V. edmandsae* by Weatherston and Percy (1968). In general, release of pheromone by the female is considered to occur during ovipositor extenstion, but as far as I have determined from the literature, only in *Orgyia antiqua* (L.) has liquid droplets been observed on the surface of the everted glands, and these, when collected on absorbent paper, elicited an intense sexual response from the male moths (Freiling, 1909).

Generally female moths remain passive or only walk a few steps before the male copulates, as occurs in *P. operculella* whereas if they are unreceptive they usually run or fly away. In some moths the female may, however, take an active part, as for example does the female *T. ni* (Hubner) (Shorey and Gaston, 1970), and *P. interpunctella* (Hubner) (Grant and Brady, 1975).

Male precopulatory behaviour

The male moth generally takes the active part in mating, but there is a paucity of information on their sexual behaviour, and in particular on their courtship. This is probably due, at least in part, to the moths being nocturnal, and this makes observations of mating behaviour difficult (Grant, 1970; Grant and Brady, 1975). Some thorough studies have been published, however, and in addition many authors have noted a few particular aspects of male sexual behaviour. A failure to mention a particular behavioural response by these latter authors does not necessarily mean that the species concerned does not exhibit it, and therefore the following account is mostly limited only to behaviour which has been described.

The responses of a male to the female pheromone apparently consist of a fixed chain or sequence of several components which are added in a fixed order, or replace one another temporally. These can be divided into preflight behaviour when the resting male first perceives the pheromone, an orientated flight towards the female, and courtship behaviour culminating in copulation. This is discussed in more detail below, and some examples are given in Table 8.3.

In many species the female sex pheromone alone is all that is required to elicit the behavioural steps of mating in the male, and this is evidenced by the wide use of behavioural bioassays. It has also been generally assumed that the initial steps in the behavioural sequence are elicited by the lowest threshold of pheromone concentration, and that each successive step has a higher threshold than the preceding one (Schwinck, 1958; Guerra, 1968; Traynier, 1968). This has also been demonstrated to be the case in *E. postvittana* by Bartell and Shorey (1969b). In some cases the pheromone is apparently species specific but many moths share the same pheromone with related species, or the males are attracted by females of other species. In these cases other factors must account for reproductive isolation, and these can include the use of more than one chemical in the pheromone, or differences in the relative quantity of chemicals in the pheromone mixture, as well as differences in release rates, circadian rhythms, and additional cues such as auditory or visual ones (Roelofs and Cardé, 1974). Habitat selection may also play a part (Krehoff, 1974), and recently Grant, Smithwick, and Brady (1975) have shown that differences in courtship behaviour, male pheromones, and mechanical and physiological barriers to insemination also play important isolating roles between *P. interpunctella* and *C. cautella*. It therefore appears likely that all of the isolating mechanisms, as listed for example by Mayr (1966) may be important in one species of Lepidoptera or another.

Preflight sexual behaviour of the male

When first exposed to the female's pheromone the resting male may bring the antennae forward, move them about (often referred to as antennal "vibration"), and vibrate their wings (Table 8.3). Presumably the wings are vibrated to warm the thorax to the required level for flight (Heath and Adams, 1967), but *P. operculella* was never observed to do this. However, it was frequently observed to bring the antennae forward and then wave them alternately before taking flight although this may be no different from normal active behaviour.

Table 8.3

Sexual behaviour reported for male Lepidoptera

Species		Preflight behaviour				Courtship								References
		antennae brought forward and, or raised	antennal vibration	wing vibration		running circling movements	abdomen curved upward	antennal vibration	rapid wing beating	evertion of scent brushes	opening of "genital claspers"	curving of abdomen sideways toward female	copulatory attempts	
Argyrotaenia velutinana (Walker)	Tortricidae	+	–	–	Orientated flight to female followed by alighting near her*	–	–	–	–	–	–	–	–	Roelofs and Feng(1967)
Acleris gloverana (Wlsm)	Tortricidae	–	–	–		+	+	+	+	–	+	–	+	Daterman(1970)
Bombyx mori L.	Bombycidae	–	+	–		+	+	+	+	–	+	–	+	Kellogg(1907);Chen & Young(1947)
Cadra cautella Walker	Pyralidae	+	–	–		+	+	–	+	+	+	+	+	Grant & Brady(1975)
Diatraea saccharalis (Fabr.)	Pyralidae					+	–	+	+	–	–	–	+	Walker(1965)
Ephestia elutella (Hubner)	Pyralidae	–	–	–		+	+	+	+	–	+	–	+	Richards and Thompson(1932)
Epiphyas postvittana (Walker)	Tortricidae	+	–	+		–	–	–	–	+	+	+	+	Bartell and Shorey(1969a)
Grapholitha molesta (Busk)	Olethreutidae	–	–	–		+	+	+	+	–	+	–	+	Dustan(1946);Roelofs & Feng(1968)
Heliothis zea (Boddie)	Noctuidae	+	–	+		–	–	–	–	+	++	+	+	Callahan(1968)
Lambdina fiscellaria lugubroas (Hulst.)	Geometridae	–	+	+		–	–	–	–	–	–	–	–	Ostaff(1974)
Mamestra configurata (Walker)	Noctuidae	+	–	+		–	–	–	–	–	–	–	–	Chisholm *et al*,(1975)
Orgyia leucostigma J.E. Smith	Lymantriidae	+	–	–		–	–	–	–	–	–	–	–	Percy *et al*, (1971)
Ostrinia nubilalis (Hubner)	Pyralidae	–	–	–		+	–	–	+	–	+	+	+	Klun(1968)
Phlogophora meticulosa (L.)	Noctuidae	+	–	+		–	–	–	–	–	–	–	–	Birch (1970a)
Phthorimaea operculella (Zeller)	Gelechiidae	+	?	No		+	+	No	+	+	+	+	+	Present study
Plodia interpunctella (Hubner)	Pyralidae	+	–	–		+	No	No	+	+	+	+	+	Grant & Brady(1975)
Prionoxystus robiniae (Peck)	Cossidae	–	+	+		+	–	–	+	–	+	+	+	Solomon & Neel(1973)
Prodenia eridania (Cramer)	Noctuidae	+	–	+		+	+	+	+	–	+	–	+	Redfern *et al*,(1970)
Tineola bidelliella (Hummel)	Tineidae	–	–	–		+	+	–	+	–	+	+	+	Roth & Willis(1952)
Trichoplusia ni (Hubner)	Noctuidae	+	–	+		–	–	–	+	+	+	+	+	Shorey *et al*,(1962);Shorey(1964)
Vitula edmandsae (Packard)	Pyralidae	–	–	–		+	+	+	+	–	+	–	+	Weatherston & Percy(1968)

**T. ni* only alights once copulation is achieved

Flight of the male towards the female

Flight by the male to the attractive female is usual in moths as evidenced both from the many observations of this, and from the great number of field trapping experiments with female moths, extracts of them, and synthetic female sex pheromones (e.g. Jacobson, 1972). The observation, in the present study, that male *P. operculella* approach sticky traps containing crude female extracts or virgin females in a lateral zigzag flight path is of particular interest because many insect species exhibit such a flight pattern when approaching a sex pheromone source (the species are listed for example by Farkas and Shorey, 1972, 1974; and Farkas, Shorey, and Gaston, 1974), and this type of flight behaviour has been proposed as a means by which the insect stays within an aerial odour trail (Farkas and Shorey, 1974). Farkas and Shorey (1974) have reviewed the mechanics of orientation to a distant pheromone source and the means by which flight is arrested. They have also proposed an integrated system for the behaviour of a hypothetical insect in response to a pheromone. This is based on "bits and pieces" of information from a variety of sources, and since it summarises much of what is known about flight orientation, a brief resumé is included here:- If the insect is stationary and perceives an above threshold concentration of pheromone, flight is initiated and it flies in the general direction of the source perhaps by anemotaxis. If the insect is a long way off it steers more or less in a straight line and turns only when in the vicinity of the boundaries of the "active space" of the pheromone. As the insect flies near to the source, the concentration of pheromone increases, and the active space becomes narrower. Consequently the angle of turning becomes smaller and a zigzag flight pattern results. Possibly the turning response, back towards the centre of the aerial trail is regulated by chemotactic mechanisms, an anemotactic response, detection of a concentration gradient of odour molecules or odour filament pulses with time, a preprogrammed turning response in which right and left turns are alternated, or a combination of these factors. As the insect progresses along the aerial trail there is also a decrease in its forward flight speed, angle of turn, and lateral amplitude of the zigzag pattern, and when the insect perceives a high concentration of odour it is stimulated to either alight or visually search for the attractive insect or for another appropriate object such as a tree trunk.

Courtship behaviour of the male

The male of most species of moth alights and then runs about searching for the female after he has flown close to her (Table 8.3) and the same behaviour is shown by *P. operculella.* One exception is the male of *T. ni* which usually remains in flight until copulation is achieved so that its courtship behaviour differs from that of other moths. A full description of this is, however, available from Shorey *et al.* (1962), Shorey (1964), and Shorey and Gaston (1970).

Once alighted the males of most species run about rapidly beating their wings in what is referred to as "circus movements" or a "copulatory dance". They may also evert scent scales and open their "genital claspers", so that their behaviour shows many similarities to that of *P. operculella* (Table 8. 3). However, the moth which apparently shows the most similarity in its courtship behaviour to that of *P. operculella* is *T. bisselliella* as described by Roth and Willis (1952). Here the male walks about "rapidly and erratically, continually vibrating or fluttering his wings ... with his abdomen extended slightly dorsad". Contact with the female takes place from any direction and the male continues to vibrate his wings the entire time. After touching the female, the male stops wing vibration, flexes his abdomen sideways, and attempts to grasp her genitalia. The photographs of courting moths included in the publication by Roth and Willis (1952) also clearly show that the male's external genitalia are extended while he searches for the female, and that the male approaches the female from behind and to one side whilst he attempts copulation. In addition, he also slightly extends the wings nearest the female so that they cover her posterior region in exactly the same manner as described for *P. operculella.*

It is interesting to note that during courtship the male *P. interpunctella* moves so that the female's antennae rest on his fore-wing scent scales (Grant and Brady, 1975), whilst the flying male *T. ni* may touch the female's pheromone gland several times with first one and then the other antennae, and sometimes also with the tarsas of one prothoracic leg (Shorey *et al.*, 1962; Shorey and Gaston, 1970). However, no similar behaviour involving antennal touching was observed to occur in *P. operculella.*

It can be noted that the courtship behaviour of the male *D. saccharalis* as described by Walker (1965) differs slightly from other moths:- The male eventually positions himself below and beside the female whilst he vigorously moves his antennae in between short bursts of wing beating. The male then mounts the the female with his head facing in the same direction as the female's, and gradually moves the front of his body laterally away from the female whilst keeping his abdomen close to hers. When his body makes an angle of about 30° antennal contact is made, and the male subsequently continues his movement until his genitalia make contact. After seizing the female's abdomen, the male moves his claspers vigorously and stimulates the female to make peristaltic-like movements with her abdomen.

Finally, the male *H. cunea*, which mates during the day, has an entirely different behaviour from that of the moths mentioned above, and it apparently lacks anything which can be described as courtship. Hidaka (1972) reported that the female's sex pheromone is only effective in a limited sphere with a radius of 2 to 3m around the female. The male flies randomly over a wide area, and when he comes into contact with an effective pheromone sphere, he seeks, in a searching flight, a white "courting-postured object" (the female calls with wings held vertically over her body). When he finds this he "leaps" on it, touches it with his antennae, and copulation then takes place.

Copulation

The copulatory position adopted in all moths is with the heads facing in opposite directions and with the wings folded in the normal manner. However, Ostaff *et al.* (1974) reported that *L. fiscellaria lugubrosa* holds the wings vertically over the body during copulation. Very little is known about the behaviour of moths during copulation: Callahan (1958) noted that during the first 20 minutes of copulation pairs of *H. zea* pushed against each other every 15 seconds or so causing an up and down movement of their abdomens and they eventually separated by turning sideways to each other and pulling apart. Walker (1965) reported that copulating *D. saccharalis* usually remained stationary but the female did sometimes pull the male after her, or even flew with the male being carried with his wings folded. Ostaff *et al.* (1974) reported that pairs of *L. fiscellaria lugubrosa* remained motionless for the duration of copulation, but immediately prior to separation, the females began walking, dragging the males behind until they released their holds. After separation the males remained motionless for about 5 minutes with their copulatory apparatus still visible, whilst the females moved about and sometimes began ovipositing almost immediately.

The length of time taken for copulation in moths varies enormously both between different species, and between different individuals of the same species. For example, copulation in *T. bisselliella* varies from 12 to 18 minutes, with a mean of 15 minutes (Roth and Willis, 1952); in *T. ni* it varies between 19 and 44 minutes with a mean time of 33 minutes (Shorey, 1964); in *D. saccharalis* it takes from 0.5 to 1 hour (Walker, 1965); in *H. zea* it varies from 1 hour to 1 hour 45 minutes (Callahan, 1958); and in *C. fumiferana* it averages 4 hours (Outram, 1968), but can vary from 3 to 7 hours depending on temperature (Sanders, 1975). In addition, Shorey (1964) reported that some pairs of *T. ni* were never observed to separate successfully, and if the male died first, the female was seen flying and ovipositing with the dead male still attached.

Location of the pheromone receptors in the male and female

In the present investigation of *P. operculella* the male receptors for the female pheromone were shown to be situated on the antennae by experiments involving the removal of the antennae from males. Removal of the antennae of *A. velutinana* (Walker) (Roelofs and Feng, 1967), *O. nubilalis* (Hübner) (Klun, 1968), and *T. ni* (Hübner) (Shorey, 1964) have also resulted in the male not responding to the female sex pheromone. However, males of *Bombyx mori* L. apparently searched for females if their antennae were removed after they were excited by the female pheromone, but they copulated only if their abdomens touched a female (Sengün, 1954). Also, Sanders (1975) reported that *C. fumiferana* males rarely copulated after the complete removal of their antennae. Further support that the receptors for the female pheromone are located on the antennae of the male is provided by the many investigations using electroantennagrams (detailed by Jacobson, 1972).

In the present study, removal of the antennae from female *P. operculella* resulted in an increase in their rejection behaviour when contacted by males, but it is perhaps surprising that the females continued to copulate. A similar rejection behaviour was observed by Birch (1970a) in female *P. meticulosa* deprived of their antennae, whereas Grant and Brady (1975) found that in female *P. interpunctella* removal of the antennae resulted in rejection behaviour but 30% copulated, and in female *C. cautella* the same treatment had no observable effect on courtship. Grant and Brady (1975) suggested that the copulations which occurred with female *P. interpunctella* deprived of their antennae largely resulted from the male trapping the female against the side of the container in which they were observed (a petri dish) and then succeeding in gaining the "head under" position. They also note that the male sex pheromone is perhaps not necessary for courtship in *C. cautella* and suggested that there may be sufficient olfactory receptors for the male pheromone elsewhere on the female's body. A probable site for these receptors, according to Grant and Brady (1975) is the flask-shaped pit found in the terminal segment of each palp. Dahm *et al.* (1971) removed both the antennae and the palps from female *A. grisella* to demonstrate the volatile nature of the male's sex attractant, so presumably removal of the antennae alone was not sufficient to eliminate the female's response. In the present study, trapping of the female *P. operculella* by the male appeared to be of minor importance during the mating of females without antennae, and it therefore appears likely that there must be receptors for the male pheromone elsewhere on the female's body, as was suggested by the latter authors for *C. cautella.*

The function of the male scent organs

The function of the wing glands of the male *P. operculella* were investigated by removing the hindwings of male moths and subsequently observing their mating behaviour. These experiments made it apparent that the glands secrete a pheromone that subdues the female escape reactions and causes them to remain stationary. Dahm *et al.* (1971) have, however, shown that the pheromone produced by the wing glands of male *A. grisella* is only effective when sound is also present, but the technique of removing only the hindwings of the males in the present study makes it unlikely that the sound of the beating wings is altered very much. Furthermore, the structure and glandular cells associated with these wing hairpencils of *P. operculella* (Chapter 10) indicates that it is likely that they are involved in dispersing some chemical, and this together with their observed use only during courtship makes the only reasonable conclusion as to their function, one of producing and dispersing a male sex pheromone.

The production of a pheromone by a male moth which acts to cause the female to remain stationary has also been attributed to a number of Noctuidae (Birch, 1970a; Clearwater, 1972; and Grant, 1970), and Grant and Brady (1975) also concluded that this was the major function of the wing glands of *P. interpunctella* and *C. cautella*. In addition, however, the male pheromones of these latter two phycitinids appears to induce the female to adopt an acceptance posture (Grant and Brady, 1975) and a similar function has also been attributed to the male sex pheromone of the plutellinid *Acrolepia assectella* Zeller (Thibout, 1972).

Other possible uses of a male sex pheromone are listed by Birch (1970c, 1974): they may constitute a species specific signal which prevents cross mating between species that use the same female sex pheromone; trigger various hormonal processes in the female such as priming oogenesis; repel other males; or repel predators. The latter two functions are suggested by the fact that many of the chemicals isolated from the male abdominal scent brushes of noctuids are low molecular weight, volatile, reactive chemicals (Jacobson, 1972; Birch, 1974) and at least one of these, benzaldehyde, is also found in defensive secretions of some arthropods (Weatherston and Percy, 1970b). Birch (1970a) has recently reviewed the possible roles and the various evolutionary theories concerning male aphrodisiac pheromones in Lepidoptera.

Other possible cues involved in mating

The demonstration that *P. operculella* can copulate in total darkness suggests that vision is not essential for mating. Vision may still, however, play some part under normal circumstances as evidenced from studies on the noctuid *T. ni*. The male of this moth can also orient to and successfully copulate in complete darkness (Shorey, 1964), but Shorey and Gaston (1970) found that visual orientation may occur at low light intensities (0.2 lux). Using dried females or black paper silhouettes as models and placing these next to spots of pheromone they found that models did not influence the frequency of orientation behaviour. The models, however, influenced the direction of orientation

(86% of all copulatory attempts were directed towards a model; 14% towards the pheromone source), and all orientation and copulatory attempts were directed towards the lower apex of the model. In addition, black was less effective than the normal moth colour, smaller silhouettes were less effective than normal moth sized silhouettes, and the shape of the model had to be varied considerably from a shape similar to a female to cause a diminution in effectiveness of direction of orientation. Doane (1968) also found that males of *P. dispar* approached and attempted to copulate with female decoys upwind, but not downwind from a pheromone source, while Traynier (1968) observed that pheromone stimulated males of *A. kuhniella* attempted to mate with clay models of females, and Sanders (1975) found that although males of *C. fumiferana* would attempt to copulate with dead males or females when in the presence of female pheromone experiments with cardboard shapes were all negative.

The observation that male *P. operculella*, when stimulated by female pheromone, will pursue and attempt to copulate with other males may also suggest a possible visual role. Males of *T. ni* will similarly try to copulate with other males when in the presence of female pheromone according to Shorey (1964) who proposed that these males move the odorous air towards each other by means of their fanning wings. Many other moths have been reported to similarly attempt copulation with other males of the same species in the presence of female pheromone. These include *A. kuhniella* (Zeller) (Traynier, 1968); *C. promethea* Drury (Soule, 1902); *G. molesta* (Busck) (George, 1965); *T. bisselliella* (Hummel) (Roth and Willis, 1952); and *M. configurata* (Walker) (Chisholm *et al.*, 1975). Shorey (1970) notes several other reports of pheromone-stimulated moths attempting copulation with each other and adds that such behaviour has also been occasionally used as the basis for female pheromone bioassays.

Many other stimuli may of course be involved to some extent in mating behaviour of *P. operculella*. The sound of the beating wings of a courting male may have some effect on the female, since the presence of sound has been demonstrated to be essential before the male sex attractant of *A. grisella* is effective (Dahm *et al.*, 1971). Tactile communication may also be a means of communication during mating in *P. operculella*. Sanders (1975) for example has recently suggested that in *C. fumiferana* the antennae are involved in a tactile role in close-range detection of other insects, and also in determining the orientation of the perceived insect so that copulatory attempts by the male are aimed at the posterior of its intended mate. Further research is, however, necessary before the relative importance of the above stimuli or other possible cues are determined in the mating behaviour of *P. operculella.*

CHAPTER 9

Bioassay of the female sex pheromone of *P. operculella* and the identification of the pheromone gland.

Introduction

The only research published on the location of the pheromone gland in *P. operculella* is a very brief study by Adeesan *et al.* (1969) who reported that it is situated on the dorsal intesegmental membrane between the 8th and 9th abdominal segments. These authors used a bioassay which involved dissecting samples from females, crushing them on filter paper discs, then introducing them into plastic cages holding unmated males and observing their behaviour. The full experimental procedure and results were not available and therefore it was decided to completely reinvestigate the identification of these glands, starting by first conclusively demonstrating the existence of a sex pheromone.

The lack of experimental detail by Adeesan *et al.* (1969) makes evaluation of their work difficult. For example they do not mention what controls they used, or whether any part of the female other than the ovipositor was attractive, if even by occasional cross contamination. In addition, they give no data on the lighting conditions under which the male moths were observed during the bioassays and this is particularly important in behavioural work.

In the present investigation an objective bioassay was developed for the sex pheromone; the behaviour selected as an indication of attraction was the upwind movement of male moths towards attractive females, but this was also supplemented by observations of the male moths at the end of the bioassay for signs of their sexual display.

Methods

Preparation of Samples

Strict cleanliness was observed during the preparation of all the samples to ensure that no cross contamination occurred between them. However, since blanks were usually used in the bioassays, they were prepared alternately with the samples and given identical treatments except for the application of the moth samples to them. This resulted in the blanks also being used to check that no cross contamination occurred during preparation.

All samples except for the frozen sections and extracts discussed below were prepared for bioassaying by crushing usually two or more specimens between a folded piece of 4.25 cm Whatman number 5 filter paper which was then opened again so air circulated over the sample. Multiple specimens were used in samples to increase the chance of detecting slight attraction. The same type of filter papers were used as the blanks for these experiments.

Moths destined to be used in samples were first anaesthetised with chloroform so they could be handled with forceps. Whole moths were immediately crushed, but when they were cut into pieces, they were first frozen rapidly in liquid nitrogen, transferred to a cryostat at -20°C and cut into the required portions with a razor-blade. The parts were then placed on filter papers and allowed to thaw before being crushed. Reproductive organs were dissected out under insect saline and as each part was separated, it was placed on filter papers in the cryostat until the samples were complete. They were then allowed to thaw before being squashed. Ovipositors were obtained by placing the anaesthetised moths between strips of plasticene on microscope slides in such a position that the tips of their abdomens were at the edge of the slides, then gently pressing another slide over them until their ovipositors extended and could be cut off. Frozen sections of the ovipositors were obtained by first dropping moths with extended ovipositors, still squeezed between slides, into liquid nitrogen, recovering them and immediately transferring them into a cryostat at -20°C where they were orientated and embedded in Lipshaw mounting fluid on a rapid freezing stage and cut into sections. The microtome knife was wiped with acetone after each section had been cut and the sections were melted onto cold strips of microscope coverslip by momentarily placing a finger underneath the glass. Strips of coverslip were also used as the blanks for these experiments. All these prepared samples were stored for up to one day at -20°C inside individual glass vials until they were required.

The extract of female moths in chloroform was prepared using the same technique as described in the methods section of Chapter 8. Samples of this extract were prepared for bioassay by using a micropipette (Hamilton Company; 0-50 ul microsyringe) to drip the required amounts onto filter papers and then they were waved about in the air for one minute to let them dry. These filter papers were then immediately sealed in their sample chambers, described below, until they were bioassayed.

Bioassay apparatus

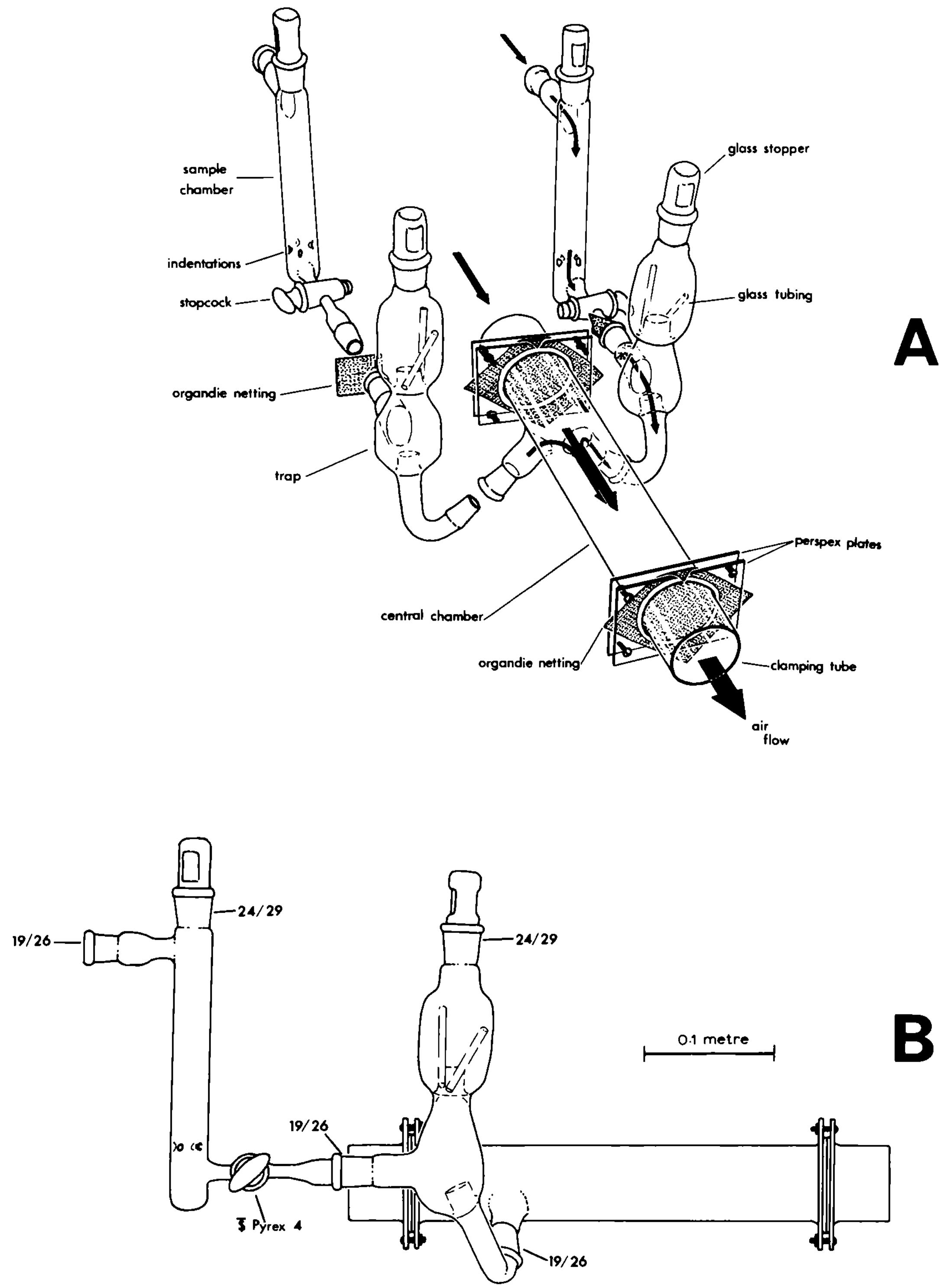
The bioassay apparatus described below was replicated 5 times and all were used simultaneously, mounted on a frame and projecting into a suction manifold. They were constructed in sections of glass 3 ± 1 mm thick, connected together with "Quickfit" ground glass joints for easy dismantling and cleaning. Their dimensions were within ± 5% of the scale drawing of Figure 9.1A unless otherwise specified in the following description.

Each bioassay apparatus consisted of a central chamber, two traps and two sample chambers (Figure 9.1 A, B). Each sample chamber was connected to one of the traps. These in turn were connected low down on opposite sides near one end of the central chamber. Organdie was fastened over each end of the central chamber and inserted between the junctions of traps and sample chambers to prevent moths from escaping from the apparatus. Suction was applied to the end of the central chamber furthest away from the trap junctions thereby causing air to flow in through the other end of the central chamber and through the sample chambers and their traps. The central chamber was also dimly illuminated from the suction end causing the test moths to congregate where air from both sample chambers blew over them.

The central chambers of each bioassay apparatus was a 30.0 ± 0.5 cm length of wide diameter glass tubing flanged at both ends and with an inlet tube projecting down 45° below the horizontal from both sides, near one end, for connecting to the traps. Two short flanged clamping tubes of the same wide diameter glass tubing were used for holding the organdie over the ends of the central chamber when fastened to it. This was accomplished by bolting together perspex plates which fitted behind the flanges of both the central chamber and clamping

Figure 9.1

Bioassay apparatus for the female sex pheromone of *P. operculella*


sample chamber
indentations
stopcock
organdie netting
trap
glass stopper
glass tubing
A
perspex plates
central chamber
organdie netting
clamping tube
air flow
24/29
19/26
24/29
0·1 metre
B
19/26
Pyrex 4
19/26

tubes. All the flanges were ground flat so the joints were almost air-tight when the organdie was in place.

The traps each consisted of two glass bulbs, one above the other, which intercommunicated through a hole with an upward projecting lip. The capacities of the upper and lower bulbs were between 95 to 120 ml and 75 to 135 ml respectively. On top of the upper bulb was a stoppered cone through which moths were removed at the end of experiments and four 6 to 8 cm long pieces of 0.5 cm internal diameter glass tubing were also placed in the upper bulb to act as additional resting sites for the moths. The lower bulb was connected on one side to the sample chamber by a horizontal tube and on the opposite side to the central chamber by a 12.5 cm long tube that extended inside the bulb as a lip and projected down 45° below the horizontal then twisted up to one side so the trap was vertical when fitted to the central chamber. The connecting tube on the trap joining the opposite side of the central chamber twisted up in the opposite direction so both traps were upwind of the central chamber inlet tubes and parallel.

Each sample chamber was a vertical tube with a stoppered cone on top through which samples were inserted, usually on filter paper. A short horizontal inlet tube that also could be stoppered, joined one side of the sample chamber near the top and a horizontal outlet tube incorporating a wide diameter stopcock joined the other side near the bottom. A ring of 4 indentations in the sample chamber wall just above the outlet tube prevented filter papers with samples from slipping down and occluding the airflow. During a bioassay, the inlet tubes were left open to allow air to be drawn through by suction from the central chamber. Originally they were intended to be connected to an air supply but this was found to be unnecessary.

Bioassay apparatus design considerations

Glass was used as the major component in constructing the bioassay apparatus because moths readily gained enough purchase even to be able to run upside down on it and it could be cleaned easily. In addition, each apparatus was also made so only glass and organdie were contacted by air being sucked through it. These features enabled the following cleaning procedure to be carried out to avoid any possibility of adsorbed odours interfering with subsequent bioassays:- The equipment was dismantled after every bioassay, the organdie was discarded and the glass sections were immersed in 4% "Decon 75" until the following day. They were then thoroughly rinsed under running tap water followed with distilled water and finally dried in an oven at 100° C. The time required for this cleaning was available because the bioassays were only started at the same time as the lights turned off each night in the room where the moths were reared.

Each bioassay apparatus was designed to prevent most moths from entering the airstreams by chance while still allowing them free admission when following an attractant. Consequently few moths were caught in the blank or non-attractive airstreams and therefore the differences between these and the attractive airstream catches were increased. This made the bioassays efficient and therefore reduced the numbers both of trials and moths required to demonstrate attraction significantly. In addition, comparisons between two or more bioassays were simplified because the blanks could be ignored.

The spatial arrangement of each bioassay apparatus resulted in low catches in non-attractive airstreams and was also responsible for the effective working of the traps. The low entry points where the traps joined the central chambers and the dim illumination from the opposite end contributed to the unlikelihood of moths entering by chance because they kept mostly to the upper surfaces nearest the light. Once moths entered the traps and adapted to the odours they usually moved into the upper bulbs where there were ample resting sites. The position of the bulbs one above the other and their relatively small entrances with upward directed lips reduced the moths' chances of finding their way back down into the central chamber again. This physical method of trapping avoided any additional odours being introduced into the airstreams and the moths could be recovered at the end of the experiment.

The physical trapping method employed in these bioassays enabled them to be run independently of an operator. Variable effects due to disturbance from an operator were therefore avoided and there was no need for the environment to be manipulated to allow observation. In addition, the bioassays could be performed in the dark during the first part of the nights when the moths usually mated (Chapter 8) as this was therefore also the time when the best responses could be expected.

Bioassay room

All bioassays were performed in a room reserved exclusively for this purpose situated on a floor without laboratories to minimise odours. A centrally located room without windows was available for all the bioassays except the first series in which the equipment was tested. This latter room had floor to ceiling windows along one side which were left uncovered to provide natural light both at night and in the day although the fluorescent ceiling lights were also used at night until the bioassays were due to start. Fluorescent ceiling lights were similarly used for daylight in the centrally located room and here dim diffused night-time illumination was produced from a lamp controlled by a variable resistance, situated under the bioassay equipment and pointed towards one wall. The bioassay equipment was placed on a table in the centre of these rooms and directed so the suction end was nearest either the windows or the wall illuminated at night. Air was sucked through it by a domestic fan inside a sealed pipe leading to a ventilator and replacement air was drawn from the room which was kept shut so that its ventilation system was isolated from the rest of the building as much as possible. The use of suction through the equipment ensured that no air could escape from it and contaminate the air in the room, while positioning the fan inside the exhaust system also ensured there were no possible odours from it.

The conditions inside these bioassay rooms closely resembled those in the darkroom where the moths were reared. The temperature was always between 19.5°C and 23°C, while the relative humidity was between 47% and 87%. The light intensity, measured in the centre of the bioassay table, vertically upwards, was 150 ± 10 lux when the fluorescent lights were on, while the lamp under the table produced 0.10 ± 0.02 lux unless otherwise stated. The natural night-time illumination was less than 0.1 lux but in this case lights such as street lamps would also have been visible to the moths through the windows.

Bioassay procedure

Live virgin moths only were used in groups of 20 to each bioassay apparatus except when specified otherwise. When their ages were not given they were between one and two days old and each moth was only used once except in the first series of bioassays when the equipment was tested.

The bioassays were timed to start at 1100 hours to coincide with the lights being turned off in the rearing room. All the moths were conditioned to the bioassay environment for at least half an hour prior to a bioassay, by being placed in the assembled equipment with the suction on but with the traps disconnected and replaced with stoppers. During the conditioning period the sample chambers were prepared in another building, then sealed with glass stoppers and carried into the bioassay room. The traps and sample chambers were fitted to the central chambers approximately 10 minutes before the bioassays were due to be started. The starting procedure was first to open the sample chamber stopcocks, then unstopper their inlets and finally leave while turning off the room lights. The room was then left unattended for half an hour or an hour while the bioassays proceeded. At the end of the bioassay periods the numbers of trapped moths were counted immediately after the lights were switched on.

The position that each bioassay and its apparatus occupied in the manifold and the allocation of samples and blanks to sample chambers were randomised. A sample known to be attractive was always used as a positive control on every night bioassays were performed. Usually these samples were crushed virgin females although portions of them were used after these were shown to contain all the attractant. The bioassay results for any night were all discarded when every one was negative, whereas they were all considered if any bioassay was positive, regardless of whether it was the control or not. Two criteria were required for a positive response:

(a) that 15% or more of the live test moths were caught in the appropriate trap;

(b) that at the end of the bioassay period, while the traps were being examined or within 5 minutes after this, at least one of the moths trapped performed sexually by running about in circles fluttering its wings as described in Chapter 8,

Results

The bioassay was given a preliminary test for half an hour with groups of 20 male moths between 6 and 7 days old and samples of 1 to 4 squashed male or female moths less than a day old. The results given in Table 9.1 showed that males were attracted to all the female samples but not to any male samples while the blanks were similarly unattractive. The male moths therefore became trapped as expected after attraction and this encouraged further work with the apparatus.

The bioassay was next tested using all combinations of sexes as live moths and samples to find if attraction occurred in any other situation apart from males to females. Groups of 20 moths were bioassayed for half an hour on 9 successive nights but the same live moths were used again each time as this increased the chances of detecting attraction. One group of males that was 7 days old to start with were given 2 squashed newly emerged females every night as a positive control. The remaining two groups of males and two of females were initially less than a day old and one group from each sex was given a sample of 2 squashed males while the other was given 2 squashed females. All these sample moths were newly emerged.

The results showed that only males older than one day were attracted to females and no clear attraction was found in any other combination (Table 9.2). In addition, no moths were trapped in any bioassay including the positive control on the 7th day so all these results could have been rejected although no cause was discovered. On the fourth day no response to the positive control was obtained even though the other group of males given a female sample still showed attraction. This latter occurrence sometimes happened in later experiments and could have resulted from the attractant becoming covered by some other portion of the moth when it was squashed.

On analysis of these catches, the numbers of young males caught responding to females were significantly different from their respective blanks, both when all nights were considered ($P < .002$) or when the 7th night was omitted ($t = 4.80$; $P < .001$) and the positive controls were similarly different from their blanks on all nights ($t = 4.26$; $P < .001$). However, no significant differences were found between the catches from both these groups of males ($P > .60$) or from males to females ($P > .20$), and females to both males and females ($P > .70$ in both cases) when all nights were taken into account.

TABLE 9.1

Responses of 20 male moths in the bioassay to different numbers of squashed male and female moths.

SAMPLE		BLANK
Number of squashed moths in each sample	Number of live moths trapped	Number of live moths trapped
1 female	9	0
2 females	9	0
4 females	7	0
1 male	0	0
4 males	1	2

TABLE 9.2

Responses of male and female moths in the bioassay to squashed moths of either sex.

In each bioassay 20 live moths were tested against 2 squashed sample moths, and the same live moths were used each day for 9 days

	Live experimental moths										
	MALE				FEMALE				MALE control		
Age of live test moths in days	Female sample	Blank	Male sample	Blank	Female sample	Blank	Male sample	Blank	Female sample	Blank	Age in days of control moths
0	0	0	0	0	1	0	0	0	12	0	7
1	6	1	0	0	0	0	0	0	13	0	8
2	14	0	0	1	0	0	1	0	6	0	9
3	8	0	0	2	0	0	0	0	0	0	10
4	10	0	1	0	1	0	0	2	11	0	11
5	4	1	0	0	0	0	0	0	11	0	12
6	0	0	0	0	0	0	0	0	1	0	13
7	2	2	0	0	0	2	0	0	6	0	14
8	15	0	0	0	0	0	1	1	13	0	15

Further evidence for the sexual nature of this male to female response was obtained by observing the spiralling and fluttering 'dance' of the males, described in Chapter 8, when the catches were being counted and the moths removed from the apparatus. This dance was frequently performed by males in female baited traps and occasionally by those that remained in the central chambers, but it was never observed from males given male samples or from females. In all subsequent bioassays at least one of the males caught in an attractive female baited trap was observed to perform this dance.

At this stage in the investigation another room located centrally in the building became available and the bioassay equipment was installed into it because the lighting could be precisely controlled. However, the light conditions under which attraction occurred were not sufficiently well known so the responses of males and females to samples of both sex were therefore investigated using half hour bioassays at light intensities of 1.0 lux, 0.1 lux (normal rearing conditions), and 0.01 lux. Again only males were caught in female baited traps whereas no attraction occurred among the other combinations under these differing conditions (Table 9.3). However, a higher than normal light intensity of 1.0 lux inhibited the responses of males to females but there was no apparent increase in response when a lower than normal light intensity was used. All subsequent bioassays were therefore performed under a light intensity of 0.1 lux.

The evidence from the experiments carried out so far indicated that only males responded to females but as final proof of this a series of half hour bioassays was performed with naive moths using both live and sample moths of different sex and age. These bioassays were run whenever sufficient moths became available and this often necessitated grouping them together because mass rearing had only recently been started. In this experiment only males aged 1 day or older were attracted to female samples and no responses were obtained from the remaining combinations (Table 9.4). However, two anomalous results were observed; in one bioassay three males were attracted to a female sample while 5 were caught in the corresponding blank trap, and later on three males were caught in a male baited trap. The latter result could have been due to chance but this was an unlikely explanation of 5 males becoming trapped in a blank. Here it was more reasonable to assume that some contamination had occurred despite the precautions

TABLE 9.3

Relationship between light intensity and the responses of male and female moths to squashed moths of either sex in the bioassay.

For each bioassey 20 live moths were tested against 2 squashed sample moths.

Light intensity LUX	Live experimental moths								Age of live male moths in days	Age of live female moths in days
	MALE				FEMALE					
	Female sample	Blank	Male sample	Female sample	Female sample	Blank	Male sample	Blank		
1.0	1 0	0 0	0	0	0	0	0	1	9-10	9-10
0.1	9 3	0 0	0	1	0	0	0	0	10	11-12
0.01	8 3	0 0	0	0	0	0	0	0	11	12-13

Table 9.4

Responses of male and female moths of different ages when bioassayed against squashed moths of both sex and of different ages.

(20 live moths bioassayed against 2 squashed sample moths)

	NUMBERS OF MALES TRAPPED				NUMBERS OF FEMALES TRAPPED				
Age of live moths (days)	Female sample	Blank	Male sample	Blank	Female sample	Blank	Male sample	Blank	Age of sample moths (days)
0	0	0							1-2
	1	0			0	0			3
0-2	3	5	0	0	0	1	1	0	0
1	11	0							1-2
	11	0							1-2
			0	1	1	0			0
			1	0	0	0			0
			3	0					0
1-8					1	1			0
2			0	0	0	0			1-2
2-9	7	0							0
3	7	0	1	1			0	0	3
							0	0	0
3-5							0	0	1-3
3-10	9	0							0
4-6	10	1					0	0	2-4
5-7	14	0							0-2
					0	0			7-10
6	9	0					1	0	1-2
6-7	9	0	0	0					0
	9	0	1	2					0
	7	0							0
7-9							0	1·2	0
7-10	12	0							0
			0	0					0-2

taken. Following this bioassay the "Decon" baths were cleaned out and replenished.

Analysis of these results showed that the catches of males attracted to females were significantly different from their blanks if the responses of newly hatched males were excluded ($t = 10.1; P<.001$) or when all results were considered ($t = 7.0; P<.001$). The catches for all other combinations showed no significant differences from their blanks with probability values of $P > 0.54$ for male to male samples, $P > 1.0$ for female to male samples, and $P > 0.45$ for female to female samples. These experiments therefore conclusively demonstrated that only male moths were attracted by the odours of females.

Once attraction had been demonstrated two additional experiments were performed to investigate how the bioassay worked.

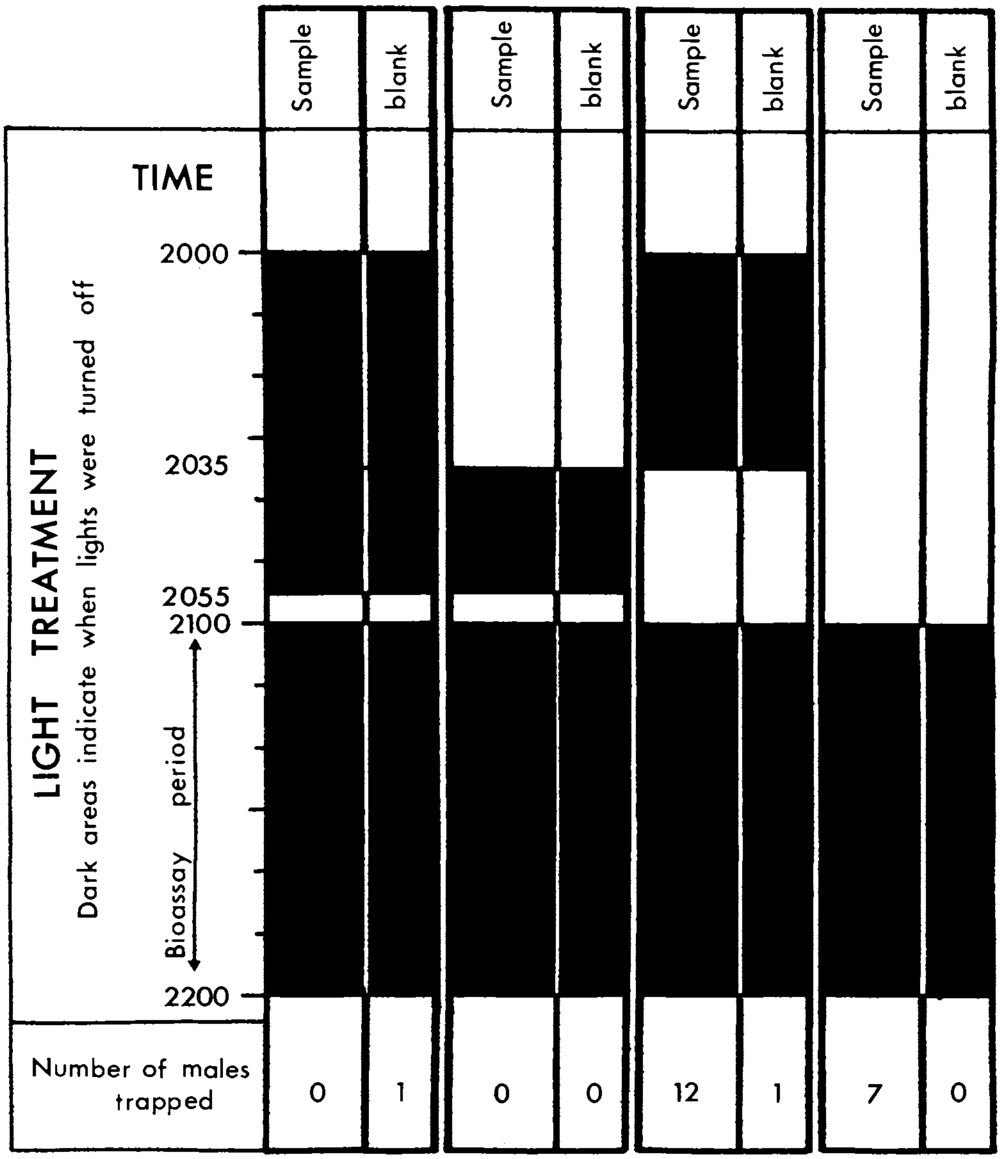
In the first of these the effects on the responses of male moths of different light treatments immediately before a bioassay were investigated to find if this was a possible explanation of why occasionally all the bioassays on a night were negative. In this experiment, none of the moths were conditioned to the complete bioassay environment before it was run but all were placed in the central chambers one hour beforehand. One group of moths was used as controls and was given constant light during this time while the other males were placed for varying periods inside lightproof bags where they were in the dark. The timing of these dark periods and the results of the bioassays are given in Table 9.5 and it can be seen that the males' responses were inhibited by darkness before a bioassay and that this inhibition could be lost if there was a sufficiently long light period between the dark one and the bioassay. This experiment demonstrated the importance of keeping the moths in constant light before running a bioassay and gives one explanation for bioassay failure if the lights were inadvertantly switched on and off without my knowledge by students or cleaning staff during the bioassay procedure. The lights were sometimes found switched off at the end of the conditioning period and in these circumstances the bioassay was cancelled.

In the second experiment the bioassay was investigated to find if it was quantitative. This was done by bioassaying different quantities of one chloroform extract of 30 females for half an hour. Different quantities of the same extract were used to ensure that precise differences were obtained in the amounts of attractant in the

TABLE 9.5

The effects of different light treatments immediately before a bioassay on the responses of male moths to females

Each group of 20 males was given 2 squashed females



	Sample	blank	Sample	blank	Sample	blank	Sample	blank
Number of males trapped	0	1	0	0	12	1	7	0

samples. This extract was reduced to 5 ml and the samples used had the equivalent amounts of attractant contained within 0.6 females, 0.12 females, and 0.06 females. The results of these bioassays given in Figure 9.2 show that there was a wide variation in the numbers of moths attracted to the same sample but after analysis significant differences were found between the 0.6 equivalent female sample and both groups of 0.06 equivalent female samples ($P < .01$ and $P < .005$). The results from no other groups of samples showed significant differences at the 5% level but this experiment did show that the numbers of moths trapped were related to the amount of attractant in the sample. However, the effective quantitative range of the bioassay was relatively small since a tenfold difference from 0.6 to 0.06 equivalent females reduced the mean catch of available males from 73% to 34.5%. Consequently it would have been increasingly difficult to detect and significantly demonstrate smaller amounts of attractant whereas much larger quantities would still give positive results that were not comparably quantitative until possibly the amount was so great that its odour inhibited or disrupted the males behaviour.

Once the bioassay had been shown to work effectively it was then used for finding the location of the attractant in the female. The bioassays were therefore used only to indicate attraction so they were all run for one hour to increase the numbers of moths that were trapped. The parts of female moths used as samples in this investigation and the sequence of their bioassay are shown in Table 9.6 together with the results. In each experiment except the last two, the remains of the moths after the samples had been removed were also tested on the same nights so complete moths were bioassayed in parts and therefore whole squashed females were frequently not used as controls. In addition, attraction was so obvious that statistical significance was not sought at each stage and instead, confirmation was obtained in the subsequent experiments.

Initially the abdomens were removed from female moths and bioassayed against what was left instead of blanks to save time. Therefore in each bioassay, an abdomen was used as a sample for one trap and the remainder was used for the other. The abdomens were found to be significantly attractive (Table 9.6) but the catches of male moths in the traps baited with the remains of the females were

TABLE 9.6

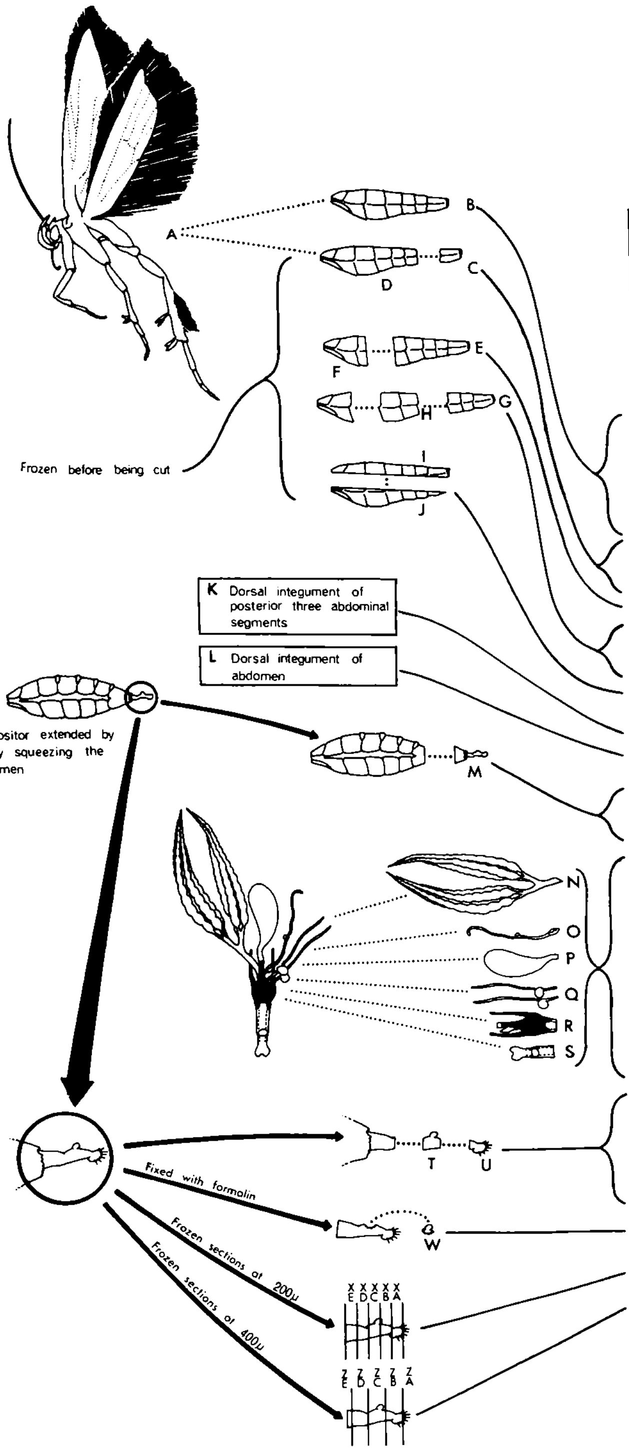
Isolation of the region containing the sex pheromone gland of *P. operculella*.

Complete moths were always bioassayed as parts so controls were not always run. When they were, each consisted of two complete squashed female moths.

The parts of the moths bioassayed are shown pictorially next to the bioassay results.

A - J: cut when frozen.
M: last part of abdomen with ovipositor extended by pressure.
N: Ovaries, lateral and median oviducts.
O: Spermatheca, spermathecal gland and associated ducts.
P: Bursa copulatrix.
Q: Accessory glands, reservoirs, and ducts.
R: Anterior region of vagina with apophyses anteriores.
S: Ovipositor and posterior region of vagina and rectum.
T.U.W: Portions of ovipositor.

XE to XA: Frozen sections of ovipositor.



Number of male moths in each bioassay	Percentage of male moths that responded																		
	Two squashed females	Blank	Key to samples	Sample	Blank	Key to samples	Sample	Blank	Key to samples	Sample	Blank	Key to samples	Sample	Blank	Key to samples	Sample	Blank	Remainder of abdomens	Blank
20			A B	0 **55**		A B	5 **75**		A B	10 **85**		A B	0 **70**		A B	5 **60**			
20	**60**	0	2× B	**25**	0	2× A	0	0											
20			2× B	**75**	10	2× B	**55**	0	2× A	5	0	2× A	10	5	2× A	0	0		
20			C	0	0	A D	**50**	0											
20			2× C	**55**	0	2× D	0	0	2× A	0	0								
19-20			2× E	**40**	0	2× E	**15**	5	2× F	0	5	2× F	0	0	2× F	0	0		
20			2× G	10	0	2× H	0	5										0	5
20			2× G	0	0													**55**	5
20			I	**40**	0	J	0	5											
19-20			2× K	0	5													**35**	0
20			2× L	5	0													5	0
20	**50**	0	3× M	**20**	0													**50**	0
20	**50**	0	3× M	**45**	0													10	0
15	**35**	0	R S	**75**	0	Q	5	0	O	0	0							10	0
20	**45**	0	R S	**80**	0	Q	0	0	O	10	0							0	0
20	**35**	0	R S	**75**	0	Q	5	0	O	0	0							10	0
15	**60**	0	R S	**60**	0	N,O P,Q	13·5	13·5										13·5	0
18	**39**	0	R S	**75·5**	0	N,O P,Q	0	0										7	0
20	0	0	2× S	**70**	0	2× R	**90**	0										**60**	0
20	**15**	**15**	S	**100**	0	R	0	0										10	0
17-20	**55**	0	S	**85**	0	R	0	0										10	0
17			U T	**35·5**	6													7	0
21			U T	**66·5**	0													0	0
17			U	0	6	T	**70**	0										0	**17·5**
21			U	8·5	0	T	**62**	0										0	10
13-15	**53·5**	0	W	**73·5**	0													**60**	0
20			XA	**75**	0	XB	**80**	0	XC	**85**	0	XD	**95**	0	XE	**85**	0		
13-16			ZA	7·5	0	ZB	**69**	0	ZC	**66·5**	0	ZD	**53·5**	0	ZE	**37·5**	0		

KEY

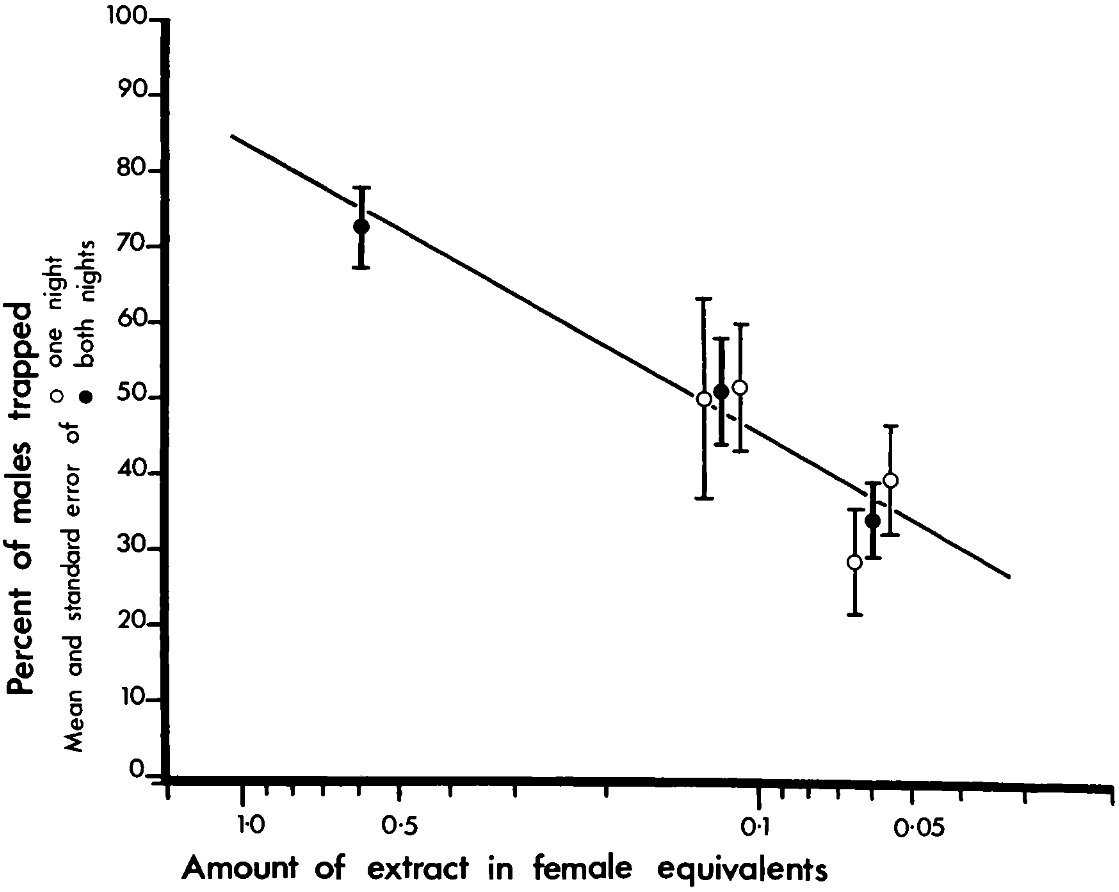
■ Greater than 15% response

······ Dissected

Figure 9.2

Relationship between the numbers of male *P. operculella* responding in the bioassay and the amount of female extract

The standard errors are indicated and the raw data is given in Appendix 2.15.


Percent of males trapped
Mean and standard error of ○ one night ● both nights
100
90
80
70
60
50
40
30
20
10
0
1·0
0·5
0·1
0·05
Amount of extract in female equivalents

no greater than could be accounted for by chance. The possibility that the rest of these females contained some attractant whose effect was masked by the relatively greater attraction of their abdomens was disproven on the next two nights when blanks were used with samples consisting either of female abdomens or the rest of their bodies.

When the abdomens were shown to contain the attractant they were then cut into various portions and bioassayed with conflicting results although the attractant appeared to be located somewhere in the posterior part. This apparant variation in position, however, suggested that it was either on one or more infolded portions of the integument or in internal organs. Attempts to remove and bioassay parts of the integument were inconclusive but when the abdomens were squeezed some dorsal intersegmental membranes including the one between the 9th and 10th segments on the ovipositor were observed to be relatively large. The ovipositors were tested first and samples were made by cutting them off with the tips of the abdomen after extending them with pressure. The two bioassays done on this also give inconclusive results so it appeared that the attractant was located in some internal organ where its position could vary. The reproductive organs were therefore next dissected into parts and these were bioassayed with the result that the attractant was found to be located in the ovipositor. However, all the samples on one night were positive probably because they became cross contaminated during their preparation, and this could also be the explanation of why both results were positive in one of the above experiments in which ovipositors and the rest of the moths were bioassayed.

Attempts were then made to locate the attractive region in the ovipositor. First extended ovipositors were removed and carefully cut with iris scissors into three parts. The bioassays of these showed that the attractant was contained within the middle region consisting of the posterior end of the 9th segment and the anterior end of the 10th abdominal segment. Its position within this region, however, could not be satisfactorily located further by dissection although after practice, the extended dorsal intersegmental membrane between the 9th and 10th segments was cut off and bioassayed, but it was felt that these results were erroneous because dissection was difficult, relatively brutal, and took so long that cross contamination almost certainly occurred. An attempt was then made to bioassay frozen sections of the extended ovipositor but most of the sections appeared to be attractive in two trials so it was again concluded that contamination had occurred during the preparation. Finally, histological methods were used to find the attractive region and these and their results are described in Chapter 10. The present experiments, however, narrowed down the location of the attractive region to a small part of the moth that was relatively simple anatomically, thereby making the histological search considerably easier.

Discussion

The presence of a sex pheromone which is produced by the female *P. operculella* to attract the male has now been conclusively demonstrated in the laboratory. This is in agreement with the conclusions drawn from field experiments and observations of the moth's sexual behaviour in the laboratory (Chapter 8).

The use of squashed moths as samples for testing in the bioassay eliminated the possibility of attraction being a response to other than olfactory stimuli. At the same time it ensured that all components of the pheromone were present. Furthermore, attraction towards a food source seemed unlikely when it was shown that only males were attracted to squashed females, whereas males of any age were not attracted to squashed males, and females of any age were not attracted to either squashed males or squashed females. However, the unequivocal demonstration that this behaviour of the males was a sexual response was made by observing their precopulatory "dances" which they performed after being attracted to the odour.

The other primary use of the bioassay was to locate the sex pheromone gland in the female, and this was accomplished with satisfactory precision. The pheromone was demonstrated to be present only in the ovipositor, and a strong indication was also obtained that it is produced in the middle third or so of the ovipositor. As far as is known to me from the literature, this portion of the moth's body containing the gland is as small as any located by bioassay. Certainly *P. operculella* is amongst the smallest moths which have been investigated in this respect, and most researchers only narrow down the location of the gland to the ovipositor and then identify it by histological methods.

Apart from the initial experiments for testing whether or not the bioassay was suitable, only two brief additional experiments were carried out to check important aspects of the moth's behaviour in relation to the bioassay. The first of these was carried out to determine the effects of different light treatments immediately before a bioassay. This demonstrated the possible detrimental effects of subjecting the moths to a dark period followed by another light period before a bioassay. This, therefore, gives one demonstration of the importance of care being taken to avoid subjecting the moths to environmental factors which they normally do not experience in their natural habitat.

The second experiment provided an indication that the moths responded to a sex attractant in the bioassay at light intensities of 0.1 and 0.01 lux, but that they did not at 1.0 lux. Since Beck (1968) reports that moonlight seldom exceeds 0.2 lux, and that starlight values are usually about 0.001 lux, this experiment tends to confirm the field observations that the males are attracted to females at the beginning of the night and not during the low light intensities at dusk. However, the use of sticky traps baited with extracts of females demonstrated that some males will respond to high concentrations of sex attractant in the field during the day (Chapter 8) so it was therefore likely that the concentrations of pheromone in the bioassay were not unduly high. There is also the chance that the pheromone was masked in some way in the particular sample given to the male moths at 1.0 lux because this experiment was not replicated.

Possibly *P. operculella* shows a behavioural response to light similar to the noctuid *Trichoplusia ni*(Hübner) which also mates at night. Shorey and Gaston (1964) tested the males responsiveness to the pheromone by bioassay at light intensities of 0.1, 0.3, and 1.0 lux and found that it was greatly inhibited above about 0.3 lux. Shorey (1966) later found that although light intensities above 0.3 lux were inhibitory some of the moths would mate at higher levels. In addition, he reported that within the range of 0.3 lux to 300 lux the proportion of moths that did mate was inversely correlated with light intensity. Further research is, however, required to determine whether or not *P. operculella* does show such a behaviour.

Most of the published laboratory bioassays for female sex pheromones of Lepidoptera have depended upon observation of male behaviour: each of the major behavioural steps leading towards copulation (described in Chapter 8) having been selected by one researcher or another as their bioassay criterion (Jacobson, 1972). For example activation from rest has been used with *Epiphyas postvittana* (Walker) and *T. ni* by Bartell and Shorey (1969a), and Shorey *et al.*, (1964) respectively; wing vibration has also been used with *T. ni* by Shorey *et al.* (1964); precopulatory "dances" have been selected for *Bombyx mori* L. by Karlson and Butenandt (1959); and copulatory movements have been used with *Porthetria dispar* (L.) by Block (1960) and with three species of *Hemerocampa* by Frech and Grisdale (1975). Upwind attraction of males to females has also been chosen as the bioassay criterion for the noctuids *Prodenia litura* (Fabr.), and *Agrotis ipsilon* (Hufnagel) (Flaschentrager and Amin, 1950), and *T. ni* (Hübner) (Toba, Kishaba, and Wolf, 1968); and for the phycitinids *Plodia interpunctella* (Hübner) and *Anagasta kuhniella* (Zeller) (Schwinck, 1953). The bioassay developed by Toba *et al.*(1968) is of interest because these authors obtained a linear response of the males over a 15-fold increase in pheromone quantity when plotted against log concentration. This is a similar effective concentration range to that obtained in the present study. Their bioassay consisted of a Y-tube and depended on the upwind migration of the male *T. ni* into one arm of the apparatus. Mayer (1973) later demonstrated that a bioassay based on upwind attraction of male moths can give a considerable improvement in the amount of information obtained when compared to other methods. He used a "tunnel olfactometer" similar to one used with mosquitoes (Mayer and James, 1964). This consisted of a long square section tube with the ends closed by fine wire mesh so as to confine the moths and to permit the suction of room air through the apparatus. In addition, it was separated into 12 equal compartments by dividers that could be raised to give the moths uninterrupted access throughout the tunnel, or quickly closed to trap them. The pheromone was dispensed in an upwind diffusion chamber and the dividers were later dropped so that the number of moths in each compartment could be counted to assess their response. Mayer (1973) used the intensively studied species *T. ni* and reported that his bioassay gave the added advantage of permitting the measurement of attraction to the pheromone over the entire range of activity from threshold to the point where the concentration of pheromone began suppressing activity. The number of moths that responded to the pheromone increased linearly over a 40 to 50-fold increase in the concentration of pheromone in the air. This corresponded to a 10^4-fold increase in the quantity of pheromone in the dispenser. In the present study a linear response was only demonstrated over a tenfold difference in the amount of pheromone extract presented to the moths, and upper and lower limits of the bioassay were not determined. In addition, the method of dispensing the pheromone was different from used by Mayer (1973) and the concentration of pheromone in the air was not determined, so the results are therefore not comparable.

During the present investigation it was evident that the responses of the male moths tended to increase as the portion of the female's body containing the pheromone gland was progressively narrowed down to a smaller and smaller region. Obviously the total amount of pheromone present could not increase in the preparations unless it was due to chance variations in the amounts present in different individual moths. It therefore follows that the pheromone was being partially masked in some way in large samples, and that the masking agent was progressively removed as the sample containing the pheromone became smaller. A likely explanation is that the pheromone is soluble in fat so that in crushed specimens containing large amounts of fat much of the pheromone dissolved and as a result became ineffective. The abdomen contains the largest stores of fat in the moth, principally in the ova and fat body, and its removal should therefore increase the amount of available pheromone in the samples, if in fact this is the correct explanation. It does, however, seem likely because Rothschild (1971-1972) reported that the pheromone of *P. operculella* is an acetate, and furthermore, it is probable that this pheromone is an acetate of a straight chain mono- or di- unsaturated alcohol of 14 to 16 carbon atoms because this is the structure of most pheromones that have been identified for female Lepidoptera (Jacobson, 1972).

Another possibility is that the masking of pheromone is due entirely to its being covered by some other part of the crushed sample and that this either blocks or slows down the rate of evaporation of pheromone into the atmosphere. Both the effects of covering a pheromone and affording it a medium in which it dissolves may act simultaneously in masking.

Generally squashed female moths or crude extracts of them are attractive to males of their own species (Jacobson, 1972) although a number of cases of masking or inhibition of the males response to the female pheromone have been reported. An extreme example, which occurs when complete moths are used as samples, was described by Kellogg (1907). He found that excised glands of *B. mori* attracted males, whereas complete mutilated females were not attractive. In some cases masking or inhibition have been traced to contaminants or impurities in the sample being tested. This has been reported for *P. dispar* (L.) (Waters, and Jacobson 1965); *Samia cyathia* (Drury) (Jacobson and Smalls, 1967); and *Heliothis zea* (Boddie), and *Heliothis virescens* (Fabr.) (Berger, McGough, and Martin, 1965). However, Shorey and Gaston (1967) reinvestigated the presence of masking compounds in crude extracts of *H. zea* and *H. virescens* using quantitative bioassays for their pheromones, and could find no evidence of their existence. There is also an indication that an additional compound may be present in the sex pheromone of *P. interpunctella* which inhibits male *Cadra cautella* (Walker) from responding to their own females, because Ganyard (1970) found that this inhibition occurred in the presence of calling *P. interpunctella* and both phycitnids use the same pheromone *cis-9, trans-12*-tetradecadien-1-ol acetate (Kuwahara, Kitamura, Takahashi, Hara, Ishii, and Fukami, 1971; brady, Tumlinson, Brownlee, and Silverstein, 1971; Dahm, Richter, Meyer, and Roller, 1971).

Masking agents or inhibitors of responses to pheromones can either be chemicals which are entirely different from the sex pheromone or chemicals that have a closely related structure. These latter compounds can sometimes be synergists, but the fact that many are potent inhibitors is interesting in that they give support to the stereochemical theory of olfaction as discussed by Amoore (1964, 1970). Presumably they have the required molecular configuration to react with the binding sites on the male antennal receptors. They do this with varying degrees of affinity, thus competing with the attractant or modulating the sensory input it produces. One example of such an inhibition is given by Jacobson (1969) who found that as little as 15% of the cis-isomer of "propylure" (10-propyl-*trans*-5, 9-tridecadien-

1-ol acetate) will completely nullify the activity of the trans-isomer of male *Pectinophora gossypiella* Saunders. Other examples include inhibitors for sex pheromones of *Grapholitha molesta* (Busck) (Roelofs, Comeau, and Selle, 1969), *Argyrotaenia velutinana* (Walker) (Roelofs and Comeau, 1971), *Choristoneura rosaceana* (Harris) (Roelofs and Tette, 1970), and *P. interpunctella* (Hubner) (Brady, 1969); and to "hexalure", a synthetic attractant of male *P. gossypiella* Saunders (Beroza, Staten and Bien, 1971).

Samples for bioassay which consist of squashed females or crude extracts of them are also likely to contain chemicals, such as food attractants or repellants, that affect males in other ways. It is therefore, perhaps, surprising that such samples elicit only sexual responses in so many cases.

Chapter 10

The male and female sex pheromone glands of *P. operculella*

Introduction

Adeesan *et al.*(1969), in a brief report, noted the existence of a female sex pheromone in *P.operculella* which attracts and excites the male, and they stated "that the tissue source of the pheromone is a gland which is in the form of a dorsal invagination of the intersegmental membrane between the 8th and 9th abdominal segments ... In the glandular region the epithelial cells of the intersegmental membrane are modified into closely packed cuboidal cells with large spherical nuclei. The cuticular covering of the intersegmental membrane extends over the glandular cells, but in this region the cuticle is uneven and varies in thickness ..." Adeesan *et al.* (1969) made no mention of a possible male pheromone although structures which may liberate such substances have been noticed in *P. operculella* by Povolný (1964). This latter author considered that the hairpencil organ on the hindwing of the male was an "osmeterisches Organ" or an organ concerned with dispensing odours, but he did not describe its structure. Povolný (1964) also noted the large macroscopically visible scales or coremata at the end of the male's abdomen but did not mention a probable function for them, although such structures are generally presumed to function in scent distribution (Tuxen, 1970).

Earlier in the present study, the female *P. operculella* was demonstrated to produce a sex pheromone which attracts the male, and the gland which produces it was shown by bioassay to be situated within the middle third or so of the ovipositor (Chapter 9). In addition, observations of the male's behaviour showed that during courtship both the coremata and the wing hairpencel organs were splayed out. Furthermore, removal of the hindwings, on which the hairpencils are located, was observed to cause a considerable reduction in the efficiency with which males initiated copulation, suggesting that these organs dispensed a pheromone which subdued or suppressed the escape reactions of the female and thereby facilitated copulation (Chapter 8). This led to the histological search for pheromone glands in both the male and female *P. operculella* which is the subject of the present chapter. Also included here is a description of the external morphology, histology, and ultrastructure of these glands.

Methods

Normal histological methods were used as described in Chapter 3.

The male pheromone glands were prepared for examination with the scanning electron microscope by affixing the wings to aluminium studs with a conducting adhesive, freeze drying them at -35°C to minimise bending and distortion of the thin cuticular structures during drying, and then coating them with a thin layer of a platinum gold alloy under high vacuum.

The female pheromone gland was prepared for examination with the scanning electron microscope using the same method as that described in Chapter 7.

The male and female sex pheromone glands were embedded and thin sectioned for examination with the transmission electron microscope by Mr N.G. Leet of Meat Research Institute, Hamilton. The female gland was first dissected away from most of the ovipositor except for the dorsal surface, and it was then processed using the normal method as described in Chapter 3. This method, however, did not prove satisfactory with the wing glands of the male because the hydrophobic cuticle reduced penetration. A number of treatments were tried to overcome this:- unsatisfactory results were obtained by cutting out a very small section of the wing containing the gland and holding it under each of the solutions. Attempts to remove the lower surface of the wing damaged the pheromone gland. Washing the moths in toluene, followed by teepol,* and then water resulted in damage to the ultrastructure. Eventually good results were obtained by the following method. A very small area of wing containing the pheromone gland was cut from the wing. This was then placed for 48 hours in 4% glutaraldehyde in CaCodylate buffer (pH 7.3) to which a trace of teepol had been added, sufficient to just wet the cuticle. Further processing was carried out by Mr Leet and this consisted of a modification of the normal procedure which is detailed in Appendix 3.2 . Briefly, the major changes consisted of adding an 0.05% aqueous solution of "Decon 75" dropwise to each liquid which did not wet the cuticle until the wing fragment submerged, of increasing the time the specimen was kept in each of the solutions to assure good penetration, and of using Spurr's low viscocity epoxy embedding medium (Spurr, 1969). Once embedded the region of the wing fragment which contained the gland cells was located by cutting sections 1 to 2µ thick on the ultramicrotome and preparing these for examination with the light microscope.

Identification of the pheromone glands

Female

In order to identify the pheromone gland of the female complete transverse and longitudinal serial sections were made of the tip of the abdomen with ovipositor extended. The anatomical structure of the ovipositor was found to be relatively simple, and consisted of an outer integument which enclosed a pair of laterally situated apophyses posteriores, a mass of muscle fibres, a dorsal rectum, and a ventral vagina (Figure 10.5). The rectum and vagina are both histologically uniform along the length of the ovipositor and since there was a strong indication that the pheromone source was situated only in the middle third of the ovipositor (Chapter 9) then it follows that it is unlikely these organs play any part in pheromone production. However, an examination of the integument revealed that the epithelium lining the dorsal sac-like invagination of the intersegmental membrane between the 9th and 10th abdominal segments on the ovipositor was much thicker than the hypodermis elsewhere in the ovipositor and abdomen (Figure 10.5). The hypodermal cells are generally flat when not actively involved in the processes associated with moulting unless they are concerned with some secretory activity (Wigglesworth, 1974). It appeared, therefore, that the dorsal invagination of the ovipositor was the pheromone gland as reported by Adeesan *et al.* (1969). It must be noted that a different terminology from that of Adeesan *et al.* (1969) is used here for the segments of the ovipositor, but as explained in Chapter 7 the musculature of the ovipositor requires investigation before the correct relationships can be determined between the segments of the ovipositor and the abdominal segments.

In addition to the histological search, the surface of the entire ovipositor was carefully examined with the scanning electron microscope but no obviously porous areas of cuticle were found. The surface structure is described in the following section on the female pheromone gland.

Male

The search for possible male pheromone glands was more difficult than the search for the female gland because, apart from the experiment involving removal of the hindwings, there was no evidence other than from morphology and histology of their possible whereabouts.

Complete transverse and longitudinal sections were made of the hindwings and these demonstrated that the cells at the bases of the hairpencil scales were different from the cells at the bases of normal scales, being bigger and having larger nuclei (Figure 10.21). The histology of these glands is described in detail in the section below on the male pheromone glands. In addition, the cuticle of the hairpencil scales was eosinophilic (Figure 10.21) and this was not observed elsewhere in the insect suggesting that this cuticle was in some way different from the rest. The fact that these hairpencil organs are displayed and opened out only during courtship, together with the observation that removal of the hindwings results in disturbance of the normal mating behaviour leads to the conclusion that these organs are male pheromone glands. Their glandular structure was also later confirmed by an examination with the transmission electron microscope (described below in the section on the male pheromone organs).

A morphological search of the forewings, abdomen and thorax of *P. operculella* indicated that the only other probable sites for male pheromone glands were the coremata. Complete transverse serial sections were made of the forewings, thorax and abdomen; and the abdomen was also sectioned longitudinally, but all these preparations failed to show any region of the integument, including that near the coremata, which was likely to be glandular. It was therefore concluded that the hindwing hairpencil organs are the only male pheromone organs of *P. operculella*.

The pheromone gland of the female

The pheromone gland of the female *P. operculella* is a sac-like enlargement of the dorsal intersegmental membrane between the 9th and 10th abdominal segments on the ovipositor (Figure 10.1). Normally, when not in use, the pheromone gland is withdrawn into the ovipositor where it forms a deep flattened invagination close to the dorsal integument of the 9th segment. When the female is calling and releasing her pheromone as described in Chapter 8, the ovipositor is fully extended at intervals, its tip is bent ventrally, and the pheromone gland is everted as a white bulbous structure. The gland can also be everted by applying pressure to the female's abdomen, and then it forms an approximately hemispherical dilation of the intersegmental membrane with a slight apical depression running transversely across it (Figure 10.1 B). The gland rolls inward as the pressure is released by forming a slit in the depression which progressively deepens until the gland entirely disappears from sight.

When examined with the scanning electron microscope the surface of the pheromone gland has a different appearance from the rest of the ovipositor. The glandular surface is irregularly thrown into small ridges or rounded hemispherical protruberances both of which have smooth crests (Figure 10.2). The depressions between them are mostly relatively smooth, but branching crevice-like infoldings also occur and these are particularly frequent in the region of the transverse apical slit in the gland (Figure 10.2 B). However, these crevice-like infoldings do not penetrate very deeply into the cuticle and no obvious pores are visible anywhere on the surface of the gland.

The surface elsewhere on the ovipositor is mostly folded into slightly wavy longitudinal grooves and ridges. Occasionally the ridges branch and anastomise but mostly they are approximately parallel and lie about 0.5 to 3µ apart (Figure 10.3 A). Generally, the sides of the grooves meet at a sharp angle, and where the ridges come close together the grooves can have the appearance of shallow pores (Figure 10.3 A). These ridges and grooves impart a striated appearance to the cuticle of the ovipositor at low magnification.

In the intersegmental membranes on the ovipositor the ridges and grooves become somewhat disrupted and irregular, whilst ventrally at the base of the ovipositor there are short spines and the surface

<u>Figure 10.1</u>

Scanning electron micrographs of the
ovipositor of *P. operculella*

The sterna are indicated by roman numerals and the terga by arabic numerals.

A. Lateral view of the entire ovipositor. Note that the genital chamber has also become everted.

B. A dorso-lateral view showing the everted pheromone gland. The material on the pheromone gland is probably some of its secretion.

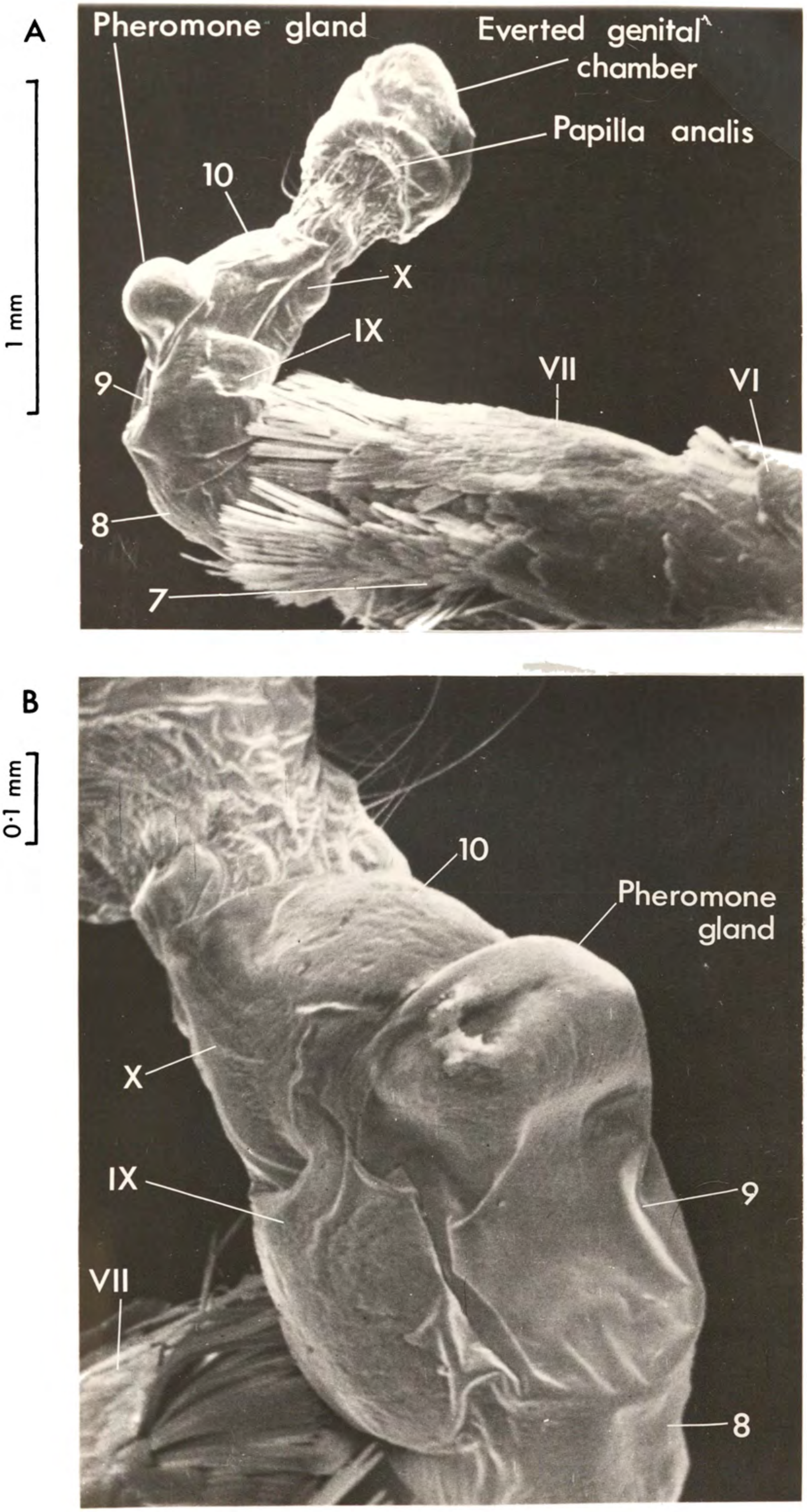

A
Pheromone gland
Everted genital chamber
Papilla analis
10
X
IX
1 mm
9
VII
VI
8
7
B
0·1 mm
10
Pheromone gland
X
IX
9
VII
8

Figure 10.2

Scanning electron mircrographs of the surface structure of the pheromone gland of the female *P. operculella*

A. Surface of the anterior side of the pheromone gland

B. Surface of the dorsal part of the pheromone gland in the region of the transverse groove (see figure 10.1 B).

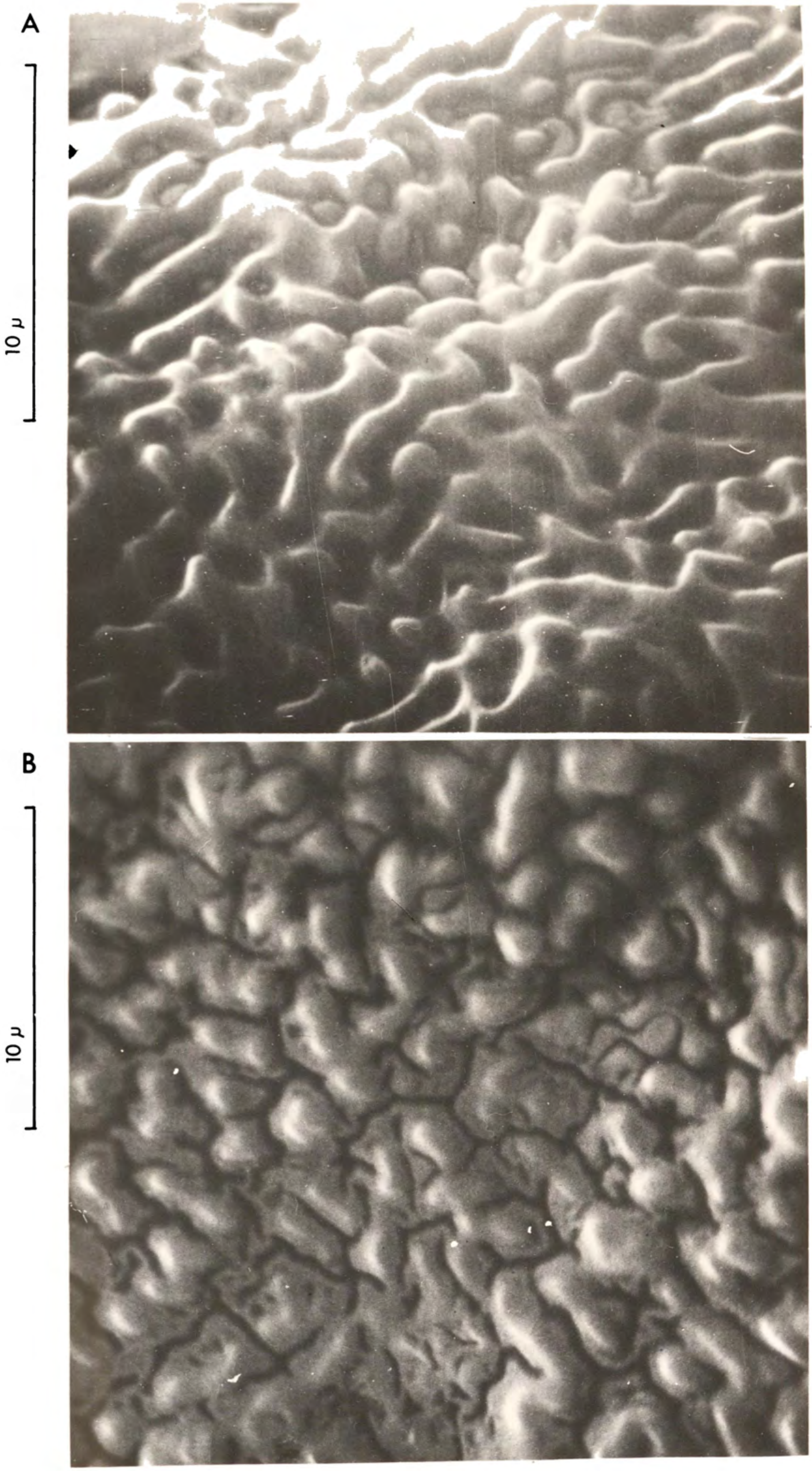
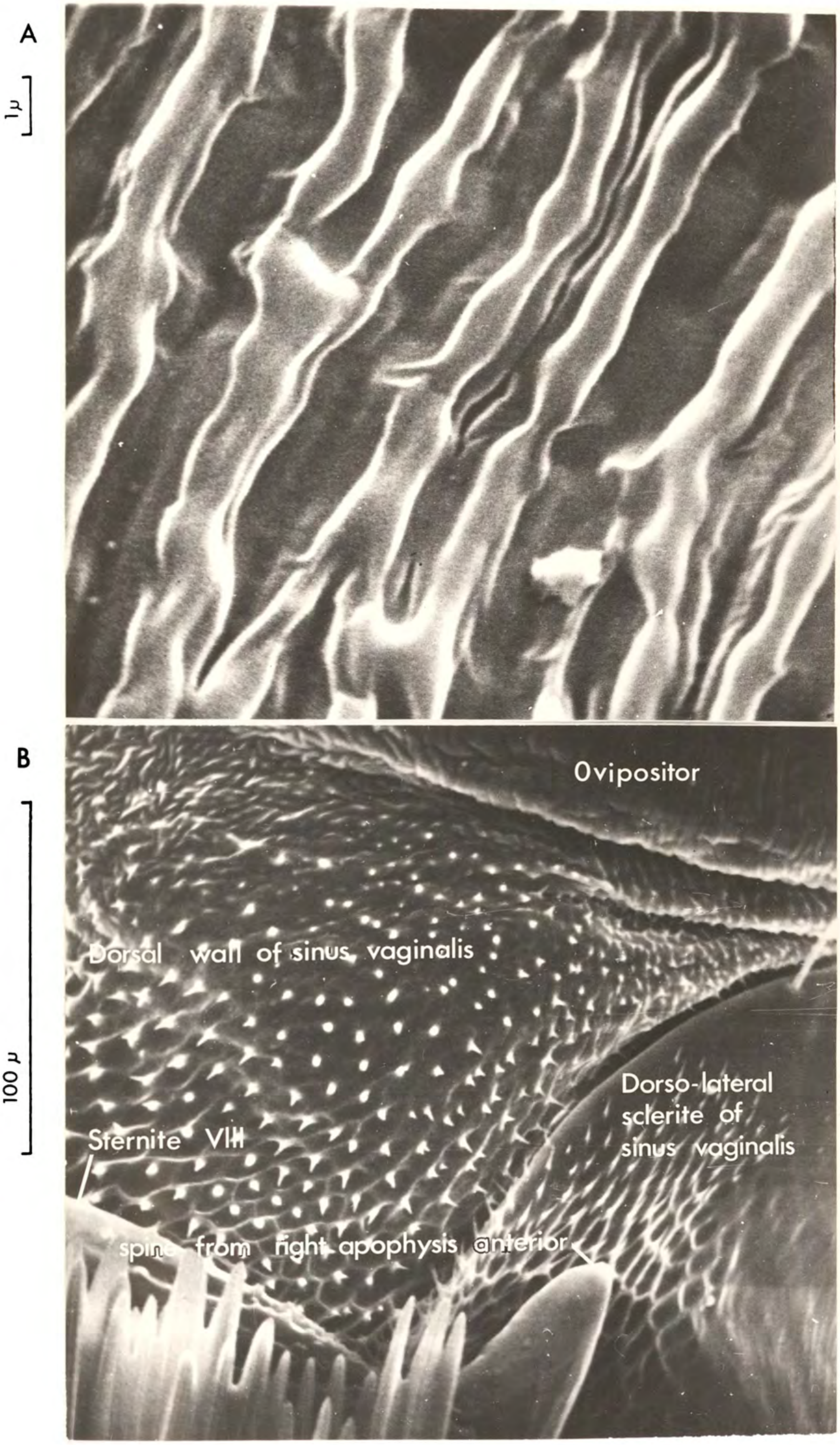

A
10 μ
B
10 μ

Figure 10.3

Scanning electron micrographs of the surface structure of the ovipositor of *P. operculella*

A. Ventro-lateral portion of the 10th sternum

B. Ventral view of the base of the ovipositor in the region of the opening to the sinus vaginalis.


A
1μ
B
100 μ
Ovipositor
Dorsal wall of sinus vaginalis
Dorso-lateral sclerite of sinus vaginalis
Sternite VIII
spine from right apophysis anterior

gradually transforms into that of the sinus vaginalis (Figure 10.3 B).

The surface of the papilla anales is exactly like that of the intersegmental membranes elsewhere on the abdomen excluding the ovipositor. It consists of large regularly spaced blister-like protruberances with smaller rounded protruberances between them (Figure 10.4 A, B). Elongate bristles also occur over the surface of the papilla anales which are probably tactile sensillae.

All the above regional differences in the surface structure of the ovipositor cuticle can also be readily observed in sections of the integument.

Histologically, the pheromone gland consists of a single layer of modified hypodermal cells which secrete a non-staining, colourless cuticle about 2 to 3μ thick (Figure 10.5). A number of fine muscle fibres also join the gland in the region of the transverse apical groove which presumably function as retractor muscles. They run anteriorly and dorsally in the ovipositor and appear to insert on the intersegmental membrane between the 8th and 9th abdominal segments. However, it is difficult to trace individual muscle fibres in serial sections because of the large number in the ovipositor, so this interpretation may not be correct. The glandular cells are cuboidal and usually 5 to 6μ tall, but can vary from 3 to 8μ. Their intercellular membranes are indistinct, their basal surfaces are somewhat rounded, and the basement membrane cannot be resolved from the cell wall by means of the light microscope. The cytoplasm is densely eosinophilic and somewhat fibrous, but occasionally clear apical vacuoles with indistinct walls and diameters of up to about 3μ are visible. The nuclei are central, rounded to oval and large, having maximum diameters of about 5 to 7μ. In addition, their chromatin is granular and evenly dispersed.

The histology of the rest of the integument of the ovipositor differs markedly from that of the pheromone gland. The cuticle is for the most part only 1.0 to 1.5μ thick, and the hypodermal cells are flattened and only about 1 to 2μ tall. Their nuclei are also flattened discs with maximum diameters of about 5 to 10μ and their chromatin stains densely. In addition, the cytoplasm which is densely eosinophilic does not contain visible vacuoles.

Figure 10.4

Scanning electron micrographs of the surface structure of the ovipositor of *P. operculella*

A. Dorsal view of the region between the 9th and 10th abdominal segments.

B. The same region as in 10.4A at higher magnification.


A
100 µ
Base of papilla analis
Vulva
Segment 10

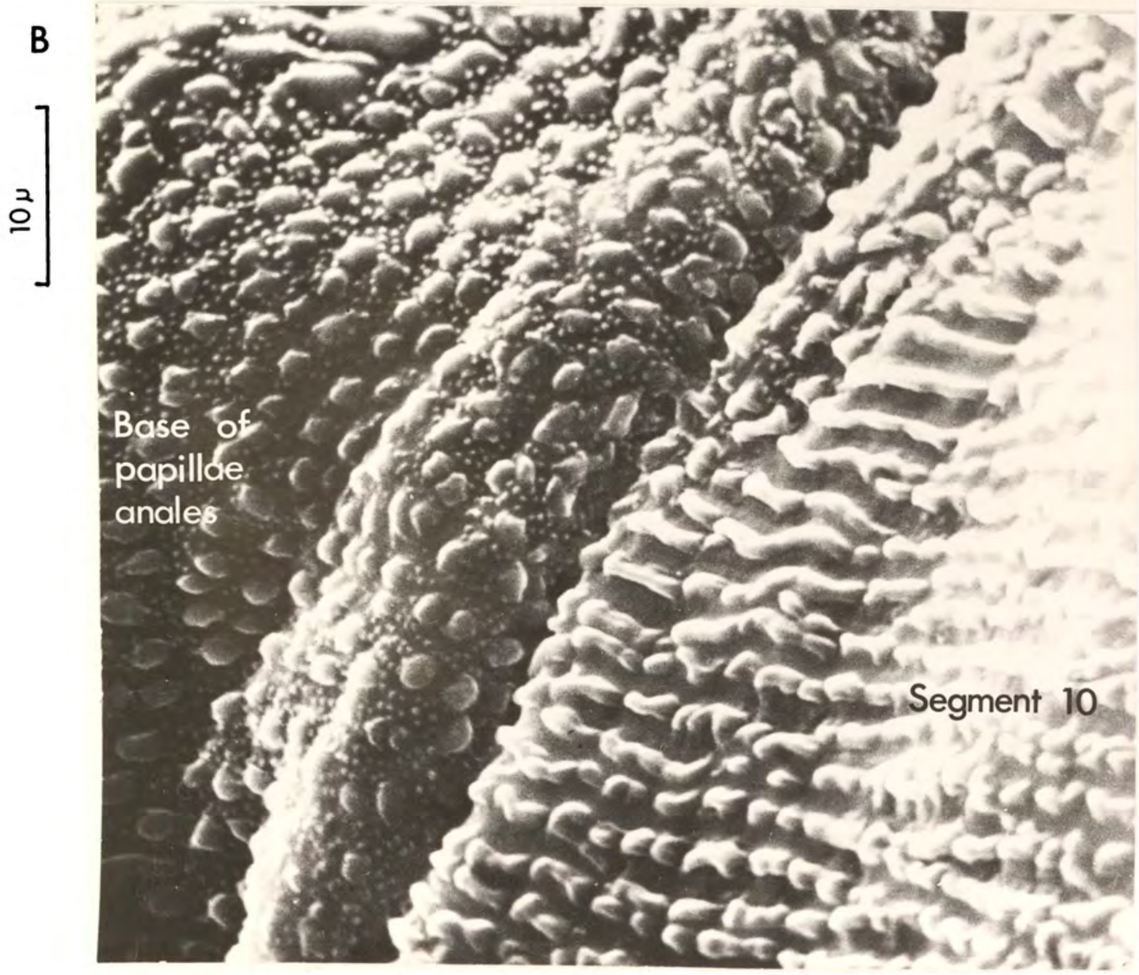

B
10 µ
Base of papillae anales
Segment 10

Figure 10.5

Histology of the ovipositor of *P. operculella*

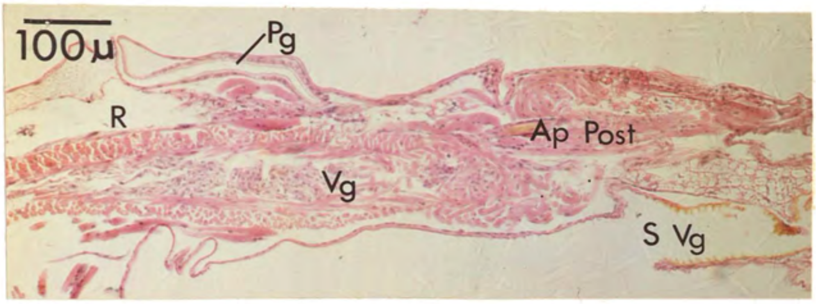
All sections stained with haematoxylin and eosin.

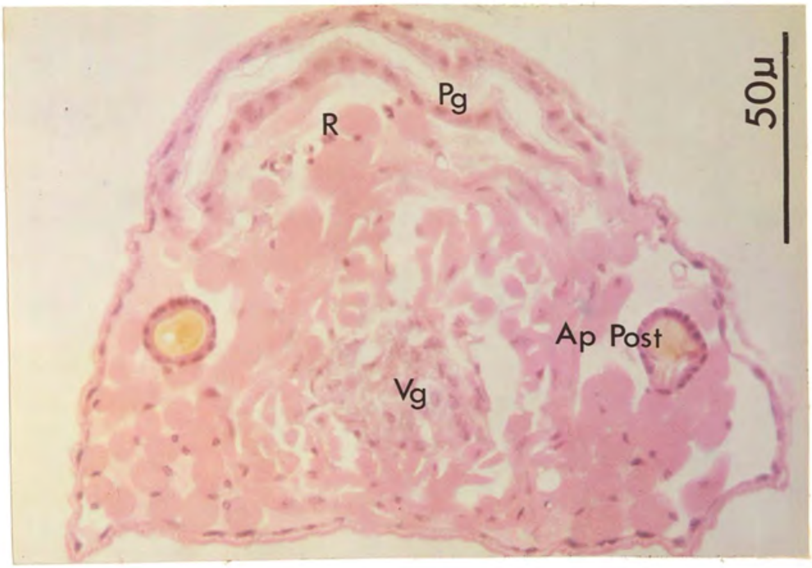
Upper: longitudinal section through the anterior portion of the ovipositor showing the withdrawn pheromone gland

Middle: transverse section through the ovipositor in the region of the pheromone gland

Lower: longitudinal section of the end of the pheromone gland at high magnification

Ap Post	apophyses posteriores
Cut	cuticle of pheromone gland
Int	outer integument
Pg	pheromone gland
R	rectum
S Vg	sinus vaginalis
Vg	vagina


100µ
Pg
R
Ap Post
Vg
S Vg


50µ
Pg
R
Ap Post
Vg


Int
Pg
Cut
m
Pg
10µ

Under the transmission electron microscope the cuticle of the pheromone gland has a complex structure consisting basically of 4 layers (Figures 10.6, 10.7). There is an outer thin electron dense layer of epicuticle followed by a thicker homogeneous layer of medium electron density which is probably cuticulin. The third layer forms the bulk of the cuticle and consists of numerous large cavities within a fibrous or irregularly granular endocuticle. In addition, finger-like extensions of cuticulin penetrate between many of the outer cavities. The cavities themselves vary a lot in size but have irregular and somewhat circular shapes. Their walls are composed of a thin layer of material which is more electron dense than the endocuticle and it can occasionally be seen to have a layered structure (Figure 10.6). A homogeneous material of light electron density occurs within the cavities which is possibly fat. Often it is partly missing from the cavities presumably from shrinkage or from loss during embedding. The inner layer of cuticle has the structure of normal endocuticle, and occasionally pore canals can be seen within it. However, these canals could not be identified in the outer three layers of the cuticle.

The gland cells, when examined with the transmission electron microscope (Figure 10.6) can be seen to rest on a basement membrane about 0.13µ thick which sometimes penetrates between the cells for up to about a quarter of the distance to the cuticle. The basal cell walls of the gland cells are more or less smooth but occasionally are raised into small folds. In contrast, the intercellular membranes appear pleated and they are also more or less straight basally, but become increasingly folded apically (Figure 10.6). Long septate desmosomes occur apically whilst tight junctions and desmosomes of the *macula adherens* type are frequent elsewhere.

The nuclei of the gland cells have the usual nuclear structure except that their envelopes are folded into low ridges, and this presumably increases their surface area (Figure 10.11).

Apically, the gland cells are bounded by a zone of microvilli which are packed together and often run horizontally to the cuticle for part of their lengths (Figures 10.6, 10.7). Their diameters vary from 0.1 to 0.2µ, and some microvilli have been followed for 1.3µ in thin sections so that they are probably somewhat longer than this. Many also have central tubules which may represent extensions of the endoplasmic reticulum.

Figure 10.6

Transmission electron micrograph of the pheromone gland of the female *P. operculella* at low magnification

The areas indicated by black boundaries are shown at higher magnification in the following three figures. Note, in particular, the basement membrane which often extends for a short distance between adjacent pheromone gland cells; the intercellular membranes which are approximately straight basally but which become complexly folded apically (one is indicated by the black dots); the apical microvilli; and the cuticle composed of 4 layers. The diagonal lines are sectioning artifacts, and the artifactual space between epicuticle and embedding resin (indicated by large black arrow) is produced by the resin not wetting the epicuticle.

Inset: A portion of the outer edge of the cuticle at high magnification. Note the thick cuticulin layer, the outer epicuticle which appears to have a very fine outer layer, and the lining of the cuticular cavity which appears to consist of a number of layers in the region near the small black arrow.

BM	basement membrane
Cul	cuticulin
Cut	cuticle
Cv	cavity in cuticle
epi	outer epicuticle layers
Mv	microvilli

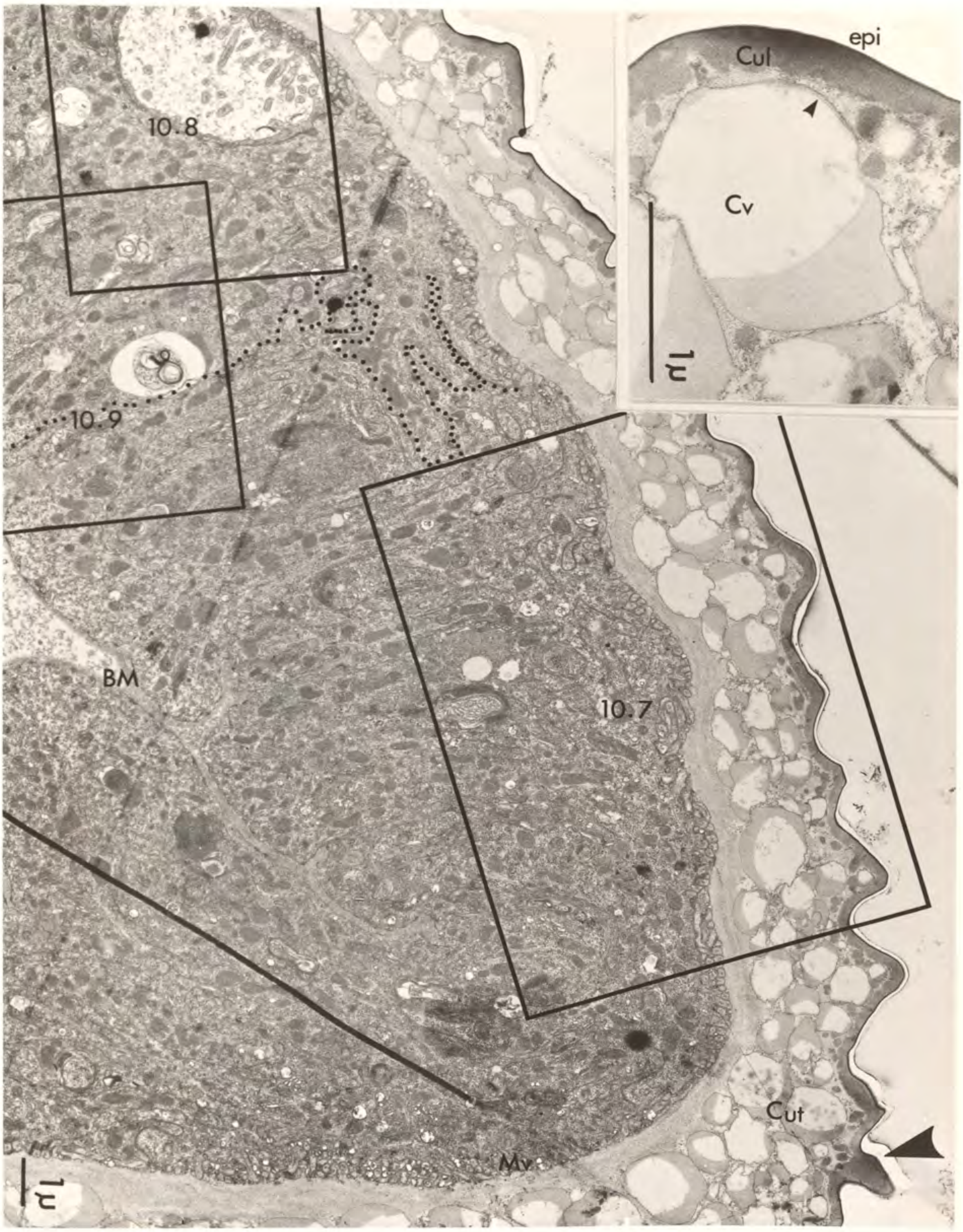

10.8
10.9
10.7
BM
Mv
Cut
1μ
epi
Cul
Cv
1μ

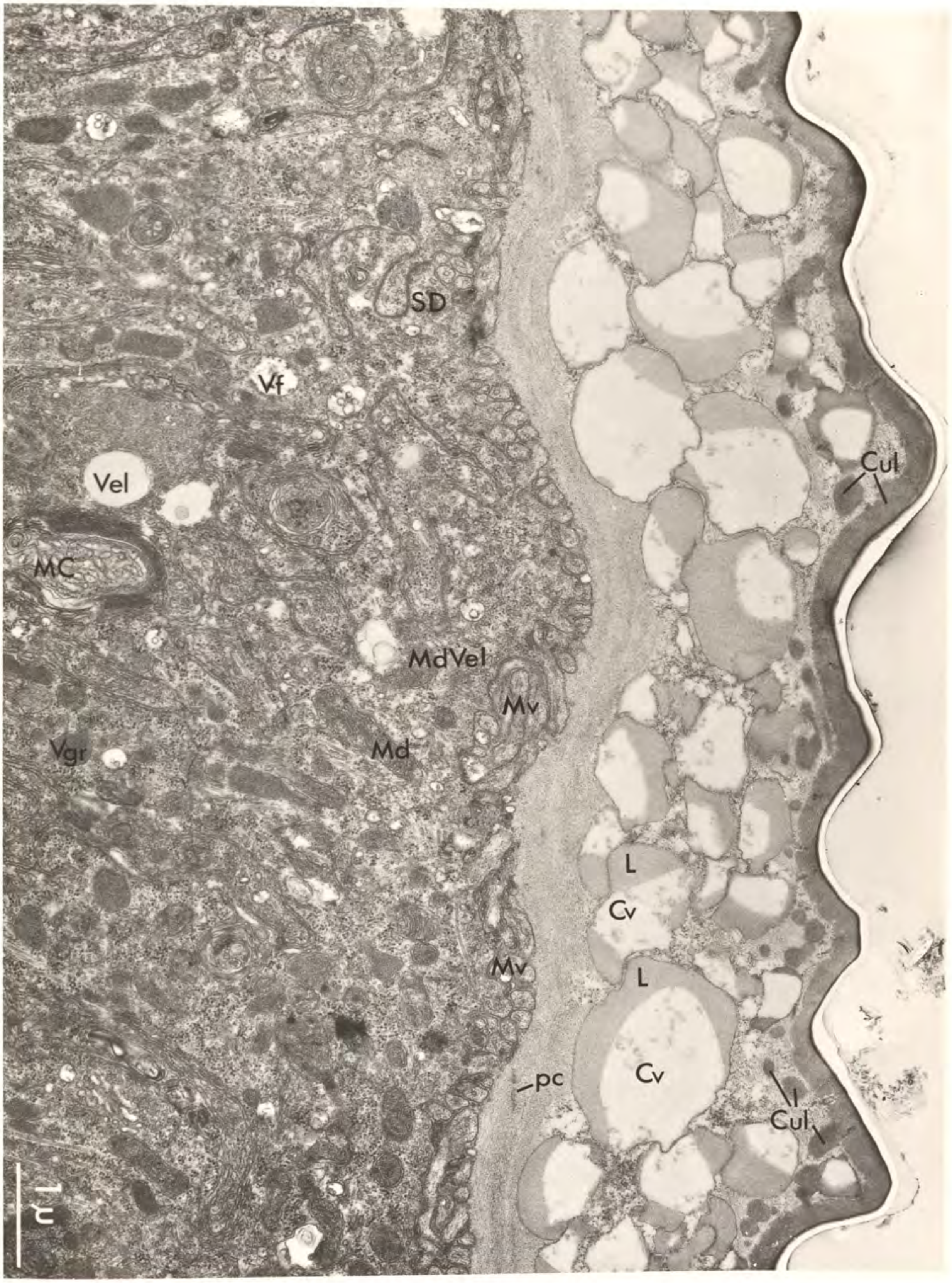
Figure 10.7

Transmission electron micrograph of the apical region of the pheromone gland cells of the female *P. operculella*

The area of this micrograph is indicated in the low magnification micrograph of Figure 10.6.

The 4 regions of the cuticle are clearly visible. There is an outer dense epicuticular layer followed by a thick layer of cuticulin, a region of endocuticle with numerous cavities partly filled with a lipid-like material, and an inner layer of dense endocuticle. The cuticulin can also be seen to penetrate between the outer cavities in the endocuticle as finger-like rods, and pore canals are occasionally visible in the inner layer of endocuticle. Lying next to the cuticle are the apical microvilli, and one septate desmosome appears as a dark intercellular junction at this magnification. The cytoplasm is packed with ribosomes, smooth endoplasmic reticulum, and microtubule-like structures (see following micrographs). A number of vacuoles are also visible which contain a variety of materials. Mitochondria are numerous, and two can be seen which are associated with small vacuoles containing complexes of membranes. In addition, to the left of the micrograph is a large membrane complex which is partly surrounded by laminae of membranes in the shape of a"J"bearing some resemblance to Golgi.

Cul	cuticulin
Cv	cavity
L	lipid-like material
MC	membrane complex
Md	mitochondrion
MdVel	mitochondrion with membrane complex
Mv	microvilli
Pc	pore canal
SD	septate desmosome
Vel	electron lucid vacuole
Vf	vacuole containing a floccular material
Vgr	vacuole containing a granular material


SD
Vf
Vel
Cul
MC
MdVel
Mv
Vgr
Md
L
Cv
Mv
L
pc
Cv
l
Cul
1 μ

The apical vacuoles observed with the light microscope can be seen with the electron microscope to be large vesicles with microvilli projecting into them (Figures 10.6, 10.8). These microvilli arise most frequently from the surface of the vesicles closest to the cuticle, and their diameters are similar to those of the apical microvilli close by them. The vesicle microvilli also have a central tubular structure within them. A clumped granular material occurs scattered sparely within the lumen of the vesicles but no opening was observed through which this material could escape from the vesicles. Possibly openings exist which are narrow and were therefore missed even though about 40 sections were examined from about the same region of gland.

The cytoplasm of the gland cells contains many small particles with diameters of about 200 to 230Å. These are probably ribosomes since glycogen should have been removed by the uranyl acetate treatment. Numerous long straight tubules are also present which resemble microtubules except that their diameters are larger and vary from about 270 to 330Å (Figures 10.7 to 10.11 inclusive). Elongate mitochondria with transversely arranged cristae occur commonly whilst other mitochondria are also found which have less regularly organised cristae and which are situated very close to electron lucid vacuoles (Figures 10.7, 10.9). In some cases these vacuoles can be seen to occupy a distention of the outer membrane of the mitochondria (Figure 10.9). A variety of vacuoles are also scattered amongst the other cytoplasmic organelles. Many of these, about the size of mitochondria, contain a granular material similar to that in the mitochondria, and in some cases mitochondria with much of this material but few cristae are also visible. This suggests that these vacuoles may be derived from mitochondria. Electron lucid vacuoles are also common, but the smaller vacuoles close to the microvilli contain a scattered floccular material. The endoplasmic reticulum is well developed and consists almost entirely of smooth membranes. Golgi is also frequently observed and in addition there are also large myelinated figures or onion bodies consisting of concentrically arranged membranes. In some cases these surround a central region of granular material similar to that within the mitochondria, whilst in others they appear to be associated with large electron lucid spaces with complexes of membranes within them (Figures 10.8, 10.9, 10.11). Some of these membranes traverse the space in an irregular or whorled

Figure 10.8

Transmission electron micrograph of the apical region of the pheromone gland cells of the female *P. operculella* showing a large apical vesicle containing microvilli

The area of this micrograph is partly shown in Figure 10.6 at low magnification.

The apical vesicle contains a floccular material and numerous microvilli project into it from its apical surface. These microvilli contain a tube of membrane which is probably an extension of the endoplasmic reticulum, and they have a similar appearance to the microvilli which lie adjacent to the cuticle (compare the microvilli indicated by black arrows). The intercellular membrane of the cell containing the vesicle also closely invests the vesicle.

The cytoplasm of these cells has a similar appearance to those in the previous micrograph. One mitochondrion which is located near the lower edge of the field is associated with a vacuole containing a granular material. Also, the dark object in the membrane complex and associated mitochondrion which occupies the upper left corner is a number of membranes lying close together.

ER	endoplasmic reticulum
G	Golgi
IM	intercellular membrane
MC	membrane complex
Md	mitochondrion
MdVgr	mitochondrion associated with a vacuole containg a granular material
Mt	microtubule-like structure
Mv	microvilli
pc	pore canal
Rb	ribosome
Ves Mv	microvilli-lined vesicle
Vgr	vacuole containing a granular material

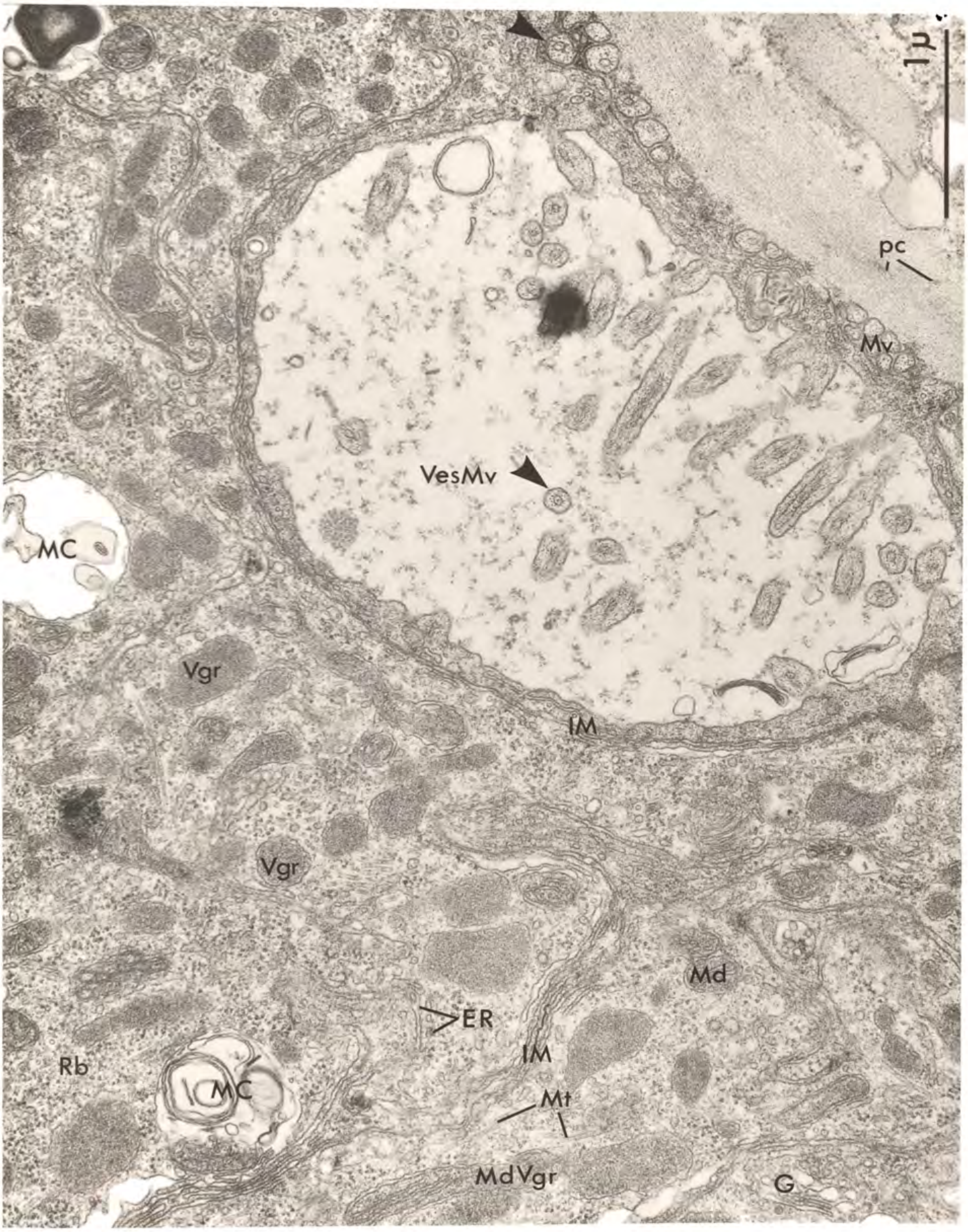

1µ
pc
Mv
VesMv
MC
Vgr
IM
Vgr
Md
ER
IM
Rb
MC
Mt
MdVgr
G

Figure 10.9

Transmission electron micrograph of the basal region of the pheromone gland cells of the female *P. operculella* showing some of the large complexes of membranes

The area of this micrograph is partly shown in Figure 10.6 at low magnification.

The two labelled membrane complexes both lie adjacent to intercellular membranes. A region of granular material similar to that found in many of the vacuoles is also associated with the largest membrane complex. Small rod-like structures in the centre of this complex are plates or tongues bounded by membrane which have become flattened until the membrane folds back closely upon itself, while the dark regions are areas of closely applied membranes. This entire large membrane complex appears to be one end of the myelinated figure shown in the following micrograph (Figure 10.10).

Note also that the two membrane complexes at the top of this micrograph appear to be associated with mitochondria.

BM	basement membrane
G	Golgi
IM	intercellular membrane
MC	membrane complex
Md	mitochondrion
MdVel	mitochondrion associated with a membrane
Mt	microtubule-like structure
N	nucleus
Rb	ribosomes
Vgr	vacuole containing a granular material

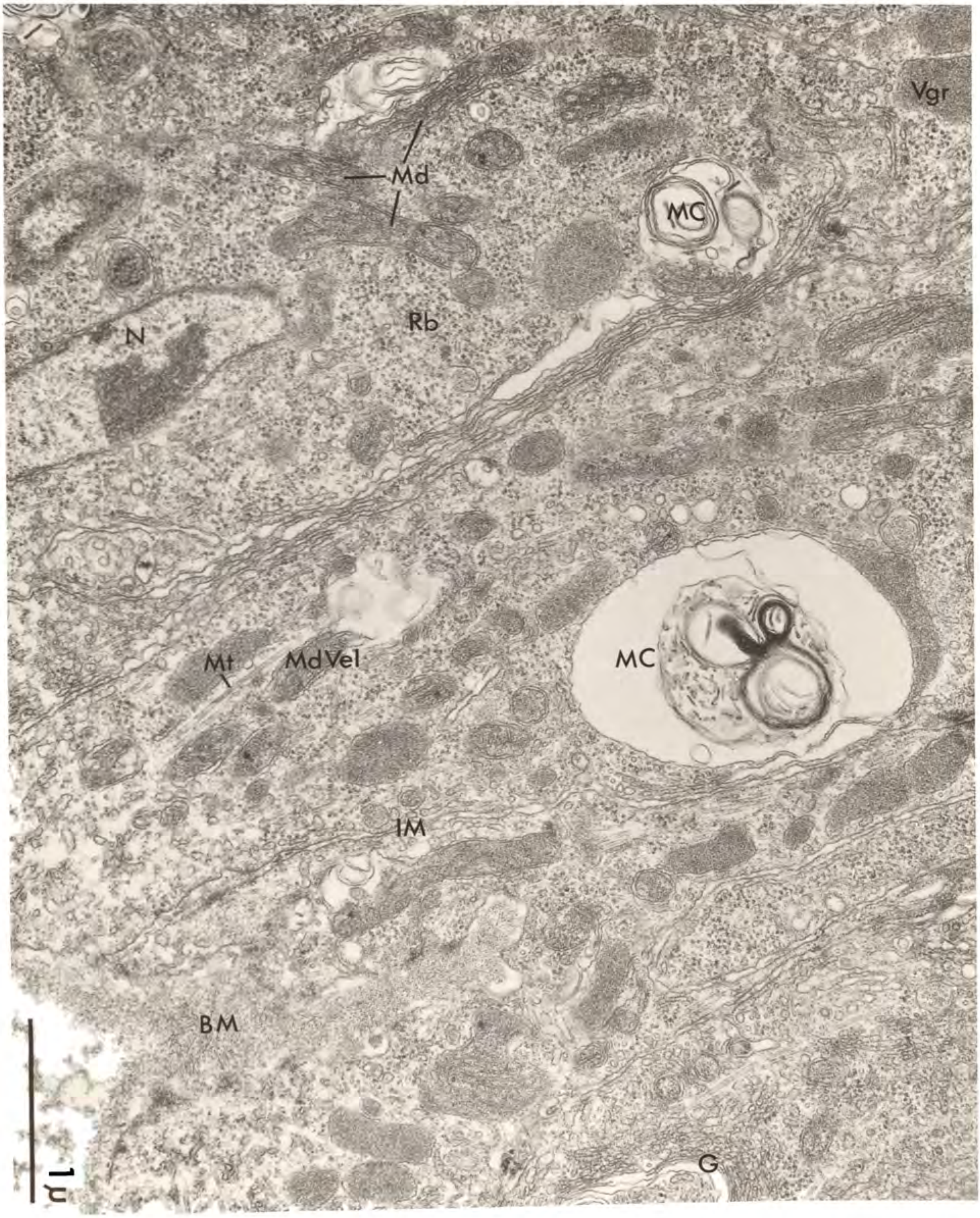

Vgr
Md
MC
N
Rb
Mt
Md Vel
MC
IM
BM
G
1 µ

Figure 10.10

Transmission electron micrographs of myelinated figures found in the pheromone gland cells of the female *P. operculella*

The myelinated figure shown in the upper micrograph was apparently associated with the large membrane complex shown in Figure 10.9, since both occupied the same relative position in sections which were very close together.

Note the electron lucid spaces associated with these myelinated figures, and the membranes crossing the one in the lower micrograph.


1 mμ

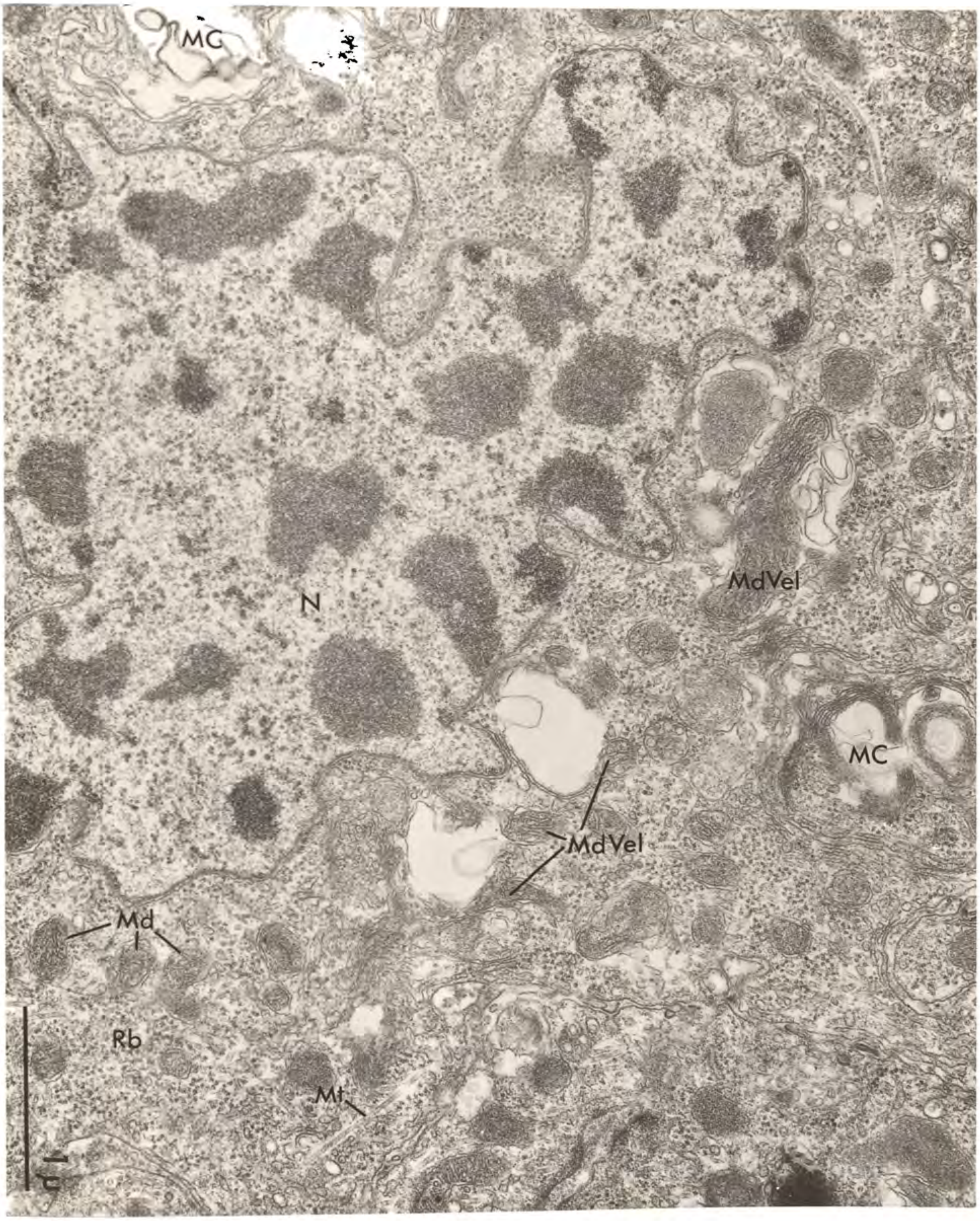

1 mμ

Figure 10.11

Transmission electron micrograph of the middle portion of pheromone gland cells from the female *P. operculella*

This micrograph shows the folded nature of the nuclear membrane, and a number of electron lucid spaces traversed by complexes of membranes. Three of these are also associated with mitochondria whilst another is associated with stacks of membranes somewhat resembling the cisternae of Golgi.

MC	membrane complex
Md	mitochondrion
MdVel	mitochondrion associated with a membrane complex
Mt	microtubule
N	nucleus
Rb	ribosomes


MC
MdVel
N
MC
MdVel
Md
Rb
Mt
1 µ

manner whilst others form folded plates or tongues in which the membranes fold back upon themselves and become closely applied to each other. Possibly these entire regions of electron lucid space are artifactual and are derived perhaps from some osmotically unstable organelle because the fixation is good elsewhere. In addition, U-shaped figures of laminated membranes also occur which either surround a central region of granular material or surround an electron lucid space containing complexes of membranes (Figure 10.7). These probably represent transverse sections of the myelinated figures described above. In some cases the spaces containing the membrane complexes appear to extend out from the open end of the "U", whilst in others the spaces appear to be associated with one side of the figure (Figure 10.10). Often they are also associated with the intercellular membranes (Figure 10.9) whilst other complexes of electron lucid spaces and membranes are associated with stacks of membranes (Figures 10.11, 10.12). However, the three-dimensional structure of all these complexes associated with stacked membranes, electron lucid spaces, and myelinated figures really needs to be worked out by serial reconstruction before the relationships between them can be clearly understood. It appears that some of the myelinated figures observed in the pheromone gland bear a close similarity to other organelles as found for example in the intrinsic secretory cells of the corpus cardiacum of *Carausius morosus* Br. and which are considered to perhaps represent mitochondria of unusual form (Smith, 1968), and to the vacuoles in the pericardial cells of *Calliphora erythrocephala* (Meig.) which contain multiple unit-membrane whorls (Crossley, 1972).

In contrast the ultrastructure of the integument elsewhere in the ovipositor is much simpler (Figure 10.13). The cuticle lacks the third layer of cavities which is present in the glandular region, and the hypodermal cells have much less well developed apical microvilli, their intersegmental membranes are largely septate desmosomes, and their basal walls are somewhat folded and rest on a basement membrane about 0.2µ thick. The cytoplasm resembles that of the gland cells in that it is packed with granules which are probably ribosomes and large micro-tubule-like structures. However, there are fewer mitochondria or vacuoles, and most of the latter are small and contain a flocculent material, although some are electron lucid. In addition, no myelinated

Figure 10.12

Transmission electron micrograph of the middle of pheromone gland cells from the female *P. operculella*

This micrograph shows a number of Golgi-like stacks of smooth endoplasmic reticulum, and three of these (marked by large X's) are on the edges of electron lucid spaces which are probably artifactual.

Md	mitochondrion
MdVel	mitochondrion associated with a membrane complex
N	nucleus
Vgr	vacuole containing a granular material

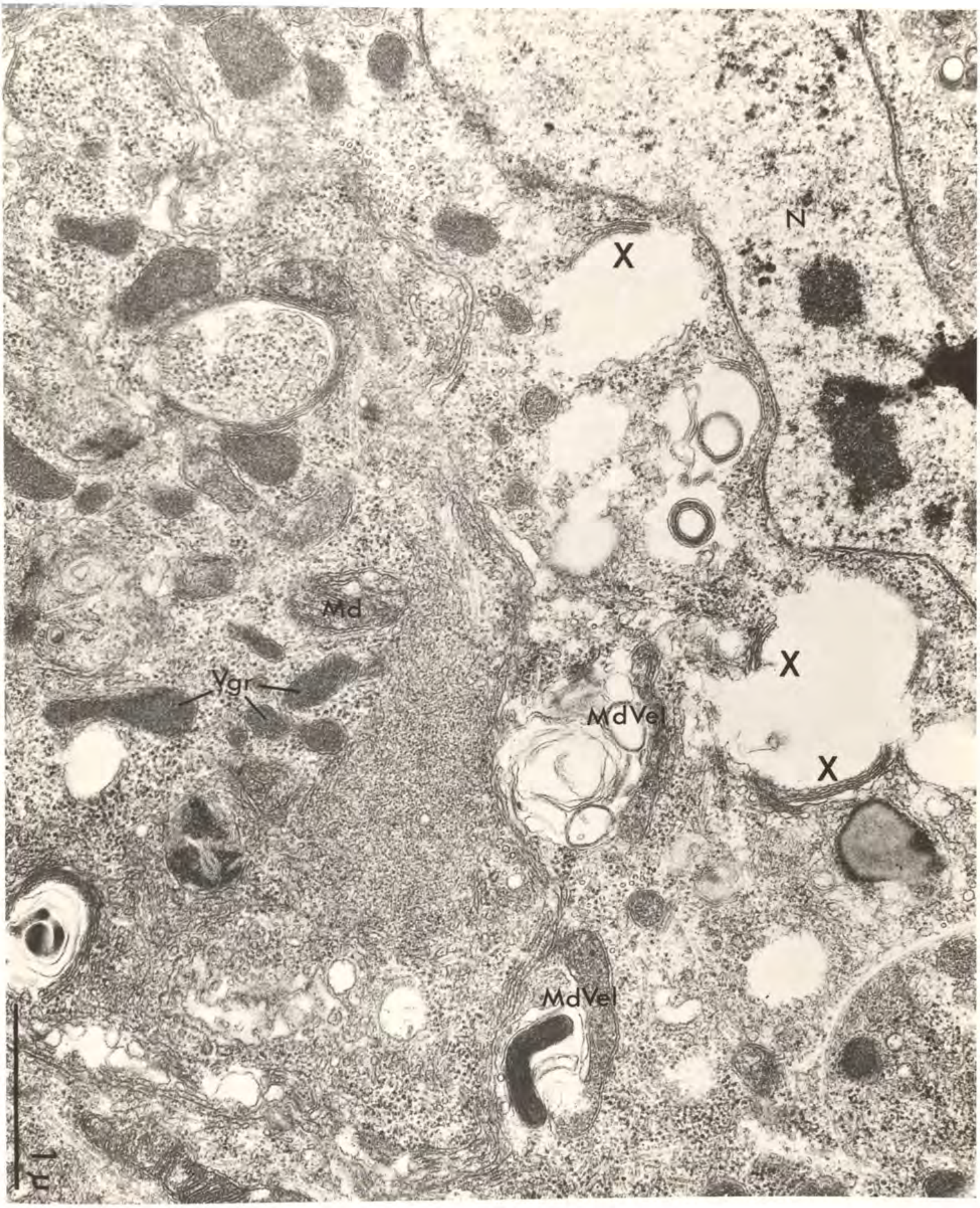
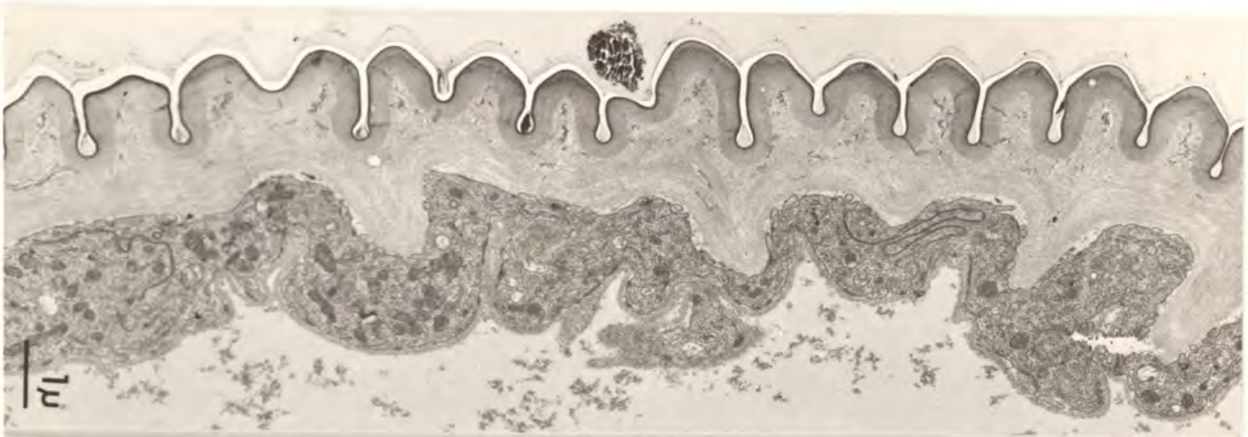

N
X
Md
Vgr
MdVel
X
X
MdVel
1 μ

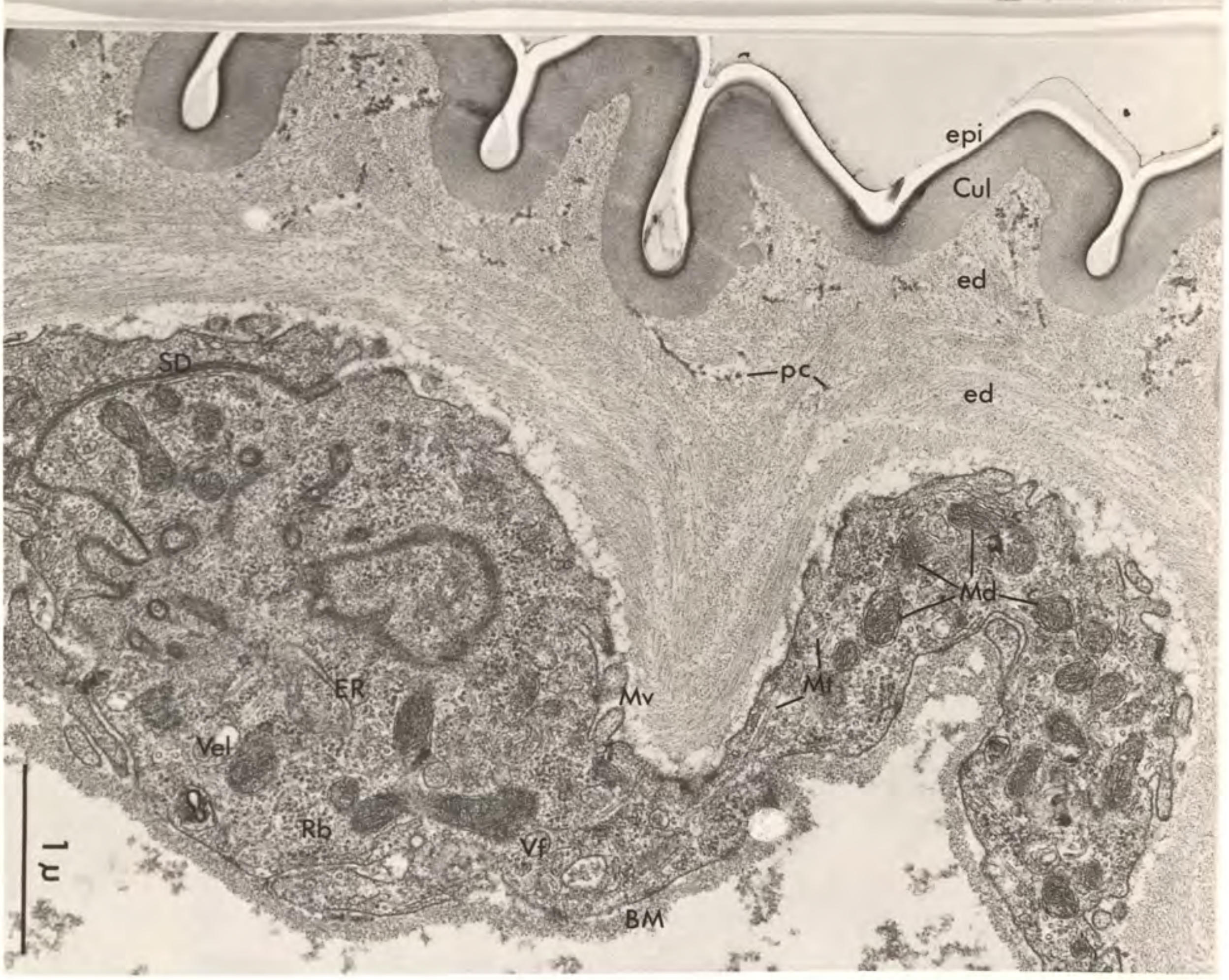
Figure 10.13

Transmission electron micrographs at low and high magnifications showing the integument of the ovipositor of *P. operculella* close to the pheromone gland

The grooves in the cuticle shown in surface view in Figures 10.3 A and 10.4 appear here in cross section. Note also that the cuticle has a very similar structure to that of the pheromone gland except that cavities are absent in the endocuticle. The hypodermal cells also rest on a thick basement membrane, and are much thinner than the pheromone gland cells. Their apical microvilli are much less well developed, their intersegmental membranes are largely septate desmosomes, and there are no large apical vesicles with microvilli. The cytoplasm is similar to that of the pheromone gland cells in that it contains numerous free ribosomes, large microtubule-like structures, mitochondria, and vacuoles containing a variety of materials. The endoplasmic reticulum, however, is not well developed, and no myelinated figures or electron lucid areas containing membrane complexes were found.

BM	basement membrane
Cal	cuticulin
ed	endocuticle
epi	outer epicuticle layers
ER	endoplasmic reticulum
Md	mitochondria
Mt	microtubule-like structures
Mv	microvilli
pc	pore canals
Rb	ribosomes
SD	septate desmosome
Vel	electron lucid vacuole
Vf	vacuole containing a flocculer material


1μ


epi
Cul
ed
pc
ed
SD
Md
Mr
ER
Mv
Vel
Rb
Vf
BM
1 μ

figures, complexes of membranes, or electron lucid spaces were observed in these cells.

The sex pheromone organs of the male

The male sex pheromone organs of *P. operculella* consist of a pair of scent brushes or clusters of long thin scales, together with shallow pouches into which these scales can be folded. Each organ is situated proximally and close to the anterior edge of a hindwing, on its dorsal surface.

Each pouch is a shallow elongate lenticular depression in the hindwing which is situated immediately distal to the base of the frenulum and anterior to the combined subcostal and radius 1 vein. The pouch extends along the anterior edge of the wing for approximately 2-4mm and reaches a maximum width of about 0.1mm. There is a gap anterio-proximally on the side of the pouch over a distance of about 0.6mm where the pouch opens anteriorly. The elongate scales of the scent brush also join the wing in the proximal 0.65mm or so of the inner surface of the pouch so that most of their insertions are opposite the anterior opening.

Each scent scale is approximately 1.0 to 1.4mm in length but all the scales of a particular scent brush are of a similar length. These scales are hollow cylinders of cuticle which have uniform diameters of between 3.2 to 4µ over most of their lengths, except that they taper somewhat at their distal and proximal ends.

A total of 14 scent brushes were carefully dissected and the number of scent scales comprising them ranged from 105 to 239, with a mean of 177.1, and a standard deviation of 35.0. There was undoubtedly some error in these counts because the scales were easily blown away by the slightest wind despite precautions, and they also appeared to gain static electrical charges which made them fly about and stick to nearby objects.

When not in use, the scales of each scent brush lie packed together in a parallel bundle within the depression formed by the pouch. Here they are normally covered dorsally by the ventral surface of the forewings both at rest or during flight. Each fore- and hindwing is coupled together in flight by a frenulum on the hindwing which hooks into a retinaculum on the forewing (Common, 1973). The frenulum is a long spike of modified scales (Figures 10.14 A, B) which arises near the base of the hindwing from its anterior edge, whilst the retinaculum consists of a single row or comb of short spines which arise ventrally from the radius vein and curve anteriorly (see Common, 1973; Figure 36.4 F). When the forewing moves anteriorly the frenulum is therefore hooked by the retinaculum thus keeping the hindwing closely applied to the ventral and posterior surface of the forewing. However, when a pheromone stimulated male approaches a female as described in Chapter 8, his precopulatory behaviour includes sliding his forewings posteriorly over his hindwings so that the leading edges of the hindwings are exposed. The scent brushes are then opened out to form a hemispherical tuft of radiating scales, which are approximately evenly spaced from each other (Figure 8. 9) and the anterior gap in the scent pouches also allows many of the scales to point anteriorly. Whilst doing this the male continues to flutter his wings and run about excitedly so that the eversion of the scent brushes is frequently very difficult to observe.

The surface of each scent brush scale, when examined with the scanning electron microscope, is found to be raised into 6 to 9 prominent longitudinal ridges with two types of smaller ridge running transversely in the spaces between them (Figure 10.15 A). The longitudinal ridges are serrated and lie about 1.3 to 1.4μ apart. They are connected by large transverse ridges which are fairly evenly spaced apart at about 0.7μ, and run at approximately right angles to the longitudinal ridges. However, these transverse ridges occasionally branch or run at an angle of up to almost 45° to the longitudinal ridges, and some vary in their spacing from almost touching each other to being almost 1μ apart. The second type of transverse ridges are only about 0.3μ long, and they lie packed together, about 0.05μ apart, on the edges of the longitudinal ridges where these join the flat surface of the scale. A series of shallow pits makes the flat surface of the scale slightly uneven, but no holes were found which penetrated deeply into the scale.

An examination of the scales found elsewhere on both male and female *P. operculella* revealed that the surface structure of the scent brush scales is quite distinct from any of the others although they all show basic similarities since most of the other scales have longitudinal

Figure 10.14

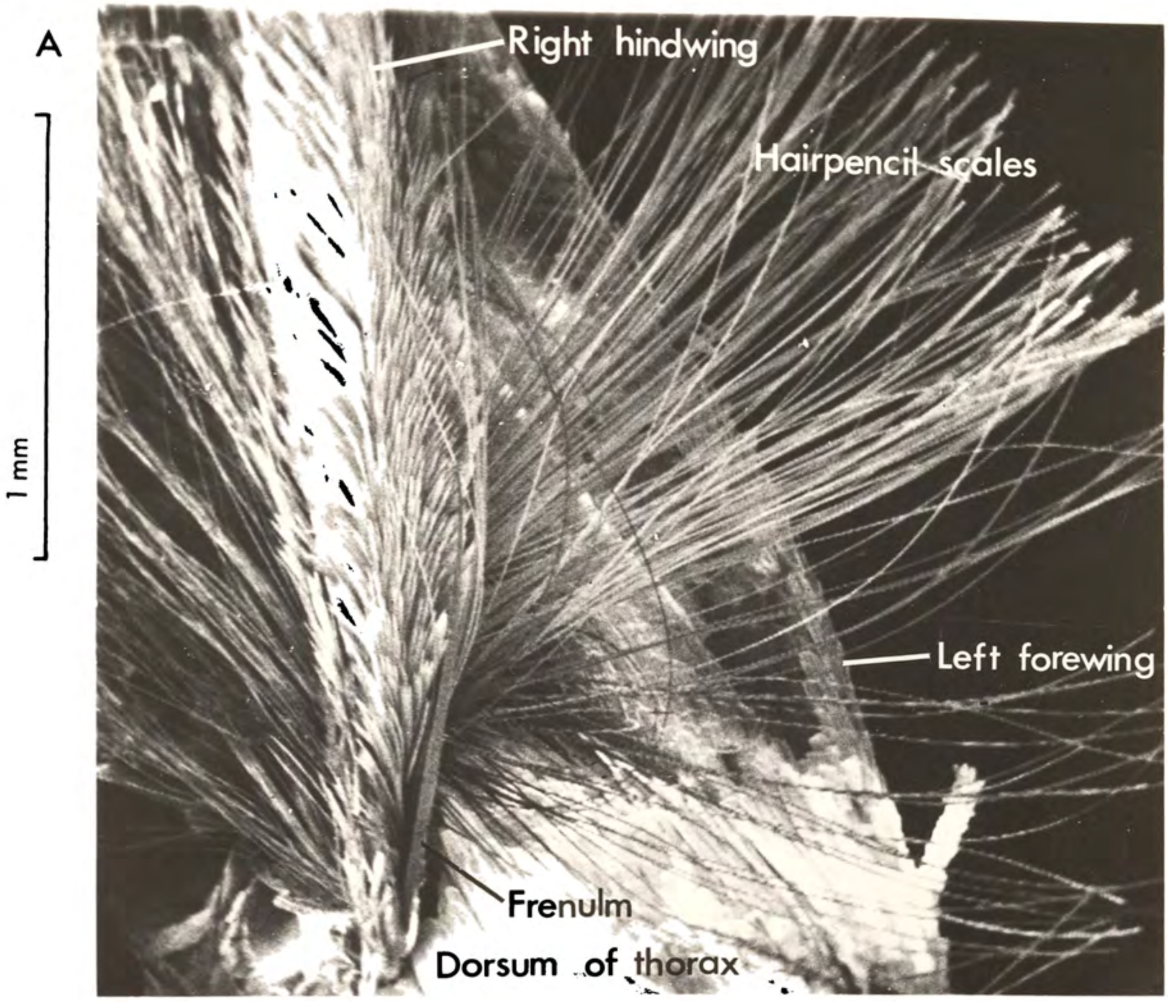
Scanning electron micrographs of the scent scale brushes on the hindwings of the male *P. operculella*

Both A and B are dorsal and somewhat lateral views of the moth looking from slightly posteriorly.
The right forewing has also been removed.

A A low magnification view showing the hairpencil or scent scales projecting from the dorsal surface of the hindwing. Note also the elongate scales, to the left of the picture, which fringe the posterior edge of the wing.

B A higher magnification view of the hindwing rotated slightly from A above, showing the anterior gap in the scent scale sheath and the bases of some of the scent scales.

A


1 mm
Right hindwing
Hairpencil scales
Left forewing
Frenulm
Dorsum of thorax

B

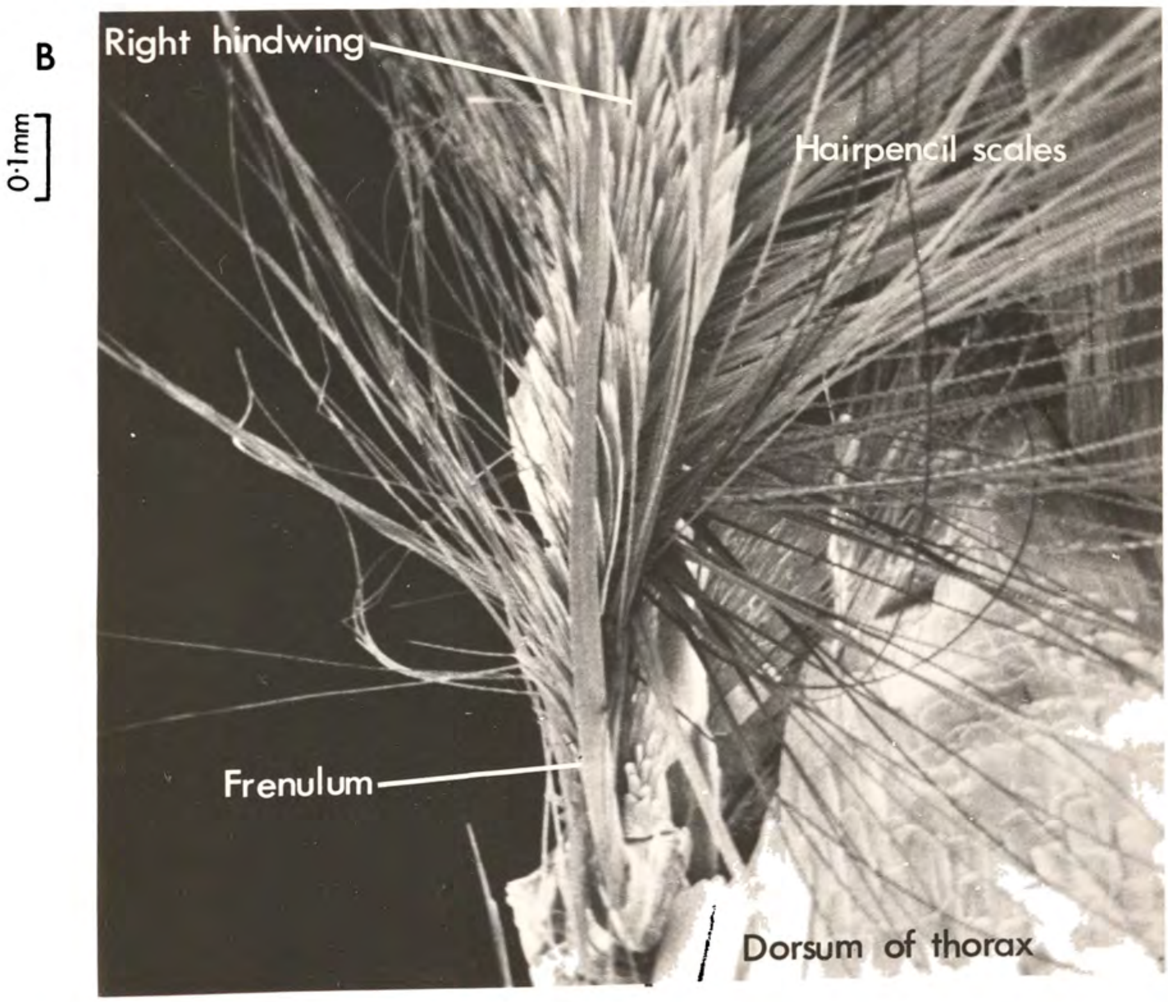

0·1mm
Right hindwing
Hairpencil scales
Frenulum
Dorsum of thorax

Figure 10.15

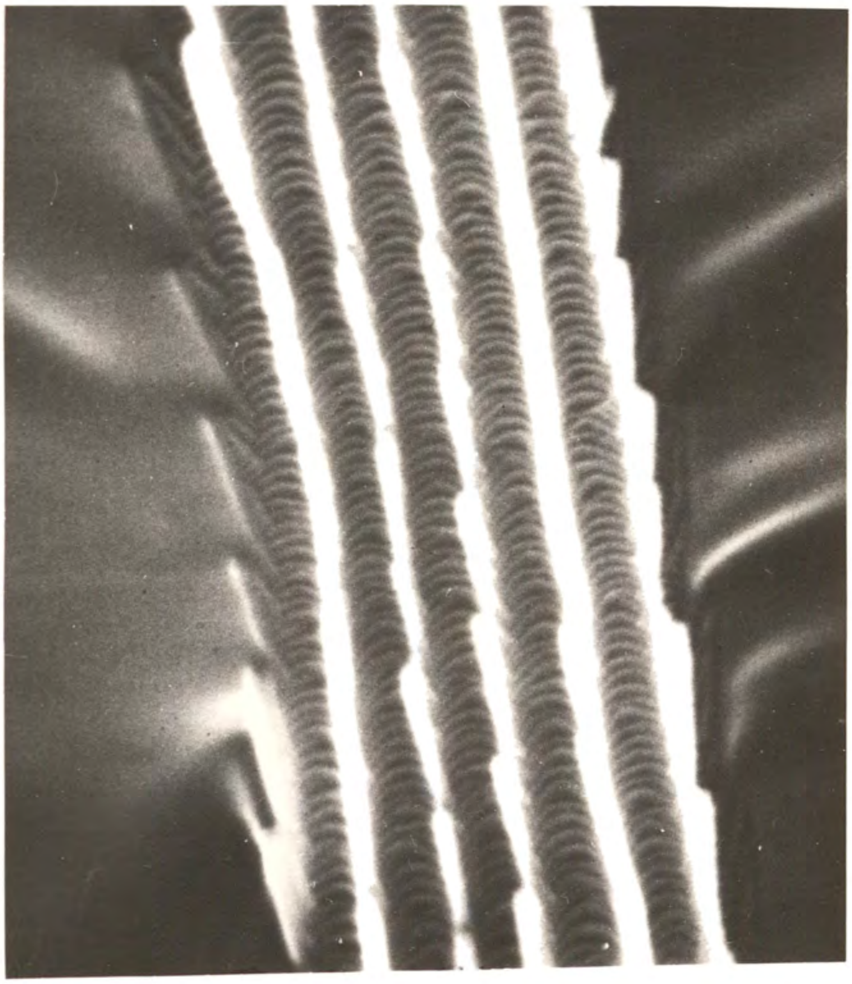
Scanning electron micrographs of the surface structure of the elongated wing scales of *P. operculella*

A. A scent scale from the male pheromone glands on the hindwings.

B. One of a series of scales which fringe the posterior and lateral edges of fore- and hindwings in both male and female. (Taken from a female hindwing).

A

B



ridges with densely packed transverse ridges running between them (Figures 10.15 B, 10.16 A). The coremata scales, however, have a surface structure closest to that of the scent brush scales, and this consists of longitudinal ridges with a complex pattern of both large and small ridges between them (Figure 10.16 B). The larger transverse ridges have cross connections between them, and additional diagonal ridges run up the longitudinal ridges. Also, the transverse ridges were never observed to be as prominently developed as those on the scent brush scales.

The only region of the scent brush sheath which differs histologically from the rest of the integument of the wing is the area at the base of the pheromone scales. Here, the cuticle is only about 0.2μ thick except where it is thickened to form the sockets into which the scent scales insert. Each socket is sunken into the wing to a maximum depth of 1 to 3μ whilst its lip is produced into a thin slightly flared hood. The edges of this hood are smooth but vary in height from being barely raised above the surface of the wing anterio-laterally (when the wing is extended laterally from the insect), to a maximum height of 10 to 11μ posterio-medially. The low portion of the hood allows the scent brush scale to fold into the sheath along the wing.

Examination of the cells in this region of the wing with the light microscope proved unsatisfactory because of the small size of the cells, and because their intercellular membranes could not be clearly distinguished (Figure 10.21). However, by using the transmission electron microscope this region was found to consist of the following layers (Figures 10.17 to 10.20 inclusive):- On the outside there was thin cuticle which apparently lacks endocuticle and has scent scale sockets arranged at intervals in it as already described. Below this is a single layer of flattened hypodermal cells and next to these is a layer of irregularly packed cells which are composed mostly of enlarged trichogens belonging to the scent scales although occasional cells of unknown function are interspersed between them. Finally there is a thin basement membrane about 350 to 750Å thick which apparently does not penetrate between the trichogens to the hypodermis. Septate desmosomes occur commonly wherever hypodermal cells meet, and where trichogens meet hypodermis near the scent scale sockets, but elsewhere intercellular junctions are infrequent and there are often large intercellular spaces

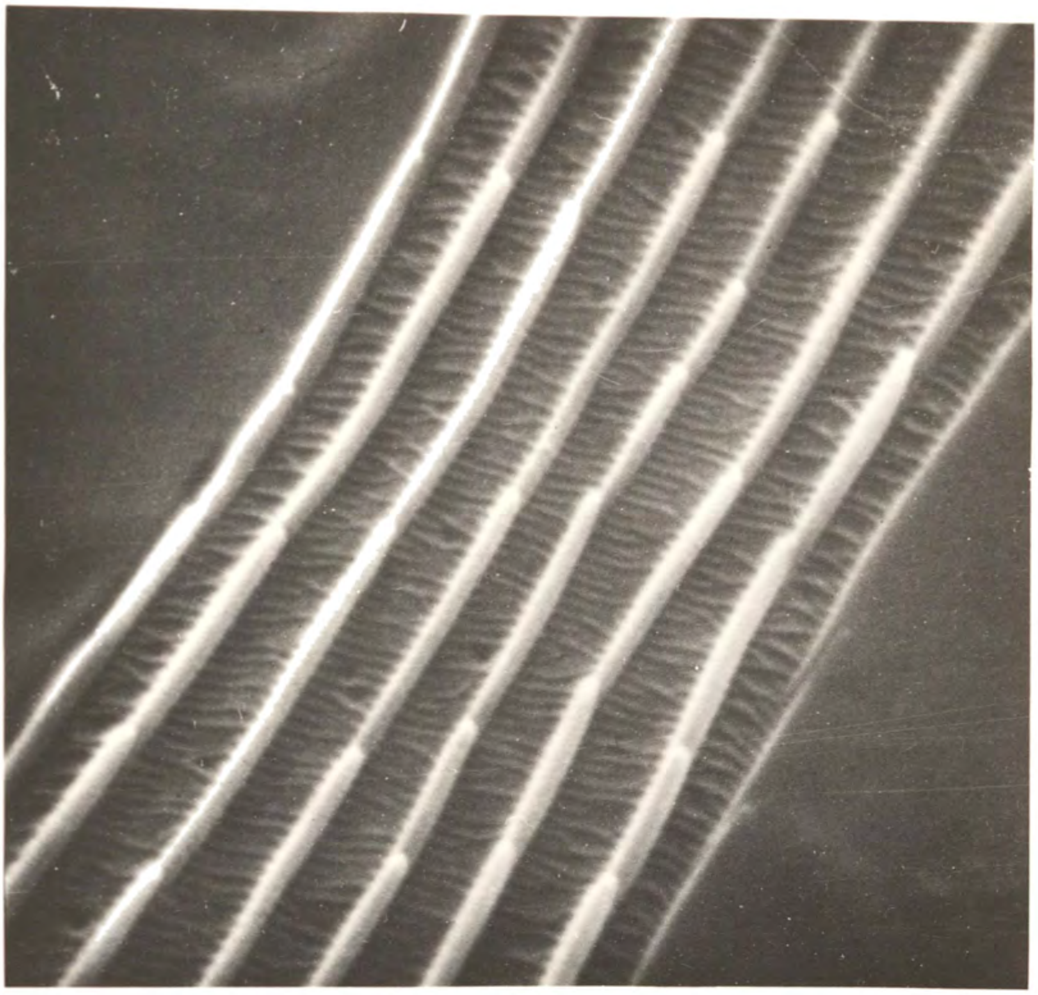
Figure 10.16

Scanning electron micrographs
of body scales of *P. operculella*

A Normal body scale of male and female. (Taken from abdomen of female.)

B A scale from the coremata of the male.

A



B

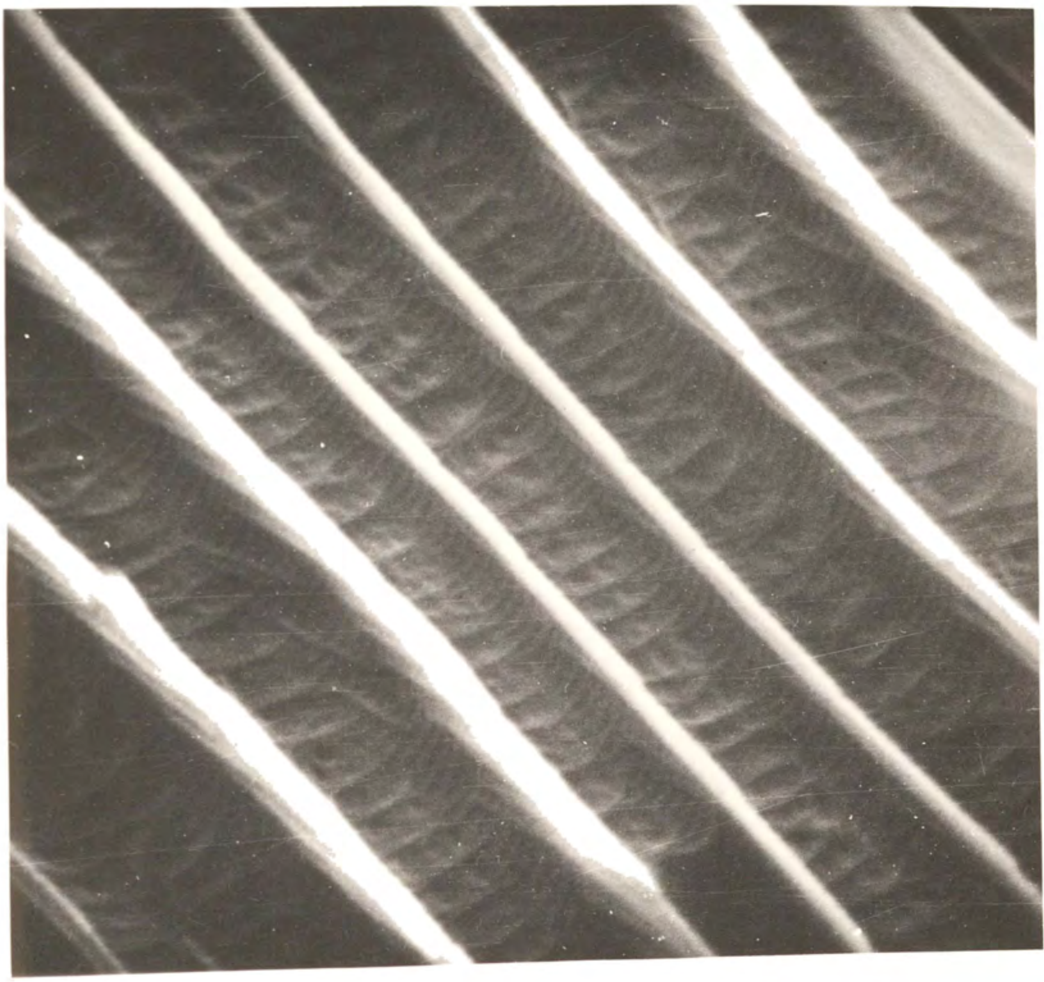


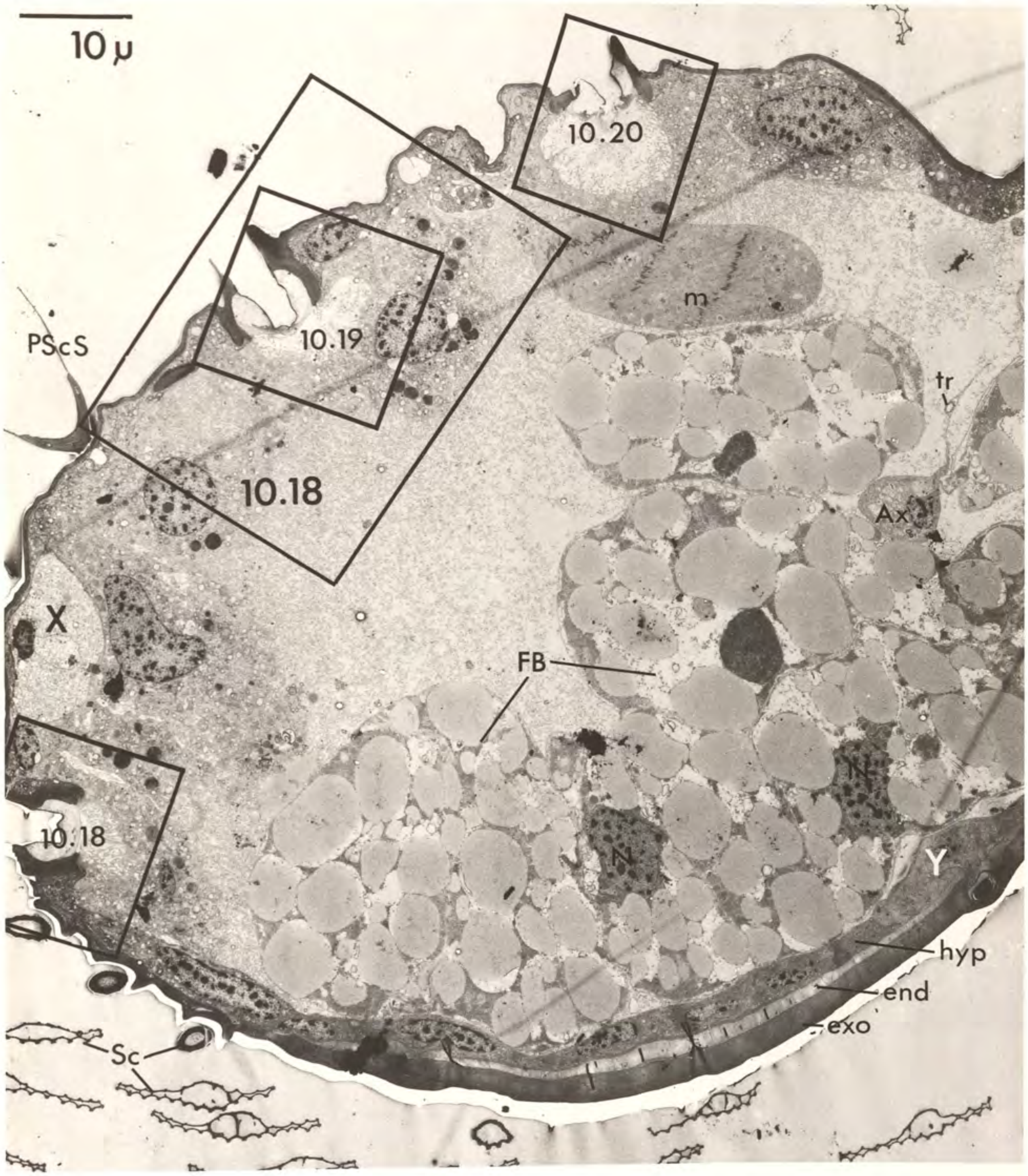
Figure 10.17

Low magnification transmission electron micrograph through the wing of a male *P. operculella* in the region of the pheromone gland

This transverse section was taken close to that shown in Figure 10.18 C. The areas shown at higher magnification in the following three figures are indicated approximately by the black boundaries.

Included in the field of this micrograph is a section through the medial hood which extends from a pheromone gland scale socket (PScS), a number of sections through normal wing scales, and a region of upper wing integument. A trichogen cell belonging to one of the normal wing scales is shown at Y, and one of the large necrotic cells of unknown function is shown at X.

Ax	nerve
end	endocuticle
exo	exocuticle
hyp	hypodermis
m	muscle
N	nucleus
PScS	pheromone scale socket
Sc	normal wing scale
tr	trachea



10 µ
10.20
10.19
PScS
10.18
m
tr
Ax
X
FB
10.18
N
N
Y
hyp
end
exo
Sc

Figure 10.18

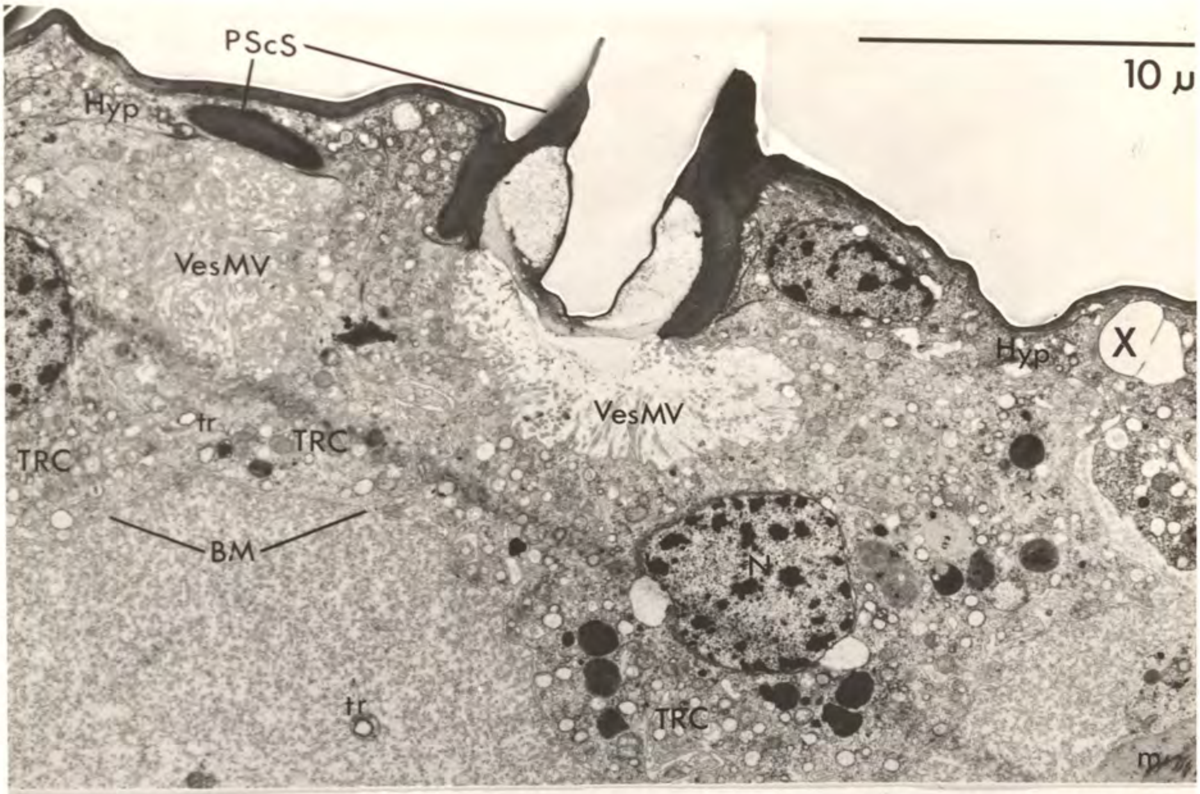
Transmission electron micrographs of
the male pheromone gland of *P. operculella*

The areas of these micrographs are shown in the low magnification electron micrograph of Figure 10.17.

The general arrangement of the cells is clearly visible in these micrographs. The cuticle is thin except where it is produced into sockets for the pheromone scales. A single layer of thin hypodermal cells lies beneath the cuticle, and this is followed by a loosely packed layer of enlarged glandular trichogen cells. These contain large nuclei and apical vesicles which are lined with microvilli, and which communicate with the lumens of the hollow scent scales. A very thin basement membrane covers the basal surfaces of the trichogen cells but does not penetrate between them.

The cytoplasm of both the glandular trichogen cells and the hypodermal cells in this region has a vesicular appearance due to the mitochondria, and numerous vacuoles within it. Some of the vacuoles are electron lucid, others have ribosomes attached to part of their membrane, while others contain a floccular material or granular materials of varying electron densities. In addition, some electron lucid vacuoles can be seen within the membranes of the nuclear envelopes, and large vesicles are occasionally visible within the hypodermal cells one of which is shown at X. Occasionally ribosome coated vacuoles can be seen opening into these vesicles (not shown on this section).

BM	basement membrane
end	endocuticle
epi	epicuticle
exo	exocuticle
Hyp	hypodermis
IS	intercellular space
m	muscle
Mb	mitochondria
N	nucleus
PScS	pheromone scale socket
Rb	ribosomes
SD	septate desmosome
tr	trachea
TRC	trichogen cell
VesMV	microvilli lined vesicle
Vqr	vacuoles containing granular material
Vf	vacuoles containing floccular material
Vrb	vacuoles coated with ribosomes


PScS
10 µ
Hyp
VesMV
VesMV
Hyp
X
tr
TRC
TRC
TRC
BM
N
tr
TRC
m


1 µ
Rb
IS
epi
exo
end
Vrb
SD
VesMV
Vgr
Mb
Vf

Figure 10.19

Transmission electron micrograph through the apical portion of a glandular trichogen cell from the male pheromone organ of *P. operculella*

The area of this micrograph is shown in Figure 10.17 at low magnification.

The vesicular cytoplasm of the trichogen cell and the adjacent hypodermal cells is packed with ribosomes and microtubule-like structures similar to that in the female pheromone gland cells, but the endoplasmic reticulum is not well developed. Occasionally tubular structures are visible within the microvilli lining the vesicle, and these are possibly extensions of the endoplasmic reticulum (arrow). Mitochondria, and different types of vacuoles are also visible as in the previous micrograph. Septate desmosomes characteristically occur where the trichogen cell joins the base of the scale socket, and some of the folds in the cell membrane of the trichogen cell wall are visible by the basement membrane in the lower left corner of the field.

BM	basement membrane
Hyp	hypodermal cell
IS	intercellular space
Mb	mitochondria
Mt	microtubule-like structure
N	nucleus
Rb	ribosomes
SD	septate desmosomes
VesMV	vesicle lined with microvilli
Vf	vacuole containing floccular material
Vqr	vacuoles containing granular material
Vrb	vacuoles coated with ribosomes
TRC	trichogen cell.

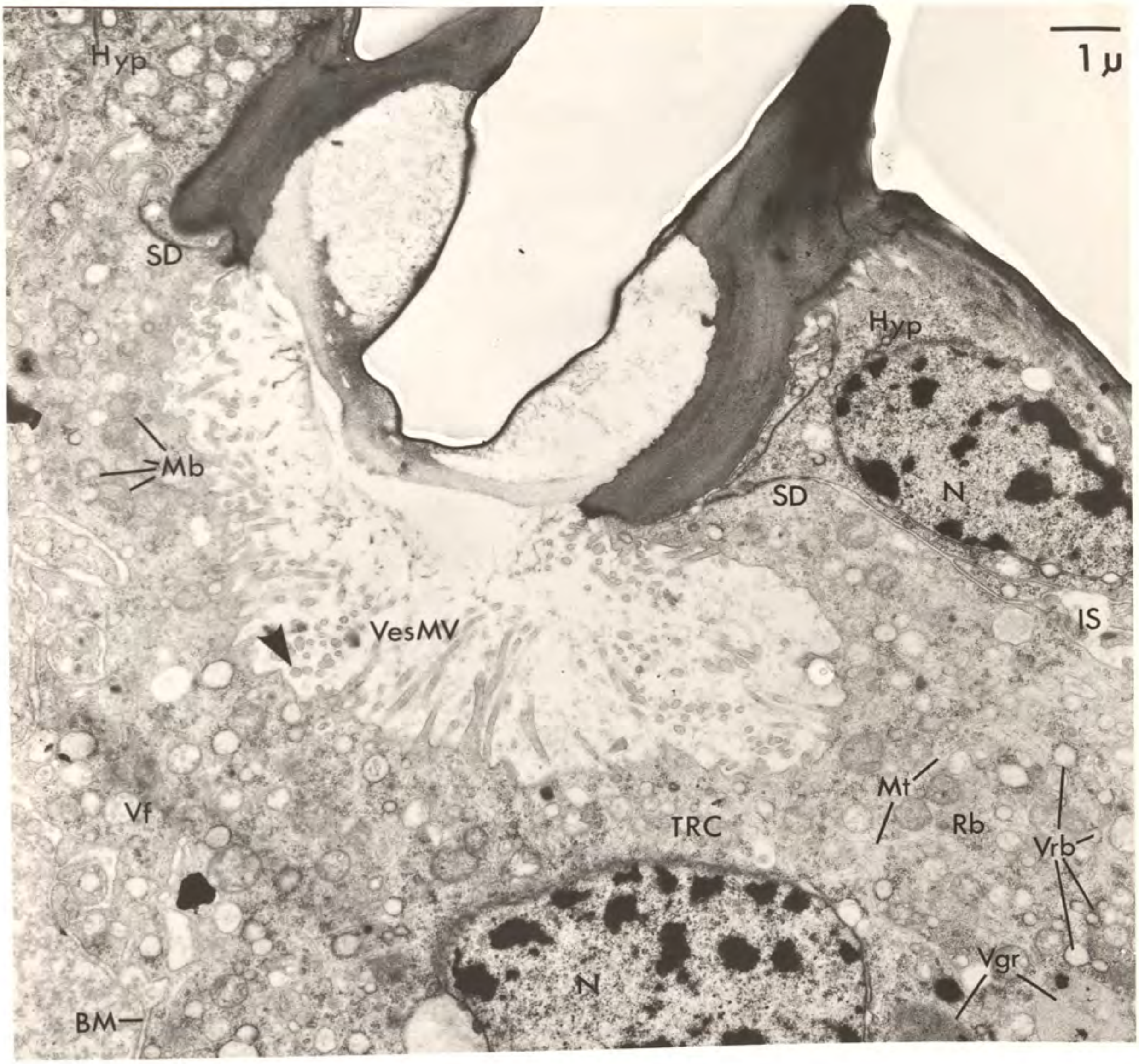

1 μ
Hyp
SD
Mb
Hyp
SD
N
IS
VesMV
Mt
Rb
Vrb
Vf
TRC
N
Vgr
BM

Figure 10.20

Transmission electron micrograph of the apical region of a glandular trichogen cell from the pheromone organ of the male *P. operculella*

The area of this micrograph is indicated in Figure 10.17 at low magnification.

This micrograph shows the same structures as Figure 10.19 and in addition part of the end apparatus of the apical vesicle is also visible but the scent scale has been lost during processing. Note also the floccular material within the vesicle.

BM	basement membrane
EAp	end apparatus
end	endocuticle
exo	exocuticle
epi	epicuticle
m	muscle
Md	mitochondria
Mt	microtubule-like structures
Mv	microvilli
Rb	ribosomes
SD	septate desmosomes
VesMV	vesicle lined with microvilli
Vgr	vacuole containing granular material
Vrb	vacuoles coated with ribosomes


1 μ
SD
epi
Vrb
exo
Rb
end
Md
EAp
SD
VesMV
Mt
Vgr
Mv
Md
BM
m

Figure 10.21

Light micrographs of the pheromone
organ of the male *P. operculella*

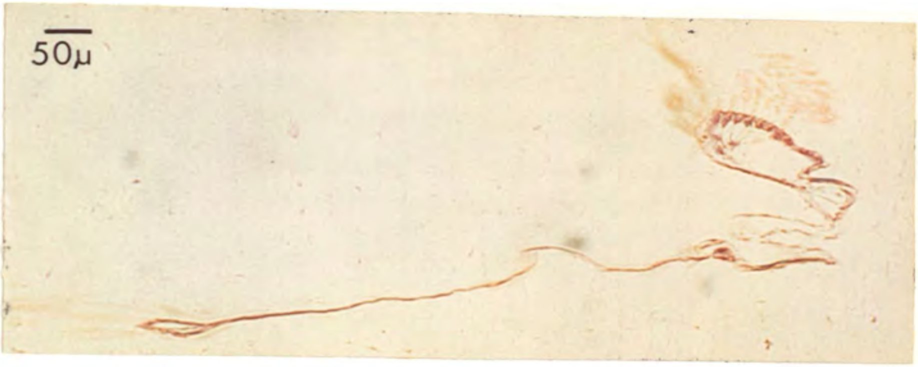
Upper: transverse section of the entire wing at low magnification showing the glandular region.

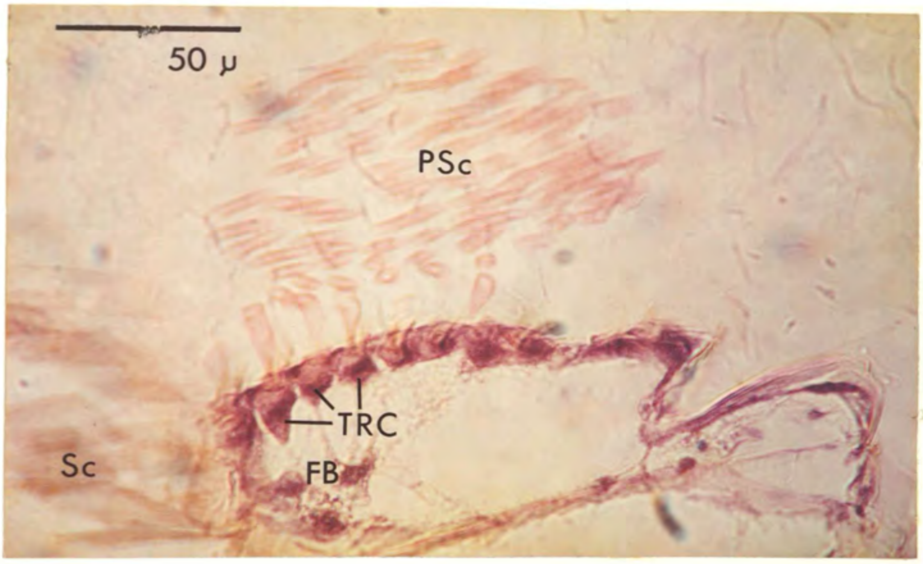
Middle: A portion of the upper section at high magnification. Note the eosinophilic scent scales and their enlarged trichogen cells.

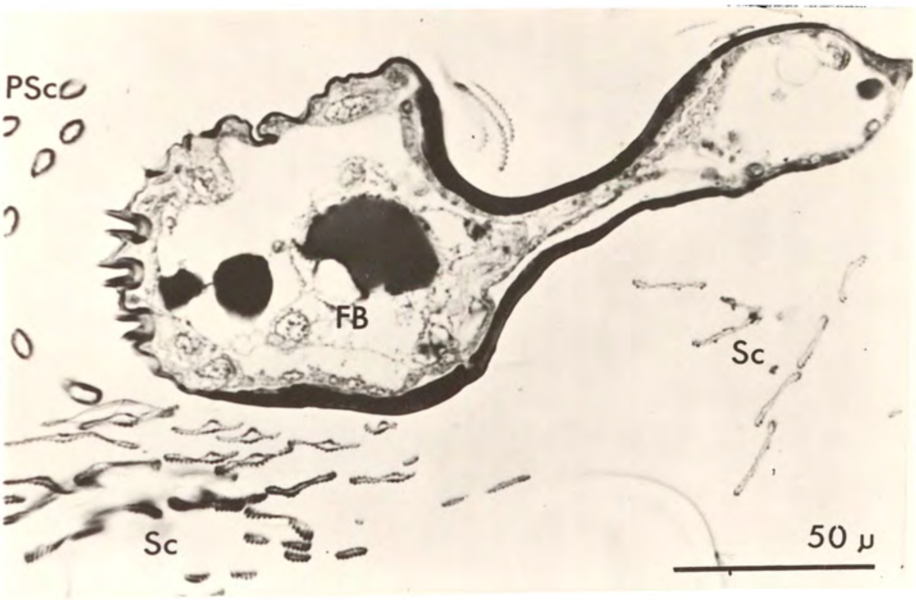
Lower: A section cut about 1μ thick with an ultramicrotome close to the region shown in Figure 10.17. Note that the arrangement of the cells in the glandular region is still not clearly visible.

Upper and middle:	Haematoxylin and eosin
Lower :	toluidine blue

FB	fat body
PSc	pheromone scales
Sc	normal wing scales


50µ


50 µ
PSc
TRC
Sc
FB


PSc
FB
Sc
Sc
50 µ

between adjacent trichogen cells.

Each scent scale is hollow and has a rounded cross section (Figure 10.21). It arises centrally from the base of a socket which is structurally similar to those from which other types of scales arise. A horizontal diaphram, or collar of fibrous material, connects the proximal edge of the scent scale to the inner edge of the socket, while the epicuticle runs upward from the end of the scale at an angle and joins the inner surface of the socket above the level of the general cuticle surface of the wing. A floccular or fibrous material is also loosely packed within the cavity formed between the collar, epicuticle, and socket (Figures 10.18, 10.19, 10.20).

The lumen of the scent scale is continuous with a hollow vesicle which is invaginated into the apical region of the trichogen cell. The wall of this vesicle joins the edge of the socket next to the junction of the fibrous collar, and numerous microvilli, which vary from about 0.075 to 0.185μ in diameter, project into the lumen of the vesicle. Some of these microvilli branch near their bases, and some can be followed for up to 2.6μ in thin sections so that they are in fact probably much longer than this. In addition, small tubules or fine filaments with diameters of about 500Å, can be seen in the centres of some of the microvilli (Figure 10.19). A floccular material which is possibly protein is irregularly scattered within the lumen of the vesicle, and becomes concentrated into a zone distal to the microtubules. Between this zone and the lumen of the scent scale is another small zone of tangled fibrills with diameters of about 270Å and these form the end apparatus.

The cell membrane of the trichogen is relatively smooth except where it is separated from the haemolymph by the basement membrane, and here it is often somewhat folded and pleated. This presumably increases the absorbtive surface area of the cell. The nucleus is rounded or oval and much larger than those of the hypodermal cells. Vacuoles are also occasionally visible between the membranes of the nuclear envelope (Figure 10.19).

The cytoplasm of each scent scale trichogen is highly vesicular, containing numerous vacuoles of varying size. Some of the smaller vacuoles are electron lucid and possibly once contained fat, whilst the others contain a finely floccular material which is possibly protein. Some of the latter also resemble coated vesicles in that they have structures resembling ribosomes regularly spaced over part of their surface. These can not be glycogen because uranyl acetate was used during fixation. The large vacuoles are basal and contain a very electron dense material which has a granular or slightly fibrous structure when examined at high magnification. Possibly this material is protein, and the structures are lysosomes. No vacuoles were observed opening into the apical vesicle.

The endoplasmic reticulum of these trichogens is only poorly developed and numerous rounded mitochondria occur scattered amongst the vacuoles. However, these mitochondria do not enter the microvilli. Free ribosomes occur frequently in the cytoplasm, as also do microtubules, although the latter are especially frequent near the apical vesicle and cell membrane where presumably they form a structural framework in the cell.

The cells of unknown function which occur scattered between the trichogen cells appear to be necrotic. They have small dense apical nuclei in which the heterochromatin is condensed and much of the euchromatin is lost. Also, these cells have a large central to basal membrane limited vesicle which is often ruptured. The cytoplasm has little structure and generally appears as a flocculent material. However, poorly developed vacuoles and mitochondria occur occasionally within it. It is not known if these cells have openings although none were observed in the 50 or so sections examined.

The hypodermal cells between the scent scale sockets secrete a thin cuticle composed entirely of epicuticle and exocuticle which is only about 0.28 to 0.47μ thick. The cytoplasm of these cells is vesicular, resembling that of the trichogen cells, and differs markedly from the cytoplasm of hypodermal cells elsewhere in the wing. Large vacuoles containing electron dense material occur very infrequently whereas vacuoles with ribosome-like bodies adhering to their membranes are particularly common. In some sections large vacuole-like structures are also visible and occasionally some of the smaller coated vacuoles can be seen to open into them. These large vacuole-like structures were not observed to open or lead anywhere although these possibilities were not discounted because of the small number of sections examined (about 50). The nuclei of these hypodermal cells also frequently show

Apart from the description by Adeesan *et al.* (1969) of the pheromone gland in *P. operculella*, the female pheromone gland has only been described for one other gelechiid, the pink bollworm moth *Pectinophora gossypiella* Saunders. It was first described by El-Sawaf, Kaschef, and Soliman (1968) as a ring gland on the 9th abdominal segment consisting of columnar cells. Later Jefferson, Sower, and Rubin (1971) stated that it is an eversible sac which is situated dorsally in the intersegmental membrane between the 8th and 9th abdominal segments. They reported that its glandular epithelium is similar to the other epidermal cells in the 8th and 9th abdominal segments, and are flattened but bulge in the region of the nuclei. Both reports, therefore, show differences with the gland in *P. operculella*.

The pheromone gland cells of *P. operculella* are obviously secretory as evidenced by their apical microvilli, the large numbers of vacuoles in their cytoplasm, and their large nuclei with folded envelopes. According to Barth (1958) the glandular cells of insects characteristically have an apical zone of parallel fibrillae and vertical striations are also visible in the apical regions of pheromone gland cells in many Lepidoptera (Gotz, 1951; Jefferson, Shorey, and Gaston, 1966; Percy and Weatherston, 1971). Many authors, however, do not mention the presence of such an apical zone in the pheromone gland cells of other female moths and therefore it is possible that some may have a similar gland cell structure to *P. operculella* which requires examination with the electron microscope before the microvilli are discerned. In *P. operculella* this probably results from the microvilli being densely packed and often running more or less parallel with the cuticle rather than being orientated at right angles to it. However, apical microvilli have been demonstrated by means of the electron microscope, to be present in the female pheromone glands of *T. ni* (Hübner) (Miller, Jefferson, and Thomson, 1967), *Bombyx mori* L. (Wakui and Sugimoto, 1969) and in *Choristoneura fumiferana* (Clemens) (Percy and Weatherston, 1971). It should be noted though, that Steinbrecht and Schneider (1964) interpreted this apical zone in *B. mori* as representing distinct foldings of the cell membrane rather than microvilli.

Many authors have noted the presence of vacuoles in the cytoplasm of the pheromone glands of other Lepidoptera. Small vacuoles have been reported to occur, for example, in these cells in *Prodenia* *litura* Fabr. (Hammad and Jarczyk, 1958); *T. ni* (Hubner) (Jefferson *et al.* 1966); *B. mori* L. (Waku and Sumimotu, 1969); *O. leucostigma* J.E. Smith (Percy *et al.* 1971); and in *C. fumiferana* (Clemens), *Choristoneura pinus* (Freeman) and *Malacosom. disstria* (Hubner) (Percy and Weatherston, 1971); whilst large vacuoles occur for example in *E. acrea* (Drury) (MacFarlane and Earle, 1970); and in *C. fumiferana*, and *C. pinus* (Percy and Weatherston, 1971).

According to Smith (1968), large nuclei are often associated with active secretory cells, and a pleated nuclear envelope also sometimes occurs in them. In addition, it is interesting that Hammad and Jarczyk (1958) obtained an indication that the nuclei of the pheromone gland cells of *P. litura* are at their greatest size and have their largest surface area when pheromone production is at its maximum during the first two days of adult life, whilst the nuclei decrease in size as pheromone production declines. Jefferson *et al.* (1966), in contrast, found that gland cells of *T. ni* contained both elongated and rounded nuclei in females of all ages, and since pheromone production increases to a plateau from about the second night on (Shorey and Gaston, 1965) they concluded that all the gland cells may not be in the most active secretory stage at the same time. This may also account for some of the differences in shape in the nuclei of the pheromone gland cells in *P. operculella* although most of the differences observed in the present investigation appeared to be attributable to local distortions of the individual cells by adjacent organs.

Although no evidence of pores in the surface of the pheromone gland of *P. operculella* could be found during the present investigation by using the scanning electron microscope, this is not conclusive evidence since the pores could either be clogged with material, or be less than about 150Å in diameter (the limit of resolution of the instrument used). Weatherston and Percy (1970a) similarly found no evidence of pores in the pheromone gland of *C. fumiferana* but they reported that there were three types of surface in the eversible gland of this insect, in contrast to the two types found in *P. operculella*. They reported that the actual glandular area has numerous spikes on the surface and it is thrown into bulges and wrinkles. Waku and Sumimoto (1969) similarly found short cuticular spikes all over the glandular surface of *B. mori*. However, no similar spikes were found in

P. operculella during the present study, so this latter insect therefore differs in this respect from both of these insects.

Weatherston and Percy (1970) proposed that in *C. fumiferana* the pheromone, after penetrating the cuticle of the pheromone gland, is retained on the surface area in the deep invaginations of the non-extruded gland and that it is exposed when the gland becomes everted in the "calling" female. These authors also noted that the surface area of the gland is not significantly increased by the spikes on the cuticle and postulated that their role may be one of retention and conservation of the pheromone on the surface of the inverted gland. The inverted gland of *P. operculella* may similarly act as a storage area for the pheromone, but it appears probable that in this insect the regularly folded surface of the 9th and 10th abdominal segments may also serve as an additional site for evaporation of the pheromone. This is supported by the observation that females with only partially extended ovipositors and without everted pheromone glands are also attractive to males (Chapter 8). If in fact the surfaces of these segments are additional evaporative areas then the fine grooves in the cuticle may act as channels along which the pheromone can run, perhaps by surface tension, to become more spread out. Possibly the small size of the ovipositor and pheromone gland of *P. operculella* necessitates this additional evaporative area.

The exact mechanism whereby the pheromone of *P. operculella* reaches the surface of the gland is not clear from the results of the present study. Steinbrecht and Schneider (1964) similarly could find no evidence of cuticular canals in the cuticle overlying the pheromone gland of *B. mori*, and this lead Steinbrecht (1964) to propose that a diffusion process took place whereby a gradient across the cuticle is maintained by evaporation of the pheromone from the gland surface. Waku and Sumimoto (1969), however, later found thin canals with a very electron-dense substance in them which were present in the endocuticle of the spikes on the pheromone gland of *B. mori*. These often penetrated deeply into the exocuticle leading these authors to conclude that although they could not define the actual point where the canals reach the cuticulin layer "it is almost certain that the canal should do so". They, therefore, postulated that these pore canals are the pathways for transportation of the pheromone to the surface. Miller *et al.* (1967) could find no obvious ducts in the endocuticle of the pheromone gland of *T. ni* but Smithwick (1970), and Percy (1974) found epicuticular filaments running through the endocuticle lamellae of the pheromone gland cuticle in *Plodia interpunctella* (Hubner) and *C. fumiferana* respectively. In *C. fumiferana* these originate at or near the tips of the microvilli and in both species these filaments form large masses beneath the epicuticle and some penetrate this layer. This cuticle structure suggests a similarity with that described for *P. operculella* in the present study although detailed descriptions from these ultrastructural studies have not yet been published. Percy and Weatherston (197) have suggested that the epicuticular filaments may represent channels for the transport and possible modification of pheromone substances passing through the cuticle. This may also apply to *P. operculella*, although it appears likely that the pheromone may be transported along the pore canals to the region of the large cavities where it is stored and subsequently released through the epicuticular filaments that penetrate between the cavities. However, no epicuticular filaments or pore canals were observed to enter the cavities but possibly the pheromone diffuses into them across very narrow regions of endocuticle. Also, the possibility must be considered that these cavities play no part in pheromone production, but this seems unlikely since they are unusual cuticular structures and they were only found in the cuticle overlying the pheromone gland. A storage function is also suggested from the observation that they are filled with a material which is possibly lipid since most female moth pheromones are acetates of fatty acids (e.g. Jacobson, 1972) but histochemical tests are necessary to confirm this. Perhaps a storage site near the surface of the cuticle is advantageous because of the small size and surface area of the gland in *P. operculella*. Such structures could also lead to a faster release rate because the path along which the pheromone is transported or diffuses is shortened.

Few ultrastructural studies of the pheromone gland cells of female moths have been carried out, and detailed descriptions have only been published for *B. mori* by Steinbrecht (1964), and Waku and Sumimoto (1969). However, from the available information concerning other species the following structural details show similarities with the pheromone gland cells of *P. operculella* as described in the present study. The presence of apical microvilli in these cells has already been discussed above but it is also interesting to note that Miller *et al.* (1967) reported the presence of fibres along the length of each villus in *T. ni* which extend into the cytoplasm, and Smithwick (1970), and Percy (1974) have both found that the microvilli of *P. interpunctella* and *C. fumiferana* respectively contain a central canal. In *C. fumiferana* this also appears continuous with the smooth endoplasmic reticulum. In *B. mori*, *C. fumiferana*, and *P. interpunctella* smooth tubular endoplasmic reticulum predominates in the adult gland, whereas rough endoplasmic reticulum is typical before emergence (Percy and Weatherston, 1974). The smooth endoplasmic reticulum is also well developed in *P. operculella* and this is indicative of lipid synthesizing cells. In addition, mitochondria and lipid droplets are numerous in the cytoplasm of *B. mori*, *C. fumiferana*, and *P. interpunctella* (Waku and Sumimoto, 1969; Smithwick, 1970; Percy, 1974), and elongate mitochondria and vacuoles have also been reported in the cytoplasm of the pheromone gland cells of *T. ni* (Miller *et al.* 1967).

A very close similarity exists between the ultrastructure of the pheromone gland cells of *P. operculella*, and those of *B. mori* as described by Waku and Sumimoto (1969). These authors reported the presence in *B. mori* of well developed Golgi apparatus, large numbers of free ribosomes, numerous mitochondria with dense matrixes and irregular cristae, large globular structures containing some lipoidal material, and myelinated figures. These myelinated figures were also observed by Steinbrecht (1964) in *B. mori*. Waku and Sumimoto (1969) reported various myelinated figures intermediate between partly myelinated mitochondria and vacuoles, and concluded that the mitochondria are the starting organelles for the production of the secretory substance and that these transform into the myelinated figures which, in turn, change into the secretory globules. Such a sequence may also occur in the cytoplasm of the gland cells of *P. operculella* although insufficient intermediate stages between these organelles were observed to confirm this.

The large apical vesicles bordered with microvilli which were observed in some of the pheromone gland cells of *P. operculella* suggest the possibility of a structure similar to that described for the male pheromone gland cells of the Mecopteran *Harpobittacus australis* (Klug) by Crossley and Waterhouse (1969a). Certainly vesicles of this type are often associated with ducts which may be cuticle lined (Crossley and Waterhouse, 1969a, 1969b) but no evidence was found of the end apparatus which frequently occurs in such gland cells. It is equally possible that the vesicles in *P. operculella* could be large infoldings of the apical surface of the gland cells perhaps analagous to the structures which occur in the ellipsoid gland cells of the osmeterium of various butterfly larvae as described by Crossley and Waterhouse (1969b). The fact that the microvilli arise predominantly from the surface of the vesicle nearest the cuticle in *P. operculella* could also suggest that the vesicle opens towards the basement membrane, rather than towards the cuticle, although this seems unlikely. Further research with the electron microscope, perhaps involving serial sections, is necessary before the correct structure can be elucidated. Nevertheless, it appears that this type of vesicle is unusual in the pheromone gland cells of female Lepidoptera.

The male

Scent glands associated with tufts or brushes of elongated hair-like scales are frequently found on male Lepidoptera. These structures have variously been termed "hairpencils", "pencils of hair", "penicilli", "rayed-hairs", and "scent-hairs" or "scent brushes" (Barth, 1937, Eltringham, 1937; Varley, 1961, 1962; Birch, 1970a). The scent organs, of which these scent brushes form a part, are highly variable in structure: generally, however, the scent brushes have pouches into which they can be retracted or folded when not in use, and the pouches frequently have areas of glandular cells which produce the scent material. Often the scent brushes are also situated on levers as in the Noctuidae (Birch, 1970a), or on extensible tubes as in Danainae (Pliske and Salpeter, 1971). Müller (1877) suggested that the brushes provide an enormous surface area for the evaporation of the scent, and thus its rapid dissemination, whilst the pockets prevent undue loss of scent when the brushes are not in use. This has generally been considered to hold true in the other Lepidoptera studied and it undoubtedly also applies to the pheromone organs in the male *P. operculella*. However, the function of the pockets in *P. operculella* for conserving pheromone is undoubtedly aided considerably by the forewings covering them. In this respect it is interesting that the wing glands of various Phycitinae, which are situated on the underside of the forewings near their leading edge, have large flaps that arise from the edges of the pockets and partially cover their openings (Barth, 1937; Grant and Brady, 1975), whereas the pockets in *P. operculella* are simple depressions. This difference may be due to the fact that the wing glands of Phycitinae are not always covered by the hindwings, whereas the hindwing organs of *P. operculella* are normally shielded by the forewings and the scent scales are entirely enclosed. McColl (1969) has emphasised that the increasing complexity of male scent organs in Lepidoptera relates to the prevention of unnecessary evaporation or secretion. It may well be, therefore, that the simplicity of these organs in *P. operculella* is due to their positioning between fore and hindwings.

The male scent organs are also very variable in position and have been reported on the abdomen, thorax, legs, or wings of various Lepidoptera (Stobbe, 1912; Birch, 1968; Jacobson, 1972). However, scent organs similar to those found on the hindwings of *P. operculella* occur on the posterior border of the hindwings of the geometrid *Hammaptera frondosata* Guerin (Barth, 1959) and, as already mentioned, on the forewings of many Phycitinae including *Anagasta kuhniella* (Zeller), *Achroia grisella* Fabr., *Aphomia gularis* (Zeller), *Cadra cautella* (Walker), *Ephestia elutella* (Hübner), *Galleria mellonella* (L.), and *Plodia interpunctella* (Hübner) (Barth, 1937). The phycitinid *Vitula edmandsae* (Packard) has tufts of scent scales on both fore and hindwings (Weatherston and Percy, 1969), and many of the above mentioned Phycitinae also have scent glands situated dorsally on the 8th abdominal segment.

It has generally been considered that the vibration or beating of the wings by the pheromone stimulated male moth, when approaching an attractive female, serves to disperse the scent and this seems very likely to occur also in *P. operculella*. In addition, the circling movements of the male probably help to ensure that the male's scent is spread in all directions and thus increase its chances of coming into contact with the female.

The wing glands of the male *G. mellonella* and *A. grisella* have been shown to produce a sex attractant for the female (Roller, *et al.* 1968; Dahm *et al.*1971), whilst those of *P. interpunctella* produce a pheromone that subdues the escape reaction of the female and thereby facilitates mating by the male (Grant and Brady, 1975). The wing gland scent of these latter moths therefore acts in a very similar fashion to that of *P. operculella*. In addition, Grant and Brady (1975) also concluded that a similar function was the major one of the wing glands of *C. cautella*, although they were unable to demonstrate it. It may also be noted that although the scent glands in male Lepidoptera are generally considered to be sex pheromone glands which produce aphrodisiac scents, this has only been demonstrated in some butterflies (Brower, Brower, and Cranston, 1965; Myers, 1972; Myers and Brower, 1969; Pliske and Eisner, 1969; Tinbergen, Meeuse, Boerema, and Vorossieu, 1942), some Noctuidae (Birch, 1970a; Clearwater, 1972; Grant, 1970), and the plutellinid *Acrolepia assectella* Zeller (Thibout, 1972) in addition to the above mentioned Phycitinids (see also Chapter 8). On the other hand, the abdominal brush organs of some male Sphingidae are everted for warning and,or defensive purposes when handled or disturbed (Birch, 1969; Grant and Eaton, 1973) but virtually nothing else is known of their function in this family. These latter observations perhaps support the suggestion by Birch (1970c) that a deterrent action of the male scent may in fact be the primitive function of male scent organs in general. This theory and others relating to the evolution of male scent functions in Lepidoptera have been discussed in detail by Birch (1970c, 1974).

It seems reasonable to suppose that the pheromone of the male *P. operculella* is secreted at least in part by the enlarged trichogen cells at the base of each of the elongated scent scales. The vesicular cytoplasm and enlarged nuclei, together with the large apical microvilli-lined vesicles at the bases of these scales support this conclusion. The scent gland cells of other male Lepidoptera are modified trichogen cells (Percy and Weatherston, 1974), and the supposed sex pheromone organs of other male Lepidoptera have been described as having prominent nuclei and microvilli-lined reservoirs (Eltringham, 1913; Percy and Weatherston, 1974). Also, in those male Lepidoptera which have been shown to produce pheromones, the nuclei of the glandular cells are enlarged: they may also be rounded to oval as in *Trichoplusia ni* (Hubner) (Grant, 1971), or irregularly branched as in *Danaus gilippus berenice* (Cramer) (Pliske and Salpeter, 1971), or they may show extreme branching as in the trichogen cells of Stobbe's organ, (an anterior invagination of the scent brush pockets of Noctuidae) in *Phlogophora meticulosa* (L.) (Birch, 1970a). Such microvilli-lined vesicles are frequently found in other insect glandular cells as already noted above in the discussion concerning the female pheromone glands. However, the apical vesicles in the gland cells of *P. operculella* possess end organs similar to those that occur in gland cells associated with cuticular ducts in *H. australis* and other insects (Crossley and Waterhouse, 1969a, and references therein), whereas Birch (1970a) found no trace of such an end apparatus in the glandular cells of Stobbe's organ in *P. meticulosa.* The possible functions of the filaments of the end apparatus have been reviewed by Crossley and Waterhouse (1969a) who note that they may have structurally bound enzymes on them, or have a valving action, perhaps by forming a sieve to separate toxic secretions from the gland cells. Such a valve function also seems possible in *P. operculella* since the male pheromones which have been identified in moths consist of simple terpenoids, aromatics and carboxylic acids (Birch, 1974) and these would be fairly toxic. It seems attractive, therefore, to suggest that the hollow shaft of the scent scale forms the major storage area for the male pheromone in *P. operculella*, and that backflow of the chemicals is prevented by the end apparatus. Perhaps structurally bound enzymes on the end apparatus filaments also form the pheromone from a less toxic precursor. In contrast, the lack of an end apparatus in the glandular cells of Stobbe's organ in *P. meticulose* is possibly related to the fact that the secretion of these cells is completely discharged soon after the adult hatches from the pupa, and that the cells are inactive thereafter.

The cytoplasm of the glandular trichogen cells under the light microscope appears homogeneous in *T. ni* (Hübner) (Grant, 1971), but the cells of Stobbe's organ in *Pseudaletia separata* (Walker) contain two types of vacuoles which contain different materials. Under the electron microscope, the cytoplasm of Stobbe's gland in *P. meticulosa* has numerous vacuoles close to the microvilli of the apical vesicle and these increase in size nearer the microvilli (Birch, 1970a). Birch (1970a) therefore suggested that these contained the substance which is secreted into the apical vesicle because he observed a material in some of the vacuoles with similar staining properties to that in the apical vesicle. Small vacuoles also occur in the cytoplasm of the scent scales of *D. gilippus* at least in the pupa when the scent scale dust (see below) is forming (Pliske and Salpeter, 1971). Presumably some of the vacuoles observed in the scent glands of *P. operculella* in the present study also contain components or precursors of the secretion in the apical vesicles, but there is as yet insufficient evidence to state that this is the case.

Little information, other than that mentioned above in connection with the apical vesicle, is available about the ultrastructure of male pheromone glands of Lepidoptera. Birch (1970a) states that the basement membrane of the glandular cells of Stobbe's organ in *P. meticulosa* is 0.05μ thick and very electron dense. A large amount of granular material, probably ribosomes, and endoplasmic reticulum is present in the cytoplasm but there is a total absence of mitochondria. Also there is a large number of microtubules in the lateral walls of these cells. In contrast, the cytoplasm of the glandular trichogens of *D. gilippus* is crowded with mitochondria and ribosomes, and microtubules are tightly packed around the microvilli-lined vesicles (Pliske and Salpeter, 1971). It therefore appears that the structure of the trichogen cells of *P. operculella* is closer to that of the gland cells of *D. gilippus* than to that of the Stobbe's organ cells of *P. meticulosa*, since the former possess mitochondria. It is also interesting to note that the relatively limited development of the endoplasmic reticulum does not exclude the possibility that this system plays some part in the synthesis of secretion according to Crossley and Waterhouse (1969a) These latter authors also mention that microtubules can have a direct strengthening role or they can act independently as templates for the oriented deposition of strengthening materials, and a strengthening function seems likely in the trichogen gland cells of *P. operculella* as well as in *D. gilippus* and *P. meticulosa*, since these cells are each somewhat like a bag surrounding a large apical vesicle.

Presumably the folds in the basal cell membrane of the secretory trichogen cells of male *P. operculella* serve to increase the surface area of the cell next to the haemolymph and facilitate greater transport of materials across it. Pliske and Salpeter (1971) make no mention of folds in this membrane in *P. gilippus*, and none occur in the walls of Stobbe's organ cells in *P. meticulosa* according to Birch (1970a). However, a thin peripheral basal zone has been described in the glandular trichogens of some male Hesperidae by Barth (1954), and male *T. ni* by Grant (1971). Grant (1971) also noted that there was a suggestion of loose microvilli within this zone in *T. ni*, but that its structure could not be determined by light microscopy. It seems possible that this zone in the gland cells of all these moths is formed by folds in the cell membrane similar to those in *P. operculella.*

The mechanism of evertion of the scent brushes of *P. operculella* was not investigated. However, the paucity of muscle fibres in the region of the bases of the scent scales suggests that direct muscular action is not involved. Possibly these muscles simply bend the cuticle of this region of the wing outward so that the scent scales become splayed. Such an action has been suggested for the muscles at the base of the scent brushes in *P. meticulosa* and *P. separata* by Birch (1970a) and Clearwater (1972) respectively. Blood pressure may also play a part in evertion in *P. operculella* and this has been suggested to cause evertion of the scent brushes in *D. gilippus* by Pliske and Salpeter (1971). In addition, it is possible that static electrical charges may help to disperse the scent scales in *P. operculella* since they seem to acquire electrical charges easily. Further research is, however, necessary to determine the exact mechanisms involved in evertion of these scales in *P. operculella.*

The pheromone of *P. operculella* presumably diffuses through the cuticle of the scent scales since no pores could be found in it. Pores do occur in the scent brush scales of the Sphingid *Manduca sexta* (Johannson) although the trichogen cells at the base of these scales atrophy soon after the adult hatches from the pupa. On the other hand no pores could be found on the pocket scales of this insect, and their trichogens are active throughout adult life so that they presumably secrete the scent (Grant and Eaton, 1973). Also, the scent brush scales in *M. sexta* resemble the long scales around the edge of the wing of *P. operculella*, whilst the scent pocket scales only have indistinct longitudinal ridges on their surfaces. The scent brush scales of *P. meticulosa*, in contrast, are much more complex than those of *P. operculella.* Birch (1970a) describes them as consisting of three regions grading from proximal longitudinal ridges with regular pits between them, to a distal region where the ridges are produced into a lattice with an irregular network of struts below. A number of other Noctuidae also have scent brush scales with a similar surface structure

(Birch, 1970a). However, the trichogens at the base of these scales in *P. meticulosa* are not glandular, but instead Stobbe's organ secretes a precursor to the pheromone and there is some evidence that a secretion from the glandular trichogens at the bases of the pocket scales is essential for the development of the scent. It is also interesting to note that the final scent found in the pockets of *P. meticulosa* is sufficient to only charge the brushes at least three times and that this limits the number of times the male can mate successfully (Birch, 1970a, 1970b).

Grant and Eaton (1973) have suggested that the highly sculptured scale surface provides a reservoir for storage of volatile secretions before the brushes are displayed, and increases their surface area to provide a large evaporative surface when the brushes are everted. Such functions may also equally well apply to the scent scales of *P. operculella*.

The fact that the scent scales in *P. operculella* stain with eosin in contrast to other cuticle may indicate some chemical difference in the cuticle although it is also likely that the secretion, or some component of it, is trapped in the cuticle and it is this which stains. Certainly the vesicle at the base of the scale is also eosinophilic. Weatherston and Percy (1969) reported that the tip of the "club pencils" of *V. edmundsae* are slightly eosinophilic, and that the scent scales on the wings contain tiny eosinophilic granules. According to Pliske and Salpeter (1971), the eosinophilic granules that occur on the outside of the scent scales of *D. gilippus* are the "hairpencil dust" particles which contain the pheromone. Recently the courtship behaviour, of Danaiinae, together with the chemicals involved and their actions have been reviewed in detail by Birch (1974).

It appears that the hypodermal cells between the scent scales in the male *P. operculella* are secretory since their cytoplasm has a similar ultrastructure to that of the glandular trichogens, and is very different from that of hypodermal cells elsewhere in the wing. Since, however, these hypodermal cells are small, and their nuclei are also small, it is probable that they may only secrete a small quantity of material. If they are in fact secretory, two possible functions for the secretion seem likely: firstly it may be a precursor of the pheromone or some component of it which is absorbed by the trichogens and further modified by them, or secondly it may be liberated onto the surface of the wing where it either reacts with the secretion from the trichogens to form the pheromone, or forms a component of the pheromone. In support of the latter possibility, Birch (1970a), as already mentioned above, obtained an indication that Stobbe's organ in *P. meticulosa* secretes a precursor to the pheromone, and that this is acted upon by another secretion which probably originates from the pocket scale trichogens to form the pheromone. Thus the contents of Stobbe's organ may be a substance which is non-toxic to the gland cells, and the toxic pheromone is formed on the outside of the cuticle. Such a mechanism could possibly also occur in *P. operculella*. In addition, the pheromones which have been identified in a number of male Lepidoptera consist of two or more components (Birch, 1974) and it is quite conceivable that the pheromone of *P. operculella* similarly has a number of components, and that these may be secreted by different cells. Perhaps the large necrotic cells of unknown function which occur between the glandular trichogen cells in *P. operculella* produce another secretion during the pupal stage, and then subsequently degenerate to form a reservoir for the secretion. Small deeply staining nuclei have also been reported to occur near the bases of the wing scent scales of male *V. edmandsae* by Weatherston and Percy (1969), and near the bases of the scent scales of *D. gilippus* by Pliske and Salpeter (1971), which possibly belong to necrotic cells similar to those observed in *P. operculella*. Further research is, however, necessary on the development and histochemistry of the various cells in the region of the scent scales before any definite conclusions can be drawn concerning their function.

In conclusion, the probable mechanisms for the production and liberation of the pheromone by the male *P. operculella* are as follows. The scent scale trichogen cells produce either the entire pheromone, a major component of it, or a precursor of it and this is stored in the hollow shafts of the scales. It diffuses through the cuticle of the scale onto its surface and a second secretion from the hypodermal cells may be added to it which may react with the scent scale secretion to form the final pheromone. The pheromone then adheres to the rough sculptured surface of the scent scales, and when the brushes are everted a large surface area of pheromone is exposed resulting in the rapid liberation of a relatively large amount of it. This is then dispersed by the beating wings and turning movements of the male, thus helping to ensure that the female is exposed to the pheromone.

SUMMARY

The reproductive organs, sexual behaviour, and pheromone glands were studied separately in the New Zealand grass grub beetle, *Costelytra zealandica* (White) (Scarabaeidae: Melolonthinae), and the potato tuberworm moth, *Phthorimaea operculella* (Zeller) (Gelechiidae: Gnorimoschemini).

In *C. zealandica*, sexually dimorphic features were noted and the male and female external genitalia were described in more detail than previously. An account was given of the muscles of the external genitalia and the mechanics of copulation: intromission was found to be effected by the internal sac when everted by fluid pumped from within the muscular sheath of the ejaculatory duct. The first account of the morphology and histology of the internal reproductive organs was given although the gonads were not treated in detail. The internal reproductive organs were found to show only minor differences from those of other Scarabaeidae; the most notable being that the male has a pair of connective tissue sheaths binding the accessory gland reservoirs to the vesiculae seminales, and the female has an anterior region in the median oviduct which is histologically similar to the lateral oviducts and the accessory glands are not differentiated into regions. A description was also included of the female accessory glands which are known to contain bacteria that produce the sex pheromone (phenol), together with a preliminary identification of these bacteria.

The first account of the sexual behaviour of *C. zealandica* was given. Precopulatory behaviour was simple: attractive females had no characteristic posture: the responses of males included flying in a horizontal zigzag and,or running towards the female whilst periodically beating their wings. Copulation lasted a mean time of 28 minutes at 12°C but was very variable. Many males remained *in copula* for some time after completing spermatophore transfer.

Mating occurred most frequently during the dusk flights, and thereafter the proportion of copulating pairs diminished until few were found after 2 to 3 hours of darkness. The beetles flew most often at dusk, less frequently at dawn, and rarely during the night. The influences of environmental factors on flight and mating were noted. Light intensity was found to have an important influence on the time when the beetles flew: peak flight occurred between 1 and 10 lux at dusk, and

apparently between 10 and 50 lux at dawn.

Sexual attraction in *C. zealandica* was investigated in the field using sticky traps. Confirmation was obtained that phenol attracts males and it was shown that extracts of females were similarly attractive. There was also an indication that phenol may be weakly attractive to females. The sex attractant was demonstrated to be most effective when located near the edges of tall edible plants where the beetles swarm. Attraction to phenol was positively correlated with temperature, and inversely correlated with wind speed.

All attempts to develop a laboratory behavioural bioassay for the female sex pheromone of *C. zealandica* were unsuccessful. Field experiments indicated that this was partly due to the beetles not being given suitable visual stimuli (probably irregular silhouettes) for them to fly towards.

In *P. operculella* sexual dimorphism and the male and female external genitalia were described in more detail than previously. The first description was also given of the morphology and histology of the internal reproductive organs. These showed close similarities with those of other Lepidoptera except that the bulla seminalis is only a dilation of the ductus seminalis.

The sexual behaviour of *P. operculella* was described for the first time. Attractive females adopted a resting posture with ovipositor extended. Male precopulatory behaviour included flying and,or running towards the female, circling movements, rapid wing beating, and everting their hindwing hairpencil organs. The hairpencil scent appeared to subdue the escape responses of the female. No other male pheromone glands were found by histological methods. Copulation usually lasted from 3 to 4 hours at 20°C but was very variable, and it took longer at lower temperatures.

The time of day when *P. operculella* was sexually active was investigated both in the laboratory and in the field. Mating occurred most frequently within one hour of the onset of darkness, and less frequently at dawn at 10°C to 20°C. At constant temperature, the proportion that mated after nightfall decreased at progressively lower temperatures, whereas the proportion that mated at dawn remained constant. Mating was almost entirely inhibited at 1°C in the laboratory whilst no attraction occurred in the field below about 10°C presumably because flight was inhibited. However, sexual activity occurred if the temperature rose provided that the rise did not coincide with the onset of darkness, or did not take place in the middle of the day. It was shown that the time of day when mating took place was determined by the female when she released her sex pheromone and that the male would respond at any time of day although this was maximal at the times when females were attractive, at the onset of darkness and at dawn.

An objective and quantitative laboratory bioassay was developed for the female sex pheromone of *P. operculella*, utilising the upwind attraction of males and their sexual displays. The presence of a female sex pheromone was conclusively demonstrated, but attraction was inhibited at 1 lux and occurred at 0.1 lux or lower. The attractant was shown to be produced in the middle third of the ovipositor by bioassay and a histological search confirmed that the pheromone gland is a dorsal sac-like region of the intersegmental membrane between the 9th and 10th abdominal segments on the ovipositor.

The male and female pheromone glands of *P. operculella* were described in detail. The cuticle of the female gland contains an unusual layer of endocuticular cavities and its surface differs from that of cuticle elsewhere. The ultrastructure of the female gland cells also differs from that of normal hypodermis in the greater development of apical microvilli and smooth endoplasmic reticulum, and in the possession of large apical microvilli-lined vesicles, enlarged nuclei, mitochondria associated with various membrane complexes, and myelinated figures of concentrically arranged membranes.

The male hindwing glands of *P. operculella* each consists of a mean of 177 elongated scent scales and a shallow pocket. The scent scales have a characteristic surface structure with longitudinal ridges about 1.35µ apart, large transverse ridges about 0.7µ apart, and small transverse ridges about 0.05µ apart. The glandular region lies at the base of the scent scales and consists of secretory trichogen cells and possibly hypodermis. Each scent trichogen has a large microvilli-lined vesicle invaginated from the hollow scent scale, a vesicular cytoplasm containing a number of different vacuoles, and an enlarged nucleus. The cytoplasm of the hypodermis cells is similarly vesicular and differs from that of normal hypodermis. Large necrotic cells of unknown function were also found occasionally between the trichogens.

Appendix 1.1

Plants eaten by adult *C. zealandica*

Introduction

Included here are observations on the plants hovered around, alighted on, and eaten by *C. zealandica* in the field, together with the results of an experiment to find which of these were also eaten in the laboratory. This was carried out to determine whether the edibility of a plant is important with respect to the hovering or alighting behaviour of the beetles. In addition, a number of other plants, not present at the locations where *C. zealandica* was studied in the field, were similarly tested for their edibility in the laboratory. This was done for its intrinsic interest since large numbers of adults were readily available for such experiments during the flight season.

Methods

Adult *C. zealandica* were collected from hedges after the dusk flights and kept in groups of 100 in plastic boxes, 30cm by 20cm by 10cm high, with tightly fitting lids. The boxes were provided with a layer of soil about 3cm deep in which the beetles could burrow. Each group of beetles was also kept only for 9 days and then discarded. Three days after their collection, 5 to 10 shoots of different plants were placed in the boxes. These were examined the following day and those that were chewed were considered to be eaten and were replaced with different plants. Those that showed no signs of having been eaten were left for a further two days and examined daily, and if, at the end of this period, they still showed no signs of having been eaten they were considered inedible and were replaced with other plants.

Results

The plants identified from the localities where *C. zealandica* was studied in the field are listed in Table A1.1 together with the behaviour of the beetles towards them and whether they ate them in the laboratory. The other plants which did not grow at these localities and which were similarly tested in the laboratory are listed in Table A1.2

Table A1.1

Behaviour associated with feeding of adult *C. zealandica* in the field.*

Plant species	Common name	Family	Edibility in Laboratory	Alighted on in field	Eaten in Field	Swarmed around
Pinus radiata D.Don	Monterey pine	Pinaceae	–	+	–	
Sequoia sempervirens Endl.	Californian Redwood	Taxodiaceae	–	+	–	
Cupressus macrocarpus Gord.	Macrocarpa	Cupressaceae	–	+	–	
Berberis vulgaris L.	Barberry	Berberidaceae	+	+	+	+
Beilschmiedia tawa (A.Cunn.) Benth.	Tawa	Lauraceae	+			
Rumex sp.	Dock	Polygonaceae	+	+		
Leptospermum scoparium Forst.	Manuka	Myrtaceae		+	+	
Metrosideros robusta A.Cunn.	Northern Rata	"	+			
Exalonia sp.		Escalloniaceae	+			
Malus "cv"		Rosaceae	+	+		
Eriobotrya japonica Lindl.	Loquat	"	+			
Prunus "cv"	Plum	"	+	+	+	+
Prunus dulcis D.A.Webb	Almond	"	+	+		
Prunus laurocerasus L.	Common laurel	"	+			
Prunus persica Batsch.	Peach	"	+	+	+	+
Rosa "cv"	Rose	"	+			
Rubus "cv"	Blackberry	"	+	+	+	
Acacia baileyana F.Muell.	Cootamundra wattle	Fabaceae	+			
Trifolium pratense P.Pu.	Red clover		+			
Sophora microphylla Ait.	Kowhai	Papilionaceae	+			
Alnus glutinosa Gaertn.	Common alder	Betulaceae	+			
Quercus robur L.	English oak	Fagaceae	+			
Citrus sp.	Grapefruit	Rutaceae	+	+		
Poncirus trifoliata R.A.F.	Japanese bitter orange		+			
Camellia "cv"	Camelia	Theaceae	+	+	+	+
Acer palmatum Thunb.	Japanese maple	Aceraceae	+	+	+	+
Ilex aquifolium L.	Common holly	Aquifoliaceae	+	+	+	+
Salix alba L.	Willow	Salicaceae	+	+	+	+
Salix matsudana Koidz	Twisted willow		+	+	+	+
Populus nigra L.	Black poplar	"	+	+	+	+
Rhododendron "cv"	Rhododendron	Ericaceae	+	+	+	
Ligustrum ovalifolium Hassk.	Oval-leaf privet	Oleaceae	+	+	+	
Viburnum tinus L.		Caprifoliaceae		+		
Leycesteria formosa Wall.		"	+			
Senecio jacobaea L.	Ragwort	Asteraceae	+	+		
Plantago lanceolata L.	Plantain	Plantaginaceae	+			

TABLE A1.2

Plants eaten by adult *C. zealandica* in the laboratory in addition to those tested in Table A1.1 above*.

Plant species	Common Name	Family	Edibility
Podocarpus ferrugineus G. Benn. ex D.Don.	Miro	Podocarpaceae	-
Podocarpus totara G. Benn. ex Don.	Totara	"	-
Hedycarya arborea J.R. et G.Forst.	Pigeonwood	Monimiaceae	+
Macropiper excelsum (Forst.f) Miq.	Kawakawa	Piperaceae	+
Melicytus ramiflorus J.R. et G.Forst.	Mahoe	Violaceae	+
Knightia excelsa R. Br.	Rewarewa	Proteaceae	+
Aristotelia serrata (J.R. et G.Forst.)W.R.B.Oliver.	Wineberry	Elaeocarpaceae	+
Weinmannia racemosa L.	Kamahi	Cunoniaceae	+
Carpodetus serratus J.R. et G.Forst.	Putaputaweta	Escalloniaceae	+
Nothofagus menziesii (Hook.f) Oerst.	Silver beech	Fagaceae	+
Cyathodes juniperina (J.R.et G.Forst.) Druce	Mingimingi	Epacridaceae	+
Dysoxylum spectabile (Forst.f) Hook.f.	Kohekohe	Meliaceae	+
Neopanax arboreum (Murr.)Allan comb.nov.		Araliaceae	+
Schefflera digitata J.R. et G.Forst	Pate	"	+
Myrsine australis (A.Rich) Allan.	Mapau	Myrsinaceae	+
Geniostoma ligustrifolium A.Cunn.	Hangehange	Loganiaceae	+
Coprosma australis (A.Rich) Robinson	Kanono	Rubiaceae	+
Coprosma robusta Raoul	Karamu	"	+
Coprosma spathulata A.Cunn.		"	-
Brachyglottis repanda J.R. et G.Forst.	Rangiora	Asteraceae	+
Vitex lucens Kirk	Puriri	Verbenaceae	+
Ripogonium scandens J.R. et G.Forst	Supplejack	Smilacaceae	+
Holcus lanatus L.	Yorkshire fog	Poaceae	+

* + = an observed positive response
- = an observed negative response
No indication = Not observed, but not necessarily a negative response

It is apparent that the imago of *C. zealandica* ate a wide variety of foliage including 49 of the 50 species of angiosperms tested, but they did not eat any gymnosperms. The beetles also showed marked differences in the amounts of various plants they consumed although no attempt was made to quantify this. It appeared, however, that in the laboratory they preferred those plants which they were observed to eat in the field. In addition, they also preferred rose although this plant was not observed to be eaten in the field. It may be significant that the rose bushes grew close together, and were overshadowed by a building so that they may not have been favourably situated for the beetles to fly towards. In addition, it was also apparent that although the beetles were often seen to fly towards gymnosperms in the field and then fly away, fewer beetles alighted on them compared with other tall plants. An exception was, however, observed in November, 1975, after the present study had been completed. This was on Mr E.R. Stack's farm near Feilding where there were a number of fields bordered almost exclusively by *Pinus radiata* D. Don. Here the beetles alighted on the pine trees in relatively high numbers of up to 10 or more on each outer cluster of needles, and this behaviour was attributed to there being a lack of other trees.

Discussion

The present investigation does provide some indication that the edibility of a plant influences the behaviour adult *C. zealandica* show towards it in the field. Thus gymnosperms which were not eaten in the laboratory were also not swarmed about or usually not settled on in the field, whereas plants which were preferred as food in the laboratory were usually swarmed about and settled on in the field. On the other hand, some factors other than edibility may influence the beetles' behaviour towards the other tall angiosperms which were eaten in the laboratory and which were usually not alighted on or eaten in the field. These plants may possibly either present an unattractive silhouette to flying beetles since they have large leaves and an open foliage, or they may not have been favourably situated for the beetles to fly towards.

It can be noted that the reports of other authors (Anon., 1927; Cockayne, 1911; Connell, 1933; Farrell and Wightman, 1972; Fenemore, 1966; Hilgendorf, 1902; Kelsey, 1951, 1957; Miller, 1924; Radcliffe and

Payne, 1969; and Thomas, 1913) support the present findings that the adult *C. zealandica* will eat a wide variety of foliage. Other Scarabaeidae which fly to trees to feed apparently also eat a wide variety of plant species as evidenced, for example, by *Amphimallon majalis* (Razoumowski) (Schwardt and Whitcomb, 1943), *Anomala aenea* Geer (Rittershaus, 1927), *Melolontha melolontha* L. (Gasser and Wiesmann, 1950; Hurpin, 1956), and *Phyllopertha horticola* L. (Rittershaus, 1927; Milne and Laughlin, 1960). In fact it appears especially advantageous that beetles which fly towards the silhouettes of trees during brief periods at dusk (see Chapter 4) should accept a wide variety of plants as food because of the restricted time available to them to find suitable host species. In addition, it is interesting that *C. zealandica* does not eat gymnosperms since this is also generally the case with other adult Scarabaeidae, although Schwardt and Whitcomb (1943), and Johnson (1954) have reported that *A. majalis* and *Polyphylla decemlineata* (Say) respectively will eat pine needles.

Appendix 1.2

Preliminary observations on the oviposition of *P. operculella*

Introduction

During studies on the longevity of *P. operculella* (Chapter 6), the opportunity was taken to determine the numbers of eggs laid per day by mated and unmated females. In addition to its intrinsic interest it was hoped that this would enable a check to be made on the success of the rearing methods by comparing total egg production with that reported in the literature. The diel pattern of oviposition was also investigated as a possible check on whether disruption of the moths behaviour was occurring during photography at night.

Methods

The methods used were those described in Chapter 6 for determining the longevity of the moths. In addition, the filter papers covering the containers were examined daily and the eggs laid on them counted and removed. The containers were also dismantled at the end of the experiments and examined for eggs laid other than on the filter papers.

The diel pattern of oviposition was determined from three groups of 10 pairs of newly emerged moths. These were kept in the observation chamber used for photographing the moths (Chapter 8; Figure 8.1). A sheet of dampened blotting paper was placed on the organdie netting and the entire apparatus was left in the room used for rearing the moths (Chapter 6). The blotting paper was then removed and examined at intervals of one hour or more for the number of eggs laid on it. Also at the end of each experiment the apparatus was dismantled and examined for eggs laid elsewhere than on the blotting paper.

Results

The numbers of eggs laid per day by each of 31 mated female *P. operculella* are shown in Figure A2.1.

It is apparent that the egg laying pattern of individual moths varied considerably. The total number of eggs laid per female varied from 19 to 199, with a mean of 99.71 (S.D. = 56.12). About half the females

Erratum

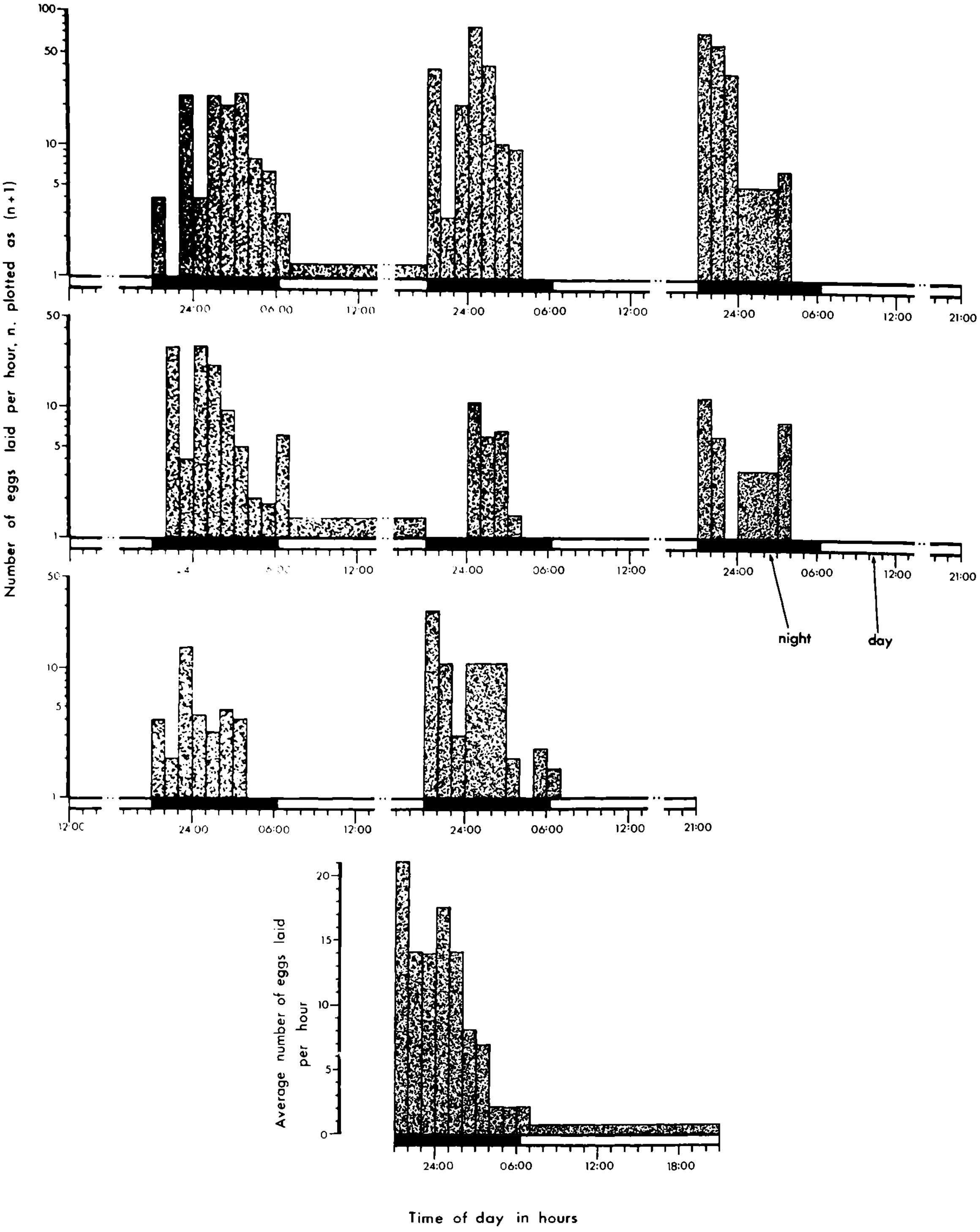
Figure A2.2

(Note; Figure A2.1 follows on page 425.)

The diel pattern of oviposition by *P. operculella*

The oviposition patterns are shown for three groups of 10 pairs of male and female moths, together with the mean oviposition pattern for all three groups.

Note that 13.06% of the total number of eggs laid are not considered here since they were not deposited where they could be counted during the experiments.


Number of eggs laid per hour, n, plotted as (n+1)
100
50
10
5
1
24:00
06:00
12:00
21:00
night
day
12:00
Average number of eggs laid per hour
20
15
10
5
0
24:00
06:00
12:00
18:00
Time of day in hours

(48.4%) began laying during the first 24 hours after they were placed with males, whereas most of the remainder (38.7%) began laying during their second day, and only a few (12.9%) started later than this. Also, the total oviposition period, including the first day of laying, varied from 1 to 17 days, with a mean of 8.3 days (S.D. = 5.0 days). On the average the females laid most of their eggs during their first day of oviposition, and the numbers laid thereafter fell rapidly so that about 80% were laid within the first 4 days of the oviposition period (Figure A2.1 C). This, together with the variation in preoviposition period noted above, accounts for the egg laying patterns for the entire population shown in Figures A2.1 A, and A2.1 B. These give the daily total number of eggs laid, and the means of the percentages of eggs laid daily by each moth respectively, and they show that both reached a peak on the third night before dropping off rapidly: about 50% of the eggs were laid within the first three days, and about 80% within the first 8 days.

In contrast the 10 virgin females examined only laid a total of 13 eggs between 4 and 23 days after they emerged from pupae (mean = 10 days; S.D. = 4.9 days).

The diel pattern of oviposition for *P. operculella* is shown in Figure A2.2. The eggs which were laid on the organdie of the observation chamber, instead of on the blotting paper, are not included, because they could not be counted until the apparatus was dismantled at the end of each experiment. They amounted to 13.06% of the total number of eggs laid.

It is apparent from Figure A2.2 that most of the eggs (98%) were laid during the night, and that during this period the pattern of oviposition showed considerable variability. Nevertheless, there was a general tendency for more eggs to be laid earlier in the night than later since almost 80% were laid in the first half of the night. However, it was concluded that the diel pattern of oviposition was not a suitable indicator of whether photography of the moths at night interfered with their behaviour patterns.

Discussion

The numbers of eggs laid by *P. operculella* during the present investigation compares well with reports of other workers. Thus Finney *et al.* (1947) noted that this insect lays between 80 and 200 eggs, and

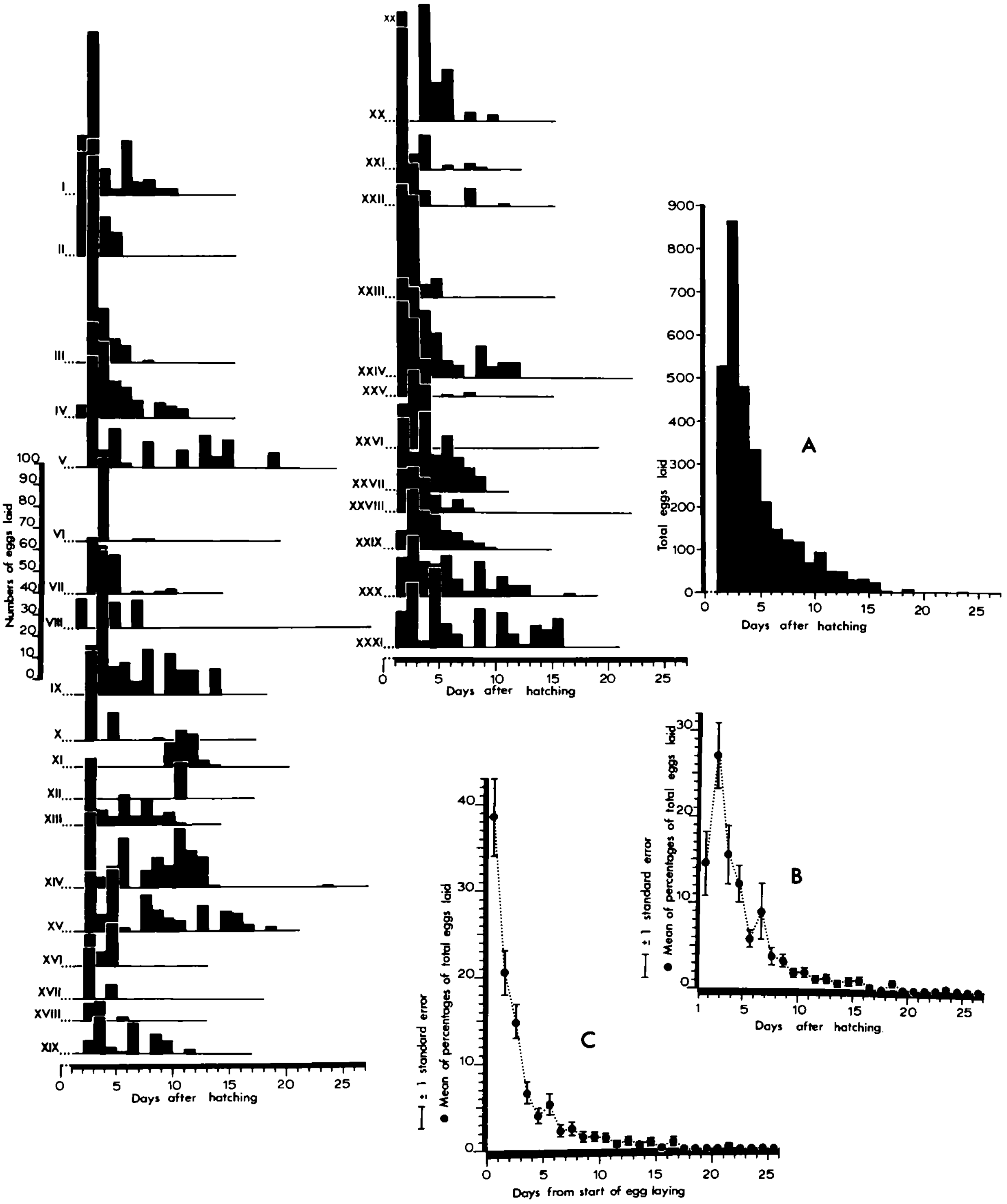
Erratum

Figure A2.1

(Note; Figure A2.2 is on page 423.)

The daily oviposition rate of *P. operculella*

This figure shows the numbers of eggs laid per day by each of 31 female moths together with the total numbers of eggs laid per day and two analyses of this egg laying behaviour: the means of the percentage of total number of eggs laid per day by each moth, and the mean percentage of eggs laid by each moth during each day of its oviposition period.


I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
Numbers of eggs laid
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Days after hatching
XX
XX
XXI
XXII
XXIII
XXIV
XXV
XXVI
XXVII
XXVIII
XXIX
XXX
XXXI
0
5
10
15
20
25
Days after hatching
A
Total eggs laid
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
Days after hatching
B
± 1 standard error
● Mean of percentages of total eggs laid
30
20
10
0
1
5
10
15
20
25
Days after hatching
C
± 1 standard error
● Mean of percentages of total eggs laid
40
30
20
10
0
0
5
10
15
20
25
Days from start of egg laying

Harwalkar, Khaine, and Rahalkar (1971) reported that they laid on the average from 76.2 to 148.1 eggs when kept at 28°C and fed 10% sucrose in water. Broodryk (1971) noted that at 21°C *P. operculella* laid an average of 71.5 eggs, with a range 0 to 232 eggs. He also determined their fecundity at 6 different temperatures between 11°C and 39°C, and found that the greatest numbers were laid at 21°C, whilst an average of 2.7 eggs per female were laid at 11°C and none were laid at 39°C. Finally, it can also be noted that Finney *et al.*(1947) found that most of the eggs were laid within the first three days of adult life (at 26.7°C), and this indicates a similar behavioural pattern to that observed in the present investigation.

It is of interest that in the present investigation, virgin females laid few eggs and that their preoviposition periods were relatively long, whereas females kept with males laid most of their eggs early in their adult lives and their preoviposition periods were usually less than 2 days. This suggests that the stimulus for egg laying is associated with copulation or the transfer of a spermatophore, such as occurs in other Lepidoptera (Outram, 1971b and references therein). Consequently it follows that most male and female *P. operculella* must therefore be able to mate within their first or second days after emerging from pupae (Chapter 8).

The diel pattern of oviposition for *P. operculella* has only been previously determined by Broodryk (1971) who kept the moths at 25.5°C and subjected them to a photoperiod of 14 hours of light to 10 hours of darkness. His results are similar to those of the present investigation except that he obtained a more pronounced peak of oviposition at the beginning of the night (about 80% of the eggs were laid in the first two hours of darkness) and no eggs were laid during the last two hours of darkness or in light (1000 lux). Broodryk (1971) also found that light intensities between 500 and 1000 lux progressively inhibited egg laying whereas low light intensities (5-8 lux) or darkness did not interfer with oviposition. This finding, therefore, appears to account for the low proportion of eggs laid in the light during the present investigation since the moths were only subjected to about 150 lux during the light periods. It would, however, be interesting to determine if high light intensities are totally inhibitory or whether it can be overridden by other stimuli such as changes in temperature as reported for *Cadra cautella* (Walker) by Hagstrum and Tomblin (1973). Certainly mating behaviour in *P. operculella* is influenced by temperature changes (Chapter 8) and so it would be especially interesting to examine this in respect to oviposition.

APPENDIX 2.1

Numbers of beetles caught in traps containing 50µl samples of 6 g/l phenol solution, blanks as empty cages, and live or squashed beetles.

DATE		BLANK SAMPLES	MALE SAMPLES	FEMALE SAMPLES	PHENOL SAMPLES
2 live virgin beetles per trap, 9 to 15 days old					
18/11/71	TRAP POSITION	G J M O S	E H L N	F I K P	
	Male catch	7 6 1 2 8	6 4 6 9	5 16 8 6	
	Female catch	1 0 0 1 0	1 1 0 2	0 2 0 1	
1 squashed virgin beetle per trap, 9 to 15 days old					
20/11/71	TRAP POSITION	E H K N Q	F J L P R	G I M O S	
	Male catch	0 0 0 0 0	1 0 3 0 0	0 0 0 0 0	
	Female catch	0 0 0 0 0	1 2 0 0 0	0 0 0 0 0	
10 live beetles per trap, collected on previous evening					
27/11/71	TRAP POSITION	E I M S	H J Q R	F L N P	G K O
	Male catch	10 11 3 8	4 5 4 8	6 2 4 4	16 19 11
	Female catch	0 2 1 0	0 0 1 0	2 0 0 0	2 1 2

APPENDIX 2.2

Numbers of beetles caught in sticky traps containing either 50 µl of 6 g/l phenol or dried samples or diethyl ether blanks of diethyl ether extracts of whole beetles.

DATE	SAMPLE PER TRAP	50ul of 6 g/l PHENOL IN WATER	DEITHYL ETHER	EXTRACT EQUIV. TO 4 MALE BEETLES	EXTRACT EQUIV. TO 4 FEMALE BEETLES
29/11/71	TRAP POSITION	E L S	H K M P	F I N Q	G J O R
	MALE CATCH FEMALE CATCH	4 12 0 0 0 0	3 4 0 2 0 0 0 0	2 2 1 3 1 0 0 0	3 1 1 3 1 0 0 0
	SAMPLE PER TRAP	50ul of 6 g/l PHENOL IN WATER	DIETHYL ETHER	EXTRACT EQUIV. TO 10 MALE BEETLES	EXTRACT EQUIV. TO 10 FEMALE BEETLES
30/11/71	TRAP POSITION	E L S	H K M P	F I N Q	G J O R
	MALE CATCH FEMALE CATCH	10 14 2 0 2 1	0 6 1 2 0 0 0 0	3 6 1 0 1 2 0 0	11 2 9 1 0 0 0 1
1/12/71	TRAP POSITION	H M Q R S	G I L P	E J O	I K N
	MALE CATCH FEAMLE CATCH	8 3 2 4 1 2 0 0 0 0	1 0 0 1 0 0 0 0	4 0 0 1 0 0	1 2 3 0 2 0

APPENDIX 2.3

Number of beetles caught in sticky traps containing dried samples of chloroform blanks or chloroform extract of whole beetles.

DATE	SAMPLE PER TRAP	CHLOROFORM BLANK									EXTRACT EQUIVALENT TO 10 MALE BEETLES								EXTRACT EQUIVALENT TO 10 FEMALE BEETLES							
23/11/72	TRAP POSITION	A	D	G	J	M	P	S	W	Z	C	F	I	L	O	R	U	Y	B	E	H	K	N	Q	T	X
	MALE CATCH	3	1	6	4	2	7	7	0	4	3	0	6	1	9	2	3	3	7	4	6	6	1	1	5	3
	FEMALE CATCH	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
24/11/72	TRAP POSITION	A	D	G	J	M	P	S	W	Z	B	E	H	K	N	Q	T	X	C	F	I	L	O	R	U	Y
	MALE CATCH	4	0	2	6	6	4	3	6	4	8	0	2	3	4	1	8	2	11	7	9	4	11	18	13	8
	FEAMLE CATCH	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0
27/11/72	TRAP POSITION	C	F	I	L	O	R	U	Y		B	E	H	K	N	Q	T	X	A	D	G	J	M	P	S	W
	MALE CATCH	3	0	0	2	3	4	9	2		6	2	7	3	1	0	8	2	4	1	4	14	24	4	5	2
	FEMALE CATCH	0	0	1	0	0	2	0	0		0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0
28/11/72	TRAP POSITION	A	D	G	J	M	P	S	W		C	F	I	L	O	R	U	Y	B	E	H	K	N	Q	T	X
	MALE CATCH	4	1	4	0	3	1	4	1		11	9	15	2	8	2	7	8	11	0	5	14	1	4	4	2
	FEMALE CATCH	0	0	0	0	0	0	0	0		0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0

APPENDIX 2.4

The times of the evening flight periods of *C. zealandica* during 1971 and 1972 together with the air temperatures and relative humidities before, during, and after the flights.

	Time of flight			Before flight			During flight			After flight		
	When 1st beetle observed flying	Approx. when last beetle stopped flying	Maximum flight period: minutes	Time	Temperature	Rel. humidity	Time	Temperature	Rel. humidity	Time	Temperature	Rel. humidity
29/10/71	1900	1920	20	1850	11		1910	10.8		1920	10.5	
6/11/71	1907	1936	29				1928	15.5	100%			
8/11/71	1910	1945	35				1923	15		1945	13	
9/11/71	1911	1932	21	1902	17	83%	1918	16	90%			
11/11/71	1914	1940	26	1906	15.5	95%						
12/11/71	1914	1938	24	1902	15	100%						
13/11/71	1909	1938	29	1906	16.5	95%				1945	15.5	100%
14/11/71	1915	1941	26	1904	14.5	95%				1945	13	90%
15/11/71	1912	1942	30	1912	15.5	80%						
16/11/71	1918	1945	27	1918	17.5	80%				1957	15	100%
18/11/71	1918	1945	27	1913	14	95%				1952	13.5	79%
20/11/71	1927	1951	24	1912	14	95%				1956	11.5	100%
21/11/71	1928	1950	22				1947	14.5	100%			
22/11/71	1927	1952	25				1950	16.25	90%			
23/11/71	1931	1955	24	1920	12.75	94%				2000	11.75	90%
24/11/71	1924	1952	28	1915	14.5	95%				1956	13	94%
27/11/71	1930	2000	30				1943	12	95%	2010	10.75	86%
28/11/71	1934	2000	26				1940	11.5	80%	2000	10.6	93%
29/11/71	1935	2007	32				1940	15.4	70%			
30/11/71	1936	1956	20				1943	13	90%			
3/12/71	1946	2010	24	1946	17.3	96%				2045	17	95%
27/10/72	1900	1918	18	1900	20.4					1900	20.4	
29/10/72	1854	1918	24							1922	20.5	
30/10/72	1906	1922	16	1859	17					1956	13	
31/10/72	1905	1925	20	1855	15.6		1915	14.8				
1/11/72	1900	1925	25	1855	17					1930	16	
5/11/72	1902	1935	33	1900	20.3		1922	18.3				
6/11/72	1904	1932	28	1856	20.3		1920	20		1940	17.6	
7/11/72	1905	1946	35									
10/11/72	1913	1940	27				1926	20.25				
14/11/72	1913	1940	27				1929	18		1952	18.6	
15/11/72	1909	1942	33				1938	17.3				
16/11/72	1912	1940	28				1922	17.8		1951	16.8	
21/11/72	1918	1957	39									
22/11/72	1921	1950	29				1948	17				
23/11/72	1926	2004	38				1945	13.5				
24/11/72	1929	1959	30							2005	9.6	
27/11/72	1924	2004	40	1900	18.5		1947	16.9				
28/11/72	1931	2007	36				1945	16.3				
3/12/72	1944	2009	25	1944	12.5		2000	12.2				
4/12/72	1935	2008	33	1922	12.7		1956	10.3				
7/12/72	1942	2006	24	1924	14.5		1958	13.1				

APPENDIX 2.5

Mean catches of male beetles from sticky traps at different positions on the hedge expressed as percentages of the mean catches of males on top of the south side of the hedge.

	NORTHERN SIDE OF HEDGE						SOUTHERN SIDE OF HEDGE					
HEIGHT OF TRAPS	2m		1m		0.15m		2m		1m		0.25m	
DATE Sample conc: g/l Phenol.	6	0	6	0	6	0	6	0	6	0	6	0
21-11-71			60.77		7.18		100.00		7.18		0.00	
22-11-71	102.78		5.56		0.00		100.00	44.44	0.00		0.00	
23-11-71	160.00	0.00	80.00	0.00	0.00	0.00	100.00	110.00	0.00	0.00	0.00	40.00
24-11-71	26.86	8.95	0.00	6.72	0.00	2.24	100.00	27.98	0.00	2.24		0.00

APPENDIX 2.6

Mean catches of female beetles from sticky traps at different positions on the hedge expressed as percentages of the mean catches of males on top of the south side of the hedge.

	NORTHERN SIDE OF HEDGE						SOUTHERN SIDE OF HEDGE					
HEIGHT OF TRAPS	2m		1m		0.25m		2m		1m		0.25m	
DATE Sample conc: g/l Phenol.	6	0	6	0	6	0	6	0	6	0	6	0
21-11-71			7.18		0.00		17.79		0.00		0.00	
22-11-71	2.78		0.00		2.78		11.11	5.56	0.00		0.00	
23-11-71	0.00	0.00	0.00	0.00	0.00	0.00	20.00	30.00	0.00	0.00	0.00	20.00
24-11-71	6.72	2.24	0.00	0.00	2.24	0.00	4.48	4.48	0.00	0.00		0.00

APPENDIX 2.7

Numbers of male and female beetles caught in sticky traps at different positions on the hedge. The traps contained 50 µl samples of water or 6 g/l phenol.

		NORTHERN SIDE OF HEDGE						SOUTHERN SIDE OF HEDGE					
	HEIGHT OF TRAPS	2m		1m		0.25m		2m		1m		0.25m	
DATE	SAMPLE; g/l PHENOL	6	0	6	0	6	0	6	0	6	0	6	0
21/11/71	TRAP POSITION			N_G N_J N_N		N_F N_I N_M		E H K		G J N		F I M	
	MALE CATCH			14 1 2		0 1 1		14 7 7		0 1 1		0 0 0	
	FEMALE CATCH			2 0 0		0 0 0		2 3 0		0 0 0		0 0 0	
22/11/71	TRAP POSITION	N_N N_I		N_G N_J		N_F N_M		I O	F M	N H K		J M	
	MALE CATCH	24 13		1 1		0 0		21 15	8 8	0 0 0		0 0	
	FEMALE CATCH	0 1		0 0		1 0		1 3	0 2	0 0 0		0 0	
23/11/71	TRAP POSITION	N_I	N_N	N_J	N_G	N_F	N_M	N F	L I	K	H	J M	O
	MALE CATCH	8	0	4	0	0	0	7 3	6 5	0	0	0 0	2
	FEMALE CATCH	0	0	0	0	0	0	0 2	1 2	0	0	0 0	1
24/11/71	TRAP POSITION	N_N	N_I	N_G	N_J	N_M	N_F	H O L	N F	H	K		J M
	MALE CATCH	12	4	0	3	0	1	52 28 54	6 14	0	1		0 0
	FEMALE CATCH	3	1	0	0	1	0	2 0 4	2 2	0	0		0 0

APPENDIX 2.8

Mean catches of male beetles from sticky traps at different positions in the field expressed as percentages of the mean catches of males on top of the hedge.

DISTANCE FROM HEDGE		0m		0.5m		1.5m		3m		6.5m		10m		20m	
SAMPLE CONC: g/1 PHENOL.		6	0	6	0	6	0	6	0	6	0	6	0	6	0
DATE	HEIGHT OF TRAPS														
12-11-71	2m	100						11.1				3.2			
	0.25m							17.5				33.3			
13-11-71	2m	100				13.1				0					
	0.25m					6.6				8.2					
14-11-71	2m	100								0				3.1	
	0.25m									6.3				6.3	
30-10-72	4m	0	0												
	2m	100	7.7					7.7	0			7.7	0		
	0.25							38.5	0			23.7	0		
31-10-72	4m		2.1	26											
	2m	100	13.4					17.6	1.0			1.0	0		
	0.25m							26.9	2.1			35.2	1.0		
6-11-72	2m	100	13	26	3.9					0	0			2.6	2.6
	0.25m			26	0					15.6	2.6			29.9	5.2
10-11-72	2m	100	34.6			4.0	2.7			0.7	0	0.7	1.3		
	0.25m					5.4	0			4.0	0	4.7	1.3		

APPENDIX 2.9

Mean catches of female beetles from sticky traps at different positions in the field expressed as percentages of the mean catches of males on top of the hedge.

DISTANCE FROM HEDGE		0m		0.5m		1.5m		3m		6.5m		10m		20m	
SAMPLE CONC: g/l PHENOL.		6	0	6	0	6	0	6	0	6	0	6	0	6	0
DATE	HEIGHT OF TRAPS														
12-11-71	2m	7.9						9.5				3.2			
	0.25m							6.3				4.6			
13-11-71	2m	8.2				6.6				3.3					
	0.25m					3.3				1.6					
14-11-71	2m	3.1								0				3.1	
	0.25m									0				0	
30-10-72	4m	0	0												
	2m	0	0					0	0			7.7	0		
	0.25m							0	0			0	0		
31-10-72	4m	0	0												
	2m	1.4	0					1.0	0			0	0		
	0.25m	0	0					1.0	0			1.0	0		
6-11-72	2m	6.5	1.3	11.9	2.6					0	0			0	0
	0.25m			1.3	0					0	1.3			1.3	0
10-11-72	2m	2.4	0.9			0	1.3			0	0	0	0		
	0.25m					0	0			0.7	0	0	1.3		

Appendix 2.10

Numbers of male and female beetles caught in sticky traps at different positions in the field. The traps contained $50 \mu l$ of either water or 6 g/l phenol solution.

DATE	DISTANCE FROM HEDGE	0m			3m			3m			10m			10m												
13-11-71	HEIGHT ABOVE GROUND	2m			2m			0.25m			2m			0.25m												
	TRAP POSITION	E	O	I	$^{3}_{P}$	$^{3}_{H}$	$^{3}_{J}$	$^{3}_{P}$	$^{3}_{H}$	$^{3}_{J}$	$^{5}_{P}$	$^{5}_{H}$	$^{5}_{J}$	$^{5}_{P}$	$^{5}_{H}$	$^{5}_{J}$										
	SAMPLE CONC: g/l PHENOL	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6										
	Male catch	17	14	32	2	2	3	1	5	5	1	0	1	8	4	9										
	Female catch	1	1	3	1	3	2	0	3	1	1	0	1	2	0	1										
	DISTANCE FROM HEDGE	0m			1.5m			1.5m			6.5m			6.5m												
13-11-71	HEIGHT ABOVE GROUND	2m			2m			0.25m			2m			0.25m												
	TRAP POSITION	E	O	I	$^{2}_{P}$	$^{2}_{H}$	$^{2}_{J}$	$^{2}_{P}$	$^{2}_{H}$	$^{2}_{J}$	$^{4}_{P}$	$^{4}_{H}$	$^{4}_{J}$	$^{4}_{P}$	$^{4}_{H}$	$^{4}_{J}$										
	SAMPLE CONC: g/l PHENOL	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6										
	Male catch	19	15	27	7	1	0	3	0	1	0	0	0	3	1	1										
	Female catch	2	0	3	2	2	0	1	0	1	0	0	2	1	0	0										
	DISTANCE FROM HEDGE	0m			6.5m			6.5m			20m			20m												
14-11-71	HEIGHT ABOVE GROUND	2m			2m			0.25m			2m			0.25m												
	TRAP POSITION	E	O	I	$^{4}_{P}$	$^{4}_{H}$	$^{4}_{J}$	$^{4}_{P}$	$^{4}_{H}$	$^{4}_{J}$	$^{6}_{P}$	$^{6}_{H}$	$^{6}_{J}$	$^{6}_{P}$	$^{6}_{H}$	$^{6}_{J}$										
	SAMPLE CONC: g/l PHENOL	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6										
	Male catch	10	10	12	0	0	0	1	0	1	1	0	0	1	1	0										
	Female catch	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0										
	DISTANCE FROM HEDGE	0m					0m				3m				3m				10m				10m			
30-10-72	HEIGHT ABOVE GROUND	2m					4m				2m				0.25m				2m				0.25m			
	SAMPLE CONC: g/l PHENOL	6	6	6	0	0	6	6	0	0	6	6	0	0	6	6	0	0	6	6	0	0	6	6	0	0
	TRAP POSITION	H	L	N	P	J	I	M	O	K	$^{3}_{P}$	$^{3}_{J}$	$^{3}_{H}$	$^{3}_{L}$	$^{3}_{H}$	$^{3}_{L}$	$^{3}_{P}$	$^{3}_{J}$	$^{5}_{H}$	$^{5}_{L}$	$^{5}_{P}$	$^{5}_{J}$	$^{5}_{G}$	$^{5}_{J}$	$^{5}_{H}$	$^{5}_{L}$
	Male catch	11	2	0	0	1	0	0	0	0	1	0	0	0	3	2	0	0	1	0	0	0	3	0	0	0
	Female catch	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	DISTANCE FROM HEDGE	0m					0m				3m				3m				10m				10m			
31-10-72	HEIGHT ABOVE GROUND	2m					4m				2m				0.25m				2m				0.25m			
	SAMPLE CONC: g/l PHENOL	6	6	6	0	0	6	6	0	0	6	6	0	0	6	6	0	0	6	6	0	0	6	6	0	0
	TRAP POSITION	H	L	N	P	J	I	M	O	K	$^{3}_{P}$	$^{3}_{J}$	$^{3}_{H}$	$^{3}_{L}$	$^{3}_{H}$	$^{3}_{L}$	$^{3}_{P}$	$^{3}_{J}$	$^{5}_{M}$	$^{5}_{L}$	$^{5}_{P}$	$^{5}_{J}$	$^{5}_{P}$	$^{5}_{J}$	$^{5}_{H}$	$^{5}_{L}$
	Male catch	40	66	35	1	12	5	4	1	1	8	9	0	1	20	6	2	0	0	1	0	0	20	14	0	1
	Female catch	0	2	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
	DISTANCE FROM HEDGE	0m				0.5m				0.5m			6.5m			6.5m				20m			20m			
6-11-72	HEIGHT FROM GROUND	2m				2m				0.25m			2m			0.25m				2m			0.25m			
	SAMPLE CONC: g/l PHENOL	6	6	0	0	6	6	0	0	6	6	0	6	6	0	6	6	0	0	6	6	0	6	6	0	0
	TRAP POSITION	O	K	E	I	$^{1}_{P}$	$^{1}_{J}$	$^{1}_{H}$	$^{1}_{L}$	$^{1}_{P}$	$^{1}_{J}$	$^{1}_{H}$	$^{4}_{F}$	$^{4}_{J}$	$^{4}_{H}$	$^{4}_{F}$	$^{4}_{J}$	$^{4}_{H}$	$^{4}_{L}$	$^{4}_{F}$	$^{4}_{J}$	$^{4}_{H}$	$^{4}_{P}$	$^{4}_{J}$	$^{4}_{H}$	$^{4}_{K}$
	Male catch	32	43	1	9	11	9	0	3	6	14	0	0	0	0	12	0	1	1	2	0	1	13	10	2	2
	Female catch	2	3	0	1	5	4	1	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
	DISTANCE FROM HEDGE	0m							1.5m			1.5m			6.5m			6.5m			10m			10m		
10-11-72	HEIGHT FROM GROUND	2m							2m			0.25m			2m			0.25m			2m			0.25m		
	SAMPLE CONC: g/l PHENOL	6	6	6	6	0	0	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0
	TRAP POSITION	G	I	K	L	E	H	J	$^{2}_{P}$	$^{2}_{J}$	$^{2}_{H}$	$^{2}_{P}$	$^{2}_{J}$	$^{2}_{H}$	$^{4}_{P}$	$^{4}_{J}$	$^{4}_{H}$	$^{4}_{P}$	$^{4}_{J}$	$^{4}_{H}$	$^{4}_{P}$	$^{4}_{J}$	$^{4}_{H}$	$^{4}_{P}$	$^{4}_{J}$	$^{4}_{H}$
	Male catch	67	80	78	72	43	24	10	5	1	2	2	7	0	1	0	0	2	5	0	0	1	1	3	4	1
	Female catch	0	3	2	2	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1

Appendix 2.11

Relationships between the number of male beetles trapped and the average temperature and wind speed during the flight periods. Each trap with 50μl of either water or 6 g/l phenol solution. Wind speeds in m/min, and temperatures in C.

A. Constants of linear regressions relating the numbers of males caught N to the temperature T or the wind speed W, using the equations

$$N = \alpha + \beta T \text{ and } N = \alpha + \beta W$$

Selection of Data		Phenol conc. g/l	Regression Coefficient β	Standard error of β	t Value	Constant α	Correlation Coefficient τ	Number of Observations
1971, Temperature with:	all days	6	2.694***	.751	3.59	-25.54	.415***	64
	-rainy days	6	2.591***	.753	3.44	-23.49	.409***	61
	all days	0	.755*	.257	2.94	-6.48	.358***	61
	-rainy days	0	.653*	.254	2.57	-4.35	.338***	53
1972, Temperature with:	all days	6	6.440**	2.35	2.74	-58.67	.361**	52
	-27/10	6	8.674***	2.36	3.67	-89.51	.476***	48
	-27/10, rainy days	6	9.574***	2.545	3.76	-99.64	.516***	41
	all days	0	.427	.246	1.74	-1.085	.200	74
	-27/10	0	.629*	.260	2.42	-3.69	.282*	70
	-27/10, rainy days	0	.470	.254	1.85	-1.63	.242*	57
1972, Wind speed with:	all days	6	-.127	.0803	-1.58	57.72	-.220	52
	-27/10	6	-.108	.0770	-1.40	60.11	-.186	48
	-27/10, rainy days	6	-.207*	.0907	-2.28	68.88	-.359**	41
	all days	0	-.0180	.0100	-1.80	6.68	-.207	75
	-27/10	0	-.0162	.0111	-1.46	6.82	-.187	71
	-27/10, rainy days	0	-.0083	.0098	-.850	5.81	-.114	58

B. Constants of linear regressions relating the numbers of males caught N to the temperature T or the wind speed W, using the transformations

$$\text{Log } (N + 1) = \alpha + \beta T \text{ and Log } (N + 1) = \alpha + \beta W$$

Selection of Data		Phenol conc. g/l	Regression Coefficient β	Standard error of β	t Value	Constant α	Correlation Coefficient τ	Number of Observations
1971, Temperature with:	all days	6	.0658*	.0285	2.31	.028	.281*	64
	-rainy days	6	.0596*	.0272	2.19	.151	.275*	61
	all days	0	.0649**	.0242	2.60	-.356	.329***	61
	-rainy days	0	.0513*	.0213	2.41	-.073	.319**	53
1972, Temperature with:	all days	6	.0673*	.0273	2.47	.219	.330**	52
	-27/10	6	.114***	.0204	5.59	-.427	.636***	48
	-27/10, rainy days	6	.118***	.0198	5.96	-.446	.690***	41
	all days	0	.0032	.168	.019	.563	.028	74
	-27/10	0	.0202	.180	.004	.344	.175	70
	-27/10, rainy days	0	.0125	.139	.090	.448	.121	57
1972, Wind-speed with:	all days	6	-.0042***	.00077	-5.55	1.63	-.613***	52
	-27/10	6	-.0037***	.00064	-5.75	1.69	-.645***	48
	-27/10, rainy days	6	-.0036***	.00063	-5.76	1.70	-.676***	41
	all days	0	-.0008	.00043	-1.86	.669	-.177	75
	-27/10	0	-.0006	.00058	-1.04	.687	-.138	71
	-27/10, rainy days	0	-.0003	.00052	-.575	.647	-.066	58

Appendix 2.11 continued

Relationships between the number of male beetles trapped and the average temperature and wind speed during the flight periods. Each trap with 50µl of either water or 6 g/l phenol solution. Wind speeds in m/min, and temperatures in C.

D. Constants of nonparametric regression methods showing the degree of association between the numbers of males caught N, with the temperature T or the wind speed W, using modifications of Kendall's τ coefficient of rank correlation.

Selection of data	Phenol conc. g/l	Variables	Modification of Method	S	$\frac{S}{\sqrt{Var(S)}}$
1971 all days	6	N,T	Burr	477	2.589**
1971 all days	0	N,T	Burr	339	2.632**
1972 -27/10	6	N,T	Sillitto	486	2.102*
1972 -27/10,rainy days	6	N,T	Sillitto	285	3.914***
1972 -27/10	6	W,T	Sillitto	368	3.620***
1972 -27/10,rainy days	6	W,T	Sillitto	238	2.691**
1973 all days	0	N,T	Burr	24	.173

C. Constants of multiple regressions relating numbers of males caught N in traps baited with 6 g/l phenol N, to temperature T and wind speed W for 1972 using the transformation

$$\text{Log}\ (N + 1) = \alpha + \beta_1 T + \beta_2 W$$

Selection of Data*	Variable	Regression Coefficient	Standard error of Regression Coefficient	t Value	Partial Correlation Coefficient	Constant (α)	Coefficient of Determination	Multiple Correlation Coefficient	F Test
all days	Temperature	.0310	.0239	1.300	0.183	1.0850	.397	.630	16.110
	Wind speed	-.00383***	.000792	-4.835	-0.568***				
-27/10	Temperature	.0817***	.0185	4.407	0.549***	0.2749	.592	.769	32.602
	Wind speed	-.00268***	.000590	-4.544	-0.561***				
-27/10,and rainy days	Temperature	.0867***	.0170	5.117	.639***	0.2278	.678	.823	40.054
	Wind speed	.00257***	.000526	-4.880	-.621***				

Appendix 2.13

Preliminary identification of bacteria in the gut and accessory glands of the female *C. zealandica*

(Carried out by Miss N.R.C. Davis)

Gram negative rods: probably *Yersinia pseudotuberculosis*

oxidase	negative	H_2S	negative
catalase	positive	indole	negative
motility	positive	KCN	negative
glucose acid	no gas	lysine	negative
sucrose	negative	phenylalanine	negative
lactose	negative	raffinose	negative
ONPG	positive	rhamnose	positive
mannitol	positive	salicin	doubtful
mannose	positive	trehalose	positive
maltose	positive	urea	positive
aesulin	positive	xylose	positive
arabinose	positive	Voges Proskaur	negative
cellobiose	positive	phanylalanine deamination	negative
citrate	negative	nitrate	positive

Gram negative coccobacilli: *Acinetobacter* species

non-motile; oxidative; indole negative; nitrate negative

Gram negative coccobacilli: *Enterobacter* species

Staphylococcus species and *Micrococcus* species

Coagulose negative

Appendix 2.14

Numbers of *P. operculella* attracted to female moths in sticky traps at various times during the day.

Numbers of moths caught

Date	Time	Temp. °C min.	max.	Each trap baited with 7 virgin females						blank traps		
17/2/72	1600	11	18.5	0	0	0	0	0	0	0	0	0
	1800	9	11	0	0	0	0	0	0	0	0	0
	1900	9	12	0	0	0	0	0	0	0	0	0
	2000	9	10	0	0	0	0	0	0	0	0	0
	2200	10	11	0	0	0	0	0	0	0	0	0
	2400	7	10.5	0	0	0	0	0	0	0	0	0
18/4/72	0200	8.5	10	0	0	0	0	0	1	0	0	0
	0400	9.5	10	0	0	0	0	0	0	0	0	0
	0600	9	10	0	0	0	0	1	0	0	0	0
	0800	9	22	5	0	0	0	0	0	0	0	0
	1900											
20/4/72	1300	18	25	5	0	1	0	22	11	0	0	0
	1330	18	26	0	0	0	0	0	1	0	0	0
21/4/72	0130	17	23	0	0	0	0	0	1	0	0	0
	0330	12	18	0	0	0	0	0	0	0	0	0
	0530	8	12	0	0	1	0	1	0	0	0	0
	0730	7	9	0	0	0	0	0	0	0	0	0
	0930	7	10	0	0	0	0	0	0	0	0	0
	1130	9	10.5	2	0	1	0	15	0	0	0	0
	1530	7.5	11	0	0	1	0	0	0	0	0	0
	1730	4.5	10	0	0	0	0	0	0	0	0	0
	1930	10	14.5	40	0	9	2	41	50	0	0	0
	2030	14.5	26	2	0	5	5	1	3	0	0	0
	2330											

Numbers of moths caught

Date	Time	Temp. °C min.	max.	Each trap baited with 3 virgin females						blank traps		
24/2/73	1835	19	21	0	0	0	0	0	0	0	0	0
	1935	15	19	1	0	0	0	29	38	0	0	0
	2035	11	15	0	0	0	0	1	6	0	0	0
	2135	10.5	11	0	0	0	0	0	0	0	0	0
	2305	11	14	0	0	0	0	0	1	0	0	0
25/2/73	2435	8	11	0	0	0	0	0	1	0	0	0
	0435	7	8	0	0	0	0	0	1	0	0	0
	0645	7	19.5	0	0	0	0	0	0	0	0	0
	0835	19.5	31	0	0	0	0	0	0	0	0	0
	1235	30	31	1	0	0	0	1	0	0	0	0
	1605	21	30	0	0	0	0	0	0	0	0	0
	1835	17	21	0	0	0	0	0	6	0	0	0
	1925	12.5	17	0	0	0	0	10	13	0	0	0
	2110			0	0	0	0	6	1	0	0	0
26/2/73	0830											
27/2/78	1800	18	22.5	0	0	0	0	0	0	0	0	0
	1935	14.5	18	5	32	4	3	38	15	0	0	0
	2030	14	14.5	0	0	0	16	2	0	0	0	0
	2045	13	14	0	10	0	1	0	2	0	0	0
	2130	13	14	0	0	0	0	0	0	0	0	0
	2230	7.5	14	0	0	2	0	0	0	1	0	0
28/2/73	0430	9	11	2	1	0	0	0	0	0	1	1
	0600	11	21	0	0	0	0	0	0	0	0	0
	0900	21	31	0	0	0	0	0	0	0	0	0
	1300	19	37	0	0	0	0	0	0	0	0	0
	1700	17	34	0	0	0	0	0	0	0	0	0
	1920	12	17	10	7	7	0	32	37	1	0	0
	2100	7	12	0	0	0	0	1	1	0	0	0
	2300	3.5	17	0	0	0	0	0	2	0	0	0
29/2/72	0800											

Appendix 2.15

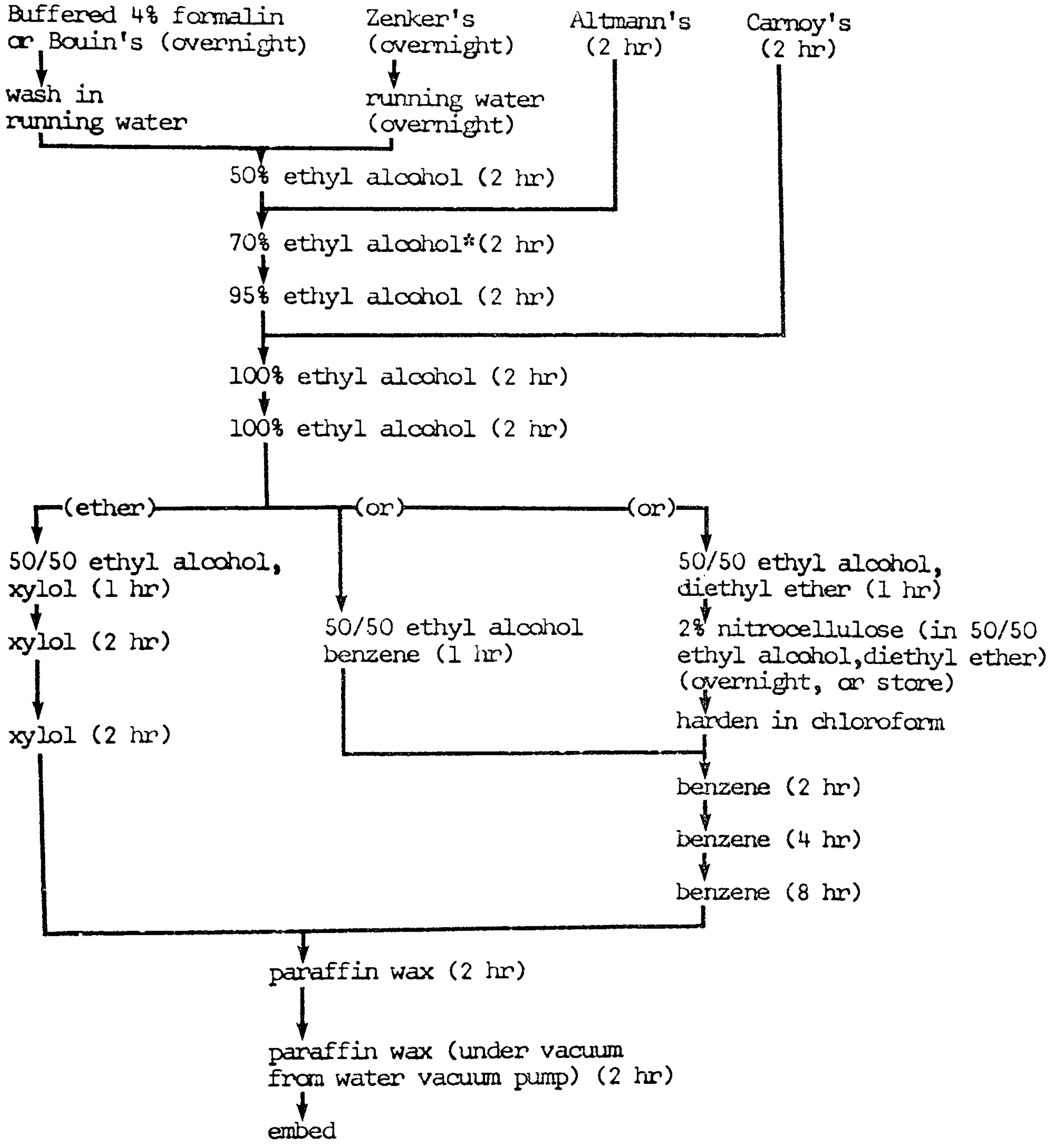
Relationship between the numbers of males responding in the bioassay and the amount of female extract.

Amount of extract equivalent females	Percent response of male moths												t-value between sample and blank
	Sample	Blank	Sample	Blank	Sample	Blank	Sample	Blank	Sample	Blank	Sample mean	S.D. Sample	
0.6	75	0	70	0	90	0	55	10	75	0	73.0	12.5	11.9***
0.12	58.3	0	57.1	0	33.3	0	11.8	0	93.3	0	50.8	13.7	3.7**
0.12	25	0	50	0	50	0	79	0	56	0	52.0	19.3	6.0***
0.06	45	0	30	0	45	0	10	0	15	0	29.0	16.4	4.0**
0.06	20	5	40	0	65	0	30	0	45	0	40.0	17.0	5.1***

** Significantly different at $P < .01$
*** Significantly different at $P < .001$

Appendix 3.1

Schedule for embedding tissue in paraffin wax or for double embedding in paraffin wax and nitrocellulose.



*with iodine if Zenker's used as fixative

Appendix 3.2

Schedule for embedding male hairpencil organs of *P. operculella* for electron microscopy

(Carried out by Mr N.G. Leet).

Initial fixation in 4% glutaraldehyde in CaCodylate buffer (pH 7.3)*

CaCodylate buffer wash:* three changes each of 1 hour

1% osmium tetroxide in CaCodylate buffer with evacuation from a water vacuum pump	24 hours
Quick rinse in water	
4% uranyl acetate CaCodylate buffer (4%)*	24 hours
75% acetone	1 hour
95% acetone	1 hour
100% acetone: three changes each of	1 hour
75/25 acetone/Spurr's**	18 hours
50/50 acetone/Spurr's	24 hours
95/5 acetone/Spurr's	24 hours
100% Spurr's with evacuation from a water vacuum pump	60 hours
Polymerisation at 60°C	

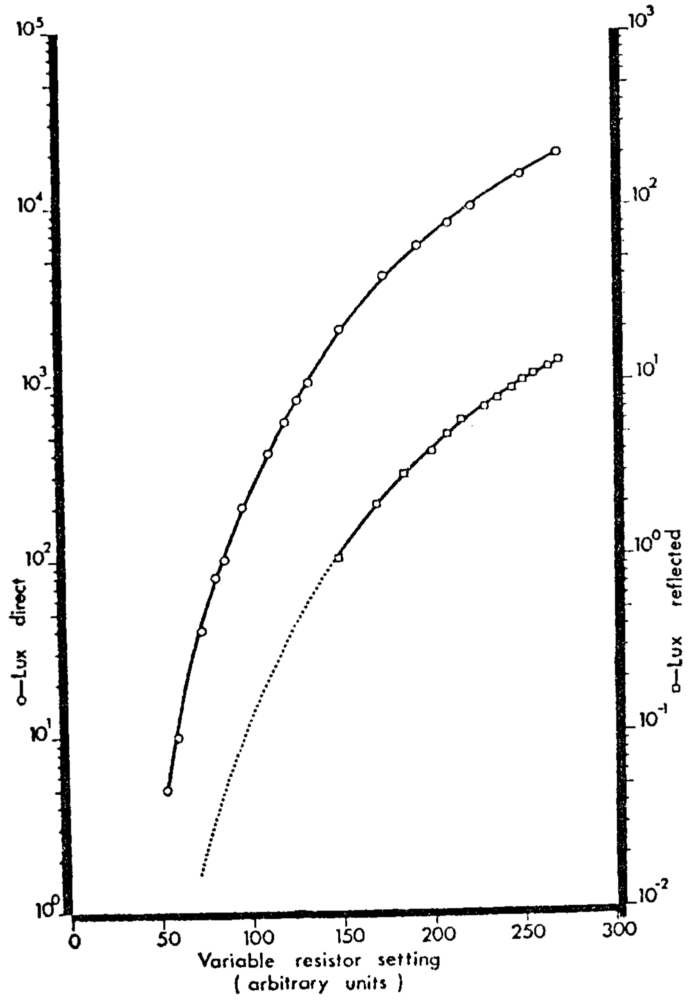
*0.05% "Decon 75" in water added dropwise until cuticle wetted

**Spurr's low viscosity epoxy embedding medium (Spurr, 1969)

Appendix 3.3

Determination of low light intensity in the bioassay room

The lux meter only had a lower sensitivity to 1 lux, and therefore a light intensity of 0.1 lux was obtained in the bioassay room by comparing the light intensity at the surface of the light bulb underneath the bioassay table (the direct light intensity) with that taken vertically upward from the centre of the bioassay table (the reflected light intensity) at different settings of the variable resistor which controlled the light bulb output. The direct light intensity was found to be a factor of about 1700 times greater than the reflected light intensity (see accompanying graph) within the measurable range of the lux meter, so this was assumed to hold true at all light intensities. The graph of reflected light intensity was accordingly extrapolated as shown, and the resistance setting was selected to give an estimated reflected light intensity of 0.1 lux (80 arbitrary resistance units).



Appendix 4

List of the insect species and their taxonomic status

Achroia grisella (Fabr.) (Lep.: Pyralidae: Phycitinae)
Acleris gloverana (Wlsm.) (Lep.: Tortricidae)
Acrolepia assectella Zeller (Lep.: Yponomeutidae)
Adoxophyes orana Fischer von Roslerstamm (Lep.: Tortricidae)
Agriphila plumbifimbriella Dyar (Lep.: Pyralidae: Crambinae)
Agrotis ipsilon (Hufnagel) (Lep.: Noctuidae)
Amphimallon majalis (Razoumowski) (Col.:Scarabaeidae:Melolonthinae)
Amphimallon solstitialis L. (Col.: Scarabaeidae: Melolonthinae)
Anagasta kuhniella (Zeller) (Lep.: Pyralidae: Phycitinae)
Anisoplia agricola L. (Col.: Scarabaeidae: Rutelinae)
Anomala aenea Geer (Col.: Scarabaeidae: Rutelinae)
Anoxa villosa Fabr. (Col.: Scarabaeidae: Melolonthinae)
Anthonomus grandis Boheman (Col.: Curculionidae)
Aphodius fimetarius L. (Col.: Scarabaeidae: Aphodiinae)
Aphodius tasmaniae Hope (Col.: Scarabaeidae:Aphodiinae)
Aphomia gularis (Zeller) (Lep.: Pyralidae: Phycitinae)
Argynnis (Lep.: Nymphalidae)
Argyrotaenia velutinana (Walker) (Lep.: Tortricidae)
Autographa californica (Speyer) (Lep.: Noctuidae)
Bolbocerosoma farctum (Fabr.) (Col.: Geotrupidae)
Bombyx mori L. (Lep.: Bombycidae)
Brachyrhinus sulcatus (Fabr.) (Col.: Curculionidae)
Cadra cautella Walker (Lep.: Pyralidae: Phycitinae)
Calliphora erythrocephala (Meig.) (Diptera: Calliphoridae)
Callosamia promethea Drury (Lep.: Saturniidae)
Carausius morosus Br. (Phasmidae: Cheleutopterae)
Ceruchus piceus Walker (Col.: Lucanidae)
Cetonia aurata L. (Col.: Scarabaeidae: Cetoniinae)
Cetonia floricola Herbert (Col.: Scarabaeidae: Cetoniinae)
Choristoneura fumiferana (Clemens) (Lep.: Tortricidae)
Choristoneura pinus (Freeman) (Lep.: Tortricidae)
Choristoneura rosaceana (Harris) (Lep.: Tortricidae)
Coccinella novemnotata (Hbst.) (Col.: Coccinellidae)
Cochliomyia hominivorax (Coquerel) (Diptera: Calliphoridae)
Colias philodice eurytheme Boisduval (Lep.: Pieridae)
Costelytra zealandica (white) (Col.: Scarabaeidae: Melolonthinae)

Cotinus nitida L. (Col.: Scarabaeidae: Cetoniinae)
Crambus harpipterus Dyar (Lep.: Pyralidae: Crambinae)
Crambus trisectus (Walker) (Lep.: Pyralidae: Crambinae)
Ctenicera aeripennis (Brown) (Col.: Elateridae)
Curculio caryal (horn) (Col.: Curculionidae)
Danais gilippus berenice (Cramer) (Lep.: Nymphalidae)
Dasychira fascelina (L.) (Lep.: Lymantridae)
Dasychira seleutica (L.) (Lep.: Lymantridae)
Dendroctonus monticolae Hopk. (Col.: Curculionidae)
Dendrolimus pini L. (Lep.: Lasiocampidae)
Desmia funeralis (Hübner) (Lep.: Pyralidae)
Diatrea grandiosella (Dyar) (Lep.: Pyralidae: Crambinae)
Diatrea saccharalis (Fabr.) (Lep.: Pyralidae: Crambinae)
Dioryctia abietella (Denis & Schiffermüller) (Lep.:Pyralidae:Phycitinae)
Diplotaxis (Col.: Scarabaeidae: Melolonthinae)
Dorcus parallelipipedus L. (Col.: Lucanidae)
Dynastes tityus (L.) (Col.: Scarabaeidae: Dynastinae)
Ephestia elutella (Hubner) (Lep.: Pyralidae: Phycitinae)
Epilachna varivestis Mals. (Col.: Coccinellidae)
Epiphyas postvittana (Walker) (Lep.: Tortricidae)
Estigmene acrea (Drury) (Lep.: Arctiidae)
Eucanthus lazarus (Fabr.) (Col.: Geotrupidae)
Galleria mellonella (L.) (Lep.: Pyralidae: Phycitinae)
Geotrupes mutator Marsham (Col.: Geotrupidae)
Geotrupes stercorosus Scriba (Col.: Geotrupidae)
Graphognathus (Col.: Curculionidae)
Grapholitha molesta (Busck) (Lep.: Tortricidae)
Gypsonoma haimbachiana (Kearfott) (Lep.: Tortricidae)
Haliocapris gigas Fabr. (Col.: Scarabaeidae: Scarabaeinae)
Hammaptera frondosata Guerin. (Lep.: Geometridae)
Harpobittacus australis (Klug) (Mecoptera: Bittacidae)
Heliothis virescens Fabr. (Lep.: Noctuidae)
Heliothis zea (Boddie) (Lep.: Noctuidae)
Hemerocampa (Lep.: Lymantridae)
Hepialus humuli L. (Lep.: Hepialidae)
Hippodamia convergens (Guer.) (Col.: Coccinellidae)
Hofmannophila pseudospretella (Staint.) (Lep.: Oecophoridae)
Holomelina immaculata (Reakirt) (Lep.: Arctiidae)
Hoplia brunnipes Bonelli (Col.: Scarabaeidae: Melolonthinae)
Hoplia caerulea Drury (Col.: Scarabaeidae: Melolonthinae)
Hoplia farinosa L. (Col.: Scaeabaeidae: Melolonthinae)

Hydrocampa nymphaeata L. (Lep.: Nymphalidae)
Hymenoplia strigosa Illig. (Col.: Scarabaeidae: Melolonthinae)
Hyphantria cunea Drury (Lep.: Arctiidae)
Hypogymia morio (L.) (Lep.: Lymantridae)
Lambdina fiscellaria lugubrosa (Hulst.) (Lep.: Geometridae)
Laspeyresia caryana (Fitch) (Lep.: Tortricidae)
Laspeyresia pomonella (L.) (Tortricidae)
Leucinodes orbonalis Guen. (Lep.: Pyralidae: Pyraustinae)
Ligyrus gibbosus (DeG.) (Col.: Scarabaeidae: Dynastinae)
Lithocolletis fitchella Clemens (Lep.: Gracillariidae)
Lucanus capreolus (L.) (Col.: Lucanidae)
Lucanus cervus L. (Col.: Lucanidae)
Lucanus placidus Say (Col.: Lucanidae)
Malacosoma disstria Hübner (Lep.: Lasiocampidae)
Mamestra configurata (Walker) (Lep.: Noctuidae)
Manduca sexta (Johannson) (Lep.: Sphingidae)
Melolontha hippocastani Fabr. (Col.: Scarabaeidae: Melolonthinae)
Melolontha melolontha L. (Col.: Scarabaeidae: Melolonthinae)
Microvalgus (Col.: Scarabaeidae: Valginae)
Musca domestica L. (Diptera: Muscidae)
Odontria (Col.: Scarabaeidae: Melolonthinae)
Onthophagus punctatus Illig. (Col.: Scarabaeidae: Scarabaeinae)
Operophthera brumata L. (Lep.: Geometridae)
Orgyia antiqua (L.) (Lep.: Lymantridae)
Orgyia leucostigma J.E.Smith (Lep.: Lymantridae)
Ostrinia nubilalis (Hübner) (Lep.: Pyralidae: Pyraustinae)
Pachypus cornutus Oliver (Col.: Scarabaeidae. Pachypodinae)
Panolis piniperda Panz. (Lep.: Noctuidae)
Passalus cornutus Fabr. (Col.: Passalidae)
Pectinophora gossypiella (Saunders) (Lep.: Gelechiidae)
Peridroma margaritosa (Haw.) (Lep.: Noctuidae)
Periplaneta americana (L.) (Blattodea: Blattidae)
Phlogophora meticulosa (L.) (Lep.: Noctuidae)
Phthorimaea operculella (Zeller) (Lep.: Gelechiidae)
Phyllopertha horticola L. (Col.: Scarabaeidae: Rutelinae)
Phyllopertha lanceolata (Say) (Col.: Scarabaeidae: Rutelinae)
Phyllophaga anxia (Le Conte) (Col.: Scarabaeidae: Melolonthinae)
Phyllotocus (Col.: Scarabaeidae: Melolonthinae)
Pinotus carolinus (L.) (Col.: Scarabaeidae: Scarabaeinae)
Platyceras virescens (Fabr.) (Col.: Lucanidae)

Platynota stultana Walsingham (Lep.:Tortricidae)
Plectris aliena Chapin (Col.:Scarabaeidae: Melolonthinae)
Pleocoma oregonesis Leach (Col.: Scarabaeidae: Pleocominae)
Plodia interpunctella (Hübner) (Lep.: Pyralidae: Phycitinae)
Podosesia syringae froxini (Lugger) (Lep.: Sesiidae)
Podosesia ayringae syringae (Harris) (Lep.: Sesiidae)
Polydrosus (Col.: Curculionidae)
Polyphylla decemlineata (Say) (Col.: Scarabaeidae: Melolonthinae)
Popilius disjunctus (Illig.) (Col.: Passalidae)
Popillia japonica Newman (Col.: Scarabaeidae: Rutelinae)
Porthetria dispar (L.) (Lep.: Lymantriidae)
Prionoxystus robiniae (Peck) (Lep.: Cossidae)
Prionus laticollus Drury (Col.: Cerambycidae)
Prodenia litura Fabr. (Lep.: Noctuidae)
Pseudaletia separata (Walker) (Lep.: Noctuidae)
Pseudolucanus capreolus (L.) (Col.: Lucanidae)
Pyronota (Col.: Scarabaeidae: Melolonthinae)
Rhopaea heterodactyle soror Blachburn (Col.: Scarabaeidae:Melolonthinae
Rhopaea magnicornis Blackburn (Col.: Scarabaeidae: Melolonthinae)
Rhopaea verreuxi Blanchard (Col.: Scarabaeidae: Melolonthinae)
Rhyacionia buoliana (Schiffermüller) (Lep.: Tortricidae)
Romaleum rufulum (Hald.) (Col.: Cerambycidae)
Samia cynthia (Drury) (Lep.: Saturniidae)
Scarabaeus sacer L. (Col.: Scarabaeidae: Scarabaeinae)
Scarabaeus semipunctatus Fabr. (Col.: Scarabaeidae: Scarabaeinae)
Serica (Col.: Scarabaeidae: Melolonthinae)
Sisyphus schafferi (L.) (Col.: Scarabaeidae: Scarabaeinae)
Spodoptera exigua (Hübner) (Lep.: Noctuidae)
Stegasta bosquella (Chambers) (Lep.: Gelechiidae)
Synanthedon pictipes (Grote & Robinson) (Lep.: Aegeriidae)
Tineola bisselliella (Hummel) (Lep.: Tineidae)
Tischeria badiiella (Chambers) (Lep.: Tineidae)
Tischeria tinctoriella (Chambers) (Lep.: Tineidae)
Trichoplusia ni (Hubner) (Lep.: Noctuidae)
Vitula edmandsae (Packard) (Lep.: Pyralidae: Phycitinae)
Zygaena (Lep.: Zygaenidae)

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