Seasonal Comparison of Lipid Composition and Metabolism in Parasitised and Non-Parasitised Clover Root Weevil (*Sitona lepidus*)

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Abstract

Since its arrival in New Zealand the clover root weevil (*Sitona lepidus* (syn. *flavescens*) (Coleoptera: Curculionidae) (CRW) has caused serious damage to New Zealand's agricultural sector. The introduction of the biocontrol agent, *Microctonus aethiopoides* has caused a significant reduction in the population of CRWs.

During their research on the CRW, AgResearch scientists discovered that the abdominal fat body and lipids present in the haemolymph in adult CRW varied with season, sex, insect age and parasitism. Parasitism has been reported to change the lipid composition of other species of insects. The purpose of the present study was to compare the chemical composition of the lipids present in parasitised and non-parasitised CRW adults, and determine how these lipids change with physiological state and parasitoid development. The investigation into how *M. aethiopoides* initiates these changes was extended to examine the roles of juvenile hormones and teratocytes in lipid regulation.

A one-step method of extraction and derivatisation was created to determine the fatty acid profile of CRWs. This method gave higher recovery percentages and higher reproducibility than the traditional two-step methods that were trialled, and enabled individual CRWs to be analysed. The fatty acid profile of the CRW was similar to that of other insects reported containing mainly the 16 carbon saturated and monounsaturated fatty acids and the 18 carbon saturated, monounsaturated and polyunsaturated fatty acids.

The one-step method was also used to track differences in the fatty acid profiles of individual CRWs with differing sex, age, parasitism and physiological state. The fatty acid profiles of male and female CRWs were similar, with only significant differences between the concentrations of the 16:1 and 18:0 fatty acids. Due to variation between individual samples in the entire sample set, no obvious correlations were found between physiological state and fatty acid composition, or distinctions between state of parasitism and fatty acid profile. Basic statistical methods were utilised initially, however, the complexity of the data set required

multivariate analysis. PCA, LDA and QDA were utilised but again no correlations or distinctions were found when the whole sample set was analysed. Results from the Gisborne subset, collected from the same location and at the same time, had reduced variation between individuals, and this allowed some distinctions to be made between parasitised and non-parasitised samples.

Teratocytes are cells that have dissociated from the serosal membrane that occur in the haemolymph of CRWs that have been parasitised by *M. aethiopoides*. The fatty acid composition of these cells was investigated using the one-step method and a MALDI-TOF spectrometry method, which detects triacylcglycerols. The fatty acid profile of teratocytes was not significantly different to that of the CRW.

Juvenile hormones (JHs) control postembryonic development and adult reproduction. They are present in all insects and JH III is the most common of the six possible JHs. The LC-MS method of Miyazaki *et al* was modified and this allowed for the determination of JH III within samples of 50 CRW adults. The comparison between parasitised and non-parasitised samples found that parasitised samples had significantly higher levels of JH III than did their non-parasitised counterparts.

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List of Abbreviations

alr	Additive log-ratio
APCI-MS	Atmospheric pressure chemical ionisation-mass spectrometry
BHT	Butylated hydroxytoluene
ca	Approximately
CI	Chemical ionisation
clr	Centred log-ratio
CRW	Clover root weevil (*Sitona lepidus*)
DAG	Diacylglycerol
DHB	2,5-Dihydroxybenzoic acid
ERMA	Environmental Risk Management Authority
ESI-MS	Electrospray ionisation-mass spectrometry
FAME	Fatty acid methyl ester
FID	Flame ionisation detection
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HASNO	Hazardous Substances and New Organisms Act
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
JH	Juvenile hormone
JHE	Juvenile hormone esterase
kgDM/ha/year	Kilograms of dry matter per hectare per year
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDA	Linear discriminate analysis
m/z	Mass to charge ratio
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight mass spectrometry
MJME/kg DM	Megajoules of metabolisable energy per kilogram of dry matter
MS	Mass spectrometry
MS^2	Tandem mass spectrometry (MS/MS)

PC	Principal component
PCA	Principal component analysis
PFP	Pentafluorophenyl
QDA	Quadratic discriminant analysis
RIA	Radio immuno assay
RF	Response factor
S/N	Signal to noise ratio
SPE	Solid phase extraction
TAG	Triacylglycerol
TFA	Trifluoroacetic acid
vs	Versus

1 Introduction

1.1 The Significance of Clover Root Weevil in New Zealand

1.1.1 Agriculture in New Zealand and the Importance of Clover

Agriculture is a fundamental industry in New Zealand. Agriculture and forestry exports represent over 60 percent of total exports.[(1)] Agriculture in New Zealand is pastoral based and the majority of livestock winter outside. White clover (*Trifolium repens*) is an essential pasture component in all farm systems. It is estimated to be worth $3.1 billion to the New Zealand economy, due largely to its nitrogen fixing capabilities and feed value.[(2)]

1.1.2 The Clover Root Weevil (*Sitona lepidus*)

The genus *Sitona* contains more than 100 species[(3)] throughout the world. It is distributed predominantly in Europe and North America, although some species occur in Asia.[(4-6)]

Figure 1.1. Adult clover root weevil (CRW). Photograph courtesy of AgResearch.

Clover root weevil (CRW), *Sitona lepidus* (syn. *flavescens*) (Coleoptera: Curculionidae) causes significant damage to clover which reduces its nitrogen fixing ability, growth rate and survival.[(2)] Adult CRW (**Figure 1.1**), feed on the foliage of *Trifolium* (clover) species, while the larvae attack the roots and root nodules (**Figure 1.2**).[(7)] CRW is not a substantial pest in the northern hemisphere,

therefore its biology and management had not been significantly reported prior to its discovery in New Zealand.[8; 9]

Figure 1.2. Damage caused to the foliage (left) and root nodules (right) of clover plants by CRW. Photographs courtesy of AgResearch.

1.1.3 Establishment of the Clover Root Weevil within New Zealand

CRW was first discovered in New Zealand in 1996,[10] but after stored samples were investigated it was found that CRW had been present in the Waikato in 1995. It is not known how or when the CRW arrived, or from whence they originated.[11; 12] CRW spread at an annual rate of between 10-70 kilometres per year throughout the North Island[9; 12-15] (**Figure 1.3**) and by 1998 CRW had been reported as far north as Whangarei, as far south as Te Kuiti and as far south east as Reporoa. CRW were found in the southern end of the North Island (Wairarapa) in 2005. CRW was first discovered in the South Island of New Zealand in January 2006.[16; 17] CRW is the second species of the genus *Sitona* Germar (Coleoptera: Curculionidae) to become established in New Zealand. *S. discoideus* Gyllenhal (lucerne weevil) was first reported in Napier in 1974, and has caused significant damage to lucerne crops.[18; 19] CRW was quick to establish in New Zealand with larval populations that had significantly higher densities than those reported in Europe.

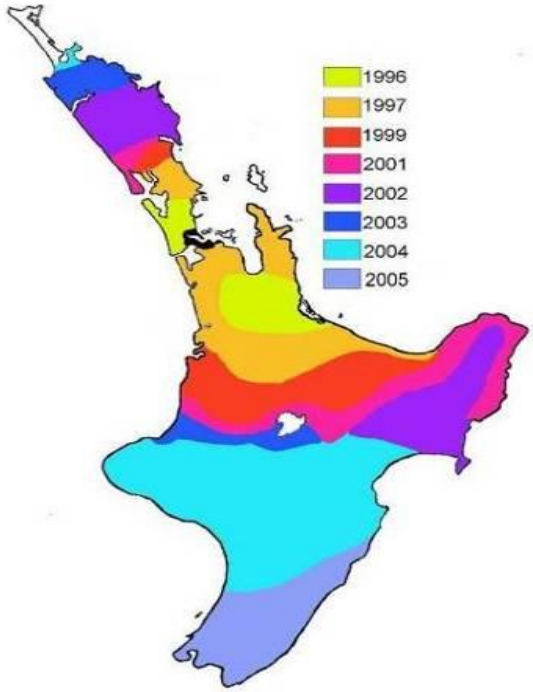


Figure 1.3. The spread of CRW throughout the North Island of New Zealand. Figure courtesy of AgResearch.

The typical densities reported in Waikato were 300 m^{-2} [10] but occasional peaks of up of to 1400 larvae m^{-2} have been reported.[17] These are far higher than the maximum reported populations of 34-90 larvae m^{-2} in England.[20; 21] The cost to New Zealand farmers was estimated to be $3 billion annually[2] due to the loss of feed and the need for additional nitrogen fertiliser.[22] Modelling a hypothetical 325 ha Waikato sheep and beef farm it was estimated that if a farmer took no remedial action, the presence of CRW would cause both a loss in pasture production (from 9200 to 7900 kgDM/ha/year) and pasture quality (from 10.5 to 10.2 MJME/kg DM). This would decrease the farm gross margin from $569/ha to $478/ha.[23] It was also estimated that the establishment of CRW would increase New Zealand's greenhouse gas emissions by 0.74 million tonnes of carbon dioxide equivalent during 2008-2012.[24]

1.1.4 Control Options

The seriousness of the threat posed by CRW meant that an effective control was of utmost importance. The widespread use of a non-specific pesticide to control CRW within New Zealand was neither environmentally or economically viable. The lucerne weevil had been controlled in Australia with insecticides directed at

adults, but this is generally unsatisfactory due to their cost and low residual effect. Use of an insecticide could also cause stock withholding issues.[25] Biological control using predators, pathogens or parasitoids was desirable. As most predators are generalist feeders, they will feed on indigenous species present in their habitat range. Therefore, most are not regarded as suitable options for introduction into NZ as classical biocontrol agents. Pathogenic diseases have been used successfully to control weevils, as well as other pasture pests.[26] The entomopathogenic fungus *Beauveria bassiana* showed potential as a suitable pathogenic disease as it controlled CRW in laboratory bioassays, but was less successful in field trials.[27] Pathogenic diseases could possibly affect indigenous species as well. A parasitoid was therefore deemed the best option for CRW control.

1.1.5 Possible Parasitoids Already in New Zealand

Introduced parasitoids have already been successful at reducing the population of two other weevils, the lucerne weevil (*S. discoideus*)[28] and the Argentine stem weevil (*Listronotus bonariensis)*[29] in New Zealand. These parasitoids were *Microctonus aethiopoides* Loan (the Moroccan biotype) and *Microctonus hyperodae* Loan respectively, and are both solitary endoparasitoids that attack the adult stage of their hosts. Both of these parasitoids were tested for their ability to parasitise CRW. It was hoped that the relatively non-host specific parasitoid *M. aethiopoides*[30] would be a suitable parasitoid for CRW, while the much more host-specific *M. hyperodae* was not expected to be a suitable parasitoid.[11] A single *M. hyperodae* was reared from CRW collected from the Ruakura Research Centre dairy farm[10] giving hope that this braconoid might be useful in controlling CRW. Unfortunately, over the following years very few *M. hyperodae* were reared from field collections of CRW, and laboratory experiments confirmed that neither *M. hyperodae* or *M. aethiopoides* were effective parasitoids for CRW.[31]

Although the established *M. aethiopoides* did not effectively parasitise CRW in New Zealand, such parasitism had been reported occurring in Europe;[4; 32; 33] up to 18% parasitism in the field had been recorded in France and Switzerland,[32] whilst in the laboratory, parasitism of *ca* 20% had been found.[34] Unfortunately

near identical methods in New Zealand gave less than 2% parasitism.[31] Phillips *et al*[34-36] reported this to be due to Moroccan *M. aethiopoides* being ineffective at parasitising CRW, rather than the New Zealand CRW being able to evade parasitism more effectively. Intra-specific differences in host preference, host range, phenology and adult morphology in *M. aethiopoides* had previously been reported,[37-39] suggesting that there are genetically distinct biotypes.[40] It was possible that these differences were due to environmental factors,[40] however, evidence of genetic variations between *M. aethiopoides* populations had been reported.[34; 41] Although parasitoids already present in New Zealand were ineffective at parasitising CRW, a number of species had been reported as parasitising adult CRW internationally (**Table 1.1**). This gave hope that a suitable biological control agent would be found.

Table 1.1. Species which have been recorded in the literature as parasitising CRW adults.[4]

Natural Enemy	Distribution	Reference
Hyalomyodes triangulifer (Loew) (Diptera: Tachinidae)	North America	Loan 1963[42]
Microctonus aethiops (Nees) (Hymenoptera: Braconidae)	Europe, North America	Mueller 1963[43]
Microctonus aethiopoides Loan (Hymenoptera: Braconidae)	Europe, North America, Australia, New Zealand	Aeschlimann 1980[32]
Microsoma exiguum Meigen (syn. *Campogaster exigua* (Meigen)) (Diptera: Tachinidae)	Europe, possibly North America	Mueller 1963[43]
Microctonus secalis (Haliday) (syn. *Perilitusceralium* Haliday (Loan 1975)) (Hymenoptera: Braconidae)	Europe	Jackson 1928[44]
Pygotolus falcatus (Nees) (Hymenoptera: Braconidae)	Europe, North America	Mueller 1963[43] Brudea 1984[45]

1.1.6 The Search for a Biological Control Agent and the Discovery of *Microctonus aethiopoides*

The search for a parasitoid for CRW led AgResearch staff and their collaborators to Europe and North America in an attempt to find a suitable biological control agent. Between 1998 and 2000, surveys of adult CRW identified five potential

braconid parasitoids, as well as a disease that infected adult CRW. *Microctonus aethiopoides* was the predominant parasitoid found and it had the highest rates of parasitism of CRW in laboratory tests with respect to the other parasitoids that had already been found.[21; 34] This European biotype, unlike its Moroccan counterpart showed potential as a biocontrol agent for CRW.[46] Samples of the European biotype from six different countries were introduced into New Zealand quarantine to determine their ability to parasitise CRW and their suitability for introduction.[33] Unfortunately the hybridisation between the Moroccan biotype already established in New Zealand and the new European biotype produced offspring that were ineffective at parasitising both the CRW and the lucerne weevil (*S. discoideus)*. This shows how important it is, when selecting a potential biocontrol agent, to ensure that the right strain is selected.[47]

Although it appeared that the European biotype would therefore not be suitable for introduction, a chance breakthrough occurred. An all-female strain of the European biotype was discovered in Ireland which reproduced via thelytokous parthenogeneticity* and was an effective parasitoid against CRW.[48] Further samples reared from CRW collected throughout Ireland all proved to be parthenogenetic, and this strain has been distinguished from other strains within the European biotype.[41] This had huge potential as a biocontrol agent due to the low risk of inter-biotype mating between the Irish thelytokous biotype and the arrhenotokous† Moroccan biotype.[49]

1.1.7 *Microctonus aethiopoides*

Microctonus aethiopoides Loan (Hymenoptera: Braconidae: Euphorine) is a solitary, arrhenotokous endoparasitoid.[41] The female *M. aethiopoides* oviposits in adult *Sitona* species and the larva develops through 4-5 instars within the active, living host. The mature larva then emerges to pupate, while the host dies.[34] *M. aethiopoides* was introduced into North America to control weevils in

*Asexual reproduction producing only female offspring.
† Asexual production of haploid males from unfertilized eggs and viable diploid females from fertilised eggs.

the genera *Sitona* and *Hypera.*[41] This parasitoid was introduced into Australia in 1977 from the Mediterranean region[50; 51] to control the lucerne weevil (*S. discoideus)*. From Australia, it was introduced to New Zealand in 1982 to also control *S. discoideus*.[52] Since its introduction into New Zealand, the Moroccan *M. aethiopoides* has been shown to attack 17 non-target species.[53]

1.1.8 Introduction of *Microctonus aethiopoides*

In 2000, *ca* 8600 CRW were collected from 15 locations in 11 European countries.[21] The *M. aethiopoides* reared from these weevils were used to infect CRW samples before sending them to New Zealand quarantine in 2000. Six "ecotypes" were established in containment to maintain genetic diversity.[33] Levels of parasitism in quarantine averaged 25% which was a lot higher than the average parasitism of 0.7% seen in field-collected CRW from Ireland.[54]

Approval for release of the Irish biotype of *M. aethiopoides* was granted under the Hazardous Substances and New Organisms (HASNO) Act in 2005. Although the Irish biotype was not technically a new organism since the Moroccan biotype was already in New Zealand, *M. aethiopoides* was classified as a risk species (at the request of AgResearch) due to the possibility of hybridisation and extension of its host range. This meant that all strains of *M. aethiopoides* (except the already established Moroccan biotype), were classified as new organisms, and hence require HASNO Act approval before introduction into New Zealand. Such approval is only obtained after a risk assessment by the Environmental Risk Management Authority New Zealand (ERMA).[55] Early 2006 saw the first releases of parasitised CRW. Around 5000 parasitised CRW were released at a total of four sites around the central North Island.[55]

1.1.9 Success of *Microctonus aethiopoides*

Early establishment was high at all release sites with all sites recording greater than 10% parasitism within six months of release, with up to 33 +/- 24%‡ at one site. By January 2007, parasitism was detected in the paddocks adjacent to the initial release sites at all of the sites. Data from the four sites combined suggested that the Irish *M. aethiopoides* might complete four generations a year in North Island. Multiple parasitoids were also commonly found within the host weevils.(55) *M. aethiopoides* was released in the South Island between August 2006 and March 2007. By May 2007 it was parasitising 4% to 14% of adult CRW.(16)

1.2 Background to Project

1.2.1 Introduction

Ruakura AgResearch staff members have been involved in CRW research and control from when the very first weevil was discovered in the Waikato in March 1996. Dr. Pip Gerard, who is the leader of the Dairy Insight and Meat & Wool New Zealand-funded release program, has been involved with CRW since its discovery in New Zealand, and led the ERMA application for the release of *M. aethiopoides.*

While studying CRW, visual observation by AgResearch researchers discovered that the appearance of abdominal fat body and the lipids present in the haemolymph in adult CRW vary with season, sex, insect age and parasitism. As adults age, male fat body increases in size and colour intensity while females become depleted (**Figure 1.4)**. However, parasitised females commence absorbance of their eggs and degeneration of their reproductive organs as soon as the parasitoid egg is laid and those containing diapausing parasitoid larvae during the winter accumulate fat in their fat bodies in a manner similar to males. It is thought that parasitism changes host endocrine regulation and the resultant fat

‡This range indicates the large natural variability present among samples.

accumulation both prolongs host longevity and provides resources for parasitoid development in the spring.

Figure 1.4. Dissected CRW samples showing a male with plentiful fat visible (left) and a female with depleted fat but visible eggs (right). Photographs courtesy of AgResearch.

1.2.2 Context of the Project

Parasitism is known to induce changes within the insect host. These changes can be caused by substances injected by the ovipositing female to regulate the host's internal environment, making it more favourable for its progeny. There is a wide range of possible effects for the host such as paralysis or changes in growth, feeding and development. There can also be changes in the substances found in the haemolymph such as solutes, proteins, carbohydrates and lipids. Metabolism and excretion can be altered as well as the endocrine and reproductive system of the host. Teratocytes (specialist cells derived from the parasitoid egg membranes whose function varies between species) and altered levels of juvenile hormones in the host have also been reported in parasitism.[56]

Parasitisation can affect the lipid levels of insect hosts. These changes depend on the host:parasite system. The adult female *Nasonia vitripennis* (a parasitic wasp) injects a venom during oviposition that causes lipid accumulation in the fat body of its host.[57; 58] In another system, the fat body of Oriental armyworm (*Pseudaletia sepata* (Mythimna)) parasitized by *Cotesia kariyai* (an endoparasitoid) was completely consumed just before parasitoid larval emergence

and the presence of teratocytes was noted; when parasitoid larvae were transplanted into the host without teratocytes this caused abnormal growth of the larvae, suggesting that teratocytes are vital to the success of this parastoid.[59] Further work on this system has found that the fat body of the host is altered by substances injected by the adult parasitoid which cause high levels of trehalose and lipids to accumulate in the host hemolymph.[60] In contrast, parasitism did not affect the total lipid and fatty acid levels of host larvae (lesser wax moth, *Achoria grisella* Fabr.*)* that were parasitised by the wasp, *Apanteles galleriae*; however, the fatty acid levels in the parasitoid increased during its growth.[61] Although each host:parasite system is different, an underlying theme is the importance of fat to the parasitoid. Parasitoid species lack adult lipogenesis, and are therefore unable to store excess energy in the form of lipid reserves. Thus being able to utilise the host's lipids and accumulate fat while growing inside the host is vital for the parasitoid's survival.[62]

In systems containing a *Microctonus* parasite and a weevil host, parasitism has been reported to cause physiological changes such as the degeneration of ovaries and retention and resorption of mature eggs,[38; 63; 64] as well as altering the protein levels in the host's haemolymph.[65] Work on parasitism by *M. aethiopoides* on three species of weevils (including CRW) reported that no parasitized female lucerne weevil (*S. discoideus*) contained eggs, whereas about 18% of non-parasitised females contained mature eggs. Teratocytes were observed in parasitised weevils of all three species and there was a positive relationship between the percentage of parasitised hosts containing teratocytes and parasitoids surviving for each species.[30]

As visual changes have been observed in the fat of parasitized female CRW, the focus of this work is the observed fat changes that occur within the CRW once parasitism occurs. No previous work has been done on the fats of the CRW nor how these change once parasitism occurs. By also investigating the composition of teratocytes that are seen in parasitised CRW and any affects on the juvenile hormone titer that parasitism causes, a greater understanding of the effects parasitism on CRW will be reached.

1.2.3 General Purpose of Project

The *Sitona* genus includes major world-wide pests of legumes but fat accumulation in parasitised weevils has not been previously reported. The purpose of the present study is to compare the chemical composition of lipids present in parasitised and non-parasitised CRW adults. From the visual observations of dissected CRW it is obvious that parasitism causes females to accumulate fat. It is hypothesised that parasitism either causes female CRW to become more like male CRW or causes the CRW host to become more acceptable for the parasitoid progeny. It has been shown that parasitoid diapause is completed by late August and therefore high fat levels in the host CRW throughout winter are likely to aid survival of host and facilitate larval development in late August/early September when host feeding activity may be slow because of low temperatures. The proportion of the CRW that is parasitised appears to increase as winter progresses while the entire parasitoid population are diapausing larvae, suggesting that parasitised CRW survive winter better than non-parasitized CRW. This has been observed at several sites where populations have been monitored regularly[(66)] and study of mortality of CRW collected from and caged individually in outdoor conditions has indicated parasitized CRW had better survival than non-parasitised CRW in winter.[(67)] Gerard hypothesizes that this is partly but not solely related to the absence of fat reserves in non-parasitized female CRW.

Ultimately, this study will assist in interpretation of field observations of both weevil and parasitoid, and contribute to the understanding of how *M. aethiopoides* ensures its larvae have sufficient food for growth and development yet optimise survival of overwintering hosts.

Agriculture is a fundamental industry of New Zealand and Biosecurity New Zealand estimates the economic cost of CRW to New Zealand agriculture as up to $3 billion annually.[(68)] The more that is understood about CRW and the introduced biocontrol agent *M. aethiopoides*, the greater the ability to increase the efficacy of controls and reduce the impact of CRW on the New Zealand economy.

1.2.4 Specific Objectives

Objective 1: Investigate the chemical composition of the lipids present in adult clover root weevil.

- The chemical composition of the CRW lipids has not been reported previously. By chemically analysing adult CRW, it will be possible to compare findings with the reported literature of other insects. This will also allow the development of an optimised method for the analysis of the lipids of CRW.

Objective 2: Determination of how clover root weevil lipids change with age, physiological state and parasitism.

- The method, which has been developed to investigate the chemical composition of lipids present in CRW, will be extended to track the changes in the lipids that occur with differing physiological states and parasitism. This information will be used to help explain the visual observations that have been made by AgResearch researchers.

Objective 3: Determination of lipids present in the teratocytes of clover root weevils.

- The role *Microctonus* spp. teratocytes play in parasitoid larval nutrition is unknown and this study would identify specific lipids that may be synthesised and/or accumulate in these cells. By determining the lipids present in the teratocytes it will be possible to determine whether these have originated from the parasitised CRW host or not.

Objective 4: Investigate the role of parasitoid-derived juvenile hormone effects in lipid regulation in parasitised clover root weevils.

- This objective will facilitate better understanding of how the observed changes in insect physiology are initiated and maintained. By investigating any changes in the juvenile hormone concentrations in parasitised CRW, it will be possible to determine if parasitism-induced changes in these levels could be responsible for any changes that occur within the host.

1.3 Scope and Limitations of Project

This project was set up as a subsection of AgResearch's established research program on the clover root weevil (CRW). The sampling methods used by AgResearch and described in **Section 3.2.2** have been developed and refined since the first discovery of CRW in New Zealand in 1996. Dissection of each CRW is required to determine its sex, physiological state and whether or not it has been parasitised as none of these factors can be identified externally, and thus this project was reliant on a supply of dissected CRW samples from AgResearch.

During the four years of this project, several factors limited the number and type of samples that could be obtained. The efficacy of the parasitoid *M. aethiopoides* exceeded the AgResearch biocontrol team's most optimistic expectations with high parasitism and marked reductions in CRW populations.[(66)] The 2008 drought conditions experienced by the Waikato region and top half of the North Island, from which most of the CRW samples originated, accentuated the CRW population decrease over the last four years.[(66)] This meant that sample sizes of CRW that were obtained were often small and frequently consisted of too few individuals to warrant inclusion in this project. In addition, not all AgResearch team members had the time to take and preserve samples for this project when doing dissections.

Twelve sites were used for sampling, however, the number of samples collected from each was not uniform due to the differences in CRW populations at each site. The composition, in regard to physiological state, of the sampled CRWs varied throughout the year and this hampered efforts to obtain replicates. For example in summer the sampled CRW were of mixed ages and hence in different physiological states and the parasitoids present were in different stages of development, whilst in winter, there was little variation (parasitised CRW all contained diapausing larvae and those few that are not parasitised are 100% reproductive).[(69)] This meant that it was not possible to obtain the desired sample sizes and replication of the different physiological states under study on almost any given date.

Although control of the sampling process was outside the control of the author, considerable effort went into ensuring that all further methods were not only accurate but also statistically valid.

2 Method Development

2.1 Introduction

Although the chemical composition of the lipids of CRW has not been reported, the topic of lipid analysis is widely covered in the literature. Gas chromatography (GC) analysis of lipids allows the complete quantitative analysis of a lipid sample in a very short time and therefore is the most utilised technique in lipid chemistry. When GC is combined with mass spectrometry (MS), both qualitative and quantitative data become readily available.[70-72]

Although triacylglycerols (TAGs) (the major lipid component of insects) can be analysed by GC,[73] it is far more common to convert the TAGs to the volatile methyl ester derivatives of the fatty acids.[71; 74] Fatty acids are usually referred to using a shorthand nomenclature. This indicates the number of carbons in the fatty acid: number of double bonds in the fatty acid. For example, hexadecanoic acid is represented as 16:0. Trivial names are also commonly used when naming fatty acids.[73; 75] The Greek letter omega (ω) is used to indicate where the last double bond is located in unsaturated fatty acids. For example if the last double bond (counting C=0 as C-1) precedes the third carbon from the terminal methyl end of the fatty acid chain, the fatty acid is labelled as a ω-3 fatty acid.
Analysis of fatty acids usually involves four steps:

1 Saponification/sample extraction followed by methylation, or transesterification.
2 GC analysis of the fatty acid methyl esters (FAMEs).
3 Identification of the FAME peaks.
4 Quantification of each fatty acid.[76]

Although GC is the most widely used technique when analysing fatty acids, other techniques such as atmospheric pressure chemistry ionisation mass spectrometry (APCI-MS)[77; 78] and electrospray ionisation mass spectrometry (ESI-MS) have proved successful.[77] High performance liquid chromatography (HPLC),[79-82] high performance thin layer chromatography (HPTLC),[83-86] matrix-assisted laser desorption ionisation-time of fight spectrometry (MALDI-TOF)[87] and nuclear

magnetic resonance spectrometry (NMR) have also been used.[88] The quality of results and the ease of use of GC when analysing lipids, however, means that it is still the most widely used technique.

2.1.1 Sample Extraction

Prior to analysis via gas chromatography mass spectrometry (GC-MS), extraction and derivatisation of the lipids are necessary .The Folch, Lees and Sloane Stanley system[89] of chloroform: methanol in a 2:1 ratio, is the most popular solvent system.[90; 91] Most reported work uses this method of extraction with solvent mixtures (differing relative amounts of chloroform and methanol) optimised for the individual situations.[70; 71; 74; 75; 89; 92-119] The Bligh and Dyer[120] method represents a modification of the Folch method that uses less solvent and a slightly different approach but it is also widely used.[121] Other methods use different solvent mixtures such as ether:methanol (3:1 v/v)[122] and ethanol:petroleum ether (3:1 v/v).[123] Dole's solvent (isopropanol: heptane:$(NH_4)_2SO_4$ 40:10:1 v/v/v) was used by Chang and Freidman.[124] Other less popular extraction methods involve various filtration steps,[125] or Soxhlet techniques.[126] Micro-extraction techniques have also been used.[127]

If necessary, lipids can be separated into classes (neutral lipids, free fatty acids and polar lipids) using columns such as aminopropyl bonded phase columns,[128] silica Sep-Pak™ columns,[129] or with thin layer chromatography plates.[84] Adsorption chromatography, such as the use of activated magnesium silicate,[130] is also commonly used to separate the extracted lipids. A separate saponification step may be needed for complex lipids.

2.1.2 Sample Derivatisation

The fatty acids in the lipid extracts must be converted to their volatile methyl ester derivatives before analysis. The commonly used methods fall under three main categories.

Acid-catalysed esterification and transesterification involves heating the lipid extracts with a large excess of anhydrous alcohol, usually methanol, in the

presence of an acidic catalyst[70; 71; 93; 112] (**Figure 2.1**). There are several ways to achieve this; hydrogen chloride gas can be bubbled through dry methanol;[70; 71; 93; 95; 97; 109; 131] acetyl chloride can be added to dry methanol[70; 71; 74; 111] or a 1-2% concentrated sulphuric acid in methanol solution can be used.[70; 71; 93; 108; 113]

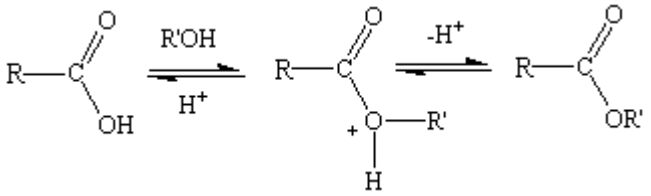


Figure 2.1. The acid-catalysed esterification of a fatty acid.

Boron trifluoride in methanol (12-14% w/v) is another transesterification method that is used extensively throughout the literature.[101-103; 106; 107; 110; 132-134] Its use, however, has been discouraged by Christie[70; 71; 110; 135] as the formation of methoxylated fatty acid artefacts is possible especially with aged reagent.[136]

Base-catalysed transesterification generally involves a basic catalyst in anhydrous methanol (**Figure 2.2**). Usually sodium methoxide[110] (obtained by dissolving sodium in anhydrous methanol) is used, however potassium methoxide[75; 100] or potassium hydroxide are also suitable.[70; 71; 76; 110; 137; 138]

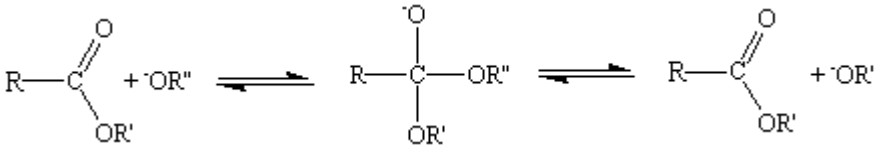


Figure 2.2. The base-catalysed trans-esterification of a fatty acid.

Although not as common as the other three methods, diazomethane is also used for esterification,[93; 106]-16 however, its toxic, carcinogenic and explosive properties make it less desirable than the other methods.[70; 71; 110; 135] Finally chloroform:methanolic HCl:cupric acetate has also been to esterify fatty acids at room temperature.[139]

An additional solvent such as toluene or tetrahydrofuran may be needed to solubilise non-polar lipids (benzene is also suitable but is very toxic).[70; 71; 110; 135]

Internal standards used include the methyl esters of 13:0, 17:0 and 19:0 as these fatty acids are rare in insect lipids.[122] These are used to ascertain losses in preparation.

2.1.3 Gas Chromatography Analysis

The volatile FAMEs are readily analysed using GC. Although flame ionisation detectors (FIDs) are commonly used in the gas chromatography of fatty acids, combining a MS with GC as stated above, allows both qualitative and quantitative results to be produced. GC-MS results have been shown to be very comparable to that of GC-FID, and the added benefit of MS detection is the identification of any unknown peaks.[140; 141]

The mass fragment data of the FAMEs are used for identification although standards should also be used to verify retention times. GC-MS of FAMEs only produces molecular ion peaks of low intensity. The characteristic intense peak at *m/z* =74 ($[CH_3OC(OH)\text{-}CH_2]^{\cdot +}$) arises by the McLafferty rearrangement (**Figure 2.3**). The $[M\text{-}31]^+$ ion representing the loss of a methoxyl group) confirms the presence of a methyl ester.[71]

FAMEs are quantified by integration of peak areas. Response factors are calculated by injecting reference FAMEs of known concentrations. The response factors of the different FAMEs have been found to be very similar to each other.[71]

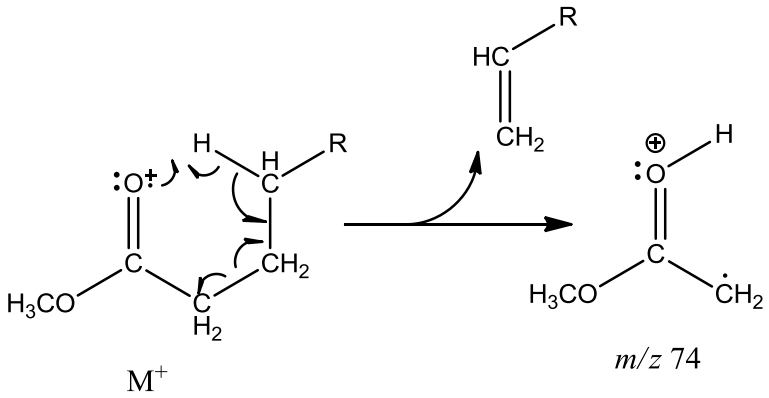


Figure 2.3. The McLafferty rearrangement of a FAME.

2.1.4 One-Step Methods

Although traditional multi-step extraction and derivatisation methods have been widely used in the literature, they are not without their drawbacks. These methods are time consuming and can require large volumes of reagents. The many steps involved can lead to mistakes in analysis, contamination and recovery losses.[142] These drawbacks increase the mass of sample required to do an analysis.

In an attempt to overcome this, several "one-step methods" have recently been reported (**Figure 2.4**[142]). Oxidation of unsaturated fatty acids can also be minimised in these new methods. Despite the findings of Mazalli *et.al*.[143] who did not find that direct methylation of the fatty acids in egg yolks was more efficient than the traditional two step (extraction then derivatisation) method, the majority of other one-step methods have proved to be successful. Lepage and Roy[144] developed a one-step method that not only significantly decreased the time taken to analyse each sample but also minimised losses and artefact formations. As a result of minimising auto-oxidation, higher 18:2 levels in the house cricket (*Acheta domesticus)* were found using their methods than in previous studies.[92; 111; 145]

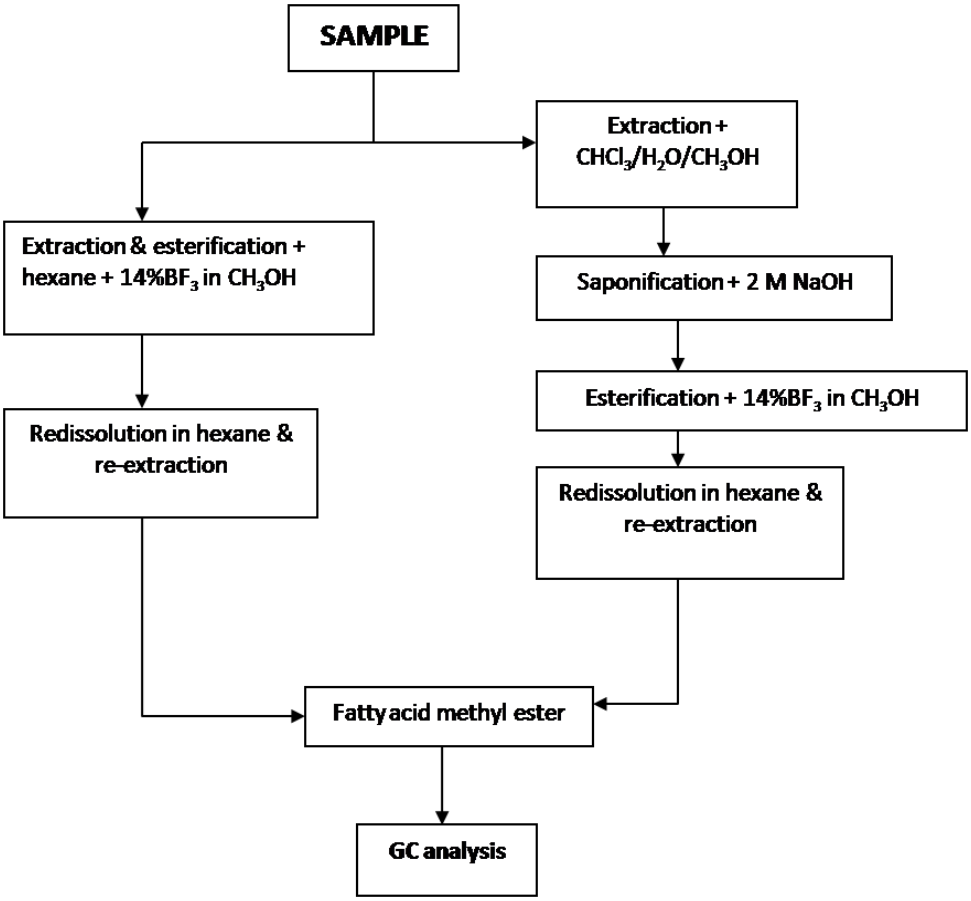


Figure 2.4. Abdulkadir and Tsuchiya's schematic comparison of procedure between their one-step method (left) and conventional (right) methods for marine fatty acid analysis.[142]

Heating infant formulae to 100°C for an hour in methanolic HCl:hexane gave higher recoveries of FAMEs than traditional methods.[146] Heating lipoprotein with methanolic BF_3:benzene at 110°C for 90 minutes gave higher recoveries of FAMEs than traditional two step methods.[147] A one-step method allowing the preparation of FAMEs without prior lipid extraction from marine animal samples has been reported to give a higher recovery than traditional methods,[142] as has a one hour direct transesterification procedure.[144]

The success of these recent uses of one-step methods highlights the potential for further development of similar methods in the area of lipid analysis. The size of insects means that losses during lipid analysis by traditional methods are particularly significant, and as a result, the development of one-step methods is particularly relevant.

2.2 General Methods and Materials

2.2.1 General Methods

Centrifugation: Centrifugation was carried out using an Eppendorf 5702 centrifuge set at 3000 rpm. No temperature control was available.
Heating Bath: Samples were heated in an oil bath on top of a magnetic stirrer. The temperature settings were verified by the use of a thermometer that had 0.1°C resolution.

2.2.2 Chemicals

Acetone (analytical grade, greater than 99.5% purity), *n*-hexane (analytical grade, greater than 98.5% purity), chloroform (analytical grade, greater than 99.8% purity), methanol (analytical grade, greater than 99.7% purity) were purchased from Ajax Finechem Pty Ltd. Distilled water was used. Butylated hydroxytoluene (greater than 97% purity) was purchased from Sigma Aldrich. Potassium bicarbonate (greater than 99% purity) was purchased from May and Baker Ltd. Sodium chloride (greater than 97% purity) was purchased from BDH. Sulphuric acid (analytical grade, greater than 97.5% purity) was purchased from Sigma Aldrich.

Dodecanoic acid, tridecanoic acid, tetradecanoic acid, hexadecanoic acid, 9-hexadecenoic acid, heptadecanoic acid, octadecanoic acid, 9-octadecenoic acid, 9,12-octadecadienoic acid, 9,12,15-octadecatrienoic acid, eicosanoic acid, methyl tridecanoate, methyl nonadecanoate (all of purity 99% or greater) were purchased from Sigma Aldrich.

2.3 Modified “Folch” Method of Extraction

As traditional multi-step methods have been widely reported for countless situations and have been successful, method development began by investigating the suitability of these methods.

2.3.1 Introduction

The Folch method is the most commonly used method for the analysis of lipids and at one time was one of the five most cited scientific publications of all time.[148] It involves homogenising the tissue to be analysed in a volume of chloroform:methanol mixture (2:1) that is equal to 20 times the volume of the tissue. After filtering and centrifuging, saline solution is added ensuring that nearly all of the extracted lipids are in the lower organic phase. From here, the extracted lipids can be derivatised using one of the derivatisation methods. The chloroform has been replaced in some later literature by dichloromethane and this reduces the many health, security and regulatory problems associated with the use of chloroform.[149]

2.3.2 Modified "Folch" Method of Extraction for Clover Root Weevils

CRWs (30) were frozen with liquid nitrogen and ground using a mortar and pestle. The pellet was transferred to a vial containing ice cold chloroform:methanol (2:1 v/v, 5 mL) solution and more of this solution was used to rinse the mortar, the rinsings were added to the vial. The vial was shaken (15 minutes) to extract the lipids and then centrifuged (20 minutes). The supernatant was collected and chilled in ice. The pellet was re-homogenised (using a mortar and pestle) with ice cold chloroform:methanol solution (2:1 v/v, 5 mL) and the process was repeated twice. Sodium chloride solution (1% w/v in water; *ca* one quarter of the total volume of the combined supernatant) was added and the vial was shaken (10 seconds) and subsequently centrifuged (20 minutes). The upper aqueous layer was removed and methanol:water solution (1:1 v/v; *ca* one quarter of the total volume of the bottom layer) was added. The vial was shaken and centrifuged (20 minutes). The top layer was removed and the remaining solution was filtered and evaporated under a stream of nitrogen before being re-dissolved in a small amount of chloroform. This method was modified further to test its ability to extract lipids from a sample of just five CRWs, with the aim of being able to analyse single CRWs.

To be able to compare the extraction methods easily, acid derivatisation was used and the samples were analysed by GC-MS. Details of these methods are discussed in **Section 2.5**.

2.3.3 Standards

As part of the investigation into this method, it was important to be able to calculate recovery losses. Two standards were selected that could be added to the sample at different stages of the preparation to calculate these. 17:0 was added at the start of the preparation and ethyl heptadecanoate (the ethyl ester of 17:0) was added just before the sample was analysed on the GC-MS. CRW samples were first analysed to ensure that they did not contain either of the standards. By comparing the peak areas of 17:0 and ethyl heptadecanoate, recovery percentages were calculated. Although ethyl heptadecanoate had proved to be an effective internal standard, the fact that it was not available commercially meant that it had to be synthesised. To simplify procedures it was decided to change to a recovery standard that was available commercially (hence also being more reliably pure) and two commonly used standards for fatty acid analysis (the methyl esters of nonadecanoic acid and tridecanoic acid) were investigated for their suitability. CRW samples were investigated to ensure that they did not contain any natural amounts of either nonadecanoic acid or tridecanoic acid. The GC spectra were investigated to ensure that the peaks corresponding to these standards did not interfere with the peaks of the fatty acids in the CRW samples. The peak of the methyl nonadecanoate had a retention time of 11.50 minutes that overlapped with the peak corresponding to the methyl ester of 18:2, which meant that accurate integration was impaired. The peak of the methyl tridecanoate had a retention time of 5.0 minutes, which was well separated from the other peaks therefore allowing accurate integration. It was decided that methyl tridecanoate was to be used as the recovery standard at the same concentration as the heptadecanoic acid.

The recovery percentage for each sample was calculated by dividing the peak area of the 17:0 FAME by the peak area of the 13:0 FAME (taking into account any difference in concentration and also the response factor of 13:0 – see **Section 2.11**) and multiplying by 100.

Solutions were accurately weighed to give concentrations of *ca* 1 mg/mL in hexane. It was found that by adding 50 µL of each standard their peak areas were in the ranges of the fatty acids analysed.

2.3.4 Results

Results from using the modified "Folch" method of extraction detailed above were not satisfactory. Results using 30 CRWs provided only five peaks that were identified as being FAMEs, and two of these had arisen from the two internal standards (**Figure 2.5**). The standards used and the recovery percentage calculation are fully described under **Section 2.3.3**. The recovery percentage of the internal standard (17:0) relative to the recovery standard (13:0) was low (less than 50%) suggesting that not all of the fatty acids present in the CRWs were being extracted.

2.3.5 Problems Encountered

After considerable refinement, there were still some problems with using this method to extract the lipids from CRWs. Even with extensive rinsing it was difficult to transfer all of the ground weevils from the mortar to the vial. This meant that losses occurred and that the recovery percentage was never greater than 50%. The method also involved a significant amount of centrifuging. Not only is this time consuming but also, as the centrifuge was not refrigerated, the samples heated up increasing the likelihood of oxidation. One of the commonly reported problems with the Folch method of extraction is its extensive use of solvents. Even with the relatively small sample size of the CRW lipid analyses, a lot of solvent was used.

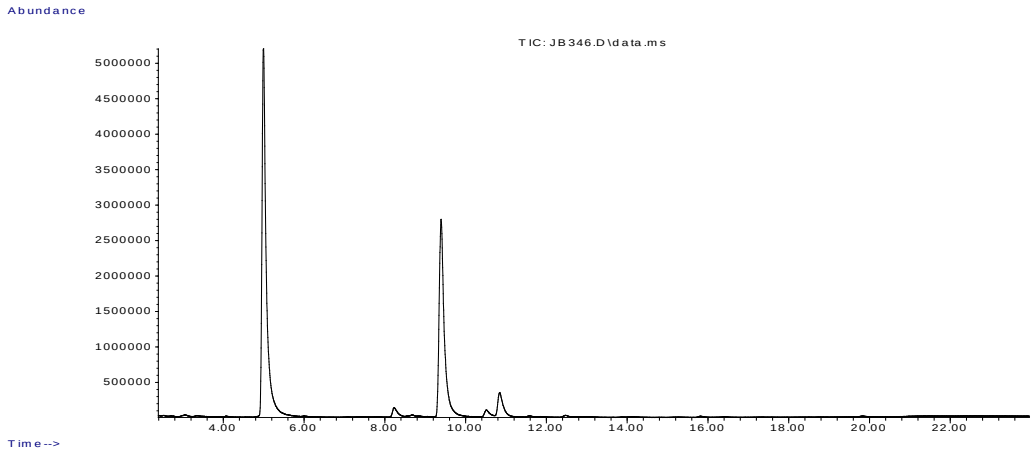


Figure 2.5. The GC chromatogram produced from the modified "Folch" method of extraction using 30 CRWs. The mass spectral traces were used to identify the five peaks as FAMEs, with the largest peak being that of the 13:0 (recovery standard) FAME and the second largest peak being that of the 17:0 (internal standard) FAME. The recovery percentage was less than 50%.

2.4 Modified "Bligh and Dyer" Method of Extraction

2.4.1 Introduction

The Bligh and Dyer method was originally developed as an economical alternative to the Folch method. It is based on the same principles as the Folch method but uses considerably less solvent (3 parts solvent (2:1 methanol:chloroform) to 1 part sample.). It was developed for the extraction of lipids from fish muscle,[120] but has been modified to analyse different tissues.[150]

2.4.2 Modified "Bligh and Dyer" Method for Extraction of Clover Root Weevils

Five weevils were weighed (*ca* 50 mg) and frozen in a vial using liquid nitrogen. The frozen weevils were transferred to a glass trituration tube that was submerged in liquid nitrogen. Weevils were crushed until well ground using a ground glass trituration rod. Lipids were extracted using methanol:chloroform (2:1, (v/v)) with a tissue:solvent ratio of *ca* 1 part sample to 3 parts solvent (v/v) (*ca* 200 µL). The sample was shaken (five minutes), chloroform:distilled water (1:1, 1 part v/v) (*ca* 100 µL) was added and the sample was filtered. The upper aqueous layer was removed. The pellet was re-extracted using the same method and the organic

layers were combined. The sample was gently blown dry under nitrogen and re-dissolved in chloroform. To be able to compare the extraction methods easily, acid derivatisation was used and the samples were analysed by GC-MS. Details of these methods are discussed later in this chapter.

2.4.3 Standards

The same standards as described above for the modified Folch method were used to calculate recovery percentages. 50 µL of each standard was used and peak areas used to calculate recoveries.

2.4.4 Results

The results from this method were similar to the results from the modified "Folch" method of extraction. Five samples with CRW numbers ranging from 5-30 (with reagents scaled to suit) were tested. Once again, only five FAME peaks were seen in the GC-MS trace and two of these were the standards. Recovery percentages were low (less than 50%), suggesting that not all of the fatty acids present in the CRW were being extracted.

2.4.5 Problems Encountered

Although this method used less solvent than the modified "Folch" method, it was still difficult to avoid losses in the preparation of samples.

2.5 Evaluation of Different Derivatisation Methods

2.5.1 Introduction

The second step, after extraction, of traditional fatty acid analysis methods is derivatisation. As fatty acids themselves have low volatility, they are commonly converted to their volatile methyl esters (FAMEs). There are several methods to achieve this.

2.5.2 Acid-Catalysed Methods

Several acid-catalysed methods have been reported; the method described here is typical. Chloroform (that the sample had been dissolved in) was evaporated and the sample dissolved in toluene (1 mL) in a test tube fitted with a condenser. Sulphuric acid in methanol (2 mL; 1%) was added. The mixture was heated (50°C) overnight in a stoppered test tube. The tube was cooled to room temperature and 5% (v/v) NaCl (5 mL) solution was added. The FAMEs were extracted by adding hexane (5 mL) and shaking the stoppered tube. The tube was allowed to settle before the hexane layer was removed using a glass pipette. A further hexane extraction (5 mL) was carried out and the hexane layers combined. The combined hexane layers were washed with potassium bicarbonate solution (4 mL; 2% w/v) and evaporated over anhydrous sodium sulphate. The solution was filtered before being dried under a stream of nitrogen.

2.5.3 Boron Trifluoride Method

Although this is technically an acid-catalysed method, its popularity means that it is usually referred to separately rather than just under the umbrella of acid-catalysed methods.

Chloroform was evaporated and sample was re-dissolved in 14% BF_3 in methanol (1 mL). The vial was gently flushed with nitrogen before being tightly sealed and heated (100°C, 30 minutes). After the vial had cooled, pentane (2 mL) and water (1 mL) was added and the vial was shaken and centrifuged. The organic layer was extracted before being evaporated and re-dissolved in hexane for GC-MS analysis.

2.5.4 Results and Conclusions

Both methods appeared to give similar results when used to derivatise the fatty acids extracted from the CRW samples using either extraction technique. However, it was difficult to compare accurately both methods due to the poor results of the extraction processes. The toxicity of BF_3 meant that sample preparation using acidified methanol was considerably safer. This, combined with the price of BF_3 and the warnings about its use from Christie[70; 71] meant that the acid-catalysed method was preferred. However, further development work into

derivatisation methods stopped once it was decided to pursue a one-step method for extraction and derivatisation.

2.6 One-Step Method for Extraction and Derivatisation

2.6.1 Introduction

To study the changes in lipid composition between CRWs of different age, sex and physiological state effectively, a method that was able to analyse single weevils was desirable. When it was found that traditional methods incurred significant losses, the newer one-step methods were investigated. The one-step method that was used was a modification of Abdulkadir and Tsuchiya's method.[142] This method sees extraction and esterification occur in a single tube with 14% BF_3 in methanol used for the esterification.[142]

2.6.2 Modified One-Step Method

As sulphuric acid in methanol was found to be the preferred method, a one-step method was developed that utilised this to esterify the fatty acids. CRWs (5) were frozen with liquid nitrogen. The CRWs were crushed using a glass trituration rod. Internal standard (50 µL) and hexane (1 mL) were added to the vial. Sulphuric acid in methanol (2% v/v, 2 mL) was added before the vial was gently flushed with nitrogen and sealed. The vial was heated overnight (50°C) with constant stirring. The vial was cooled to room temperature before being neutralised with potassium bicarbonate solution (4%, 2 mL). The recovery standard (50 µL) was added and the FAMEs were extracted for GC-MS analysis using hexane (1 mL).The sample was analysed using the GC-MS method described below. The method was further developed to be able to analyse a single weevil. This meant that the volume of standards could be reduced to 30 µL each.

2.6.3 Standards

The same standards, as described above for the modified Folch method, were used to calculate recovery percentages. 30 µL of each standard was used and peak areas used to calculate recoveries.

2.6.4 Initial Results

The results obtained from the one-step method were very promising. Not only was it a simpler method involving significantly less steps than traditional methods, the recovery percentages achieved were also considerably higher. The higher recovery percentages (see **Section 2.3.3** for explanation of recovery percentage) meant that the less of the internal standard (17:0) was being lost throughout the extraction process, therefore, indicating that the extraction process was more efficient than the earlier methods trialed. Up to ten peaks identified as FAMEs (using MS data) appeared in the GC traces of individual CRW samples. However, peak shape and separation needed to be improved and the method statistically tested before it could be used in the rest of the project.

2.7 Different GC-MS Conditions

For the peak areas of the FAMEs to be accurately integrated, it is important that they were fully resolved from one another and that peak shape was appropriate. Many components of a GC-MS system can be optimised for a particular system to ensure this.

2.7.1 GC-MS Set Up

Samples were analysed using an Agilent 7890A GC system connected to an Agilent 5975C MS system with an inert MSD triple-axis detector. A 10 µL syringe was used with 5 µL injections. Helium was used as the carrier gas at a flow rate of 1.41 mL/min. Two washes pre-injection with hexane and two washes post-injection with acetone (both at the maximum volume of the syringe) were carried out with each injection. The detector had limits of low mass 42 and high mass 450. The threshold was 150 counts with 3.52 scans per second. The MS source temperature was set at 230°C and the MS quad temperature was 150°C.

The temperature program of the column can be altered to give the best separation of signals. A program was designed (**Table 2.1**), which achieved optimal

separation. The splitless time was 0.15 min. Varying the column flow rate was not investigated as the temperature program achieved the required separation.

Table 2.1. The GC temperature program used for analysis.

	Rate (°C/minute)	**Value (°C)**	**Hold (minutes)**	**Total run time (minutes)**
Initial		60	0.3	0.3
Ramp 1	20	100	0	2.3
Ramp 2	6	200	5	24.0

2.7.2 Column Types

The type of column used to separate the sample within the GC has a major effect on the appearance of the chromatogram produced. Most laboratories have a general "all-purpose" column that is for the general running of samples. Usually this column is of low polarity and is relatively robust. The Zebron ZB5 column is such a column and was initially trialled to test its suitability. Initial results were not promising as there was a lot of tailing and peaks were not sharply resolved from one another.

The GC column of choice for fatty acid chemists is one that has both polar and polarisable components. The Supelco SP2330 is in this category and is commonly used for the analysis of fatty acids.[80; 151; 152] This column's stationary phase is 80% biscyanopropyl and 20% cyanopropylphenyl siloxane. This column (15 m x 250 µm x 2 µm) was trialled with the CRW samples and produced far better results than the general column. The peaks were well-resolved single component peaks and this allowed for accurate integration (**Figure 2.6**).

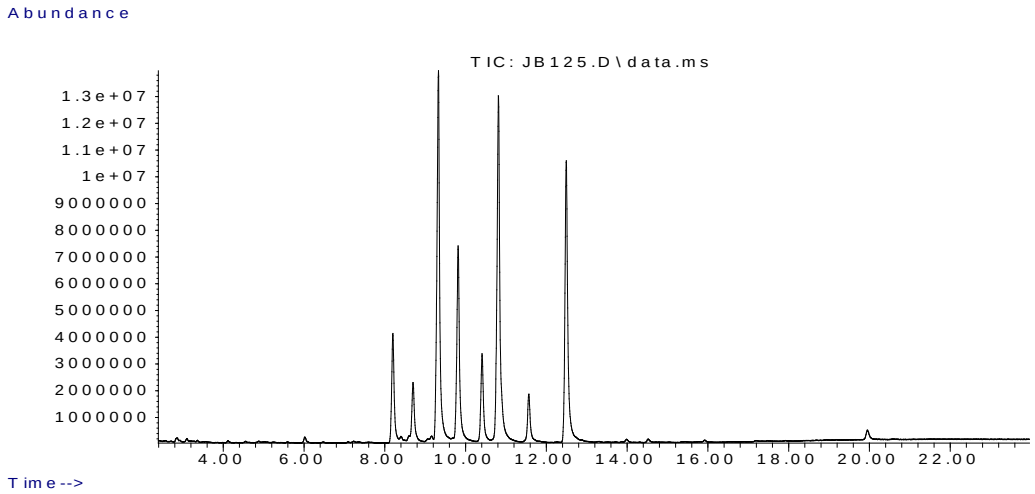


Figure 2.6. GC trace of a single CRW sample produced by the one-step method and analysed on a Supelco SP2330 GC column.

2.8 Use of an Anti-Oxidant

To determine whether significant oxidation of the CRW samples was occurring, an experiment was set up to compare results from samples that had an anti-oxidant added and samples that did not. Butylated hydroxytoluene (BHT) is the anti-oxidant that is commonly used in fatty acid analysis,[71; 74] at a concentration of *ca* 25 mg/L. Sufficient BHT was added to hexane to ensure at least this concentration was present throughout the method. Ten CRW samples with the BHT-stabilised hexane and ten with non BHT-stabilised hexane were analysed using the one-step method.

As it was difficult to compare peak sizes of the unsaturated FAMEs due to the natural variation seen between samples, the absence of any oxidation products(e.g. dicarboxylic acids)in either set of spectra was taken to indicate that no or minimal oxidation had occurred.[153] The benefit of using a MS detector is that the identity of every peak can be found allowing one to check for oxidation products in every spectrum. It is likely that the gentle flushing of the vial with nitrogen before heating provides enough protection against oxidation hence BHT was not used in subsequent analyses. The majority of one-step methods reported do not use an anti-oxidant as the methods themselves reduce the sample's exposure to air,[92; 142;

[145] however, it was necessary to check as the oxidation of the CRW samples may have significantly reduced the sizes of the fatty acid peaks.

2.9 Identifying the Fatty Acid Methyl Esters

The FAMEs were initially identified by their MS data. The software uses a mass spectral library to suggest possible matches and gives the percentage certainty of the match. Peaks were identified with a minimum of 80% certainty (although this was usually a lot higher). As well as comparing mass spectral traces, the presence of an ion with a *m/z* value that matches the molecular ion *m/z* value of a certain FAME can be used as extra identification. The molecular ion *m/z* values of the FAMEs that were expected to be present were calculated (**Table 2.2)**, and used to verify identifications. **Figure 2.7** is an example of the identification of a FAME (9,12,15-octadecatrienoic acid methyl ester) using the presence of the molecular ion on the MS trace as extra verification (the spectral library also identified this).

The mass spectra of saturated FAMEs are expected to contain the $[M-31]^+$ ion (loss of a methoxyl group), the $[M-43]^+$ ion (loss of a propyl group (carbons 2 to 4)) and the $[M-29]^+$ ion (loss of carbons 3 to 4). Also there is a series of related ion clusters differing by 14 atomic mass units due to the loss of neutral aliphatic radicals ($[(CH_2)_nCOOCH_3]^+$), with *m/z* 87 being the most abundant ion.[71] The mass spectra of monoenic FAMEs are expected to contain the $[M-32]^+$ ion (loss of a methoxy group plus a hydrogen) and the $[M-116]^+$ ion (loss of the fragment containing the carbonyl group followed by cleavage between carbons 5 and 6, and addition of the rearranged hydrogen atom). Additionally there is a series of ion clusters following on from the $[M-116]^+$, each 14 atomic mass units less than the previous (loss of additional CH_2 groups).[71]

Table 2.2. The molecular ion *m/z* values of the FAMEs commonly found in insect samples.

Standard	Molecular ion mass
12:0	214
13:0	228
14:0	242
16:0	270
16:1	268
17:0	284
18:0	298
18:1	296
18:2	294
18:3	292
20:0	326

The mass spectra of dienoic FAMEs are expected to contain ion clusters in which the most intense ion has the formula $[C_nH_{2n-3}]^+$, while the corresponding trienoic FAMEs produce most intense ions of the formula $[C_nH_{2n-5}]^+$ with the ion at *m/z* 79 as the base peak. The 18:3 FAME present in the CRW samples was identified as methyl 9,12,15-octadecatrienoate (α-linolenate or 18:3 (ω-3)) rather than methyl 6,9,12-octadecatrienoate (γ-linolenate or 18:3 (ω-6)) due to the presence of the ion *m/z* = 108 (characteristic of polyunsaturated FAMEs with the *n*-3 terminal moiety) (**Figure 2.7**).[71; 154]

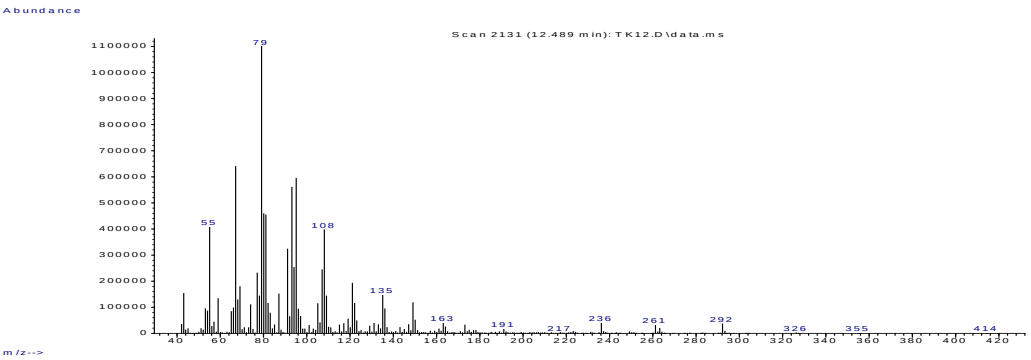


Figure 2.7. The mass spectrum of the peak identified as 9,12,15-octadecatrienoic acid methyl ester (retention time 12.54 minutes). The molecular ion (*m/z* = 292) is seen, the base peak is *m/z* 79 and the most intense ions in the clusters have the general formula C_nH_{2n-5}; the ion *m/z* 108 denotes an ω-3 fatty acid.

Although the MS provides qualitative results, it is good practise to verify all predicted identifications with the retention times of standards. Hence, the retention times of the major fatty acids present were verified by analysing pure samples of the fatty acids predicted by the MS spectral patterns (**Table 2.3**). When comparing the retention time results to the FAMEs identified in the samples, the retention times were very similar and only varied by +/- 0.2 of a minute. The mass spectral data was the same between the standard and the sample.

Table 2.3. The retention times of standard FAMEs.

Standard	Retention time (minutes)
12:0	4.4
13:0	5.0
14:0	6.1
16:0	8.3
16:1	8.8
17:0	9.4
18:0	10.5
18:1	10.9
18:2	11.6
18:3	12.5
20:0	14.6

2.10 Verification of the Method

2.10.1 Introduction

To ensure that the one-step method was valid it was thoroughly tested as described in the following sections. These tests would also ensure the statistical validity of the method.

2.10.2 Recovery Percentage

It was important that a high percentage of the fatty acids present in the CRW samples were extracted and derivatised by the one-step method. The recovery percentage of the method was calculated by comparing the peak area of the

internal standard (17:0) with the peak area of the recovery standard (13:0) taking into account slight differences in concentration (both standards were accurately weighed to *ca* 1 mg/mL and 30 µL of each were used). The average recovery percentage over all of the trial samples was 89%, which was far higher than recovery percentages achieved with the previous methods trialled (less than 50%).

2.10.3 Reproducibility

As the method would be used on single CRWs, it was not possible to do replicates of each individual sample, as every CRW had natural variation therefore the chances of finding two or more CRWs that were the same were very slim. Therefore, to test the reproducibility experiments were conducted on CRWs that had been halved longitudinally and the results from each half were compared to ensure that the method yielded reproducible results. In the first instance, individual CRWs were cut in half using a scalpel. However due to the hard exoskeleton it was difficult to accurately cut the CRW in half. Instead, the individual CRWs were crushed in hexane using a trituration rod. An equal amount of the hexane was transferred to two clean vials and the method was followed from this step. The results from both halves (labelled Half 1 and Half 2) were analysed and compared to each other to see if they were significantly different. This was done for 20 individual CRWs (20 pairs of halves). The peak areas of each fatty acid for each half were recorded and this was the data used for statistical analysis. Raw data for the peak areas is in **Appendix 9.1.1**.

A nested ANOVA[§] was conducted to determine the size of the variation between each fatty acid within a pair of halves, relative to variation of each fatty acid between different samples.[(155)] This analysis determined the percentage of the total variation that could be attributed to the differences between halves and also the percentage of the total variation that was due to differences between the 20 CRW

[§]A nested ANOVA is the correct form of analysis rather than a paired t-test. The nested ANOVA takes account of the fact that the measurements are paired within weevils but, unlike for a paired t-test, the weevil halves "A" and "B" do not line up between weevils as they would if they were say A=top vs B=bottom. In this situation comparing A_{mean} with B_{mean} as in a t-test would be meaningless

samples. As the data is compositional data rather than binomial data, no transformation was required for ANOVA of individual percentages.[156] The percentage of total variation that was due to differences between halves for each fatty acid (**Table 2.4.)** was small (less than 5% for any of the fatty acids). The error introduced by the method is significantly less than the variation between samples. Hence, the one-step method is very reproducible. Raw data from the nested ANOVA is in **Appendix 9.1.2**.

Table 2.4. The percentage of the total variation that was due to differences between halves in the nested ANOVA.

Fatty acid	Percentage of total variation due to halves
12:0	1.92
13:0	3.67
14:0	1.31
16:0	0.19
16:1	0.35
17:0	2.83
18:0	0.11
18:1	0.34
18:2	0.42
18:3	0.34

Although the percentage of total variation caused by differences between halves is small (all less than 5%), the fatty acids with the two highest percentages are the two standards (13:0 and 17:0). This suggests that error from adding the standards is greater than the error introduced by the rest of the method. This was noted and particular care was taken when adding the standards throughout the rest of the project. The pipettes were calibrated regularly to help reduce error. However, the most likely cause of higher values for the two standards is that the variation between the 20 pairs of halves is small for the standards thus meaning any variation between halves contributes more than when the variation between samples is much higher. Apart from the standards, the remaining fatty acids all have 98% or more of total variation arising from differences between the 20 pairs

of halves. The fatty acid with the next highest percentage of total variation due to halves was 12:0, which is routinely the smallest peak. A possibility of the cause of this variation is discussed under **Section 2.10.4**.

2.10.4 Reproducibility of the GC-MS Method

Manual integration was used to integrate the peak areas throughout the project as this allowed for the most accurate baseline identification. Reproducibility of GC-MS integration was tested by running the same sample ten times and comparing results. The average peak areas, standard deviation and coefficient of variation percentage were calculated for each FAME over the ten replicates (**Table 2.5**). The coefficient of variation percentage was calculated (**Equation 2.1**), as was the average coefficient of variation percentage for all FAME peaks for each of the five samples (**Table 2.6**).

$$CV\% = \left(\frac{\sigma}{\mu}\right) * 100 \qquad \textbf{(2.1)}$$

The coefficient of variation percentage for each FAME peak and the average coefficient of variation percentage for each sample were small (less than 5%) which indicated that the methods used were very reproducible. The fatty acid with the highest coefficient of variation percentage was 12:0. This was likely because it was the smallest peak and was more difficult to distinguish from the baseline than any of the other peaks. This introduces greater variation between replicate integrations.

2.10.5 Reproducibility of the Method over Different Days

As it would be impossible to analyse all samples on one day, it was important to assess that the reproducibility of the GC method over time. To do this one CRW sample was injected each day for fourteen consecutive days and the results analysed. The average coefficient of variation over all of the peaks in the fourteen injections was 2.6%, which is very small. This sample was stored at -18°C between analyses. Results were manually integrated and compared. Throughout the project, this sample was periodically analysed to ensure that only minor variation was arising from the GC-MS.

Table 2.5. Average peak areas, standard deviation and coefficient of variation percentage for each FAME over the ten replicates.

Fatty acid	Average peak area	Standard deviation	Coefficient of variation percentage
12:0	783485	116257	3.5
13:0	170047701	2135934	1.3
14:0	2824520	38952	1.5
16:0	70105596	443675	0.8
16:1	61395730	494466	1.1
17:0	139091525	1927634	1.5
18:0	29720060	238291	1.5
18:1	203596628	1875197	1.1
18:2	16902079	461229	2.7
18:3	85576668	1391336	1.6

Table 2.6. The average coefficient of variation % for all FAME peaks for each of the five samples.

Sample	Average coefficient of variation percentage
One	1.9
Two	2.1
Three	2.2
Four	1.7
Five	1.4

2.10.6 Bulk Extraction

A bulk extraction of 40 CRWs was completed using the one-step method (with reagents scaled to suit) to ensure that no FAMEs were being missed in the single CRW samples due to their concentration being too low. No extra FAMEs were detected in this bulk extraction that had not been detected in any of the single CRW samples.

2.11 Response Factors

The GC-MS response factor (RF) of an analyte is the ratio of the size of the peak produced and the concentration of the analyte relative to that of the respective ratio of the internal standard. Although analytes with similar structures usually have similar response factors,[157] it can be inaccurate to assume this is always one. In a lot of literature, the response factors of FAMEs are assumed to be equal and therefore are not calculated.[71; 157; 158] However, although the response factors are usually similar, there is often slight variation especially with increasing chain length (usually an increase in response factor) and the occurrence of double bonds (usually a decrease in response factor).[159] When GC-FID and GC-MS analyses were compared it was found that the variability of RF was less for MS detection compared with FID detection.[140] To ensure accuracy, response factors were calculated (**Equation 2.2)**, for the FAMEs usually found during insect lipid analysis and identified in the CRW samples using the GC-MS system.

$$RF_{(FA)} = \frac{\frac{A_{(FA)}}{A_{(17:0)}}}{\frac{M_{(FA)}}{M_{(17:0)}}} \qquad \textbf{(2.2)}$$

Where:

$RF_{(FA)}$ is the response factor of the fatty acid

$A_{(FA)}$ is the integrated peak area of the fatty acid

$A_{(17:0)}$ is the integrated peak area of the internal standard, heptadecanoic acid

$M_{(FA)}$ is the mass of the fatty acid

$M_{(17:0)}$ is the mass of the internal standard, heptadecanoic acid

At least five samples of differing ratios of the standard and sample were analysed. Graphs were plotted (see example for 14:0, **Figure 2.8**; other graphs in **Appendix 9.2**) and the response factors and the coefficient of the linear regression (R^2) were calculated using Microsoft Excel 2007 (the linear trend line was set to go through zero) (**Table 2.7**). The calculated response factors were in line with those reported for GC-MS.[140]

The GC-MS used for this project was brand new at the start of the work and used almost exclusively for this project. This meant that the MS was exceptionally clean throughout the project and that cleanliness had no bearing on the response factors of the analytes. The MS was also auto-tuned weekly, and was regularly serviced by an Agilent technician.

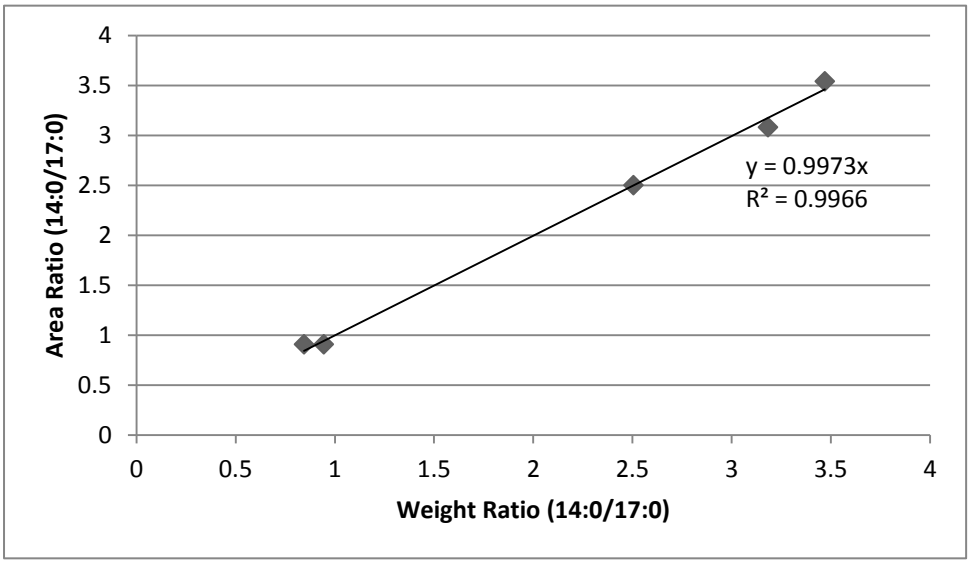


Figure 2.8. Response factor graph for the standard 14:0.

Table 2.7. Calculated response factors and coefficients of determination R^2 values for the FAMEs commonly found in insect lipid analysis.

Standard	Response factor	R^2
13:0	0.98	0.9968
14:0	0.99	0.9967
16:0	1.02	0.9994
16:1	0.98	0.9936
18:0	1.10	0.9989
18:1	0.98	0.9948
18:2	0.82	0.9920
18:3	0.77	0.9940

2.12 Corrected Peak Areas

The recovery percentage (calculated for each sample) and response factors were used to correct the peak areas of every sample. As the response factors were only calculated for the common fatty acids found in the CRW samples, the response factors of the minor fatty acids were assumed to be one as commonly occurs in the literature.[71; 158] The low concentrations of these minor fatty acids meant that any error introduced by this assumption was small.

2.13 Discussion and Conclusions

Insect lipid analysis is widely covered in the literature and results are commonly reported as the fatty acid profile of different insects.[71; 74] As the fatty acid profile of CRWs had not been reported, traditional methods of insect lipid analysis were first investigated to test their suitability. The small size of CRWs, as well as the goal to be able to analyse individual weevils meant that previous methods of fatty acid extraction and derivatisation provided recovery percentages that were too low to allow these methods to be suitable.

The one-step method of extraction and derivatisation that was developed proved to be very successful. Recovery percentages (averaging 89%) and reproducibility (percentages of the total variation that could be attributed to the differences between halves were less than 5%; average coefficient of variation percentages were less than 5%) were high and good peak separation was achieved with the SP2330 GC column. Recovery percentages are not commonly reported as they are taken into account in the results reported. It is difficult to differentiate between the variation due to the reported methods and the variation due to natural variation between insects. Grapes *et al* reported that the coefficient of variation of up to 50% seen in the analysis of house cricket (*Acheta domesticus)* was due to the variation between insects.[111] The only accurate way of comparing two methods is to test them both with the same subjects. When a conventional method was trialled alongside their developed one-step method, Abdulkadir *et al*[142] found that the conventional method had coefficients of variation of up to 70% whereas the one-step method had coefficients of variation of *ca* 5-10% and the recovery

percentage of the one-step method was at least 30% higher than the conventional method. Lepage and Roy's[144] one-step method was found to give recovery percentages that were 3.9-20.1% greater than the Folch method. The results achieved using the one-step method developed in this chapter therefore indicates that is comparable to previously published one-step methods. However, by testing traditional methods alongside the developed one-step method, as was done in this chapter, one can be even more confident of its effectiveness than just comparing it to published methods alone.

Thorough testing was carried out to ensure that this method would be robust enough to be used throughout the project and that the results produced would be valid for statistical analysis. Retention times and response factors were calculated using standards for the FAMEs most commonly identified in insect lipid analysis and identified in the CRW samples. The success of the one-step method of extraction and derivatisation meant that it was used throughout the project.

3 Fatty Acid Profile of the Clover Root Weevil

3.1 Introduction

Fatty acids are compounds of basic significance in biology. Their roles in metabolic energy storage, cell and biomembrane structure, and regulatory physiology appear to apply, in a general way, to most organisms including insects.[160] In insects fatty acids play vital roles at all life stages and in processes such as growth, metamorphosis, reproduction, energy production and flight.[98] As discussed earlier (**Section 2.1**), the lipids of insects are largely comprised of esterifed fatty acids termed triacylglycerols (TAG), as lipids are stored in this form.

The presence of the specialised fat body organ in insects highlights the importance of fat during the life processes of insects.[161] Fat bodies are the main store for insect lipids[107; 162] and the composition of the fat body is a result of different processes including the storage of dietary lipids, *de novo* synthesis, degradation and subsequent release for mobilisation to sites where they can be metabolised.[102] The metabolism of lipids will be discussed further below.

While the chemical composition of the lipids of the CRW has not been previously reported, the composition of lipids is documented for a large number of insect species.[99; 162; 163] The majority of reported literature regarding insect lipids focuses on the qualitative and quantitative description of the fatty acids present in the extracts.[73; 75]

3.1.1 Insect Lipid Composition

There are certain fatty acids that are dominant in all insect lipids. In most insects, because they are non-homiothermic, unsaturated acids predominate over saturated; the lower melting point of unsaturated fatty acids means that they remain liquid to lower temperatures. This was true of the 80 species of insects reported by Young.[92] Unsaturated acids with 18 carbon atoms were the dominant

components together with lesser levels of saturated fatty acids of which palmitic acid (16:0) was only significant contributor. Oleic acid (18:1) ranged in from 19 to 60% contribution.[92] In insects, the dominant fatty acids were 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid) and 18:3 (α-linolenic acid).[74] Other fatty acids found in lower % contributions in some species of insects include 10:0, (capric acid), 12:0 (lauric acid) and 14:0 (myristic acid).[121] Reports of insect-derived polyunsaturated fatty acids beyond 18:3 are rare, as are reports of unsaturated fatty acids longer than 20 carbons. Fatty acids 20:0 and longer were likely to be precursors for the biosynthesis of eicosanoids and hormones.[164; 165] Traces of 15:0, 17:0 (margaric acid) and 19:0 fatty acids have been found in some insects, but these were also rare.[92]

There have been extensive reports on the fatty acid composition of individual species of insects and a common factor between these reports has been the dominance of the 16:0, 16:1, 18:0, 18:1 and 18:2 fatty acids.[74; 75; 95; 97; 98; 100; 101; 107-112; 122; 166-175] Barlow[121] analysed the lipids of 30 species of insects (including species from the Orders Coleoptera, Neuroptera, Trichoptera, Homoptera, Diptera, Lepidoptera, Hymenoptera, Orthoptera and Arachnida) and reported that the 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2 fatty acids accounted for over 80% of the total lipids for each species. Thompson[176] presented an analysis of previous reports, including Barlow's findings, as a summary of the fatty acid composition of seven insect Orders. Downer[177] has summarised these findings as a table (**Table 3.1**). Although this offers a useful general summary, the lack of a standardized procedure means that the results for each Order have come from a variety of experimental procedures for the extraction and estimation of lipids.[177] The results of each Order were also highly influenced by the particular species that were included. For example, the predominance of 14:0 fatty acid in Hemiptera may result from the many representatives of the family Aphididae that were included in the database from which the compilation was made. If instead, the relatively low percentages of l4:0 fatty acid that were detected in such Hemipteran families as Cecopidae, Cicadellidae, Eriosomatidae, Jassidae, Lygaeidae, Myridae, Membracidae and Reduviidae were included, then the results would have been different.[177]

Table 3.1. Downer's summary of Thompson's review of the fatty acid composition of seven insect Orders.[177]

Order	Fatty Acid% Contributions							
	14:0	**14:1**	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**
Coleoptera	1	0	20	5	5	38	19	10
Lepidoptera	1	0	26	6	2	32	8	22
Hemiptera	42	1	20	2	3	16	9	1
Orthoptera	2	0	29	3	8	32	12	9
Diptera	3	1	23	26	4	28	12	2
Hymenoptera	2	0	16	4	4	46	11	16
Dictyoptera	3	0	34	7	4	42	1	0

3.1.2 Main Contributors to Whole Body Fatty Acid Composition

When whole body insect samples are analysed for their fatty acid composition there are four main contributors to the whole body fatty acid profile.

(a) Fat Body

In insects, the major organ involved in metabolism is the fat body and this organ is the most important in both the synthesis and storage of lipid, and the supply of lipid to the haemolymph, for instance during flight.[178] In some insect species the weight of this organ constitutes up to 50% of the fresh weight of the insect.[102; 179] The storage of this lipid (as TAG) is mainly the result of the transfer of dietary fat from the midgut to the fat body during the feeding period but lipid storage from *de novo* lipid synthesis also contributes.[178; 180] These processes will be further discussed below. When whole body fatty acid analyses are conducted for insects, the greatest contribution comes from the fat body, due to the storage of potential metabolic energy as TAG within the fat body.[173; 181]

(a) Cuticle

Fatty acids and the products of their metabolism, particularly hydrocarbons and wax esters, are the main components of the cuticular lipids that cover the epicuticular surface. These lipids prevent desiccation, allow chemical communication and reduce the penetration of chemicals and toxins. They also provide a barrier to reduce susceptibility to fungal infection and reduce entry

of infectious microorganisms.[182; 183] Cuticular lipids are an integral part of an insect's physiology,[184] especially for insects such as scale insects and bees. In insects such as the woolly apple aphid (*Eriosoma lanigerum*) and the giant whitefly (*Aleurodicus dugesii*) secreted wax is observed and therefore influences the lipid profile of the whole insects.[185-187]

Free fatty acids are common in the cuticle, with even carbon number components ranging from 14 to 20 being most prevalent.[188] Extensive *in vivo* study has established the role of fatty acids as precursors to waxes.[189] Investigation into the cuticular fatty acids of the honey bee, (*Apis mellifera*) found that the dominant fatty acid was 18:1, with amounts of 16:0 and 16:1 fatty acids also present. Traces of docosanoic acid (22:0) and lignoceric acid (24:0) were also found.[189] Analysis of the cuticular fatty acids extracted from pine tree lappet (*Dendrolimus pini*) larvae indicated a composition of 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids, where 18:1 fatty acid was found to be dominant. This was different to results obtained from exuviae, suggesting that the fatty acid profile of the cuticle was subject to change during the lifecycle.[182; 190] The four fatty acids dominating the bluebottle blowfly (*Calliphora vicina*), pine tree lappet and greater wax moth (*Galleria mellonella*) larval cuticle were 16:0, 18:0, 18:1 and 18:2 fatty acids, although traces of some of the 5 to 20 carbon atom fatty acids were also found.[190] Little is known of the functional significance of these free fatty acids,[188] although it has been suggested that the free fatty acids serve a defensive role.[190] *In vitro* tests proved that the presence of 16:0, 18:0, 18:1, 18:2 or 18:3 fatty acids in culture media inhibits fungal growth and reduces conidia production.[191] Free fatty acids from the insect cuticle have also been implicated in the inhibition of fungal propagule germination.[192]

(b) Eggs

When a female insect contains eggs, the fatty acids present in the eggs can contribute to the whole body fatty acid profile. Often insects are not dissected before sampling so it can be impossible to determine whether eggs are present or not. Lipids are an important component of eggs. For example, in tobacco

hornworm lipids represent around 40% of the dry weight of a mature oocyte.[193] Lipids are mainly stored as triacylglycerol (TAG) in insect oocytes[193-195] The ability of insect oocytes to obtain fatty acids by *de novo* synthesis is very small. In tobacco hornworm and yellow fever mosquito (*Aedes aegypti),* about 1% of total lipids were synthesized by oocytes in vitro. Therefore, most of the lipids in oocytes must originate from the diet and/or storage tissues such as the fat body.[196-198] The eggs of the Neuropteran *Lertha sheppardi* differed from the other life stages, with lower proportions of 18:2, and higher proportions of 16:1, 18:0 and 18:3 fatty acids. This suggests an accumulation of energetic and structural reserve materials during embryonic development.[199] However the fatty acid composition of the eggs of the field cricket (*Gryllus campestris*) showed decreased amounts of 16:0, 18:0, 18:1 and 18:3 fatty acids and increased amounts of 16:1, 18:2 and 20 fatty acids.[200] The lipids in eggshells are the main protection from water loss.[201] Fatty acids in eggs have also been implicated as sensory aids to ensure dispersal of eggs.[202]

(c) Hemolymph

The fatty acids found in an insect's hemolymph are largely in the form of diacylglycerols (DAG) and usually attached to a lipophorin.[173; 203] Reasons for this will be discussed below. In the hemolymph of the tawny earwig (*Labidura riparia*) the dominant fatty acids were myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0); the monounsaturated fatty acids palmitoleic acid (16:1) and oleic acid (18:1); and the polyunsaturated fatty acids linoleic acid (18:2) and linolenic acid (18:3). The percentage compositions of total and triacylglycerol fatty acids in hemolymph do not change markedly during the reproductive cycle with 18:2, 18:1 and 16:0 fatty acids being the major components. Only traces of lauric (12:0) and arachidic (20:0) acids were detected.[204]

3.1.3 Lipid Metabolism

Although the lipid composition is reported for many insects, much of what is known about insect lipid metabolism is based on results of laboratory studies of relatively few insect species that are easy to rear, in particular tobacco hornworm

(*Manduca sexta*).[180] Although this gives a broad understanding of lipid metabolism in insects, one must be cautious at drawing general conclusions. To fully understand the lipid metabolism of a particular species, several generations must be raised on artificial diets.[180] A task which is not only outside the scope of this project but judging by the lack of results published, even beyond the scope of most entomology investigations.

(a) Incorporation of Dietary Lipids

In insects, midgut cells produce lipases that hydrolyze dietary lipids,[180] however, enzymology of lipid metabolism in insects is virtually unknown.[160] Unlike mammals, insects lack bile salts and therefore require other mechanisms to facilitate lipid solubilisation.[178] These include the use of luminal glycolipids and the formation of fatty acyl-amino acid complexes. Absorbed fatty acids and partial acylglycerols are then converted into intestinal DAG, TAG and phospholipids. Two pathways may be involved - the monoacylglycerol pathway (acylation of 2-monoacylglycerol) and the *de novo* or phosphatidic acid pathway (acylation of *sn*-glycerol-3-phosphate).[180] There has been evidence of both pathways occurring in insects.[178] However, the contribution that each pathway makes has only been established in larval tobacco hornworm (*M. sexta*) which indicated that it was the largely the phosphatidic pathway.[205] Earlier work suggested that dietary TAGs were absorbed directly into insect's hemolymph either unchanged or resynthesized from products of their hydrolysis.[206] This work was later disputed by Chino and Downer using the same insect species previously investigated.[207] Since then, numerous *in vivo* and *in vitro* studies using several insect orders have all concluded that DAG is the main lipid in insect hemolymph after lipid digestion.[56; 205; 208-214]

Unlike in vertebrates, dietary lipids (as DAG) are added directly to the existing lipophorin (a lipoprotein) in the hemolymph. Lipophorin cycles between the midgut, where it picks up lipids, and the fat body, where lipids are delivered and stored. However, lipophorin does not enter the midgut cell of the fat body cell during this process, instead acting as a transport shuttle. The mechanisms involved in the transfer have not been fully investigated, although a model is offered by Canavoso *et al*[180](**Figure 3.1**).

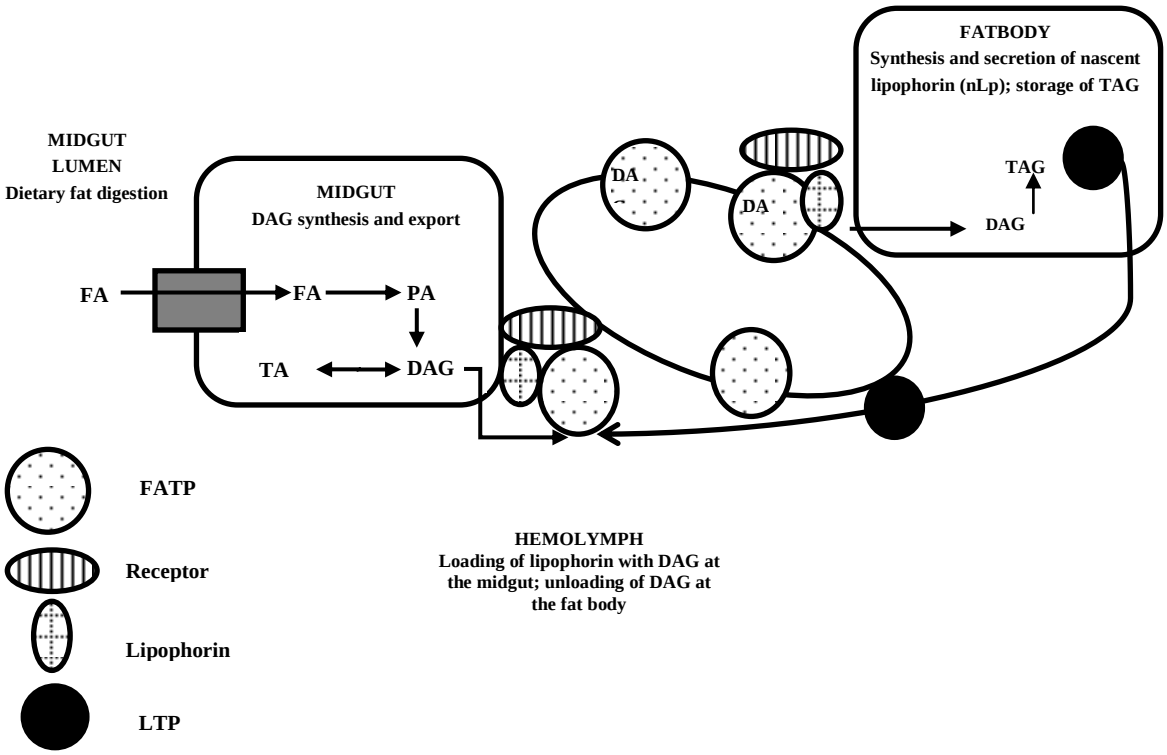


Figure 3.1 Scheme showing the absorption of fatty acid FA into the midgut enterocyte, the synthesis of DAG in the midgut cell, and the transport of DAG to the fat body for storage after. FATP = fatty acid transporter; LTP = lipid transfer particle.[180]

(b) Influence of Dietary Fatty Acids

Incorporation of dietary lipids is the major contributor towards the lipids of insects, therefore, the fatty acid composition of insects is highly influenced by the fatty acid composition of the insect's diet.(174) The lipids of the common house cricket (*A. domestica*) were reported to reflect the dominant lipids in their food.(122) The shield bug (*E. integriceps*) feeds on wheat and as a result has high levels of saturated and unsaturated 16 and 18 fatty acids,(109) while the fatty acid profile of the boll weevil was found to be very similar to that of its natural diet (cotton).(215) Insects with different functional feeding groups were investigated and their fatty acid composition differed predictably due to different diets.(216) Ogg *et al*(170) studied the fatty acid profiles of five species of beetle at different life stages. Although the profiles were very similar regardless of which life stage the beetle was in, there were some minor differences. It was suggested that these differences in the fatty acid profiles of different life stages of beetles, could be caused by a difference in diet for each life stage.(170) Numerous studies have

shown that the fatty acid compositions (rather than total fat contents) of whole insects can be altered by changing the levels of dietary polyunsaturated fatty acids. By increasing the levels of polyunsaturated fatty acids, an increase in the levels of polyunsaturated fatty acids in the tissues is seen, and this is coupled with a decrease in the levels of monounsaturated and saturated fatty acids.[173; 217-226] Unusual lipid constituents in the diet may also be incorporated into fat body fatty acids.[171]

There have been a few reported exceptions to the consensus that the fatty acid composition of insects is largely derived from their food sources. Exceptions include aquatic insects and some Antarctic beetles that are high in unsaturated fatty acids containing 20 carbons. It is thought that the high levels are not caused from diet alone.[216] Instead, it is likely that habitat temperature affects the fatty acid composition of insects (via *de novo* synthesis) with the fats of insects living in a hot climate having a higher melting point and a lower iodine number than those insects living in cold regions.[94; 174] However, as CRW originates from temperate to cool Northern hemisphere zones, this phenomenon is not likely to be seen in CRW.

Other differences between the fatty acid profiles of insects and their diets have been reported. Results from the cockroach (*Blaberus discoidalis)* suggest that the occurrence of fatty acids in these insects is independent of their diet, with the fatty acid patterns of insects fed on a fat-free diet being similar to control insects.[171] Insect cell lines are able to maintain fatty acid compositions that are considerably different to the fatty acid composition of their growth media.[226-230] The fatty acid profile of granary weevil, (*Sitophilus granarius*) was different from that of its diet (wheat),[231] as were the fatty acid compositions of the corn weevil (*Sitophilus zeamais*) and rice weevil (*Sitophilus oryzae* L.).[232] It is likely that the *de novo* synthesis of lipids contributes to these differences.

(c) De novo synthesis

The fat body is the site of *de novo* synthesis of fatty acids from excess carbohydrates and certain amino acids.[173; 180] Fatty acids are synthesized by head-to-tail condensation of two-carbon units. Two activating effects are applied

to make the acetate group sufficiently reactive. A thioester group increases reactivity and converting the acetate group into a malonate also increases reactivity. The combined effects of thioester and additional carboxylate are used to activate the CH_3 portion of acetic acid for condensations.[180] N-carboxybiotin is used as a carrier of CO_2 and from biotin, the carboxyl group is transferred to acetyl coenzyme A, converting it to the much more reactive malonyl coenzyme A. An acetyl group is transferred from the coenzyme A to acyl carrier protein (ACP) and from there to β-ketosynthase. Condensation between acetate and malonate occurs, followed by reduction of the keto-group, dehydration of the alcohol and reduction of the double bond. The butyl group moves to the β-ketosynthase and is replaced by another molecule of malonate on ACP. Condensation occurs again and the cycle continues until the chain reaches the required length. The enzymes of fatty acid biosynthesis are held together in a complex known as fatty acid synthetase. The growing chain remains attached to this complex, passing from one enzyme to the next. When the chain has grown to 16 or 18 carbons it becomes detached from the complex. It requires 64 individual steps from acetic acid to make a molecule of stearic acid.[180; 233]

Radiolabelled investigations have determined that all insects examined to date are able to synthesize fatty acids *de novo*.[160] Examination of the destination of the label has determined that the primary products of *de novo* fatty acid synthesis are 16:0, 18:0 and 18:1.[160] Results for some Coleoptera species are shown in **Table 3.2**.[177] Although differences between species are apparent, the 16 and 18 carbon saturated and monounsaturated fatty acids are the dominant fatty acids made *de novo* in these species and it is likely that other species in Coleoptera will have this same dominance. Differences in the absolute amounts of these compounds reported in the various studies may reflect the duration of the experiment. The longer the duration, the longer the time available for elongation and desaturation of the fatty acids that are formed initially. This is substantiated by the fact that in silkworm (*Bombyx mori*), the incorporation of label into 18:0 and 18:1 fatty acids relative to 16:0 fatty acid increases with experimental time.[177; 234] Insects that have l2:0 and 14:0 fatty acids in their tissue lipids are also able to biosynthesize them *de novo*.[160]

Table 3.2 Radiolabelled investigations of the products of *de novo* fatty acid synthesis in some Coleoptera species.[177]

Species	Developmental stage	Substrate	Percentage of substrate incorporated into FA					Reference
			14:0	16:0	16:1	18:0	18:1	
Oulema melanopus	adult	[1-^{14}C]acetate	0	25	3	9	60	Lamb and Monroe[235]
Lyctus planicollis	larva (60d)	[U-^{14}C]glucose	0	7	15	2	63	Mauldin *et al*[236]
	adult (75d)		0	8	12	2	65	
Anthonomus grandis	aseptic adults	[1-^{14}C]acetate	2	24	9	18	23	Lambremont[237]
	normal adults		3	29	9	14	25	

Double bonds are introduced by desaturase enzymes that can remove hydrogen atoms from an unactivated alkyl chain with precision of position and stereochemistry. It has been shown in a number of studies on whole insects that long-chain saturated fatty acids may be directly desaturated to the monoenic equivalents.[177] The desaturase activity was found within the microsomal fraction and had a requirement for oxygen. This is similar to desaturases from yeasts and higher animals. In addition to the direct desaturation pathway, evidence has been obtained for an alternative route for the biosynthesis of palmitoleic and oleic acids in mitochondria of the fruit fly (*Drosophila melanogaster*), which was similar to the monoene synthetic pathway reported for microorganisms.[177] Linoleic and linolenic acids are made by plants by further desaturation of oleic acid. It was previously thought that no animal, including insects, could synthesise either 18:2 or 18:3 fatty acids. However opinion is now divided as to whether some or all insects can.[180] The biosynthesis of 18:2 and 18:3 fatty acids will be further discussed below. Most insects are able to elongate 18:2 and 18:3 fatty acids into longer-chain fatty acids, for example, arachidonic acid.[178; 238]

Branched fatty acids do sometimes occur in small amounts in insect lipids. Their synthesis begins with the amino acids valine and isoleucine. Both isobutyric and 2-methylbutyric acid have been reported as defence compounds among insects. The kapra beetle (*Trogoderma granarium*) (same Order as CRW) and other species of *Trogoderma* use derivatives of the *anteiso*-unsaturated acid (*R,Z*)-14-

methyl-8-hexadecenoic acid, as part of the female-produced sex attractant. Methacrylic and isobutyric acids were found in the defensive secretion from the pygidial glands of the carabid beetle (*Scarites subterraneus*).[233; 239] Small amounts of odd numbered fatty acids are sometimes found in insect lipids. These are biosynthesized starting from a propionic acid group to which are added acetate units in the usual way.[233]

(d) Biosynthesis of Polyunsaturated Fatty Acids

It was once thought that insects, like all other animals, require a source of polyunsaturated fatty acids in their diets and either linoleic or linolenic acids had been shown to satisfy this need in most insect species.[180] In Coleptera, a lack of polyunsaturated fatty acid caused slow larval growth and decreased adult fecundity, although the precise role of the polyunsaturated fatty acid has yet to be established.[240] However, in the last two decades, the biosynthesis of 18:2, 18:3 and other polyunsaturated fatty acids in insects has been reported.[173] The earliest reports of this *de novo* synthesis of polyunsaturated fatty acids were criticised because they failed to eliminate the possible contribution that symbionts could make to the observed biosynthesis. However, it was demonstrated that the synthesis of 18:2 fatty acid occurred in termites that had been defaunated by treatment with carbon dioxide or penicillin-streptomycin, hence removing all possible symbionts.[241] The potential role of microorganisms in linoleate production was further ruled out by studies using isolated tissue under axenic conditions from house cricket (*Acheta domestica*) an insect which does not contain intracellular microorganisms.[164]

Some insects contain a Δ-12 desaturase enzyme and can therefore biosynthesize 18:2 fatty acid. This enzyme has been characterized from house cricket (*Acheta domestica*) and American cockroach (*Periplaneta americana*). In contrast to the plant Δ-12 desaturase, which converts 18:1 fatty acid esterified in a phospholipid as substrate to 18:2 fatty acid, the insect Δ-12 desaturase uses oleoyl-CoA as substrate. Radiolabelled [2-^{14}C] acetate was used to show that silver whiteflies (*Bemisia argentifolii)* synthesize linoleic acid and linolenic acid. Studies of the fatty acid biosynthesis in cecropia moth) (*Hyalophora cecropia*) revealed that although this insect can convert radiolabeled acetate to palmitate, palmitoleate,

stearate, and oleate, radioactivity was never detected in either linoleate or linolenate.[179] 32 species of insects were investigated and the ability to biosynthesize 18:2 fatty acid was only apparent in eight species.[242] This investigation included three species of Coleoptera (**Table 3.3**), none of which were able to biosynthesize 18:2 fatty acid, however, there have been reports of Coleoptera being able to synthesise 18:2 fatty acid.[243] Insects that cannot biosynthesis 18:2 and 18:3 fatty acids require a dietary source.[180] Mosquitoes have an essential fatty acid requirement that is not met by either 18:2 or 18:3 fatty acids.[164] A number of insect species, including representatives of both those that do and do not produce 18:2, elongate and desaturate 18:2 and 18:3 to 20:4 and 20:5 fatty acids respectively.

Some insect species have been shown to metabolize 20:4 to prostaglandins and other eicosanoids.[164] These are molecules of particular biological significance in insects and other animals.[173] They are oxygenated metabolites of certain polyunsaturated fatty acids and hence require polyunsaturated fatty acids as precursors.[177] They are important in insect immunity, stress responses and reproduction. They have also been implicated in host-parasite relationships, particularly those involving ticks.[244]

Table 3.3. Results from an investigation into the ability of three Coleoptera species to biosynthesize 18:2.[243]

Species	**Common name**	**Incubation time (h)**	**Percentage distribution of radioactivity**					
			14:0	16:0	16:1	18:0	18:1	18:2
Hippodamia convergens	Convergent lady beetle	2	5	29	19	29	18	0
Dermestes maculatus	Hide beetle	2	0	24	0	68	8	0
Tenebrio molitor	Yellow mealworm	2	0	15	0	48	37	0

(e) Dietary Requirements of Insects

All insects require sterol in their diets.[245] Cholesterol is the major sterol found in insects and most species of insects have adapted to transform a wide range of dietary sterols into cholesterol.[245; 246] Cholesterol is utilised to produce the molting hormones, ecdysone or 20-hydroxyecdysone. Carotenoids and some fat-

soluble vitamins are also required from the insect's diet but their exact need is unclear.[180]

(f) Lipid Utilisation

Lipids are mobilized from the fat body as DAG (in contrast to free fatty acids as in vertebrates). This is induced by two types of hormones: adipokinetic hormone and octopamine.[180] The initial event in the lipolysis of the fat body TAG involves hydrolysis of the long-chain fatty acylglycerol esters by the action of lipases.[178] Once DAG has formed it is attached to a pre-existing lipophorin in the hemolymph and transported to the tissues by a shuttle system that is similar to the one described above for transport of lipids to the fat body.[180]

3.1.4 Lipid Metabolism in Coleoptera

There are few reports on the specific fat metabolism of Coleoptera. Radiolabelled sodium acetate was used to investigate the *de novo* biosynthesis of fatty acids in the red and gray sunflower seed weevils (*Smicronyx fulvus* and *S. sordidus* respectively, same Family as CRW).[247] Both weevils had very similar results with five fatty acids being produced *de novo*. These were 14:0, 16:0, 16:1, 18:0 and 18:1 fatty acids. Oleic acid was a major product, suggesting that its precursor 18:0 is very quickly desaturated. There was no evidence of 18:2 or 18:3 fatty acid synthesis in either weevil.[247] These results are similar to those presented in **Table 3.2** with 18:1 being the major product. The incubation time was 16 hours which is the likely reason that 18:1 fatty acid is the major product rather than 18:0 fatty acid as shown for the species in **Table 3.3** which had an incubation time of two hours.

In further studies on the same species of weevils, radiolabeled 18:0 fatty acid was used to determine whether dietary fatty acids were directly incorporated into TAGs or whether they were oxidized to acetyl-CoA and resynthesized into new fatty acids.[247] The radiolabeled 18:0 fatty acid was incorporated into several species of triacylglycerol; however, there was no transfer of the radioactive label to non-oleic fatty acids. Therefore, it was concluded that dietary fatty acids were directly incorporated into TAGs.[247] This work was expanded to determine the fate of radiolabeled triolein (TAG with three 18:1 fatty acid moieties). It was

found that triolein was not directly stored in the fat body but instead hydrolysed by lipases and reesterified to glycerol along with other fatty acids. It was not catabolized to acetyl-CoA for resynthesis into new fatty acids.[242] The fatty acid compositions of both weevils were the result of both *de novo* synthesis and incorporation from dietary fatty acids.[247] It is possible that the lipid metabolism of CRW will be similar to these weevils.

3.1.5 Differences between the Fatty Acid Compositions of Different Species

Although most of the fatty acid compositions of different insects are similar,[173] differences can sometimes be used to distinguish between species or even subspecies. As discussed, Diptera have been reported to have exceptionally high levels of 16:1 fatty acid, aphids can have up to 80% of 14:0 fatty acid, while coccids have high levels of 10:0 and 12:0 fatty acid.[89] It has also been suggested that fatty acid composition is characteristic of lineage. The fatty acid profiles of different subspecies of fruit fly (*Drosophila melanogaster)* revealed that each species subgroup was characterised by a distinct fatty acid composition.[94] Samples within bumble bee species were very similar while there was diversity among species.[74] However, the fatty acid composition of similar species can sometimes be very similar. The lipid content and fatty acid profiles of the Nigerian raffia palm weevil (*Rhynchophorus phoenicis)* and the oil palm weevil (*R. ferrugineus*) were investigated as potential sources of essential fatty acids for dietary purposes. The fatty acid profiles of both weevils were very similar.[104] Several species of edible insects commonly found in Thailand had similar fatty acid profiles.[108] 58 genera of aquatic insect had similar fatty acid profiles and the biggest differences among the orders were in the polyunsaturates.[216]

3.1.6 Fatty Acid Profile of Weevils

All reports on the fatty acid profile of weevils fit within the general insect profile. When the fatty acid profiles of the corn weevil (*Sitophilus zeamais*) and rice weevil (*Sitophilus oryzae* L.) were investigated eight fatty acids were found (12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3). 16:0 and 18:1 were the dominant fatty acids in both weevils contributing up to 70% of the total in the rice weevil and 73% in the corn weevil. 18:2 fatty acid was the next most dominant in both

species contributing 15% and 13% respectively.[232] The fatty acid profile of the granary weevil, (*Sitophilus granarius*) was largely made up of 18:1, 16:1 and 18:2 fatty acids.[231] In the boll weevil (*Anthonomus grandis*) seven fatty acids (14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3) account for 98% of the total fat with *ca* 62% of the boll weevil's fatty acids being unsaturated.[215] Frampton[248] found the fatty acid profile of the lucerne weevil (*S. discoideus*) to conform with that which was reported for Coleoptera. 14:0 (traces), 16:0 (18%), 16:1 (7%), 18:0 (7%), 18:1 (35%), 18:2 (6%) and 18:3 (24%) fatty acids were found in the control field sample.[248]

3.1.7 Context of the Present Research

In order to draw any conclusions about changes to the fat of CRW that may occur during parasitism and the extent of any such changes, one must first determine both the fatty acid profile and the fat concentration of the CRW. The fatty acid content of the CRW is previously unreported and determining it would also allow for comparisons with other weevils and insects, as well as comparisons with its diet.

Whole body fatty acid analysis was chosen for this work as visual changes in the fat of dissected CRW had been observed, therefore suggesting that significant changes in the bulk fat of CRW were taking place. Also a large amount of the reported work focuses on the whole body fatty acid analysis of insects, therefore this would allow for comparisons with other species.

3.1.8 Expected Fatty Acid Profile of the Clover Root Weevil

The fatty acid profile of most insects is dominated by the 16 and 18 carbon saturated and unsaturated fatty acids; therefore, it is likely that the fatty acid profile of CRW will also be dominated by these. Work on other species of weevils (Argentine stem weevil,[249] lucerne weevil,[248] Egyptian alfalfa weevil[101] corn weevil,[232] rice weevil,[232] granary weevil[231] and boll weevil[215]) has also found the dominance of the 16 and 18 carbon saturated and unsaturated fatty acids.

The fatty acid composition of white clover (*T. repens*) will be likely to contribute to the fatty acid composition of CRW due to the influence diet generally has on the fatty acid composition of insects.[170; 174] When the fatty acid composition of five species of *Trifolium* wasinvestigated, 12:0, 14:0, 16:0, 18:0, 18:1, 18:2, 18:3, 20:0 fatty acids were found in all species.[250; 251] 18:3 was the dominant fatty acid with 16:0 and 18:2 fatty acids next.[251] Therefore, it is likely that these fatty acids will be present in the fatty acid composition of CRW. However, some weevils (corn weevil,[232] rice weevil,[232] Argentine stem weevil[249] and granary weevil[231]) have shown differences in fatty acid composition from their respective diets so there may be differences in the CRW, although the profiles of other weevils (boll weevil[215] and lucerne weevil[248]) have been very similar to their respective diets. Goldson *et al*[249] suggested that the oleic acid that was the dominant fatty acid in diapausing Argentine stem weevil (*L. bonariensis*) came from bacterial (*Enterobacter* species) biohydrogenation of linoleic acid; linoleic acids makes up 72% of the ryegrass that the Argentine stem weevil feeds upon. It is possible that a similar phenomenon could happen within CRW.

The *de novo* synthesis of fatty acids is also likely to contribute to the fatty acid composition of CRW. Although there have only been a few reports of 18:2 fatty acid biosynthesis in Coleoptera,[243] it is likely that the de novo synthesis of 14:0, 16:1, 16:1, 18:0 and 18:0 fatty acids will contribute to the fatty acid profile of CRW.

3.2 Methods and Materials

3.2.1 General Methods and Chemicals

As described in **Chapter 2**.

3.2.2 Clover Root Weevil Sampling Methods

The CRWs were collected as part of AgResearch's routine sampling. AgResearch scientists sampled CRW from a total of twelve different sites throughout New Zealand using a method developed when CRW was first discovered in New

Zealand.[252] The majority of the sites were based in the Waikato and upper North Island regions, although other sites were used to get sufficient samples.

A modified blower vac (Homelite HB 180 V Blower Vac) was used to suck samples up from a transect at the sampling site. The length of the transect used varied from 30 m to 200 m depending on the abundance of weevils. The adult CRW were sorted from the other insects collected and stored at -20 °C.[55; 253]

3.2.3 Dissection

Individual CRWs were dissected following AgResearch's protocol. The weevils were mounted in wax, with the dorsal side uppermost and abdomen free. The weevils were dissected in aged tap water (aging stops bubbles forming). The elytra** were removed and the abdomen opened by grasping the sclerotised first tergite†† and pulling towards the rear end of the beetle. It was possible to tell immediately if a parasitoid larva was present by the appearance of the fat and the presence of teratocytes. The presence of parasitoid eggs was indicated by appearance of new fat and egg resorption in females, but nothing similar showed in male weevils. Weevil sex, reproductive state, parasitoids present and fat abundance and colour score were recorded (see **Section 4.2.2**).

When CRW of known physiological state were required for fatty acid analysis, the researcher would place each individual dissected weevil in hexane (1 mL) in a vial, ready for analysis. The availability and physiological state of fresh weevils would depend on season and researcher workload. All attempts were made to analyse samples as soon as possible after dissection but if there was any delay, samples were stored at -18°C until analysis.

** Wing cover
†† Hardened plate on first segment of abdomen

3.2.4 Fatty Acid Analysis

164 individual CRW were extracted and derivatised using the one-step method described in **Chapter 2**. Several further bulk extraction and derivatisations, with reagents scaled to suit, were also undertaken. Samples were analysed in batches as soon as possible after dissection. Extraction and recovery factors were applied as described in **Section 2.12.** A summer scholarship student assisted in carrying out some of the extractions for the weighed subset but all samples were processed by the author.

3.2.5 Weighed Subset

A subset of 22 individual CRW samples was weighed prior to dissection and their weights recorded. This subset was used to determine fatty acid concentration of CRW.

3.2.6 Standards

As in **Chapter 2**, the two standards used were heptadecanoic acid (internal standard) and methyl tridecanoate (recovery standard). These standards were accurately made up to solutions of concentration *ca* 1 mg/mL

3.3 Results for Clover Root Weevil

3.3.1 Fatty Acids Present in Clover Root Weevils

The one-step method of extraction and derivatisation developed in **Chapter 2** was used to investigate the fatty acid profile of CRWs. The results from the 164 individual CRW samples found up to 11 fatty acids (plus the two standards) **(Table 3.4**) detected as their corresponding FAMEs. Of these 11 fatty acids detected, only eight were found in every sample and these eight made up the large majority (at least 99%) of every sample.

The eight fatty acids found in every sample were the 16 (16:0 and 16:1) and 18 (18:0, 18:1, 18:2 and 18:3) carbon fatty acids found in large quantities in all insect lipids, as well as the 12:0 and 14:0 fatty acids. The three other fatty acids detected

in trace amounts have all been mentioned as occurring in insect lipids before but are much less common than the other eight fatty acids detected.[92; 121]

3.3.2 Composition of Fatty Acids in Clover Root Weevils

(a) Average Composition of Fatty Acids Found in all Clover Root Weevils

The content of each of the fatty acids present in all of the individual CRW samples was expressed as a percentage of total fatty acids present. Total fatty acids were calculated by adding all corrected peak areas (**Equation 2.3)** corresponding to FAMEs (excluding the standards). The average composition for each fatty acid was calculated over all CRW samples (**Table 3.5**). The corresponding standard deviations and coefficients of variation were also calculated.

Table 3.4. The 11 fatty acids that were detected in the CRW samples.

Retention time (minutes)	Fatty acid identified	Present in all samples
4.1	12:0	Yes
6.1	14:0	Yes
6.5	12-Methyl-14:0	No
7.1	15:0	No
8.3	16:0	Yes
8.8	16:1	Yes
10.5	18:0	Yes
10.9	18:1	Yes
11.6	18:2	Yes
12.5	18:3	Yes
13.7	20:0	No

Table 3.5. Average composition, the standard deviation and coefficient of variation percentage of fatty acids found in all CRW samples.

Fatty acid	Average composition percentage	Standard deviation of composition	Coefficient of variation percentage
12:0	0.680	1.09	161
14:0	1.14	1.70	149
16:0	14.6	3.82	26.2
16:1	9.74	5.65	58.1
18:0	8.22	5.54	67.4
18:1	38.8	8.48	21.9
18:2	6.17	4.69	76.1
18:3	20.7	7.74	37.4

The coefficients of variation (and corresponding standard deviations) calculated were large (ranging from 26% to 161%) representing the large variation between individual CRW samples. The two fatty acids with the lowest average composition percentage (12:0 and 14:0) have the highest coefficient of variation percentage.

(b) Fatty Acids Identified in Some Clover Root Weevils

The maximum percentage of each of the three fatty acids that were only found in some of the CRW samples was calculated, as was the percentage of CRW samples in which they were found (**Table 3.6**). All three of these fatty acids have been found in insect samples before but their presence is much less common than the eight fatty acids that were found in all of the CRW samples.[92; 164; 165]113 Eicosanoic acid (20:0) which was found in the most samples and at the highest concentrations of the three minor fatty acids, is the most biologically significant; the occurrence of 20:0 within insects has received more attention since the 1980s.[164; 165] It has been proposed that this has a prostaglandinogenic role in some insects.[194] 20:0 was reported to occur in boll weevils (*Anthonomus grandis*) at slightly higher levels (1% of total fatty acids) than found here.[254] The reason for the presence of the branched methyl 14:0 and the 15:0 fatty acids in insect lipids is not entirely understood,[113] although the presence of 15:0 was also

reported in another species of weevil (the boll weevil) at very low levels (less than 0.5% of total fatty acids).[254] No other occurrences of these fatty acids have been reported for Coleoptera. No obvious correlations between the presence of these three minor fatty acids and any other factor such as parasitism, sex, size or physiological state were discovered.

Table 3.6. Fatty acids identified in some CRW samples, the percentage of samples that they were found in and the maximum percentage composition.

Fatty acid	Percentage of samples present in	Maximum percentage composition
12-Methyl-14:0	22	0.15
15:0	32	0.11
20:0	44	0.32

(c) Percentage of Body Weight

The results from the weighed subset of CRW samples were used to calculate the concentrations of each fatty acid as an average percentage of body weight (pre-dissection) of the individual CRW (**Table 3.7**). The average concentration for each fatty acid (percentage of pre-dissection body weight) was calculated over all samples in the weighed subset; these ranged from 0.1% to 2.97%.

Total concentration of fatty acids (as percentage of body weight) of each CRW sample was calculated by adding the concentrations (percentage of body weight) of each of the eight fatty acids for the individual CRW sample. **Figure 3.2** shows the total percentage of fatty acids for each of the 22 individual CRW samples. The range for total percentage of fatty acid was between 3.52% and 9.72% of body weight, with the mean being 7.12%. The standard deviation was 1.68% and the coefficient of variation percentage was 23.4%. This was within the range which would be expected for insects as the majority of insects contain 3–15% fatty acids,[101; 103; 104; 255] although some have up to 37% fat depending on what caste they are in.[175; 256] The results found for the CRW were very similar to results for both the pecan weevil (*Curculio caryae*) which was found to have 5.8% (male) and 8.2% (female)[257] and the lucerne weevil (*S. discoideus*) which normally has fat levels between 8–15% , although this can reach 22%.[248]

To further investigate the weighed subset, a scatterplot of the body weight of the CRW sample (mg, pre-dissection) *versus* total fatty acid peak area (total peak area of peaks corresponding to FAMEs but not including standards) was plotted (**Figure 3.3**) and the R^2 value calculated. A positive correlation (R^2 equal to 0.617) was found, indicating that CRW samples with higher body weights had higher amounts of total fatty acids present.

Table 3.7. Average percentage of body weight for each fatty acid in the CRW samples of the weighed subset.

Fatty acid	Average percentage of body weight	Standard deviation of percentage body weight	Coefficient of variation percentage
12:0	0.010	0.0032	30.2
14:0	0.044	0.012	26.1
16:0	1.16	0.30	26.1
16:1	0.860	0.41	47.9
18:0	0.325	0.19	59.5
18:1	2.97	0.71	24.0
18:2	0.265	0.09	35.1
18:3	1.48	0.48	32.6

This work was extended to look at the relationship between body weight and concentration of each of the individual fatty acids. Scatterplots for peak area of each individual fatty acid versus body weight were plotted and the results summarised (**Figure 3.3**). A positive correlation was found for each fatty acid. The value for 12:0 fatty acid was noticeably lower than the other fatty acids but this is in line with what was found in **Section 2.10.4**, where the 12:0 peak was found to have the highest coefficient of variation percentage (largely due to its small size). Only one of the R^2 values is higher than the R^2 value for the scatterplot of bodyweight *versus* total fatty acid peak area (**Table 3.8**). It is likely that the reason for this is that the significance of the variations among each of the fatty acids is reduced when all the fatty acids are combined.

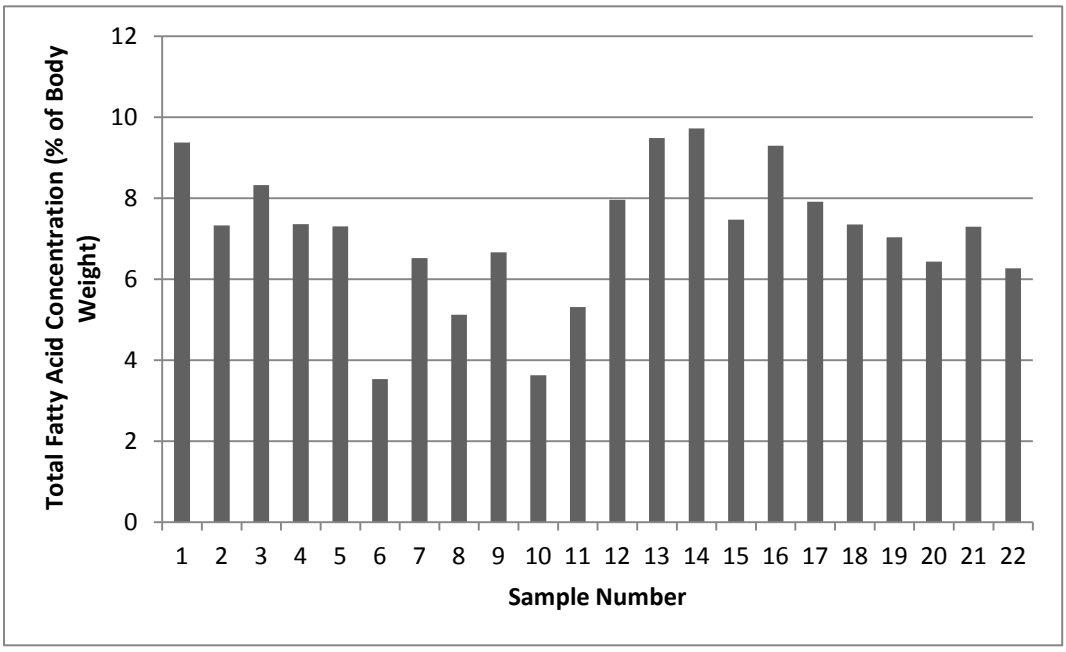


Figure 3.2. Total contributions of fatty acids (as percentage of body weight) for the 22 CRW samples of the weighed subset.

Further correlations from the weighed subset are discussed in **Section 4.4.3**, including investigations into the relationship of total peak area and differing physiological states.

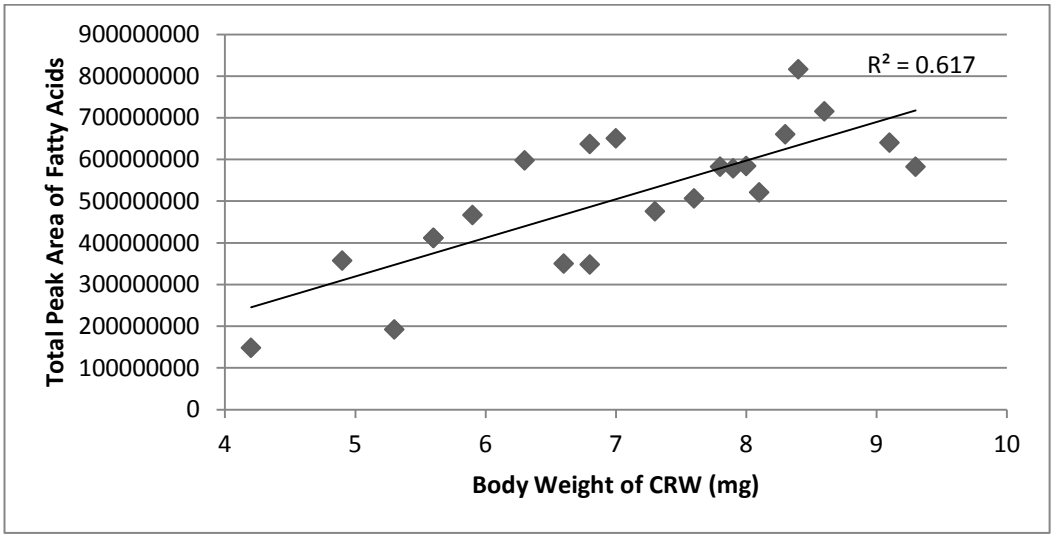


Figure 3.3. Scatterplot of body weight of CRW (mg, pre-dissection) *versus* total fatty acid peak area (excluding standards) with the value of R^2 displayed. Microsoft Excel was used to create the regression line. The equation of the linear regression line is y = 92,000,000x-140,000,000.

Table 3.8. R^2 and regression equations (calculated by Microsoft Excel 2007) for each of the scatterplots of body weight versus the peak area of a fatty acid.

Fatty acid	R^2	Regression equation
12:0	0.12	y = 56000x + 326000
14:0	0.50	y = 551000x - 733000
16:0	0.39	y = 11,372,000x + 1,979,000
16:1	0.49	y = 18,557,000x - 67,563,000
18:0	0.56	y = 4,662,000x-12,084,000
18:1	0.65	y = 42,396,000x – 85,917,000
18:2	0.53	y = 3,125,000x – 4,547,000
18:3	0.39	y = 13,466,000x+2,596,000

3.3.3 Errors

It was not possible to carry out replicates, as individual weevils were dissected, recorded and analysed. Testing during the development of the method (**Section 2.10.3**) found that the average variation between halves of the same CRW sample accounted for less than 5% of the total variation. When this is compared to the large standard coefficients of variation percentages seen for each fatty acid (ranging from 26.2 to 161%), it is likely that the variation due to the experimental process (i.e. the variation between halves contributes less than 5% of total variation) is far outweighed by the variation between samples.

3.4 Discussion and Conclusions

3.4.1 The Fatty Acid Profile of Clover Root Weevil

The fatty acid profile of CRW is very similar to that of other insects and is within what is expected for an insect's fatty acid profile.[121; 216; 258; 259] The dominant fatty acid in CRW was oleic acid (18:1 fatty acid), with an average composition of nearly 38%. This fatty acid is commonly the most prevalent within insect lipids,[100; 110] The CRW results are similar to the results for Coleoptera summarised in **Table 3.1**, however there are key differences. The most striking differences are between the % contributions of 18:2 and 18:3 fatty acids, with

CRW having substantially less 18:2 fatty acid (6.2% *vs.* 19%) but substantially more 18:3 fatty acid (20.7% *vs.* 10%) than the reported average for Coleoptera. However, when the results from Thompson's review[176] are scrutinised, it is found that the reported high average value for 18:2 fatty acid (19%) in Coleoptera, is likely to be skewed by certain species and certain families having far greater values than others. For example the 18:2 fatty acid compositions of both fiery hunter (*Calosoma calidum*) and goldenrod soldier beetle (*Chauliognathus pennsylvanicus*) were higher than 50%, while all species that were tested belonging to family Scarabaeidae were higher than 30%. When the results from the four species of Curculionidae that were analysed in the review are examined, the values for 18:2 fatty acid composition range from 8.4% to 14.0%.[176] Although the value found for CRW is still lower than this range, this shows that the species from the family to which CRW belongs, are likely to have lower amounts of 18:2 fatty acid than other families in the same order.[176] When the 18:3 fatty acid compositions reported for the Curculionidae species were examined, the range was 3.9%-23.1%,[176] which range the value for CRW fell within, but this also highlights the variation within families.

The fatty acid profile of CRW was similar to that of other weevils. 16:0 and 18:1 fatty acids were the most dominant fatty acids in both the corn weevil (*Sitophilus zeamais*) and the rice weevil (*S. oryzae*),[232] while in the CRW 18:1, 18:3 and 16:0 fatty acids were the most dominant. The high content of 18:3 fatty acid in CRW with respect to the other weevils is likely to be caused by the high content of 18:3 in the clover diet of CRW (see below). A difference in diet is also the likely reason why the fatty acid profile of the granary weevil (*Sitophilus granarius*) (18:1, 16:1 and 18:2 fatty acids most dominant)[231] is different to that of the CRW. The fatty acids 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 were found to be dominant in all three types of weevil,[232] and also in the boll weevil (99%).[215]

The fatty acid profile of CRW might be expected to be similar to that of the lucerne weevil (*S. discoideus*)[248] as both weevils are from the same genus (*Sitona*). Frampton[248] reported the fatty acid profile of the lucerne weevil (*S. discoideus*) to contain 14:0 (traces), 16:0 (18%), 16:1 (7%), 18:0 (7%), 18:1

(35%), 18:2 (6%) and 18:3 (24%) fatty acids.[248] When these results are compared to the results for the CRW, they are very similar (as both sets of results came from different authors using different methods etc., statistical comparison is not valid). 12:0 fatty acid is not reported in Frampton's results and only traces of 14:0 fatty acid were found. In an attempt to compare the fatty acid profile of CRW with lucerne weevils, 50 lucerne weevils (*S. discoideus*) were analysed by the one-step method as part of a collaboration with J. Dohmen-Vereijssen from AgResearch, Lincoln. The weevils were dissected in the same way as the CRW and fact sheets completed. Unfortunately, their sample vials were sealed with parafilm, which dissolved and produced phthalate contamination in the GC spectra. This meant that the GC spectra could not be integrated accurately due to some overlapping peaks; however, the qualitative fatty acid profile of these lucerne weevils showed that it was similar to that of CRWs. The presence of 12:0 and 14:0 fatty acids were detected which was in contrast to Frampton's results as only traces of 14:0 fatty acid and no 12:0 fatty acid were found in lucerne weevils by the latter. This could have been due to a sensitivity issue. Frampton's control population of lucerne weevils[248] afforded generally similarly results to those reported in **Table 3.5**.

Although comparison with the literature on other insect's fatty acid compositions is a worthwhile exercise and a good starting point for any investigation, one must be cautious about drawing any exact comparisons. There is no standardized procedure for insect lipid investigation and therefore, every author uses their own method for the extraction and estimation of insect lipids. Also the nature of lipid metabolism in insects can be vagarious and some authors even warn against using a representative composition, stating that fatty acid patterns are not fixed or descriptive elements of insects but can be strongly influenced by diet and development.[177] The issue of recovery factors is also very rarely addressed and although one would hope that as part of good scientific practise these were taken into account in each experimental work, without specific acknowledgement of them it is difficult to determine the accuracy of reported experimental work. Some reported work has recovery factors of less than 75%,[254] which highlights the need to take recovery factors into account.

The three fatty acids found only in some CRW samples, have all been reported as occurring in insects, although they are far less common than the eight fatty acids that dominated the CRW samples.[92; 164; 165] Although bulk analyses were done in an attempt to ensure that any trace fatty acid that may have been missed in individual samples were identified, these three fatty acids were not always present in the bulk samples. It is possible that these fatty acids are actually present in all CRW but at such low levels that they are not always detected, even in bulk analysis. However, further work would be required to prove this. The presence of these three minor fatty acids was not obviously correlated to any factor such as parasitism, sex, size or physiological state.

Average concentrations for each fatty acid and total percentage of fat fell within the range expected for insects, with most insects containing 3–15% fat,[101; 103; 104; 255] although some insects have been reported as having up to 37% fat depending on what caste they are in.[175; 256] Lucerne weevils (*S. discoideus*) were found to be between 8–15% fat, although this reached 22% during post-eclosion‡‡ feeding.[248] Total FAME (excluding standards) peak area was found to be positively correlated to body weight of CRWs indicating that larger insects contained more fat.

3.4.2 Reasons for Fatty Acid Profile

(a) Diet

The main source of fatty acids for most insects is their diet[170] and the fatty acid composition of CRW is similar to that which has been reported for the fatty acid composition of the major component of its diet, white clover leaves (*T. repens*).[250; 251] However, a major difference between the fatty acid profiles of CRW and white clover is the high % contribution of 18:2 fatty acid in white clover leaves compared to the relatively low % contribution in CRW and the correspondingly low 18:1 fatty acid % contribution in leaves but high 18:1 fatty

‡‡ The emergence of an adult insect from a pupa or larvae from an egg.

acid % contribution in CRW. 18:3 (*ca* 60%), 16:0 (*ca* 12%) and 18:2 (*ca* 10%) are the three dominant fatty acids in white clover leaves and although 18:3 and 16:0 feature in the top three fatty acids present in the CRW, 18:2 fatty acid contributes less than 10%. The fatty acid composition of 18:1 is low in white clover leaves (less than 10%) but is the major fatty acid in CRW. Goldson *et al*[249] found a similar result when the fatty acid composition of the Argentine stem weevil (*L. bonariensis*) was compared to that of its ryegrass diet. This was attributed to extensive biohydrogenation of linoleic acid (18:2 fatty acid) by bacteria found in the alimentary tract of the weevils. This could explain both the lack of 18:2 fatty acid and the abundance of 18:1 fatty acid in CRW, when compared to the fatty acid profile of white clover leaves. A similar phenomenon was also seen in the Egyptian alfalfa weevil (*Hypera brunneipennis*)[101] and the alfalfa weevil (*Hypera postica)*, where both insects contain far more 18:1 fatty acid than the foliage on which they feed. Lambremont *et al*[215] reported that the boll weevil (*Anthonomis grandis*) was able to desaturate both 16:0 and 18:0 fatty acids to their respective monounsaturated fatty acids, and this could also be happening within the CRW, although the 18:0 fatty acid level within white clover leaves[251] is similar to what is found in the CRW. From this evidence, it is possible that biohydrogenation similar to that described by Goldson *et al*[249] is occurring within CRW, increasing their relative amounts of 18:1 fatty acid with respect to their white clover diet. However, *de novo* synthesis of 18:1 fatty acid is also likely to be occurring and this will be further discussed below. 18:2 fatty acid is of particular biological significance in insects (and all other animals); therefore, it is likely that it is being preferentially used by CRW reducing its contribution to the fatty acid profile. Without detailed experimentation involving labelled substrates, it is not possible to determine the exact origin of the fatty acids present in CRW.

Traces of both 15:0 and 20:0 fatty acids have been reported in white clover,[250] and it is possible that the presence of these fatty acids in some of the CRW is due to incorporation from the diet. The incorporation of unusual lipids from a dietary source has been reported.[171]

(b) De novo Synthesis

As in other insects, including other weevils, it is likely that the fatty acid profile is a combination of incorporation of dietary lipids and *de novo* synthesis.[247] Without further detailed study on the lipid metabolism of the CRW, which is outside the scope of this current investigation, one can not accurately deduce CRW's ability for *de novo* synthesis. Instead, it is only possible to infer what CRW's ability to synthesis fatty acids *de novo* may be, based on examples from the literature and CRW's fatty acid profile. The predominance of 18:1 in the fatty acid profile of CRW may be contributed to by *de novo* synthesis, as 18:1 fatty acid along with 16:0 and 18:0 fatty acids, are the primary products of insect's *de novo* fatty acid synthesis.[173] Due to the substantial difference between the 18:1 fatty acid composition of CRW and the 18:1 fatty acid composition of its diet, it is likely that at least some *de novo* synthesis occurs in CRW, as it does in most animals, including insects.[199] 14:0, 16:0, 16:1, 18:0 and 18:1 fatty acids were all found to be produced *de novo* by the red and gray sunflower weevils, and all are found in CRW suggesting that they may also be synthesised *de novo*.[247] It was found that a significant portion of these weevils' fatty acids came from carbohydrates from photosynthesis by the host plant and therefore it was suggested that an active fatty acid synthase and desaturase system converted the acetyl-CoA from these carbohydrates to fatty acids, mainly 18:1 fatty acid.[247] The ability of insects to synthesise 12:0 fatty acid *de novo* has also been reported and this may also occur within CRW.[160] The biosynthesis of polyunsaturated fatty acids has not been frequently reported for species of Coleoptera,[243] and without further experimentation it is not possible to determine whether CRW is capable of synthesising polyunsaturated fatty acids.

It is likely that the branched 14:0 fatty acid found in some CRW is synthesised *de novo* as no branched fatty acids have been recorded in clover lipids.[250; 251] The synthesis of most branched fatty acids begins with the precursor amino acids valine and isoleucine.[233] The 15:0 fatty acid found in some CRW could also have been synthesised, as odd-numbered fatty acids can be synthesized from a propionic acid starting group to which acetate units are added.[233] However since

15:0 fatty acid has been reported in clover, further experimentation would be required to prove its origin.

3.4.3 Whole Body Analysis

The fatty acid composition of whole insects is affected by four main components, but the main influence is from the fat body as this is the largest store of fat within an insect's body.[74; 178] Contributions from the fat associated with the cuticle, hemolymph and eggs (if present) also contribute to the fatty acid profile. As there are no particular fatty acids that are only associated with one of the main contributors, it is difficult to determine the source of the fatty acids when doing a whole body analysis. 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids are the dominant fatty acids of all four contributors.[189; 200; 204]

Whole body fatty acid analysis is commonplace, with the majority of literature utilising this technique.[142] Although this method is quick and reduces the chance of oxidation, information is lost about the special features of individual tissue patterns as these are not observed in whole body fatty acid analyses. It is therefore, difficult to determine the physiological significance of fatty acids at the tissue and cellular level, when using whole body data.[103] Whole body analysis was used in this study as visual differences had been observed in the lipids of dissected CRW that were in different physiological states. This was the basis of the rationale that there would be chemical differences in the whole body lipids of CRW, therefore a method was developed to analyse whole body samples and this was also used in this chapter. Specific tissue or lipid analysis was beyond the aims of this work. Also whole body analysis reduces any potential oxidation that may occur in dissected tissue samples. The vast majority of published work on insect fatty acids also uses whole body analysis so by utilising this method, comparisons can be made with other published work.

3.4.4 Significance of Whole Body Fatty Acid Profile

Although there is general agreement regarding insects fatty acids functions, it is still difficult to assign a physiological significance to particular fatty acids.[173; 204] The fatty acid of most reported biological significance for insects, when the eight

dominant fatty acids are taken into account, is 18:2 fatty acid.[3b] One of the major functions of 18:2 and unsaturated fatty acids in general, is that they serve as a structural component of membranes, maintaining proper fluidity and permeability. 18:2 fatty acid is also a precursor to the physiologically important arachidonic acid (20:4 fatty acid), which is itself a precursor to the eicosanoids, including prostaglandins.[170; 173; 199; 260] The significance of these elongation/ desaturation pathways in which eicosanoids are made, is that insect cells have the ability to generate particular fatty acid compositions that are required to meet cellular and organismal needs.[75] Due to the biological significance of 20:0 fatty acid it is likely that it is present in all CRW, although it was only detected in some samples. Polyunsaturated fatty acids are also used as a flight energy source.[261]

When the significance of the three minor fatty acids that were only present in some CRW samples is considered, it is likely that the presence of 20:0 fatty acid is linked to the biosynthesis of prostaglandins. However, the biological significance of 15:0 fatty acid and the branched 14:0 fatty acid is more difficult to infer. The presence of odd-numbered fatty acids is rarely reported in insects, although some authors believe that they are in fact a fairly common component of insect lipids, just missed in analysis or reporting.[109] There have been cases however, when an odd-numbered fatty acid has been reported although mass spectral verification of identity or the like has not been used. When identifications are carried out using only retention time it is possible that unsaturated even-numbered fatty acids are misidentified as the saturated fatty acid with one less carbon.[180] Also whole body analyses may mean that trace amounts of odd-numbered fatty acids are swamped out. The occurrence of odd-numbered fatty acids is more commonly reported in specific tissues or lipid types. For example in the phospholipids of mealworm beetle (*Tenebrio molitor*),[262] specific tissues of cicada (*Tibicen dealbatus*)[263] and two species of chinch bugs (*Blissus leucopterus leucopterus* and *B. iowensis*).[264] It is possible that if in the current study we analysed specific lipid classes or tissues, the presence of odd-numbered fatty acids would have been greater. Probably as a consequence of the lack of reporting of odd-numbered fatty acids, the biological significance of odd-numbered fatty acids has not been thoroughly investigated and is therefore, is not understood.[109]

Apart from the significance of individual fatty acids, the fatty acid profile of an insect can give an indication of which fatty acids are stored and which are used preferentially, especially when compared to the profile of their diet. In *L. sheppardi* the major fatty acid was 18:1 fatty acid which made up between 38% and 45% of the fatty acid composition, but this component was only 3% of the dietary source for this insect. It was inferred that *L. sheppardi* was able to store 18:1 fatty acid and utilize 18:2 and 18:3 fatty acids preferentially as the insect lipids were higher and lower respectively in these acids than in the diet. These authors then suggested that *L. sheppardi* is able to modify its fatty acid compositions, probably to suit its physiological requirements.[199] It is likely that the CRW preferentially utilises 18:2 fatty acid while storing 18:1 fatty acid, as there are differences between its fatty acid profile and that of its diet. As 18:2 fatty acid is particularly significant,[3b] it is not surprising that CRW utilises this fatty acid. A similar situation was reported for the beehive honey moth (*Vitula edmandsii serratilineella*) as both the larvae and adult contained relatively high levels of 16:0, 16:1 and 18:1 fatty acids, even when these acids were not predominant in the dietary lipid.[265]

3.4.5 Data Limitations

Due to the limited availability of CRW samples, the weevils analysed had come from multiple sites over different seasons, therefore, one would expect variation between samples. This was seen as large standard deviations and coefficients of variation percentage were found. As the experimental error within the method was proven to be relatively small (variation between halves contributed less than 5% of total variation), this variation has arisen largely from variation within the CRW samples. If a greater number of samples were available, it would have been beneficial to be able to sort samples into age, season and site and report the fatty acid composition for each subgroup. However due to the nature of the sampling procedure used, the outcome is truly representative of CRW in general. Further attempts at analysing subgroups are discussed in **Chapter 4**.

Another possible reason for the large variation is the fact that individual insects were analysed. As the majority of literature does not report on individual insects

but instead much larger sample sizes, it is possible that a lot of the variation between individuals is averaged out. However, for the data to be accurate for use in **Chapter 4**, individual analyses were necessary in this work.

4 Changes in the Fatty Acid Profile of Clover Root Weevil During its Lifecycle

4.1 Introduction

The lifecycle of an insect involves distinct developmental stages. As these stages progress, two types of lipid changes are possible, in the total amount of lipids present and the fatty acid composition of these lipids. By investigating these changes, insights into the importance of different lipids to the insect can be gained. While this study represents the first investigation into the lipids of the CRW, the lipid metabolism of many other insect species during their life cycle has been investigated.[173; 266-270]

4.1.1 Changes in the Total Amount of Lipid

The majority of insects for which lipid data has been reported have changes in their total lipids during their lifecycle. Lipids are accumulated during some stages and utilised in others. During dormant states such as aestivation and diapause, insects consume stored lipid. The lipid composition of adult Egyptian alfalfa weevil (*Hypera brunneipennis*) declined by *ca* 50% of body weight by the end of aestivation,[101] and the gall moth (*Epiblema scudderiana*) also had a decrease in total lipids during diapause.[166] It is crucial to survival that prior to entering dormant states, lipids are accumulated. Documented examples of this include the adult butterflies (*Aglais urticae* and *Inachis io)*.[271]

Lipids are also used in other stages of the insect lifecycle such as transformation from juvenile to adult. A reduction of total lipids was seen in adult pecan weevils (*Curculio caryae*) compared to larvae. During this transformation lipids were reduced from 41% in larvae to 6-8% in newly-emerged, unfed adults.[257] However, despite this reduction in total lipids, the ratio of oleic acid (18:1 fatty acid) to linoleic acid (18:2 fatty acid) was 2:1 in all lifecycle stages of the pecan weevil.[103] Not all insects use lipids during pupal-adult transformation. Studies on

the total lipid changes of the male cecropia moth (*Hyalophora cecropia*) found that it utilises little, if any, lipids during the pupal-adult transformation.[272] During the pupal period of the blow fly (*Lucillia sericata*), lipid content increases once the biosynthesis of fatty acids commences on the ninth day.[273]

Other processes in the lifecycle of insects have been found to affect the total lipid content. The fat body of the Colorado beetle (*Leptinotarsa decemlineata*) decreased during the last two larval moultings, suggesting that lipids were involved as an energy source or building material.[163] Lipids were accumulated by the female madeira cockroach (*Leucophaea maderae*) during the 20-day period of oogenesis.[§§] Accumulated lipids were found to be a major substrate for the developing embryos during the following two months of embryogenesis[***].[274]

The effect of imposed starvation on insects reduces the total amount of lipid present, as would be expected. Canavoso *et al*[102] investigated the starvation-induced changes in the lipid content of the fat bodies of three types of reduviid bugs. After 30 days of fasting between one half and one third of the amounts of lipids that were found on day ten were still present. Starvation also caused the dry weight of the fat body and the total lipid content to decrease in adult tobacco hornworn (*Manduca sexta)* while the haemolymph lipid concentration dramatically increased.[275]

4.1.2 Changes in the Fatty Acid Composition

The fatty acid compositions of insects are not fixed and some change seasonally. Influences on the composition include development,[173; 266-270; 276] diet and diapause status.[110] Although there is general agreement about the importance of

[§§]creation of an ova (egg cells)
[***]formation and development of an embryo

fatty acids to insects, it is difficult to assign a physiological significance to the fluctuations in particular fatty acids over the lifecycle of an insect.[107; 173; 277]

During the development of insects, changes in the fatty acid composition are possible. The fatty acid profiles of adult and larval southern corn rootworm (*Diabrotica undecimpunctata*) adults differ, possibly due either to the impact of developmental changes or different diets or a combination of both.[170] Differences between larval and adult fatty acid profiles were also seen in the western corn rootworm (*Diabrotica virgiferavirgifera*) and the banded cucumber beetle (*Diabrotica balteata*).[170] However, there are many species of insects that have no changes in their fatty acid composition during their lifecycle. No differences were reported in the fatty acid profiles of eggs, all nymphs and male and female adults of the field cricket (*Gryllus campestris*)[98; 278] or the house cricket (*Acheta domesticus*).[111; 279] The tobacco hornworn (*Manduca sexta*)[28] and Egyptian alfalfa weevil (*Hypera brunneipennis*)[101] also had constant lipid profiles throughout their lifecycles. Although silver whitefly (*Bemisia argentifolii)* had different amounts of lipids in instars of different ages and adults, the fatty acid composition was similar throughout all ages.[97]

A large number of insects experience changes in fatty acid composition during diapause. Changes in the proportions of 18:1 and 18:3 fatty acids were related to diapause in the silkworm, *(Bombyx mori*),[280] while during diapause in the south western corn borer (*Diatraea grandiosella*), the % contribution of oleic acid (18:1 fatty acid) in the fat body dropped significantly.[281] Diapause caused the fatty acid composition of the codling moth (*Cydia pomonella*) to change, with an increase in the ratio of unsaturated to saturated fatty acids from 1.72 in non-diapause larvae to 2.63 in diapause larvae.[110] A rise in the concentration of unsaturated fatty acids was also seen in the lucerne weevil (*S. discoideus*) during aestivatory diapause, although the concentration of 18:1 fatty acid remained relatively constant.[248] Any differences seen are likely to be caused by a change in the metabolism during diapause, although very few specific examples are given.

A decrease in the temperature has been found to affect the fatty acid composition of some insects. The proportion of unsaturated fatty acids in two species of cold-hardy gall insects increased over winter.[166] It is likely that this increase in unsaturation helps maintain fluidity within the insect's membranes during the colder weather.

Even after thirty days of fasting the relative fatty acid composition of triatomine bugs was unchanged.[282] Similar results were also seen in reduviid bugs.[102]

There is very little information available regarding the changes in the fatty acid composition of the host as a result of parasitism. Nurullahoglu *et al*[61] have reported that the fatty acid composition of the wax moth (*Achroia grisella*) remained the same even when it was parasitised by *Apanteles galleriae* (a solitary endoparasitoid). Parasitism of the flesh fly (*Sarcophaga bullata)* by the jewel wasp (*Nasonia vitripennis*) caused a lipid accumulation in the fat body, although changes in the fatty acid composition were not investigated.[283]

4.1.3 Changes in the Fatty Acid Composition During the Lifecycle of Weevils

The changes in the fatty acid composition of weevils (Family Curculionidae) during their lifecycles follows the same pattern as insects in general, in that some weevils experience changes and some do not. Dormancy states such as diapause and aestivation have an effect on the fatty acid composition of some weevils. The fatty acid composition of the Argentine stem weevil (*Listronotus bonariensis*) was found to change during diapause. Diapausing weevils had more oleic acid (18:1 fatty acid) and less palmitic acid (16:0 fatty acid) than reproductive weevils.[249] Goldson *et al*[249] suggested that oleic acid is preferentially oxidised during times of high metabolic activity (such as during reproductive activity) causing the difference between the reproductive and diapause states. The boll weevil (*Anthonomus grandis*)[215]and the alfalfa weevil (*Hypera postica*)[284]also undergo similar changes in their fatty acid compositions during diapause. However, in contrast, Frampton[248] found diapausing lucerne weevils (*S. discoideus*) to have a lower percentage of oleic acid than any of the other physiological groups sampled,

although the results were somewhat variable. When the fatty acid compositions of different ages of Egyptian alfalfa weevils (*H. brunneipennis*) were compared, very few changes were seen. Although the total lipid content changed, the lipid composition of pre-aestivating and aestivating adult were very similar.[101] This was similar to that which was seen between the larvae and adults of pecan weevils (*Curculio caryae*), as although the total amounts of lipids were different, the ratio of oleic acid (18:1 fatty acid) to linoleic acid (18:2 fatty acid) was 2:1 in all stages of the weevil.[103; 257] As all reported changes in Curculionidae appear to relate to dormancy states, it is difficult to predict what changes, if any, will occur in CRW which has no dormancy period.

4.1.4 Differences in the Fatty Acid Profiles of Sexes

Although some species of insects exhibit differences in the fatty acid profiles of the sexes, in the majority of insect species the sexes have similar fatty acid profiles.[95] The fatty acid composition of adult periodical cicadas (*Magicicada septendecim*) was similar between males and female,[112] as it was in the shield bug (*Eurygaster integriceps*).[109] The fatty acid profiles of both sexes of the southern corn rootworm (*Diabrotica undecimpunctata),*western corn rootworm (*Diabrotica virgiferavirgifera*) and banded cucumber beetle (*Diabrotica balteata*) were similar to one another.[170] The fatty acid composition of adult male and female of the northern corn rootworm were also relatively similar, except for males having slightly lower 18:1 fatty acid (31% *vs*. 44%) and higher 18:2 fatty acid (36% *vs*. 25%) than females. No reasons were suggested for these differences.[170]

Where differences exist, few reasons are given for the differences found between the fatty acid profiles of male and female insects of the same species. Adult female periodical cicadas incorporated just over five times more radioactive labelled acetate than males, indicating that they biosynthesised fatty acids at a greater rate, although the biological basis of this large difference is unclear.[112] It is possible that some of the difference seen is due to the production requirements of eggs for females. In *Lertha sheppardi* the higher level of 16:1 fatty acid in adult females when compared to males was suggested to be related to the need for

accumulation of sufficient energy and a carbon reservoir in the developing new vitellum[†††].[(277)] It has been reported that male Coleoptera use 18:1 and 18:2 fatty acids to make pheromones.[(285)]

4.1.5 Context of the Present Research

The lipids of CRW have been observed to change in appearance with differing sex, insect age and parasitism, although any chemical changes occurring in the lipids of CRW have not been previously reported.

Parasitism can affect the lipid content of a host insect,[(57; 58)] and because of these visual observations by AgResearch scientists, parasitism by *M. aethiopoides* is hypothesised to affect the lipids of the CRW. Once parasitised, female CRW absorb their reproductive organs and begin accumulating fat. It is not known whether parasitism causes the female CRW to become more like a male CRW (male CRW accumulate fat from the start of their adult lives) or that it is just that lipids are not being utilised for egg production. Nor is it known if the parasitoid induces this change in the host environment to provide a favourable food resource for the parasitoid's progeny or to ensure the host has enough resources to stay alive until the parasitoid larvae have completed development.

The purpose of the work described in this chapter was to determine how the chemical composition of CRW lipids change with age, physiological state and parasitism.

4.2 Methods and Materials

4.2.1 General Methods and Chemicals

General methods and chemical sources are as described in **Chapter 2**.

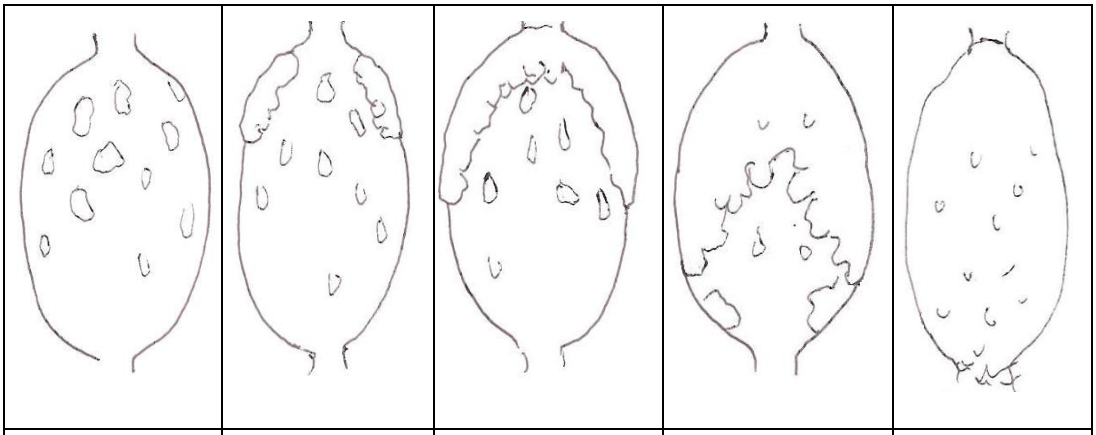
[†††] egg yolk

4.2.2 Differences in Fatty Acid Composition between Clover Root Weevils of Different Physiological States

The 164 CRWs that were utilised in **Chapter 3** were used for this work. These consisted of 15 parasitised and 38 non- parasitised males and 26 parasitised and 85 non-parasitised females. In order to summarise the visual differences and hence compare differences between individual weevils, fact sheets had been used to record visual observations for each individual CRW that was dissected for the fatty acid analysis as described in **Chapter 3**. Scales were developed to describe the amount of flight muscle, the sexual maturity, the amount of fat and the colour of the fat. The presence of eggs (CRW), teratocytes and oil were noted as was whether or not the CRW had been mated. The number and stage of parasitoid larvae present was also noted as was the month and location from which the samples were collected. The factors on which each dissected individual CRW was scored are summarised in **Table 4.1.** and **Figure 4.1-Figure 4.5**. Only whole numbers were used when scoring any of the factors. As the literature focuses on changes in the fatty acid composition or ratios, the majority of CRW samples were not weighed prior to analysis. This meant that results for each fatty acid were recorded as a percentage of total fatty acid present rather than as an absolute concentration.

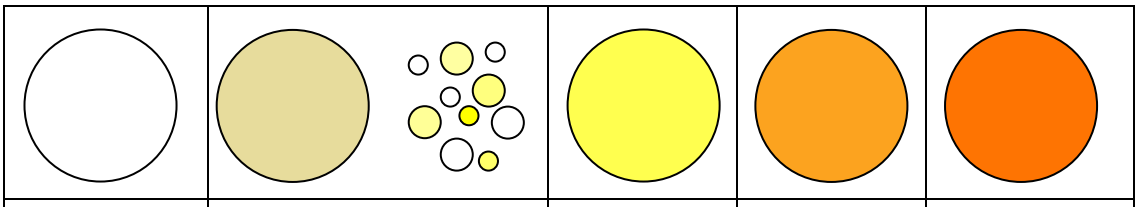
Table 4.1. Factors on which each dissected individual CRW was scored.

Factor	Scale/description
Location collected	Name of the site where the sample was collected.
Month collected	Calendar month when the sample was collected.
Sex	Male or female.
Flight muscle present	Amount of flight muscle present on a visual scale of 0 (no flight muscle) to 3 (mature flight muscle).
Sexual maturity	Males 0 (immature) to 3 (fully developed testes) Females 0 (no development) to 5 (fully developed ovaries containing mature eggs)
Eggs	Presence (1) or absence (0) of eggs.
Mated	Presence (1) or absence (0) of sperm in spermatopohore
Parasitoid	Presence (1) or absence (0) of parasitoid/s.
Teratocytes	Presence (1) or absence (0) of teratocyte/s.
Amount of fat	Visual scale of amount of fat present from 0 (no fat) to 5 (fat covering entire midgut)
Colour of fat	Visual scale of colour of fat present from 0 (colourless) to 5 (orange-red).
Oil	Presence (1) or absence (0) of oil.
Other comments	Any other comments about the physical appearance of the dissected CRW.



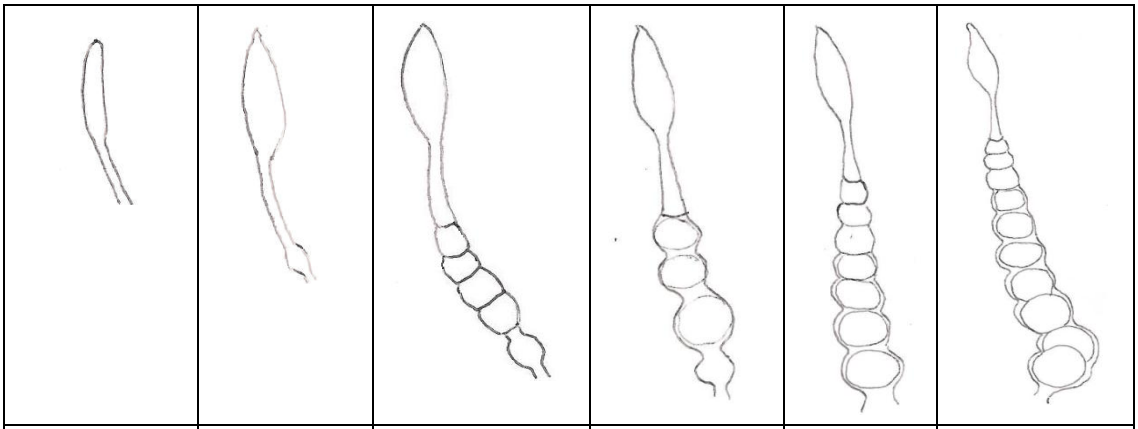
1	2	3	4	5
Scattered empty fat bodies.	Fat on shoulder of mid gut	Fat covering top of mid gut	Fat covering over half of mid gut	Fat covering entire mid gut

Figure 4.1. Diagrams describing how dissected CRW were scored for fat score



1	2	3	4	5
White or clear	Muddy or mix of yellow and white	Clear yellow	Orange	Reddish orange

Figure 4.2. Diagrams describing how dissected CRW were scored for fat colour



0	1	2	3	4	5
No development	Swollen germanium	Full length of abdomen with differentiation	Egg development started	Eggs reaching full size but still yellow	Ivory-white eggs present ready to be laid

Figure 4.3. Diagrams describing how dissected female CRW were scored for reproductive development.

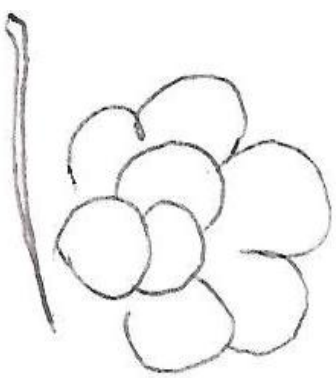
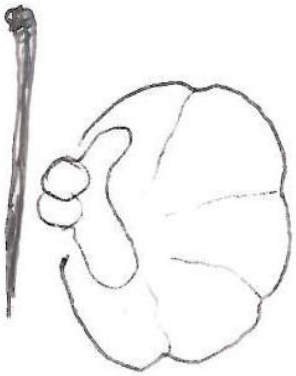
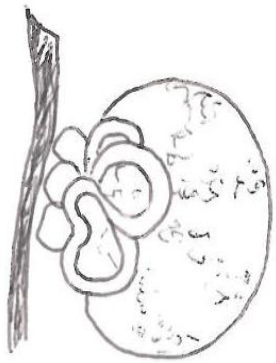
		
1 Little chitinisation of apodemes. Testes in loose lobes	**2** Testes no longer in lobes. Accessory gland starting to show.	**3** Robust apodemes. Thick white accessory glands.

Figure 4.4. Diagrams describing how dissected male CRW were scored for reproductive development.

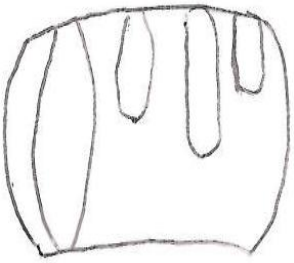
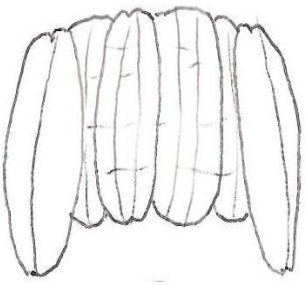
		
1 Small muscle formations visible	**2** Partially formed muscles	**3** Large muscles with multiple strands visible

Figure 4.5. Diagrams describing how dissected CRW were scored for wing muscles.

4.2.3 Gisborne Sample Set

In order to eliminate any variation between the samples that was caused from them being collected in different locations during different months, the 56 CRW samples from Gisborne collected in August were treated as a subset. By treating this group as a subset it would be possible to determine if variation caused by differing months and location of collection interfered with the analysis of the full sample set. This subset was the largest sample set that was collected from the same location during the same month.

4.2.4 Weighed Subset

A subset of 22 CRW samples that were weighed prior to dissection was investigated to determine whether concentrations (as percentage of body weight) of each fatty acid influenced any of the physiological states, or could be used as a predictor for parasitised and non-parasitised individuals.

4.2.5 Statistical Analysis

(a) Data Used

The corrected peak areas for each fatty acid from the chromatogram were converted to percentage compositions (as in **Chapter 3)** by dividing the peak area for each fatty acid present in the sample by the total of all the areas of the fatty acids present (excluding standards). This fatty acid composition data was subjected to the statistical analysis using Minitab 16 that follows. Originally, all of the fatty acids that were present in any of the CRWs were included. However, this resulted in a significant number of zeros in the data set as there were three fatty acids that were only found in some of the samples. The three fatty acids (12-methyl-14:0, 15:0 and 20:0) were only detected in 22%, 32% and 44% of CRW samples respectively. It is possible that they were present in all CRW samples, however, bulk extractions typically failed to detect them. Since the foregoing three fatty acids represented less than 0.01% of total fatty acids in the CRW samples in which they were identified, it was decided to perform statistical analyses using only the eight fatty acids that were present in all CRW.

(b) Initial Survey of Differences between Clover Root Weevils of Different Physiological States using Scatterplots

Scatterplots were created of the composition of each of the eight fatty acids against each of the visual observation scores (flight muscle, sexual maturity, egg, mated, fat, colour and oil) to determine whether any trends existed. Each scatterplot was divided into two plots – parasitised and non-parasitised. The regression lines and coefficients of determination were calculated using Minitab 16.

(c) Differences between Male and Female CRW

The differences between male and female insects of the same species have been investigated for many insects, therefore, the differences between the fatty acid composition of male and female CRW samples were investigated as a starting point.[286] This was done by comparing the average percentages of each of the eight fatty acids for male and female. The data were split into either male or female and no consideration was taken of the remaining information on the sample factsheet as previous reported investigations had taken no account of differing physiological states while investigating the effect of sex.[111] The standard deviations and coefficients of variation were calculated using Minitab 16. This data was plotted in two separate pie graphs to compare visually the differences.

(d) The Data Matrix

For the full sample set the data matrix has eight columns recording the fatty acid composition for each of the 164 weevils. The compositional data has been normalised so that each row adds to 100%. Further columns contain categorical variables such as sex, location or parasitism, which may be used to subdivide the cases for more detailed analyses. The other columns that contain the numerical scales can also be used for further subdivision. The data matrices for the Gisborne sample set and the weighed subset are identical to that of the full sample set except there are 56 and 22 weevils respectively rather than 164.

(e) ANOVA

Although ANOVA (analysis of variance) has not been used in the analysis of insect's lipids previously, it has been used to discriminate between different species, varieties, geographical origins and crop years for the lipids of wheat[287] and also between lipids in different species of seafood.[114] ANOVA can be used not only for analysing experimental data but also for simple descriptive models of the relationships between potential explanatory factors and a response variable with observational data.[155] Two-way ANOVA was used to test for any effects of sex or parasitism on fatty acid percentage (or any interaction between such

predictors). As visual differences in the fat accumulation of female CRW were observed once they were parasitised, this ANOVA was used to determine whether there was any interaction between sex and parasitism affecting the fatty acid composition. This ANOVA ignored all explanatory factors except sex and parasitism.

As there had been no attempt to ensure that the same proportion of parasitised weevils of either sex was present in the sample being assessed, an unbalanced ANOVA was performed with sex fitted before parasitism and the interaction of these two factors was investigated. Parasitism and sex are both two-level factors (parasitised/non-parasitised and male/female, respectively). Effects were evaluated for significance at the 5% level. Use of correction methods such as the Bonferroni correction were not needed as the use of ANOVAs was used to produce an indication of which differences/relationships were worth further investigation, rather than to draw water-tight conclusions, that is it is exploratory.

One-way ANOVA was used after chemometric analysis to investigate variations in the first principal component (PC1) with location and to investigate differences between parasitised and non-parasitised CRW samples in the weighed sample set. The significance level that was adopted for ANOVAs was 5%. As the data is compositional data rather than binomial data, no transformation was required for ANOVA of individual percentages.[156]

(f) Unsaturated/saturated Ratio

The total saturated/total unsaturated fatty acid ratio was investigated for the CRW samples. The saturated/unsaturated fatty acid ratio (total peak area of saturated fatty acids divided by total peak area of unsaturated fatty acids) was calculated for all samples and this was plotted against the physiological states with the data split into parasitised and non-parasitised. The regression equations and coefficients of variation were calculated for the saturated/unsaturated ratio *versus* the physiological state score (taken to be numerical).

(g) Total Percentage of Body Weight

To investigate the effect of total fatty acid concentration (as percentage of body weight), the concentrations of each fatty acid for each individual CRW sample were added as in **Chapter 3**. Scatterplots for each physiological state *versus* total fatty acid concentration (percentage of body weight) were plotted. A one-way ANOVA was used to determine whether there were significant differences in the total fatty acid concentration (as percentage of body weight) between parasitised and non-parasitised samples. The significance level that was adopted for one-way ANOVAs was 5%.

4.2.6 Multivariate Methods

Univariate statistical methods were utilised initially, however, the complexity of the data set required multivariate analysis. Three multivariate methods (principal component analysis (PCA), linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA)) were used and calculations were carried out using Minitab 16 Statistical Software and GenStat Statistical Software. For details of these analyses, how they were applied in this case and the results see **Appendix 9.3**. The statistical analysis was conducted by the author, under the supervision of Dr. Ray Littler, Statistics Department, The University of Waikato.

Canonical analysis was used after these three multivariate methods. Canonical analysis takes linear combinations of the data to successively maximise the variation. It describes how LDA achieves its classification by using the ratios of the maximum variation between groups and that of within groups. As the LDA and QDA for the Gisborne sample set had some success in predicting whether a CRW sample was parasitised or non-parasitised, the alr values were used for canonical analysis. Like PCA, the contributions made by each variable to the analysis can be compared by the size and value of their coefficient. A regression equation and canonical variates, termed “G Scores” were calculated and to investigate further the ability of the G Scores to be able to distinguish between parasitised and non-parasitised, a two-sample t-test was carried out with parasitised as the response.

A two-sample t-test is a special case of an one-way ANOVA.[155] It is the case where the single predictor factor has only two levels (parasitised and non-parasitised).[155] To illustrate the ability of the canonical variates to separate the data into parasitised and non-parasitised, a box plot of the canonical variate for each data point, with the data split into parasitised and non-parasitised was plotted. A ternary plot visually depicts the ratios of three variables as positions inside an equilateral triangle. The proportions of the three variables must sum to a constant (usually one or 100%). Each side of the triangle represents a proportion of 0% while the point of the triangle directly opposite the side represents a proportion of 100%. By plotting three variables, sometimes it is possible to group the data into distinct groups i.e. parasitised and non-parasitised. To investigate if this would work for the Gisborne sample set the three fatty acids (12:0,14:0 18:0) whose alr values were identified as contributing the most (84.5%) to the canonical analysis regression equation were combined to produce a ternary plot.

4.3 Results

4.3.1 Initial Survey of Differences between Clover Root Weevils of Different Physiological States using Scatterplots

Scatterplots (**Appendix 9.4.1**), linear regression trend lines and coefficients of determination (R^2) (**Appendix 9.4.2**) were used to search for useful relationships and to determine any differences between parasitised and non-parasitised CRWs. From the scatterplots the following were noticeable:

- No parasitised female CRW was scored as fully sexually mature – parasitism prevents maturation and causes mature females to revert to a non-reproductive state.
- Parasitised colour scores are much less scattered than non-parasitised – CRW are long-lived and male CRW accumulate fat from the time they emerge, whether they are parasitised or not, so colour intensity is an indication of age. However, female CRW only accumulate fat once parasitised. Therefore, the fat bodies prior to parasitism are generally colourless and as fat accumulated after parasitism is of more recent origin

than that in males, it rarely develops the same intensity. This is especially true in summer when parasitoid larval development takes only three weeks.

- No parasitised CRW scored 0 for fat, unlike non-parasitised CRW– once a female CRW is parasitised it starts accumulating fat.

Only very weak correlations were seen between fatty acid composition and physiological state, and as the coefficients of determination were so low it was not possible to detect valid differences between parasitised and non-parasitised specimens. As no strong correlations were found between fatty acid composition and physiological state, no conclusions could be drawn about how physiological state influences fatty acid composition. All of the scatterplots showed considerable noise due to the large variation between individual CRWs.

4.3.2 Differences between Male and Female Clover Root Weevils

(a) Full Sample Set

The results are shown in **Table 4.2** and **Figure 4.6** and **Figure 4.7**. Although some of the components appeared to differ between sexes, the large coefficients of variation percentages (and corresponding standard deviations) meant that it was difficult to visually compare the values accurately.

Table 4.2. Average % composition of total fatty acids, standard deviation and coefficient of variation for female (F) and male (M) CRW samples.

Fatty acid	Average composition		Standard deviation		Coefficient of variation	
	F	**M**	**F**	**M**	**F**	**M**
12:0	0.57	0.92	0.98	1.27	172	139
14:0	0.90	1.63	0.74	2.75	81.6	169
16:0	14.5	14.2	3.82	3.81	25.8	26.8
16:1	10.5	8.17	5.50	5.71	52.4	69.9
18:0	7.59	9.55	5.25	5.94	69.2	62.2
18:1	39.6	37.1	8.01	9.23	20.2	24.9
18:2	5.80	6.96	5.00	3.91	86.3	56.2
18:3	20.3	21.5	7.29	8.60	36.0	40.0

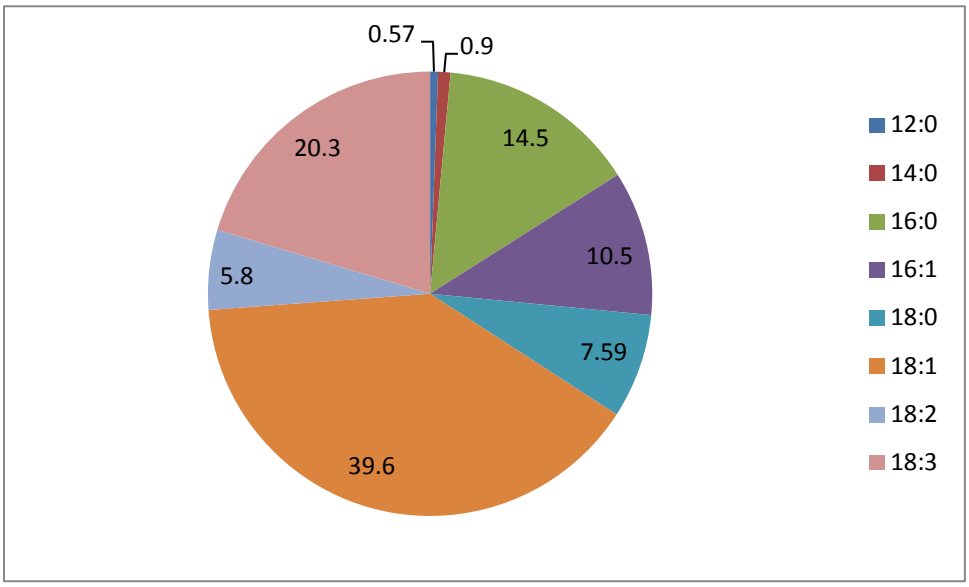


Figure 4.6. Average % composition of each of the eight fatty acids for female CRW samples.

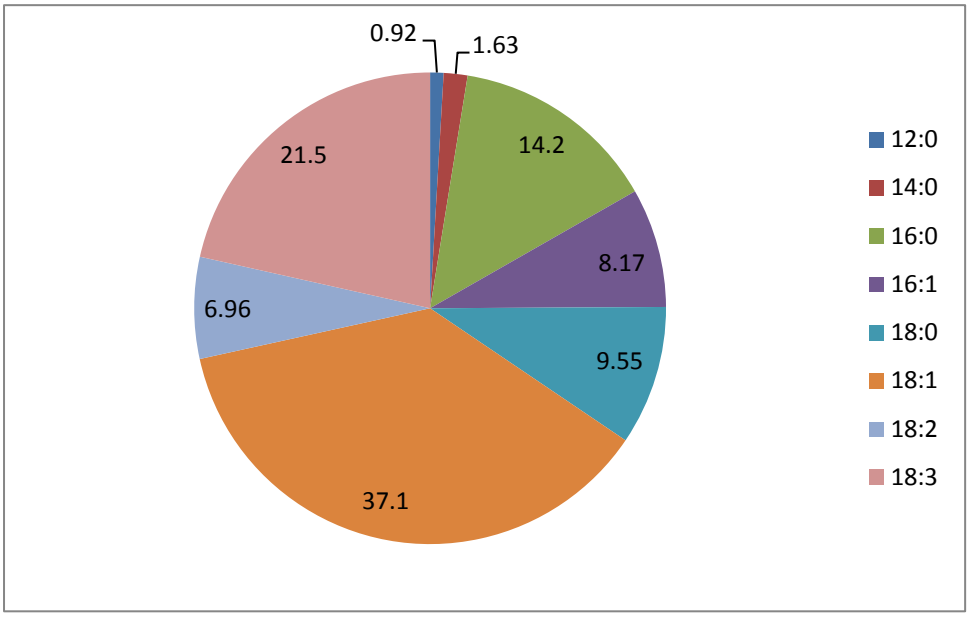


Figure 4.7. Average% composition of each of the eight fatty acids for male CRW samples.

(b) Gisborne Sample Set

The results are shown in **Table 4.3**, **Figure 4.8** and **Figure 4.9**. Although some of the components appeared to be quite different, the large coefficients of variation percentages (and corresponding standard deviations) meant that it was difficult to visually compare the values accurately.

Table 4.3. Average % composition, standard deviations and coefficients of variation for female (F) and male (M) CRWs from the Gisborne sample set.

Fatty acid	Average composition		Standard deviation		Coefficient of variation	
	F	**M**	**F**	**M**	**F**	**M**
12:0	1.03	1.52	1.51	1.76	146.3	116.2
14:0	0.91	1.65	0.65	1.95	71.34	118.0
16:0	11.5	12.5	2.25	3.82	19.5	30.6
16:1	7.99	5.68	3.37	3.50	42.1	61.6
18:0	9.54	9.12	6.29	6.08	65.9	66.7
18:1	38.2	36.7	10.38	11.4	27.2	31.1
18:2	6.79	6.71	5.05	2.58	74.4	38.5
18:3	24.0	26.2	6.38	7.90	26.6	30.2

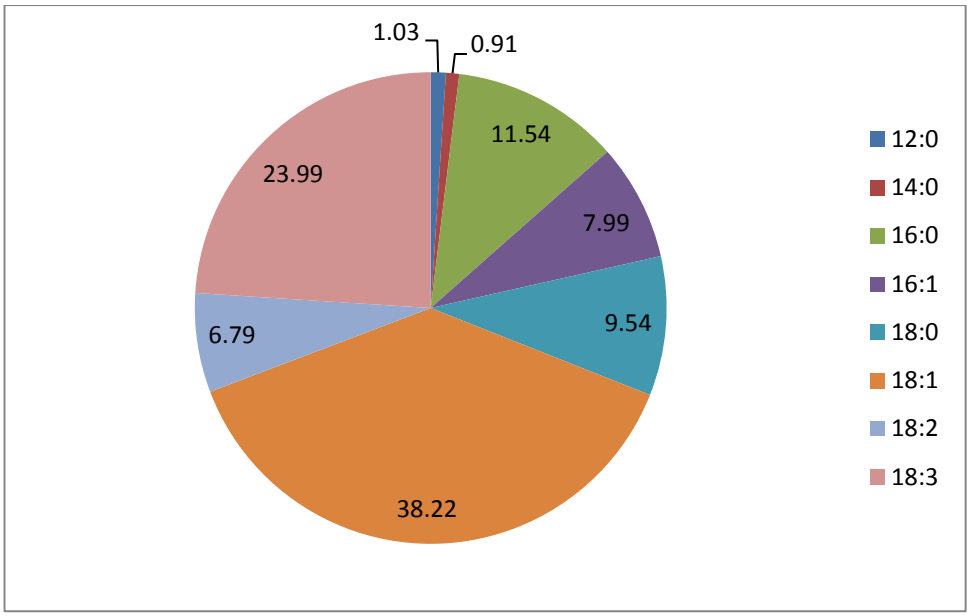


Figure 4.8. Average % composition of each of the eight fatty acids for female CRWs of the Gisborne sample set.

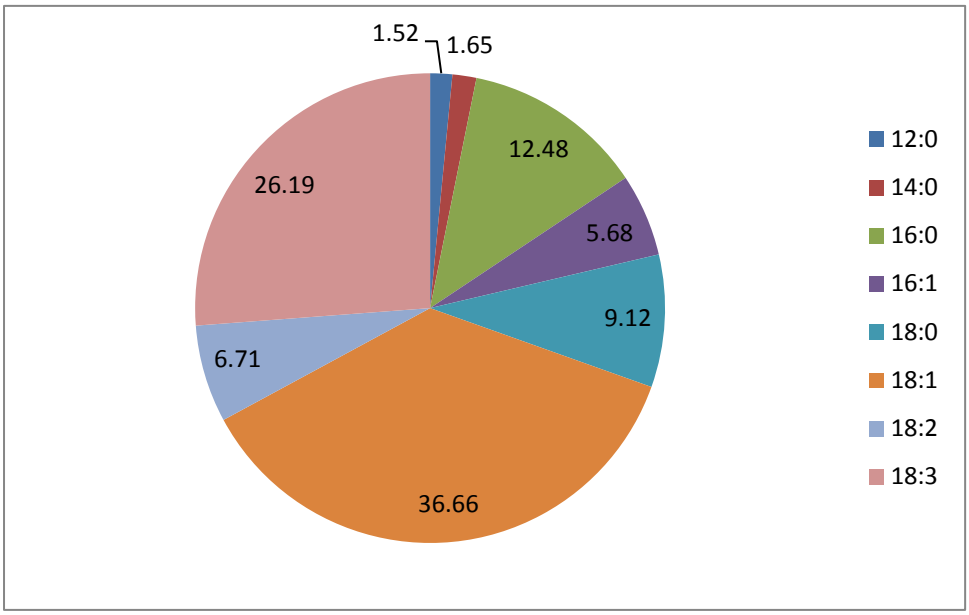


Figure 4.9. Average % composition of each of the eight fatty acids for male CRWs of the Gisborne sample set.

4.3.3 ANOVA to Investigate Influence of Sex and Parasitism on Fatty Acid Percentages

(a) Full Sample Set

The results for each fatty acid are shown in **Table 4.4**, while raw data is in **Appendix 9.4.3**. Significant differences between sexes were found for 14:0, 16:1 and 18:0 fatty acids; therefore, the null hypothesis, that there was no significant difference between male and female samples, had to be rejected for these fatty acids. Males had significantly more 14:0 and 18:0 fatty acid than females, while females had significantly more 16:1 fatty acid than males. In no case did parasitised or the sex*parasitised interactions have a p-value of less than 0.05. There was no evidence from these analyses that parasitism had an effect on the levels of any particular fatty acid for either sex of CRW.

Table 4.4. The influence of sex and parasitism on % fatty acid results in the full sample set. p values that are significant (at the 95%) level are bolded.

Factor	DF	Value	Fatty acid							
			12:0	**14:0**	**16:0**	**18:0**	**16:1**	**18:1**	**18:2**	**18:3**
Sex	1	F	3.77	6.78	0.86	4.62	6.20	3.25	2.20	0.92
		p	0.054	**0.010**	0.355	**0.033**	**0.014**	0.101	0.140	0.340
Parasitised	1	F	2.49	0.24	3.22	1.53	1.19	1.18	0.50	3.69
		p	0.116	0.625	0.075	0.218	0.277	0.279	0.481	0.057
Sex* Parasitised	1	F	0.04	2.84	0.98	1.24	0.20	2.72	0.45	0.00
		p	0.835	0.094	0.325	0.267	0.658	0.101	0.505	0.971
Error	160									
Total	163									

(b) Gisborne Sample Set

The results for each fatty acid are shown in **Table 4.5**, while raw data is in **Appendix 9.6.1.** Significant differences between sexes were found for 14:0 and 16:1 fatty acids, therefore the null hypothesis, that there was no significant difference between male and female samples, had to be rejected for these fatty

acids. When these results were compared with those for the differences between sexes it was apparent males had more 14:0 fatty acid and less 16:1 fatty acid than females. Significant differences between parasitised and non-parasitised samples were found for 12:0 fatty acid and 18:0 fatty acid, therefore, the null hypothesis that there was no significant difference between parasitised and non-parasitised samples had to be rejected for these fatty acids.

When the fatty acid composition means for parasitised and non-parasitised CRW samples of the Gisborne sample set (**Table 4.7**) were determined it is found that parasitised CRW samples have less 12:0 fatty acid but more 18:0 fatty acid than non-parasitised CRW samples. The only fatty acid percentage for which a significant difference in the interaction between sex and parasitism was found was 18:3 fatty acid. This indicates that for this fatty acid the effect of parasitism is different for male and females. A scatterplot of 18:3 fatty acid *versus* parasitised (assumed to be numerical) with data grouped into male and female is shown in **Figure 4.10** Regression equations and R^2 coefficients are given in **Table 4.6.** Although the regression equations are different, R^2 values are so low that the equations are just about meaningless, therefore no conclusions can be drawn from the data.

Table 4.5. The influence of sex and parasitism on fatty acid percentages results in the Gisborne sample set. P values that are significant (at the 95%) level are bolded.

Factor	DF	Value	Fatty acid							
			12:0	**14:0**	**16:0**	**18:0**	**16:1**	**18:1**	**18:2**	**18:3**
Sex	1	F	1.25	4.20	1.30	0.06	6.22	0.29	0.00	1.50
		p	0.268	**0.045**	0.260	0.802	**0.015**	0.591	0.944	0.226
Parasitised	1	F	4.83	2.03	30.17	5.34	1.36	1.50	1.42	1.73
		p	**0.032**	0.161	0.686	**0.025**	0.248	0.225	0.240	0.194
Sex* Parasitised	1	F	0.020	0.42	0.00	0.2	2.93	2.81	0.56	7.50
		p	0.896	0.522	0.948	0.660	0.093	0.100	0.456	**0.008**
Error	52									
Total	53									

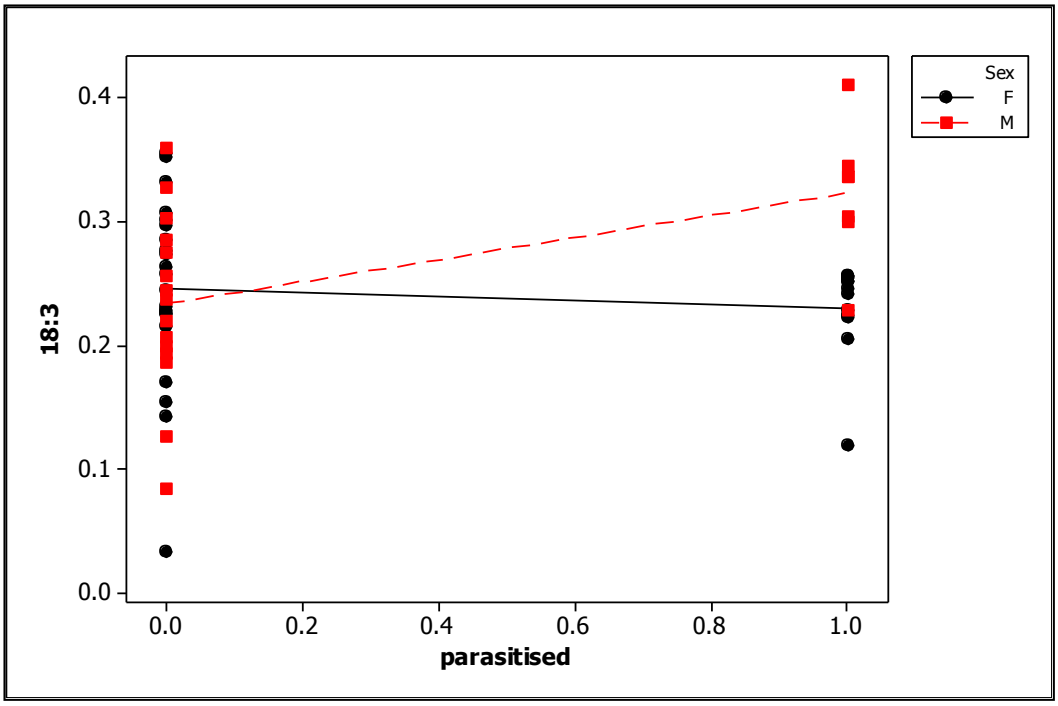


Figure 4.10. Scatterplot of 18:3 *versus* parasitised with data grouped into male and female.

Table 4.6. Regressions equations and coefficients of determination (R^2) for the relationship between 18:3 and parasitised for the Gisborne sample set for male and female.

Sex	Regression equation	R^2
M	18:3 = 0.2334 + 0.08939Parasitised	0.29
F	18:3 = 0.2453 - 0.01548Parasitised	0.01

Table 4.7. The fatty acid composition means for parasitised and non-parasitised CRW samples of the Gisborne sample set.

Fatty acid	Non-parasitised mean	Parasitised mean
12:0	0.016	0.006
14:0	0.014	0.008
16:0	0.120	0.117
16:1	0.067	0.079
18:0	0.107	0.068
18:1	0.363	0.401
18:2	0.072	0.058
18:3	0.240	0.264

4.3.4 Saturated/Unsaturated Fatty Acid Ratio Changes

Figure 4.11 is the scatterplot of the saturated/unsaturated fatty acid ratio *versus* fat score (0-5) (see **Figure 4.1** for description of levels). The regression equations and coefficients of variation (R^2 values) were calculated (**Table 4.8**) and the low R^2 values indicated that no relationship between saturated/unsaturated fatty acid ratio and fat score of these samples was present. Similarly low R^2 values were found for other physiological states.

Table 4.8. Regressions equations and coefficients of determination (R^2) for the relationship between saturated/unsaturated ratio and fat.

Parasitised	Regression equation	R^2
0	Sat/unsat = 0.441-0.03485Fat Score	0.090
1	Sat/unsat = 0.392-0.02562Fat Score	0.016

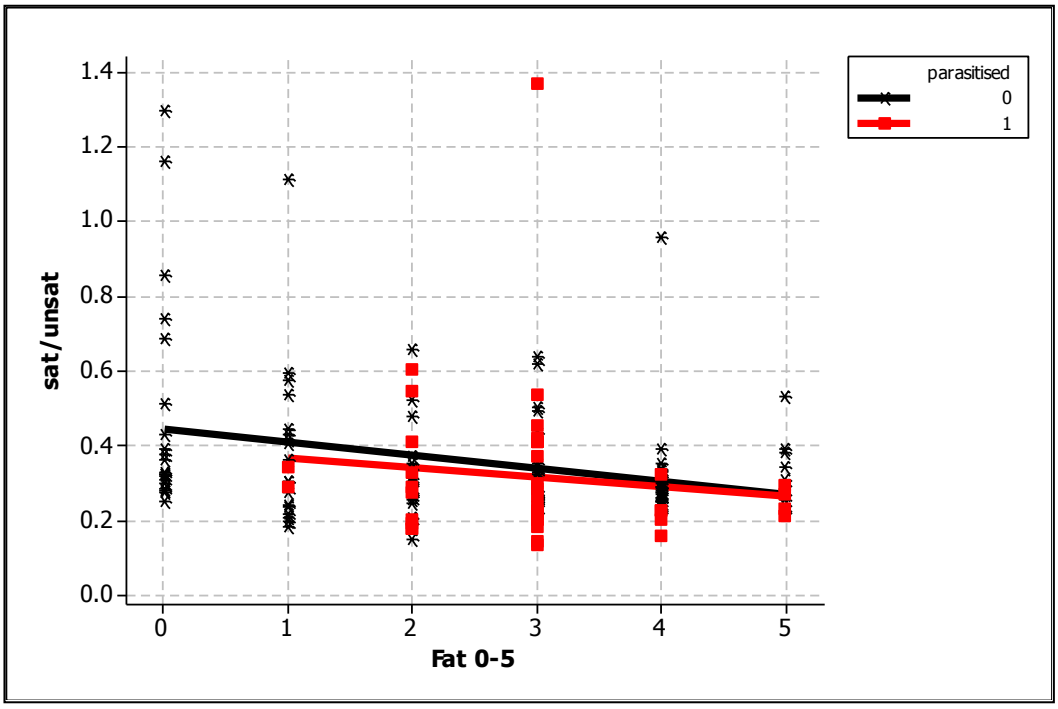


Figure 4.11. Scatterplot of the saturated/unsaturated ratio *versus* fat with the data grouped into parasitised and non-parasitised. Linear regression lines have been added.

4.3.5 Multivariate Analysis of Differences in Fatty Acid Composition between Clover Root Weevils of Different Physiological States

Principal component, linear discriminant and quadratic discriminant analyses (see **Appendix 9.3**) did not permit the differentiation of parasitised and unparasitised CRW in either the full sample set or the Gisborne sample set. However, using the Gisborne sample set improved the outcomes for multivariate analysis and this indicates that tighter control of sampling could produce better results.

4.3.6 Canonical Analysis

(a) Canonical Variates – G Scores

A regression equation and canonical variates, termed "G Scores" were calculated using GenStat software. **Equation 4.1** is the G score regression equation that was determined for the Gisborne sample set

$$\begin{aligned} G\ Scores = {} & -1.92373 - 1.22791\ 12{:}0alr + 1.3024914{:}0alr + 0.4988516{:}0alr \\ & - 0.729633\ 16{:}1alr - 1.55502\ 18{:}0alr + 0.79525\ 18{:}1alr \\ & + 0.65985\ 18{:}3alr \end{aligned} \quad \textbf{(4.1)}$$

Inspection of **Equation 4.1** reveals that alrs of 12:0 fatty acid and 18:0 fatty acid make large negative contributions, while the alrs of 14:0 fatty acid and 18:1 fatty acid make a large positive contribution. These contributions are summarised in **Table 4.9**.The R^2 column indicates how much of the total alr variation arises from either a single fatty acid designated by a "X" in **Table 4.9** or multiple fatty acids.

(b) Two-Sample t-Test

The p-value of 0.000 (**Table 4.10**) shows that there is a significant difference between the G Score values of parasitised and non-parasitised and that the null hypothesis, that there is no difference, must be rejected. The box plot below (**Figure 4.12**) shows that the upper quartile of the G scores for the non-parasitised data is below the lower quartile for the parasitised data. Therefore 75% of the non-parasitised data have values for G scores that are lower than 25% of the parasitised data.

Table 4.9. Summary of the contribution of each alr in the canonical analysis.

Number of Variables	R^2	12:0alr	14:0alr	16:0alr	16:1alr	18:0alr	18:1palr	18:2alr
1	46.1					X		
1	43.1	X						
2	62.0	X				X		
2	53.2			X		X		
3	84.5	X	X			X		
3	71.4	X		X		X		
4	92.5	X	X			X	X	
4	85.7	X	X	X		X		
5	95.8	X	X		X	X	X	
5	93.1	X	X			X	X	X
6	99.3	X	X		X	X	X	X
6	97.3	X	X	X	X	X	X	
7	100.0	X	X	X	X	X	X	X

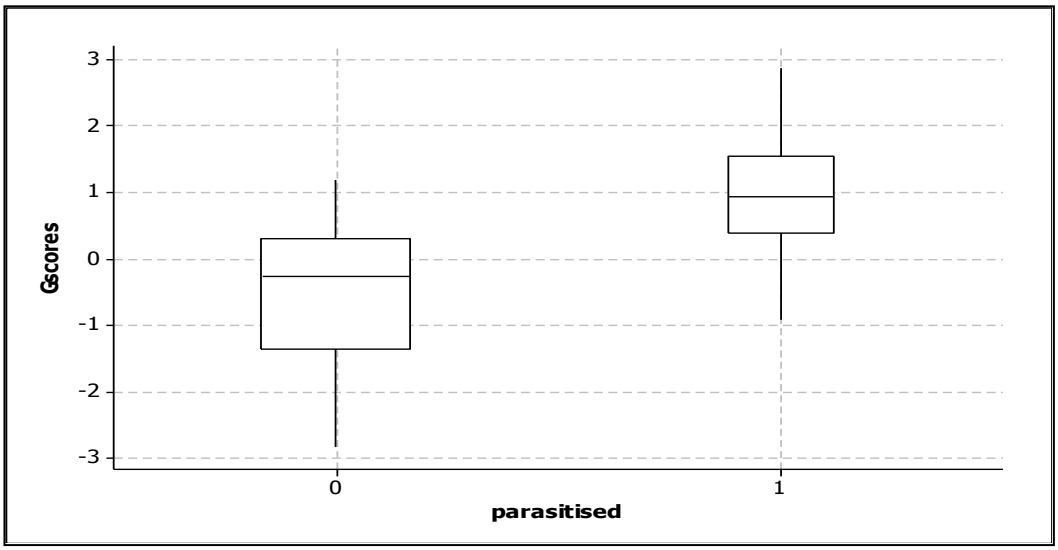


Figure 4.12. Box plots of the G Scores for the Gisborne sample set with the data separated according to whether it is parasitised (parasitised = 1) or non-parasitised (parasitised = 0).

Table 4.10. Two-sampled t-test results for G Scores with parasitised as the response.

Parasitised	N	Mean	Standard deviation
0	37	-0.22	1.01
1	19	0.98	0.99

Estimate for difference= -1.54

95% Confidence interval for mean difference: (-2.11, -0.977)

t-Test of mean difference = 0 (*vs* ≠ 0): T-Value = -5.47p Value = 0.000

4.3.7 Ternary Plots

The data points were either labelled P (parasitised) or N (non-parasitised) (**Figure 4.13**). As the relative level of 18:0 fatty acid contributions was much bigger than the 12:0 fatty acid and 14:0 fatty acid contributions, this skewed the data towards the W corner. Since data points arising from parasitized and non-parasitised groups overlapped, no distinction between these two groups could be made

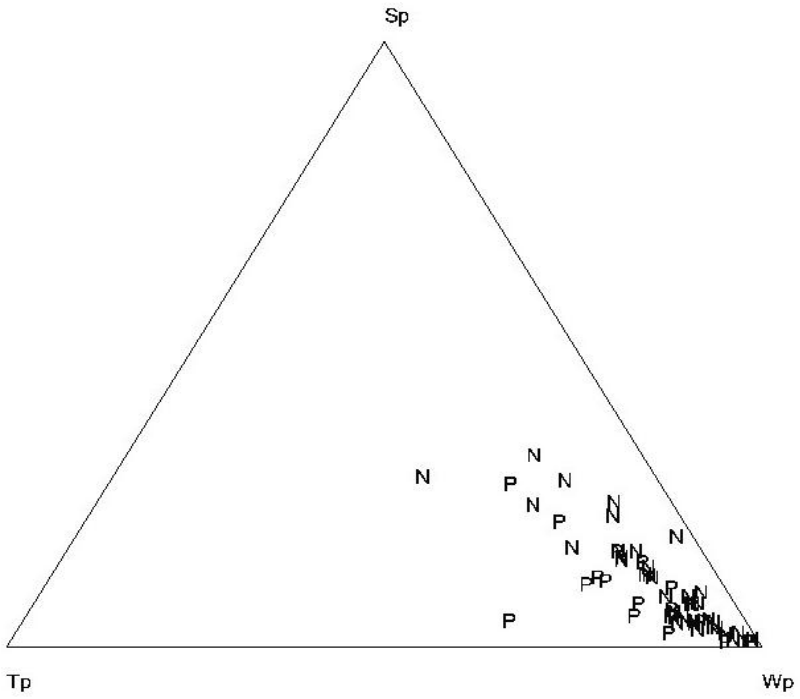


Figure 4.13. Ternary plot depicting the ratios of the fatty acid components of 12:0 (Sp), 14:0 (Tp) and 18:0 (Wp), with data labelled as parasitised (P) or non-parasitised (N).

4.4 Results from the Weighed Subset

4.4.1 Differences between Parasitised and Non-Parasitised States

As the weighed subset were all females, no two-way ANOVA to determine interactions between sex and parasitism was possible. Instead an one-way ANOVA was used for each fatty acid to determine whether there were significant differences in each fatty acid component occurred between parasitised and non-parasitised samples (**Table 4.11**). For full ANOVA results see **Appendix Error! Reference source not found.**. As the calculated p-values were all larger than 0.05,the null hypothesis (that there was no significant difference between parasitised and non-parasitised samples) had to be accepted for all of the fatty acid percentages at the 95% significance level.

Table 4.11. One-way ANOVA results for differences between parasitised and non-parasitised CRW samples in the weighed subset.

Fatty acid	Non-parasitised mean	Parasitised mean	p Value
12:0	0.0016483	0.0014051	0.453
14:0	0.006210	0.006484	0.636
16:0	0.16561	0.16177	0.748
16:1	0.11255	0.13233	0.350
18:0	0.04470	0.04562	0.920
18:1	0.41197	0.43675	0.153
18:2	0.038591	0.033129	0.211
18:3	0.21872	0.18252	0.109

4.4.2 Chemometric Analysis of the Weighed Subset

PCA and LDA (see **Appendix 9.3.3**) did not permit the differentiation of parasitised and unparasitised CRW in the weighed subset. Due to the small sample size of parasitised CRW in the weighed subset (six samples) and the correlation between some of the fatty acids, QDA could not be calculated. QDA

requires different variance–co-variance matrices and a sufficient sample size to calculate these matrices.

4.4.3 Total Percentage of Body Weight

(a) Scatterplots

Total percentage of fatty acid *versus* fat score and total percentage of fatty acid *versus* sexual maturity results are presented in **Figure 4.14** and **Figure 4.15** respectively. No correlations with coefficients of determination were seen (**Table 4.12**). The small sample size also meant that correlations were affected by outliers. One would have expected a correlation between the visual fat score (fat (0-5)) and total fatty acid concentration (as % of body weight), however, it appears that the correlation is affected by two outliers that have lower than expected fat scores for their respective total fatty acid concentrations (as % of body weight)..

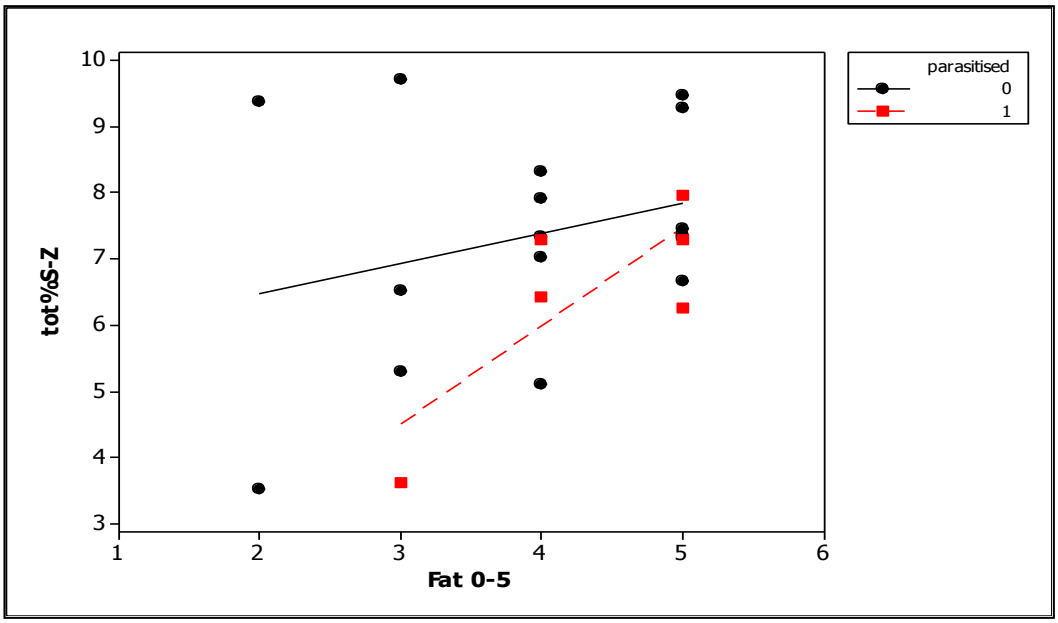


Figure 4.14. Scatterplot of percentage of body weight (tot% S-Z) *versus* fat (0-5) with a straight line plot added. Data is grouped into non-parasitised (parasitised = 0) and parasitised (parasitised = 1). Linear regression lines have been added.

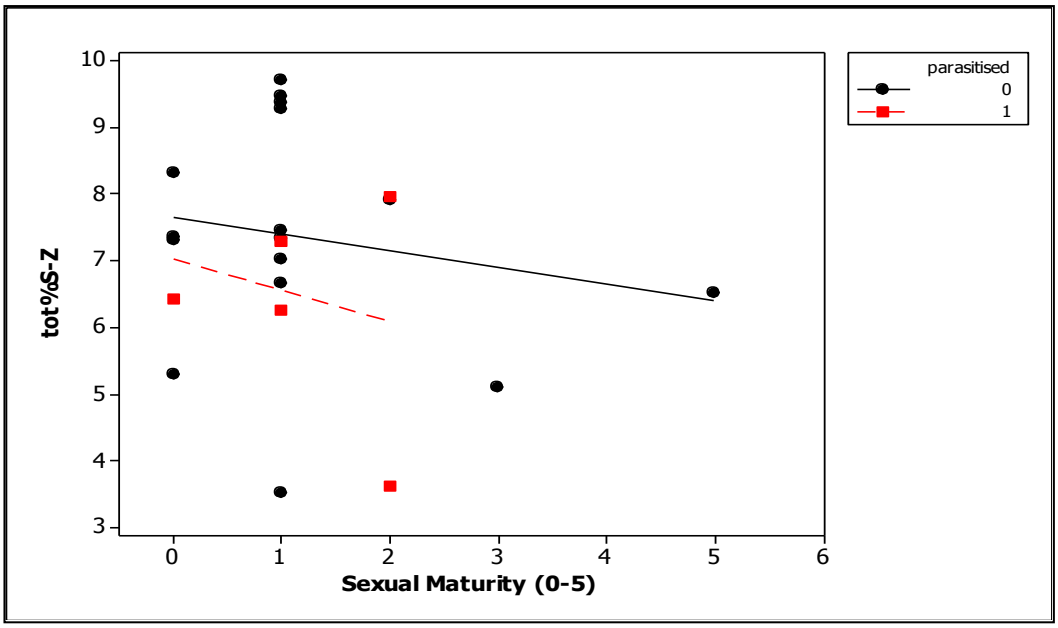


Figure 4.15. Scatterplot of sexual maturity (0-5) *versus* fat percentage of body weight (tot%S-Z) with a straight line plot added. Data is grouped into non-parasitised (parasitised = 0) and parasitised (parasitised = 1). Linear regression lines have been added.

Table 4.12. Regressions equations and coefficients of determination (R^2) for linear regression lines relating fat percentage of body weight (tot% S-Z) to fat score (fat) and sexual maturity.

Regression Equation	**R^2**
Tot% S-Z = 0.057 + 1.482(Fat)(Parasitised)	0.625
Tot% S-Z = 5.587 + 0.4502(Fat)(Non-Parasitised)	0.077
Tot% S-Z = 7.026 - 0.4696(Sexual Maturity)(Parasitised)	0.053
Tot% S-Z = 7.659 - 0.2529(Sexual Maturity)(Non-Parasitised)	0.035

(b) Differences between Parasitised and Non-Parasitised States

The ANOVA results are presented in **Table 4.13**. For full ANOVA results see **Appendix 0**. As the calculated p-value was larger than 0.05, the null hypothesis (that there was no significant difference between parasitised and non-parasitised samples) had to be accepted at the 95% significance level.

Table 4.13. One-way ANOVA results for differences in total fatty acid concentration (as percentage of body weight) between parasitised and non-parasitised CRW samples in the weighed subset.

Source	DF	F	p Value
Parasitised	1	1.21	0.285
Error	20		
Total	21		

4.5 Discussion and Conclusions

4.5.1 Correlations between Fatty Acid Composition and Physiological State

The nature of CRW lifecycle is such that different physiological stages are present at any one time. This makes the investigation of CRW-more difficult than the investigation of other insects that have synchronised lifecycles. The sample set used in this investigation consisted of weevils of different ages, seasons, sex, locations and parasitism, causing large variation between samples. The use of individual samples, while useful for recording all physical features of each CRW, also increased variation. Therefore, it is likely that variation between individual samples had a substantial affect on the ability to deduce correlations from the data.

Frampton[248] was able to track changes in the fatty acid profile of lucerne weevils (*S. discoideus*) during different life stages, however these experiments used 100 weevils per sample and therefore, were less affected by the individual variation between weevils.The life cycle of the lucerne weevil is more highly synchronised than that of the CRW, and this would have also contributed to the lack of variation.[288] Lipsitz *et al*[279] used sample sizes of between 300 and 900 randomly selected individual insects from a single population to decrease genetic variability, while their test subject (house cricket, *Acheta domesticus*) also has a more synchronised lifecycle than CRW. To reduce variation an increased number of

CRW could be used per sample, however, this would significantly increase the number of CRW needed for sampling and lose the benefits of individual samples.

The use of whole body analysis may have also affected the ability to deduce correlations as in some other insects, whole body analysis has masked fatty acid composition changes. No appreciable differences in the fatty acid composition of total lipid extracts in different ages of the house cricket were found however, considerable differences in different ages were observed in the fatty acid composition of the neutral phospholipid lipid fractions.[279] The investigation of the fatty acid composition of different tissues throughout the lifecycle of the tobacco hornworm (*M. sexta*) found that some tissues experienced changes in fatty acid composition while others did not. It is possible that individual tissues or lipid classes in CRW experienced a change in fatty acid composition during the lifecycle but these changes were hidden by tissues or lipid classes that did not have changes.

When this variation is taken into account, it is possible that the CRW has a relatively constant fatty acid composition and that the lack of correlations between lifecycle and fatty acid composition is typical of this species. Other insects have been found to have constant lipid composition throughout their lifecycle.[98; 101; 111; 172; 278] A constant fatty acid composition throughout an insect's lifecycle indicates that all fatty acids are utilised at similar rates throughout its life.[102] This was illustrated by the remarkable drop in lipid stores induced by fasting in adult triatomine bugs (up to 70%), without significant changes in fatty acid composition.[102]

No correlations between the presence of oil droplets and fatty acid composition were found. It is possible that these were lost in the water that was used to dissect the CRW and not enough were transferred when putting dissected specimens into the vials. The dominant lipids found in the haemolymph of insects are the usual 16 and 18 carbon fatty acids that dominate insect lipids,[204] while the presence of

oil drops has not been previously reported. In future work it would be worthwhile to attempt to extract the oil droplets and analyse their fatty acid composition.

When, at the commencement of the investigations repoprted here, visible differences were observed in fat in dissected CRW of different physiological states (e.g. the differences in fat colour), it was a reasonable assumption that there would be differences in the fatty acid compositions of CRW in different physiological states. However, based on the results obtained during this investigation it is likely that the visible changes in fat colour are caused by other biochemical changes. Other causes of fat body colour changes have been reported. The occurrence of a biliverdin-containing, very high density lipoprotein during the last larval stage of the bollworm (*Heliothis zea*) was suggested as the reason for the change in colour of the fat body to a dark blue colour.[289] In two different families of Lepidoptera, two distinct types of fat body have been identified which have different functions and structures.The fat body histotypes are tan and white in the Indian meal moth (*Plodia interpunctella*),[290] and blue and white in the corn earworm (*Helicoverpa zea*).[289; 291] No reasons were given for the differences and the fatty acid composition of the different coloured fat bodies was not investigated. Other possible causes of the differences in fat body colour of CRW include the build up of uric acid[292] or carotenoids.[293] Based on the colour changes seen in dissected CRW it is likely that carotenoids are involved, although further investigation would be required to verify this.

4.5.2 Male and Female Clover Root Weevil Fatty Acid Profiles

The fatty acid profile of male and female insects of the same species is usually very similar.[170; 254] This was true for the CRW examined in this investigation, with five of the eight fatty acids showing no significant difference (at the 95% level) between male and female samples. Significant differences between the male and female components of 14:0, 16:1 and 18:0 fatty acids for CRW samples were found in the full sample set, with females having more 16:1 fatty acid and less 14:0 and 18:0 fatty acids than males. In the Gisborne sample set the same significant differences were found for 14:0 and 16:1 fatty acids only.

The most striking difference in the lifecycle of most male and female insects is that females have to lay eggs. It is likely that a difference between the sexes may be linked to this. In the Neuropteran, *L. Sheppardi* the higher level of 16:1 fatty acid in adult females when compared to males was suggested to be related to the need for accumulation of sufficient energy and a carbon reservoir in the developing new vitellum,[277] and it is possible that this is also the case in CRW. The eggs of the Neuropteran, *Lertha sheppardi* discussed above and field cricket (*Gryllus campestris*) had higher levels of 16:1 fatty acid than other life stages of the respective insects, so it could be that the presence of eggs in some of the female CRW samples influenced this result.

It is also possible that the lesser % contribution of 14:0 fatty acid seen in female CRW with respect to male CRW is also related to egg production. Female ticks (*Boophilus microplus*) utilised 14:0 fatty acid during the time period that the oocytes matured.[294] Male Coleoptera have been reported to use the unsaturated 18 carbon fatty acids in pheromone production,[285] and it is possible that the cause of the higher 18:0 fatty acid component in males with regard to females in the full sample set is linked to the production of pheromones. 18:0 fatty acid may be dehydrogenated to form the unsaturated 18 carbon fatty acids,[177] therefore, a reservoir of 18:0 fatty acid may be needed for pheromone production. Since pheromones are usually only present in small quantities in insects,[288] it seems unlikely that this would have a significant effect on the amount of 18:0 fatty acid present. Further work is required to determine if the differences in 18:0 fatty acid seen in the full sample set were genuine since they were not seen in the Gisborne sample set.

4.5.3 Differences between Parasitised and Non-parasitised States

No significant differences in fatty acid composition were found between parasitised and non-parasitised CRW in the full sample set or in the weighed subset. The fatty acid composition of the wax moth (*Achoria grisella*) remained the same even after parasitism,[61] so it is possible that the lack of differences between the fatty acid compositions of parasitised and non-parasitised CRW in the full data set is genuine. Due to the ability of parasitoids to influence the internal

environment of its host[295] and the two significant differences in the fatty acid compostions of parasitised and non-parasitised CRW in the Gisborne sample set, it may be that variations between individual samples in the full sample set contributed to the lack of differences found. Parasitised samples from the Gisborne sample set had less 12:0 and 18:0 fatty acids than the corresponding non-parasitised samples. Although it is difficult to assign the biological significance of specific fatty acids,[296] it is likely that the reduction in levels of 12:0 and 18:0 fatty acids in parasitised CRW makes the host environment more favourable for the parasitoid.

No significant differences between parasitised and non-parasitised CRW were found when total fatty acid % contribution (as percentage of body weight) was investigated. This is different to the visual observations that parasitism causes fat accumulation in female CRW. It is possible that the fat that accumulates in the parasitised female fat body (i.e. the highly visible fat in a dissected CRW) is a similar amount to the fat in the eggs of non-parasitised females (not as visible in a dissected CRW) i.e. the same amount of total fat but in different locations. When the data obtained is visually analysed, it is apparent that individual variation between samples and the small sample size has affected this analysis.

In another venom-based parasitism situation, parasitism of the flesh fly (*Sarcophaga bullatal)* by the jewel wasp (*Nasonia vitripennis*) caused a lipid accumulation in the fat body, although changes in the fatty acid composition were not investigated.[58] A proposed pathway for this lipid elevation was presented[283] and injection of wasp venom into the host stimulated fatty acid synthesis in the host fat body. The elevation of host lipids may be a strategy employed by the female wasp to maximize the fly as a resource for progeny production.[297]

4.5.4 Statistical Analysis and Ability to Predict Parasitism

The multivariate analysis of the full sample set and the weighed subset did not - contribute to the goal of being able to distinguish between parasitised and non-parasitised CRWs or relating fatty acid composition to physiological state.

However the corresponding analysis of the Gisborne sample set was more successful. This shows the potential of these techniques when variation is reduced and large samples from one location are used. A cause of the large confidence intervals in the analysis of differences between locations was the small sample sizes at some of the locations. Analyses of larger sample sets would be needed to unequivocally determine whether or not parasitised and non-parasitised states could be deduced from fatty acid analyses

4.5.5 Limitations of the Current Study

The tracking of how the fatty acid composition of CRWs changed with differing physiological state and parasitism failed to provide any statistically significant distinctions for the full sample set. Variation due to the multiple sampling sites and dates meant that correlations between fatty acid composition and differing physiological or parasitism states were not seen. The ability to analyse individual weevils meant that the results were affected by the variation between individual weevils.

The nature of the sampling process was dictated by agencies external to this project and the project was reliant on available samples. This meant that experiments could not be designed to specifically test any hypotheses. It was not possible to get sufficient samples for each of the range of physiological factors that were recorded. The Gisborne sample set reduced the variation caused by different sample sites and different times of collection but more sets like this would be needed for valid conclusions to be drawn.

4.5.6 Recommendations for Future Work

Any future work investigating changes of fatty acid profiles during insect's lifecycles needs to be specifically designed to overcome the difficulties that were faced in this project. A different species with a well synchronised life cycle or easily reared to produce large batches of uniform insects at the same stage would make this type of study much easier. Sufficient sample supply needs to be obtained and sampling protocols must eliminate as much variation between

samples as possible. To have improved the current work with CRW it is recommended the following should have been done:

- Less sampling sites should be used and each of them sampled regularly. This would reduce variations caused by different sites and seasons. Significantly more CRW should be sampled. This would give a much better spread of data including a full range of physiological states.
- Ideally CRW should also be reared in the laboratory. This would increase the number of samples available and also allowed for control of some physiological states. Currently the artificial rearing of CRW is difficult, if not impossible as dietary requirements change with instar.
- Both bulk samples and individual samples should be used. Although the one-step method proved to be successful when applied to individuals, bulk sampling will eliminate individual variations.
- A larger weighed subset should be used since this will allow for a more reliable analysis of the total fatty acid concentration as percentage of body weight.

5 The Teratocytes of the Clover Root Weevil

5.1 Introduction

It is essential for all endoparasitoids, including *M. aethiopoides,* to ensure that the growing larva can obtain nutrients without killing its host prematurely. There are several ways in which a parasitoid can achieve this, including teratocytes (**Figure 5.1**). Teratocytes are cells that have dissociated from the serosal membrane enveloping the parasitoid embryo.[245; 298] These cells become dispersed in the haemolymph of the host, often increasing in size and developing microvilli on the surface.[298; 299] Teratocytes are specific to braconid and scelionid parasitoids,[300; 301] and appear in CRWs that have been parasitised by *M. aethiopoides*.[299]

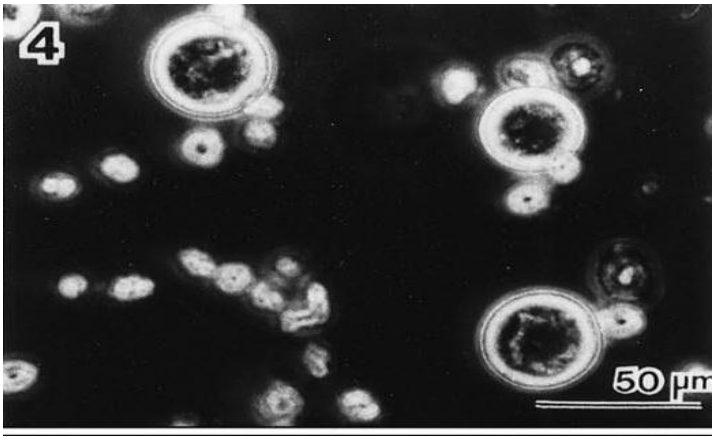


Figure 5.1. Teratocytes in the *Cotesia kariyai* (an endoparasitoid)*:Pseudaletia separatea* (oriental armyworm)[‡‡‡] system under a light microscope four days after parasitism.[302]

The presence of teratocytes has been reported in more than 40 species,[301] although the most extensively studied parasitoid: host system involving teratocytes is that of the braconid wasp (*Microplitis croceipes*)*:* tobacco budworm

‡‡‡ Systems are labelled parasitoid: host

(*Heliothis virescens*).[303] Most literature describing teratocytes has focussed on parasitoids of immature stage hosts, especially Lepidoptera[§§§] larvae.[299]

5.1.1 Role of Teratocytes

The developing parasitoid must compete with the host for nutrients; however, for most parasitoids the survival of the host is crucial for the development of the parasitoid. The parasitoid needs to keep the host sufficiently viable so that it can effectively feed, avoid predation and perform other critical survival functions while the parasite matures. Therefore, the parasitoid must minimise its impact on the host, although effects on the host physiology and metabolism are inevitable.[304]

It is widely accepted that teratocytes have an important, if not essential, role in successful parasitoid development;[300; 305; 306] however, the precise way in which this is accomplished is very likely to be species-specific.[59; 298] They may be nutritive, immunosuppressive,[228,] or secretory[229] and can be involved in regulating host development.[299; 300; 305; 307; 308] Teratocytes have been reported to affect the fat body proliferation[59; 298; 305; 309] and the concentration of juvenile hormone present in the host.[301; 305; 310; 311] In the parasitic wasp (*Cotesia kariyai*): Oriental armyworm (*Pseudaletia separata*) system, paraffin sections of the parasitized host suggested that the teratocytes attach to the outer sheath of the fat body and secrete an enzyme that makes a hole in the matrix of the fat body. This would allow the growing parasitoid larva to ingest the contents of the fat body.[59]

Teratocytes increase in size while the parasitoid larvae are developing within the host, however, their total number often decreases. They become highly polyploid,[305] disperse in the haemolymph of the host and develop microvilli as well as increasing in size.[208] Teratocytes have been found to affect the host's lipids. When teratocytes were transplanted into hosts, the lipid concentration of the host's haemolymph increased compared to controls.[309; 312] Zhang *et al*[298; 303]

[§§§] Order of insects containing moths and butterflies.

studied the braconid wasp: tobacco budworm system and found that the perivisceral fat bodies of parasitised, and teratocyte-injected larvae were of smaller size than in non-parasitised hosts. The number of teratocytes also decreased with the development of the parasitoid and the authors suggested that this was because the parasitoid was consuming the teratocytes.[313]

Teratocytes are able to synthesize and then release substances that allow the parasitoid to break down its host's tissue and then utilise them for itself. Teratocytes have been hypothesised to secrete numerous substances such as encapsulation-inhibition agents,[203] fungicidal substances,[314] juvenile hormone,[315] inhibitors of juvenile hormone (such as juvenile hormone esterase),[316-319] proteases,[233] phenoloxidase inhibitors,[181] molecules that suppress ecdysteroid production[207] and parasitism-specific proteins.[298; 304; 305; 320; 321] They are also capable of producing substances that affect the host's immune and endocrine system, as well as metabolism.[307]

5.1.2 Teratocytes of Euphorinae

The majority of the literature concerning teratocytes describes systems where the host is in an immature stage, especially Lepidoptera larvae.[299] A notable exception is the work by Okuda's group who have investigated the braconid parasitoid *Dinocampus coccinellae* which parasitises adult Coccinellidae[****]. Both this parasitoid and *M. aethiopoides* are in the same subfamily, Euphorinae. It was suggested that the teratocytes of *D. coccinellae* serve a nutritive function for the growing larvae as they are consumed as the larvae grow.[321-323] This suggestion together with visual observations of parasitised CRW, indicate that it is possible that the teratocytes of *M. aethiopoides* may also serve a nutritive function. As observed by Barratt and Sutherland[299] in Moroccan *M. aetheopoides*, Irish *M. aetheopoides* teratocytes follow a general pattern of increasing in size and decreasing in number as the parasitoids develop. This suggests that teratocytes are consumed by the developing larvae. In Irish *M. aetheopoides* the number and size

[****] A family of beetles commonly known as ladybirds.

of teratocytes is dependent on how many larvae are present with teratocytes being distinctly more numerous and smaller as larval number increases. Initially Irish *M. aetheopoides* teratocytes are transparent but as they enlarge the contents become white with large transparent oil droplet inclusions.[324] As the *M. aetheopoides* parasitoid larvae appear to feed on the teratocytes, it is possible that the teratocytes both accumulate nutrients from the haemolymph and synthesize specific nutrients to match larval developmental requirements.

5.1.3 Composition of Teratocytes

Early studies of the composition of teratocytes found that teratocytes were largely comprised of proteins and lipids, with some glycogen.[56; 325] There is only one report concerning the fatty acid composition of teratocytes. This work by Cohen and Debolt[326] investigated the fatty acid composition of teratocytes in a host that were caused by two different parasitoids. Teratocytes from western tarnished plant bug (*Lygus hesperus*) parasitised by the braconid wasps *Peristenus stygicus* and *Leiophron uniformis* were analyzed and compared. The presence of the fatty acids 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 and 20:3 in the teratocytes of both species was reported. The fatty acid profiles of teratocytes from both parasitoid species were similar except for myristic acid (14:0 fatty acid) which was found in significantly higher concentrations in teratocytes of *P. Stygicus*, and linolenic acid (18:3 fatty acid) which was found in significantly higher concentrations in teratocytes of *L. uniformis.* As the fatty acid composition of the teratocytes differed in spite of growing in the same host, the authors suggested that teratocytes are not formed by passively absorbing the fatty matter from the body fluid of the host but that material is selectively absorbed to match species-specific larval requirements.

5.1.4 Context of the Present Work

The biological control community has many gaps in its knowledge of the nutritional needs of immature parasitoids. Given the important of teratocytes in the establishment and development of parasitoids within their hosts, better understanding of their composition may help casting light upon a range of aspects from host specificity and larval survival through to adult fitness and fecundity.

Knowledge of teratocyte composition may even facilitate in the development of *in vitro* rearing programs for similar braconid species, greatly reducing the cost and increasing year round availability of these biocontrol agents.[327] By determining the fatty acid composition of the teratocytes of CRW, it may be possible to gain an insight as to what teratocytes contain and from where this originates. The development of suitable methods for teratocyte analysis may also provide potential methods for the analysis of teratocytes from other species, especially as this area of work is largely unreported.

5.1.5 Investigation Methods

(a) One-Step Method for Extraction and Derivatisation of Lipids

The first investigations of fatty acids in teratocytes used simple histochemical techniques, but these methods gave qualitative rather than quantitative results.[56; 325] In Cohen and Debolt's[326] work the fatty acid composition of extracted teratocytes was analysed by base-catalysed derivatisation to FAMEs.[326] In light of this report, the one-step method already developed to investigate the fatty acid composition of CRWs had potential to be modified to suit the analysis of teratocytes.

(b) MALDI-TOF Spectrometry Method

MALDI-TOF spectrometry was seen as another feasible method of analysis. Although it had not been reported as a method to investigate the composition of teratocytes, the direct analysis, often with no derivatisation needed and the small sample size requirements, offered by MALDI-TOF were likely to suit the small size of teratocytes. MALDI-TOF has been used previously for the investigation of fatty acids[328] and also of insect lipids.[212]

MALDI-TOF allows for intact triacylglycerols (TAGs)[329; 330] to be analysed rather than single fatty acids. TAGs are composed of three esterified fatty acids attached to a glycerol backbone (**Figure 5.2**).[331] A drawback with using MALDI-TOF to analyse complex TAGs such as those found in insect lipids is isotope

interference and differences in the response factors for TAGs of different molecular weights. These problems will be discussed further below.

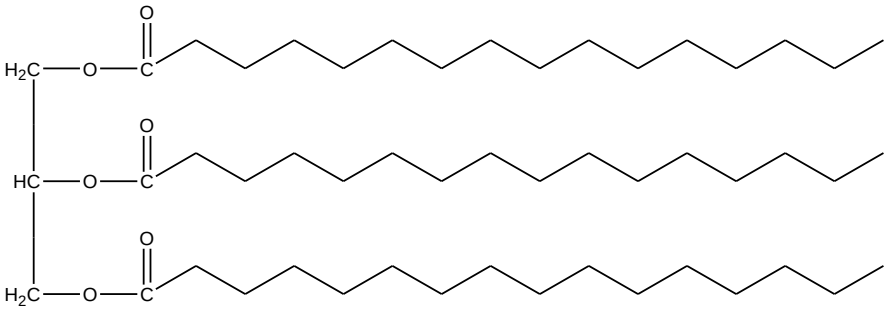


Figure 5.2. A representative TAG, containing three esterified fatty acids attached to a glycerol backbone.

5.2 Methods

5.2.1 General Methods

5.2.2 One-Step Method for Extraction and Derivatisation of Lipids

GC-MS: GC-MS was carried out as described in **Chapter 2**.

(a) MALDI-TOF Spectrometry Method

A Bruker Autoflex II TOF/TOF™ MALDI-TOF spectrometer was used.

5.2.3 Chemicals

(a) One-Step Method for Extraction and Derivatisation of Lipids

All chemicals involved with the one-step method of extraction and derivatisation are as described in Chapter 2.

(b) MALDI-TOF Mass Spectrometry Method

2,5-Dihydroxybenzoic acid (DHB) (analytical grade, greater than 99% purity) was purchased from Sigma Aldrich. Acetonitrile (HPLC grade, greater than 99.99% purity) was purchased from Honeywell Burdick and Jackson. Sodium chloride (greater than 97% purity) was purchased from BDH. Trifluoroacetic acid (TFA) (analytical grade, greater than 99% purity) was purchased from Sigma Aldrich.

5.2.4 Extraction of Teratocytes

Teratocytes were removed from dissected CRWs at AgResearch. CRW samples were dissected in agitated water. Initially filter paper was trialled to see if it could be used to isolate teratocytes from the dissection medium. However, the teratocytes could not be separated from the filter paper. This meant that the filter paper had to be kept in the sample during analysis, which produced peaks that interfered with the GC spectrum (**Figure 5.3)**. Teratocytes were eventually collected by suctioning them up with a fine glass pipette and transferring them into a glass vial. Glass must be used as they adhere to plastic.

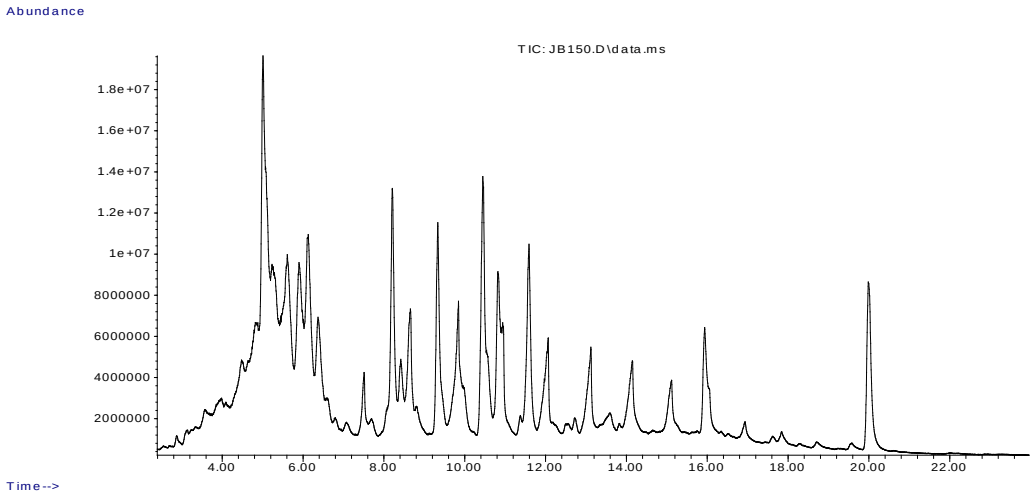


Figure 5.3. GC spectrum of a teratocyte sample including filter paper obtained using the one-step method of extraction and derivatisation. Contaminating peaks from the filter paper partly overlapped FAME peaks.

For analysis via the one-step method for extraction and derivatisation, as much water as possible was suctioned out with a pipette after the teratocytes had settled to the bottom of the vial. Hexane (1 mL) was added and the vial gently shaken to ensure that the teratocytes were dispersed in the hexane. For analysis via MALDI-TOF there was no need to remove the water from the teratocyte sample and instead the teratocytes were stored in water for analysis. Analysis was carried out as quickly as possible after extraction to ensure the integrity of the sample. If there was any delay the teratocyte samples were refrigerated at 4°C, however analysis was always carried out within 24 hours.

As many teratocytes as possible were collected from batches of CRW samples. Due to their tiny size, the teratocytes from different CRW within each batch were combined. Therefore, the teratocyte composition results obtained are representative of a range of samples. This is similar to the work of Cohen and Debolt[326] who pooled teratocytes from five to ten hosts.

Similarly, it was not possible to quantify accurately the amount of teratocytes in each sample. Counts were not a good measure due to the huge variation in teratocyte size and it was beyond the scope of this project to develop a method to weigh them. Teratocytes are tiny, biological samples that are very fragile. It would not be possible to dry them without causing them to rupture, therefore accurate weighing would not be possible.

5.2.5 One-Step Method for Extraction and Derivatisation of Lipids

The one-step method **(Section 2.6)** was investigated to determine whether it would be effective in analysing the lipid contents of teratocytes. Ten samples of teratocytes were analysed. These teratocytes came from the same batches of CRW as the 164 CRW analysed for the results discussed in **Chapters 3** and **4**.

The component of each of the fatty acids present in the teratocyte samples was expressed as a percentage of total fatty acids present in each sample. As in **Chapter 3** peak areas were corrected for recovery percentage and response factors (**Equation 2.3)**. Total fatty acids were calculated by adding all peak areas corresponding to FAMEs (excluding the standards). Individual FAME peak areas were divided by the total FAME peak area and multiplied by 100 to give the percentage composition. A two-sampled t–test was used to compare the means of the fatty acid compositions of the teratocyte samples with the corresponding values for the CRW samples described in **Chapter 3**.

5.2.6 Comparison with Parasitoid Larvae

To compare the results from the teratocytes with the fatty acid composition of the parasitoid larvae, five samples of individual *M. aethiopoides* larvae were analysed using the one-step method. These samples were removed from five CRW that

were in the batch of CRWs from the Manawatu region on the 22nd of February 2010. The CRWs from which they were removed were not analysed. All larvae were second instar. Once removed the parasitoids were placed in hexane (1 mL) ready for analysis.

The average fatty acid composition, standard deviation and coefficient of variation percentage was calculated for the larvae and a two-sampled t-test was used to determine if the results for the larvae were significantly different from results reported for CRW.

5.2.7 MALDI-TOF Spectrometry Method

A MALDI-TOF method that had been optimised for the analysis of TAGs was used. This method was developed by Meenakshi Batra in this Department in 2011.

(a) Sample Preparation

DHB has been reported to give the best sample preparation for the analysis of TAGs, and the use of an organic solvent means that the solvent evaporates quickly to give a thin layer of very small DHB crystals.[330] DHB was dissolved in acetonitrile:TFA (0.1 $molL^{-1}$) (1:2, 50 µL) to form the matrix. The matrix (0.5 µL) was spotted onto the target followed by the teratocyte sample (0.5 µL) and NaCl solution (1 $molL^{-1}$, 0.5 µL). This was done in triplicate and the spots were dried.

(b) MALDI-TOF Settings

A reflector method was used with the reflector set at 20 kV, the polarity set to positive and pulsed ion extraction of 70 ns. Laser frequency (10 Hz), laser power (45%), laser attenuator (offset = 64%, range = 20%), mass range (*m/z* 400 – 1200) and ion source (19 kV) had all been optimised for analysis of TAGs. Each spectrum was a sum of 210 shots.

iii. Prediction of Possible TAGs Present in Teratocytes

The possible fatty acid combinations present in the TAGS of the teratocytes were calculated using the five dominant fatty acids (only five fatty acids were considered due to there being 56 possible combinations if six fatty acids were

considered) found in the one-step analysis (16:0, 16:1, 18:1, 18:2 and 18:3 fatty acids). Although the choice of only five fatty acids would introduce a modest error (especially as the 18:0 component was only slightly less than the 18:2 component), the total combined percentage of the fatty acids not included was *ca* 8%, it was likely that the error due to isotope interference would be larger. The order of the fatty acids does not matter and repeats are possible so the number of possible combinations was calculated (**Equation 5.1** and **Equation 5.2)**.

$$Number\ of\ Combinations = \frac{(n + r - 1)!}{r!\,(n - 1)!} \qquad \textbf{(5.1)}$$

Where n = number of fatty acids (= 5) and;

r = number fatty acids needed (=3)

Therefore:

$$Number\ of\ Combinations = \frac{(5 + 2 - 1)!}{3!\,(5 - 1)!} = \frac{5040}{144} = 35 \qquad \textbf{(5.2)}$$

The *m/z* values for the sodiated pseudomolecular ions, $[M+Na]^+$, of the 35 combinations were calculated (**Table 5.1**). $[M+Na]^+$ ions are usually the most abundant ions.[328; 332] The *m/z* values of the pseudomolecular ions were calculated as shown in (**Equation 5.3**).

$$\mathrm{Mw(TAG)} = \mathrm{Mw[glycerol} - 3(\mathrm{OH})] + \mathrm{Mw}\left[\left(\mathrm{FA_1} + \mathrm{FA_2} + \mathrm{FA_3} - 3(\mathrm{H})\right)\right] + \mathrm{Aw[Na^+]} \qquad \textbf{(5.3)}$$

Where Mw = molecular weight

Aw = atomic weight

TAG = triacylglycerol

OH = molecular weight of hydroxyl

H = atomic weight of hydrogen

FA_1 = fatty acid 1

FA_2 = fatty acid 2

FA_3 = fatty acid 3

The calculated *m/z* values correspond to the most abundant isotope peak (A0) for each TAG. The ^{13}C-containing A+1 peak is commonly ignored in the MALDI-TOF analysis of TAGs for simplicity, although this does mean that there is a slight over-representation of the TAGs containing more of the smaller (C16) fatty

acids.[333] This is because the distribution of the total intensity values amongst isotopes increases as the size of the TAG increase from 0.55/1 (A+1/A0) for C51 (containing three C16 fatty acids) to 0.62/1 for C57 (containing three C18 fatty acids). For practical reasons, the contributions from all isotopes are ignored for the MALDI-TOF analysis of TAGs. In theory, the majority of TAGs would have contributions coming from the A+2 isotope peak of adjacent less-saturated members of the same carbon number TAG series. These contributions can enhance the measured TAG values by *ca* 20% of the most abundant isotope peak of the member in the TAG series that is two mass units less. It would be very difficult to eliminate this contribution experimentally, therefore it is common to ignore these contributions especially in complex spectra such as those arising from insect lipids.[212; 330]

Table 5.1. Calculated *m/z* values for the pseudomolecular ions $[M+Na]^+$ of the TAGs produced from the 35 possible combinations of the five fatty acids: L= linoleic acid (18:2), Ln = linolenic acid (18:3), O = oleic acid (18:1), P = palmitic acid (16:0), Pm = palmitoleic acid (16:1).

TAG	*m/z*	TAG	*m/z*	TAG	*m/z*
PmPmPm	823.7	PPO	855.7	OOP	881.8
PmPmP	825.7	LnLnPm	871.7	LnLnLn	895.7
PPPm	827.7	LnLnP	873.7	LnLnL	897.7
PPP	829.	PmLLn	873.7	LLLn	899.7
PmPmLn	847.7	LLPm	875.7	LnLnO	899.7
PPmLn	849.7	PmOLn	875.7	LLL	901.7
PmPmL	849.7	PLLn	875.7	OLLn	901.7
PPLn	851.7	LLP	877.7	OOLn	903.7
PPmL	851.7	PmOL	877.7	LLO	903.7
PmPmO	851.7	POLn	877.7	OOL	905.8
PPL	853.7	POL	879.7	OOO	907.8
PPmO	853.7	OOPm	879.7		

Each peak from the experimental MALDI-TOF spectrum that had an *m/z* value that matched one of the expected *m/z* values (**Table 5.1**) was labelled and its intensity recorded. Total intensity was calculated by the summation all of the

intensities of the identified TAG peaks. The percentage of total intensity was also calculated for each peak

5.2.8 Comparison between the Results of the Two Methods of Analysis

To be able to compare the results from the two methods, the results from the one-step method are modified to include only the five fatty acids that were represented in the MALDI-TOF analysis. This meant that only the five fatty acids included in the MALDI-TOF analysis were used for comparison.

5.2.9 Analysis of Clover Root Weevil Fat Bodies

It was important to ascertain that any differences between the two methods of analysis were not simply arising because the MALDI-TOF method was analysing only the intact TAGs, whereas the one-step method was analysing all of the fatty acids present. To do this is was necessary to analyse a lipid sample of a CRW using MALDI-TOF. As it was not possible to analyse a whole CRW using MALDI-TOF, a sample of fat bodies (site of the largest amount of fat within a CRW) from CRWs was analysed using the MALDI-TOF method. The aim of this was to provide a fairer comparison with the results of the teratocyte analysis by MALDI-TOF.

The fat bodies of ten parasitised CRWs were removed from dissected samples and placed in water (0.5 mL). The sample was analysed using the same MALDI-TOF method as the teratocytes sample.

5.3 Results

5.3.1 One-Step Method for Extraction and Derivatisation of Lipids

(a) Fatty Acid Profile of Teratocytes

The average composition for each fatty acid as well as the corresponding standard deviations and coefficients of variation percentages are shown in **Table 5.2**.

(b) Comparison with Fatty Acid Profile of the Clover Root Weevil

Raw data for the two-sample t-test is in **Appendix 9.7.1**, while the p-values are shown in **Table 5.3**. As all the p values were larger than 0.05, no significant difference was found between the means at the 95% confidence level. Therefore, there were no significant differences between the means of any of the fatty acid components of the teratocyte samples and the CRW samples at the 95% confidence level. These results indicate that the contents of the teratocytes may have originated from the lipids of the CRW host.

Table 5.2. Average composition, standard deviation and coefficient of variation of fatty acids found in the teratocyte samples plus the results from the two-sample t-test to compare the mean fatty acid compositions for the teratocyte samples and all of the CRW samples. This test calculated the p-value (at the 95% confidence level).

Fatty acid	Average composition	Standard deviation	Coefficient of variation %	p Value
12:0	1.27	1.11	87.8	0.136
14:0	1.81	1.78	98.6	0.274
16:0	15.4	6.08	39.4	0.676
16:1	8.88	2.55	28.7	0.368
18:0	5.34	4.64	86.9	0.088
18:1	37.1	10.7	28.8	0.634
18:2	5.85	2.47	42.2	0.712
18:3	24.3	13.2	54.4	0.408

(c) Comparison with Parasitoid Larvae

The average composition for each fatty acid, as well as the corresponding standard deviations, coefficients of variation percentages and p-values from the two-sampled t-test are shown in **Table 5.3**. Raw data is in **Appendix 9.7.2**.

No significant differences (at the 95% confidence level) were found between the CRW samples and the parasitoid larvae for six of the fatty acids (12:0, 14:0, 16:0, 18:0, 18:1 and 18:2 fatty acids). However, significant differences (at the 95% confidence level) were found for 16:1 and 18:3 fatty acids. The larvae had

significantly less 16:1 fatty acid and significantly more 18:3 fatty acid than the CRW samples.

Table 5.3. Average fatty acid composition, standard deviation and coefficient of variation percentage for *M. aethiopoides* larvae plus results from a two-sample t-test comparing the CRW samples and the parasitoid larvae. This calculated the p-value (at the 95% confidence level). Fatty acids that are significantly different at the 95% level are bolded.

Fatty acid	Average composition	Standard deviation	Coefficient of variation %	p Value
12:0	0.91	0.91	84.9	0.476
14:0	1.81	1.81	82.1	0.288
16:0	15.8	15.8	25.4	0.473
16:1	7.48	7.48	19.6	**0.006**
18:0	5.61	5.61	78.6	0.181
18:1	32.3	32.3	24.2	0.074
18:2	9.82	9.82	54.9	0.129
18:3	26.3	26.3	21.1	**0.0350**

5.3.2 MALDI-TOF Spectrometry

Each peak in the spectra produced (

Figure 5.4 and

Figure 5.5) that had an *m/z* value that matched one of the expected *m/z* values (**Table 5.1**) was labelled and its intensity recorded (**Table 5.4**). The percentage of total intensity for each peak is shown in **Table 5.5**.

To convert the results to a format that would be comparable with the results from the one-step method of extraction and derivatisation, the fatty acid profile of the teratocyte sample was calculated from the MALDI-TOF TAG results. For example, the relative proportion of 16:0 fatty acid (P) in the TAG PPO is 2/3 and this TAG's relative intensity is 0.7% (**Table 5.4**). Therefore this TAG's contribution to the fatty acid component of 16:0 is 0.5%. This calculation was carried out for every TAG and the total components for the five fatty acids were calculated (**Table 5.5**). When there was more than one possible TAG for a single

m/z value, the relative intensity was split over the possible TAGs before being split into contributions from each fatty acid. For example for the TAG with a relative intensity of 1.2%, the calculation split the 1.2% over the two TAGs so that each had 0.6%, before further splitting this into each fatty acid's contribution. Although splitting the contribution over the possible TAG combinations for a given *m/z* value may introduce error, the size of the relative intensities for all of the *m/z* values with more than one TAG possibility was small hence reducing this error. The highest relative intensity of any *m/z* value with more than one TAG possibility was 7.2%. This meant that each of the nine fatty acids was assigned 0.8% (theoretically, the contribution of each of the nine fatty acids to the *m/z* intensity could have ranged from 0-2.4%). The relative intensities of the TAGs with more than one possibility were considerably smaller than the *m/z* value with the highest intensity, thus the amount of error introduced by splitting the relative intensity is small. This is a common approach when TAGs are analysed using MALDI-TOF.[212; 330]

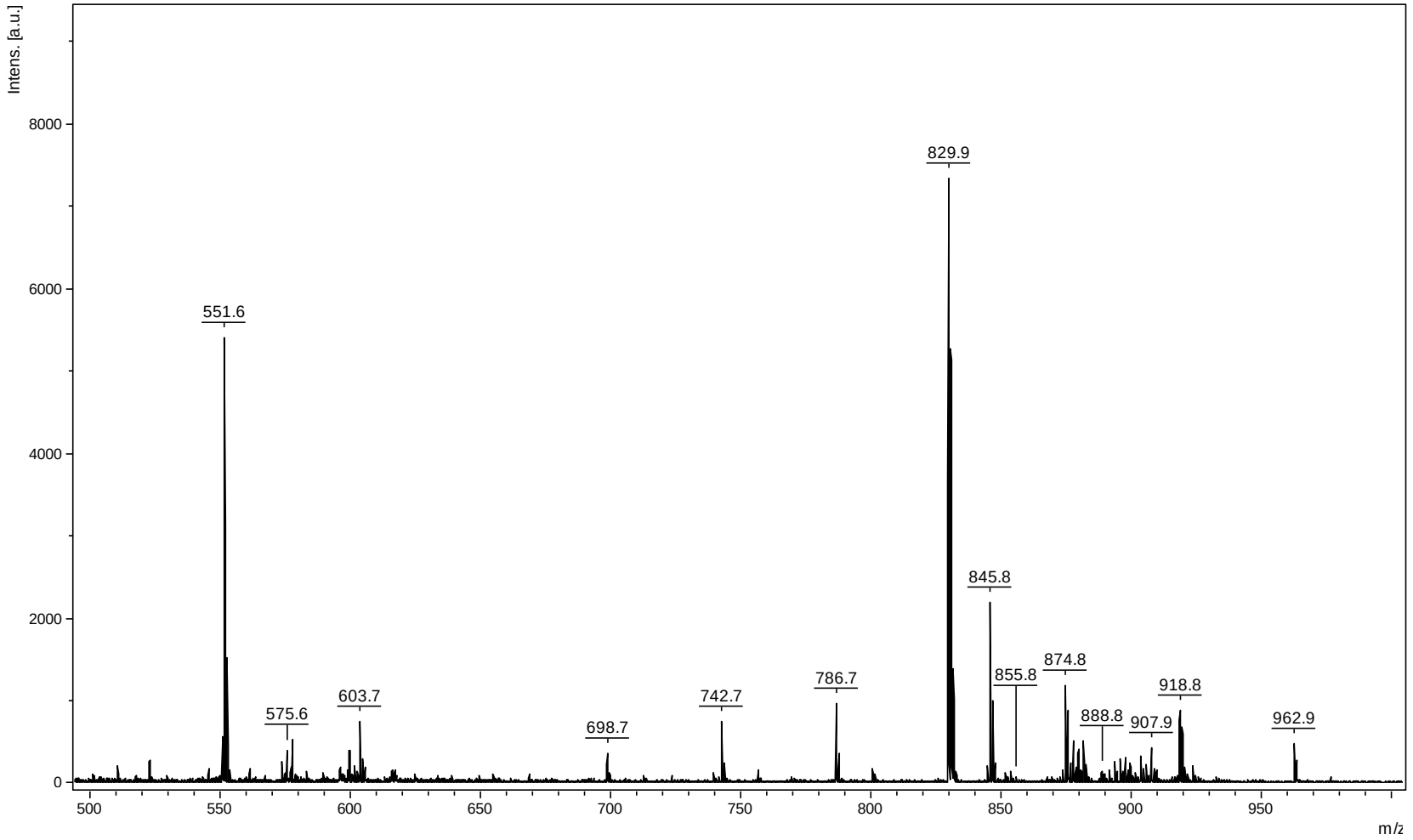


Figure 5.4. MALDI-TOF spectrum of a teratocyte sample.

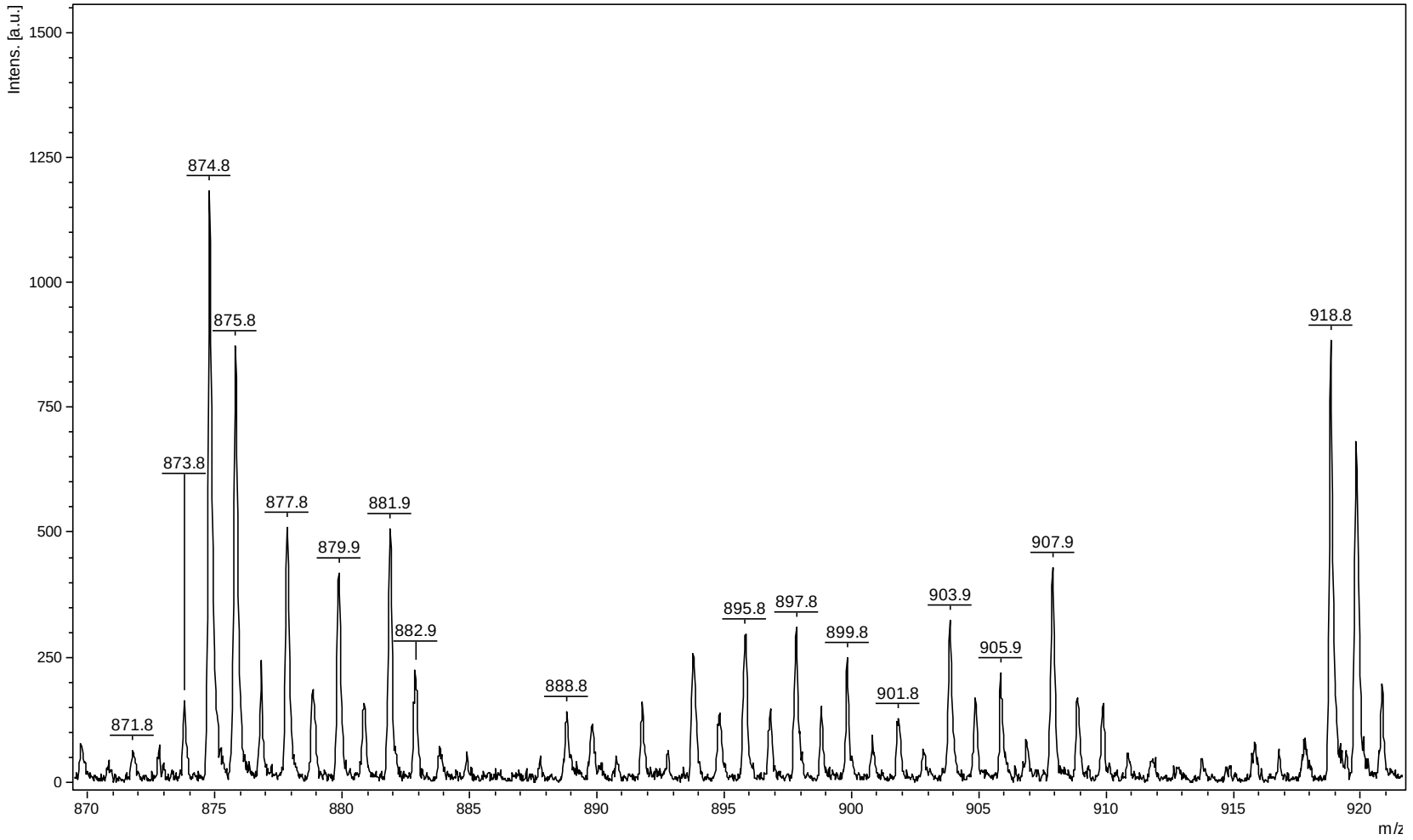


Figure 5.5. Expanded MALDI-TOF spectrum showing *m/z* values in the region 870 -920 of a teratocyte sample.

Table 5.4. The *m/z* value of each TAG identified in the teratocyte sample, its calculated *m/z*, its intensity and its percentage of total intensity as calculated by MALDI-TOF. L = linoleic acid (18:2), Ln = linolenic acid (18:3), O = oleic acid (18:1), P = palmitic acid (16:0), Pm = palmitoleic acid (16:1)

m/z	Calculated *m/z*	TAG/s	Intensity	Percentage of Total Intensity
829.9	829.7	PPP	7340	60.2
851.8	851.7	PPLn, PPmL, PmPmO	126	1.0
853.9	853.7	PPL, PPmO	147	1.2
855.8	855.7	PPO	80	0.7
871.8	871.7	LnLnPm	64	0.5
873.8	873.7	LnLnP, PmLLn	163	1.3
875.8	875.7	LLPm, PmOLn, PLLn	874	7.2
877.8	877.7	LLP, PmOL, POLn	510	4.2
879.9	879.7	POL, OOPm	418	3.4
881.9	881.8	OOP	506	4.2
895.8	895.7	LnLnLn	297	2.4
897.8	897.7	LnLnL	311	2.6
899.8	899.7	LLLn, LnLnO	250	2.1
901.8	901.7	LLL, OLLn	126	1.0
903.9	903.7	OOLn, LLO	324	2.7
905.9	905.8	OOL	219	1.8
907.9	907.8	OOO	428	3.5

Table 5.5. The fatty acid composition percentage of the teratocyte sample as calculated by the MALDI-TOF methodology.

Fatty acid	Composition percentage
16:0 (P)	64.3
16:1 (Pm)	3.6
18:1 (O)	13.3
18:2 (L)	18.6
18:3 (Ln)	10.2

Table 5.6. Composition percentage of teratocytes sample analysed by the one-step method and modified to include only the five fatty acids represented in the MALDI-TOF analysis.

Fatty acid	Composition percentage
16:0	16.9
16:1	9.7
18:1	40.5
18:2	6.4
18:3	26.6

5.3.3 Analysis of Clover Root Weevil Fat Bodies

The spectra produced showed peaks within the TAG region (**Figure 5.6**). Each peak that had an *m/z* value that matched one of the expected *m/z* values (**Table 5.1**) was labelled and its intensity recorded, and the percentage of total intensity was calculated for each peak (**Table 5.7**). The results were converted to a format that would be comparable with the results from the one-step method of extraction and derivatisation (**Table 5.8**).

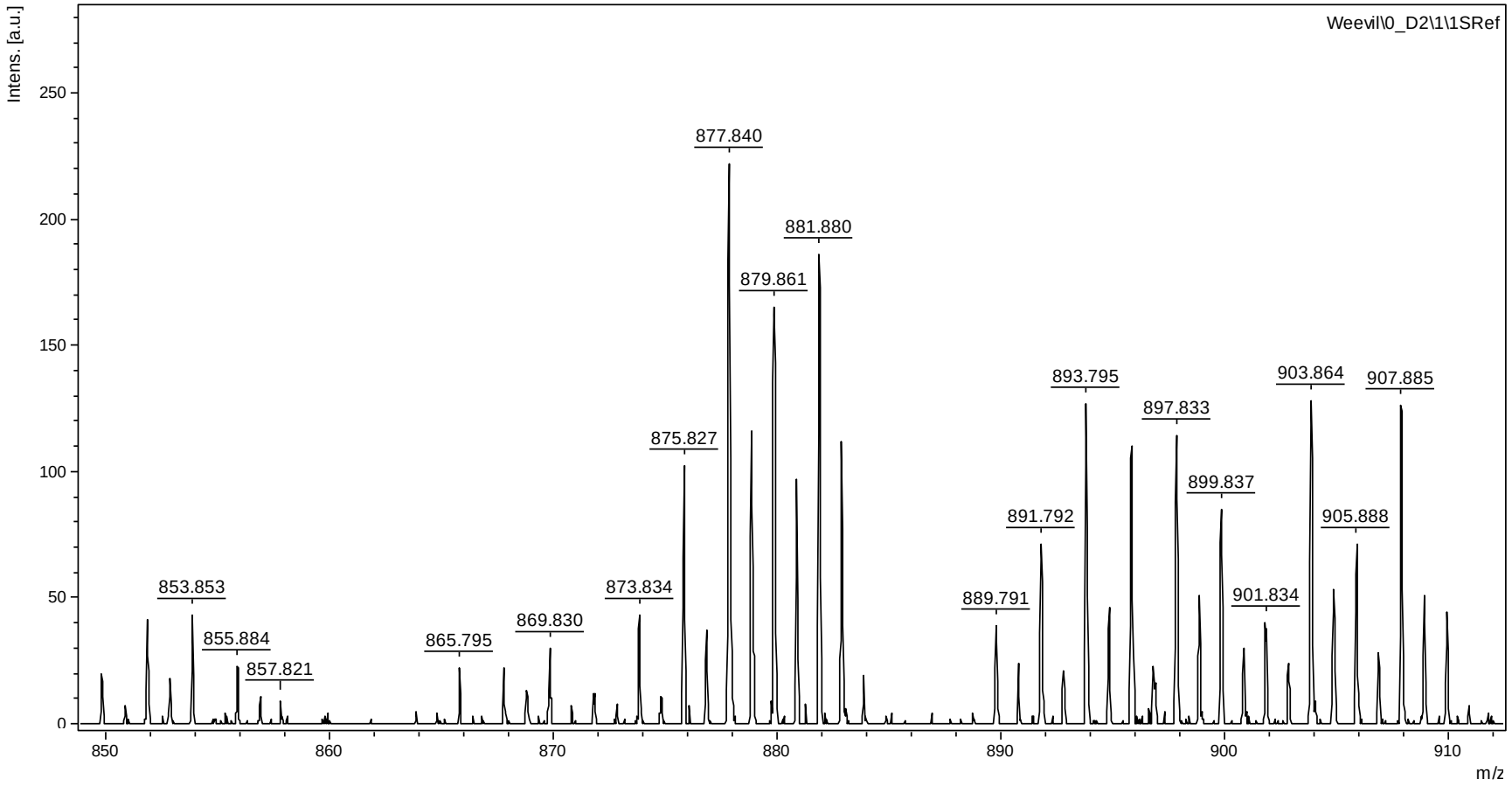


Figure 5.6. Expanded MALDI-TOF spectrum showing *m/z* values in the region 850-910 of a fat bodies sample.

Table 5.7. The *m/z* value of each TAG identified in the fat body sample, its intensity and the percentage of total intensity as calculated by MALDI- TOF. L = linoleic acid (18:2), Ln = linolenic acid (18:3), O = oleic acid (18:1), P = palmitic acid (16:0), Pm = palmitoleic acid (16:1)

m/z	Calculated *m/z*	TAG/s	Intensity	Percentage of Total Intensity
829.9	829.7	PPP	43	2.7
851.8	851.7	PPLn, PPmL, PmPmO	41	2.6
853.9	853.7	PPL, PPmO	48	3.1
855.8	855.7	PPO	23	1.5
871.8	871.7	LnLnPm	22	1.4
873.8	873.7	LnLnP, PmLLn	43	2.7
875.8	875.7	LLPm, PmOLn, PLLn	102	6.5
877.8	877.7	LLP, PmOL, POLn	222	14.1
879.9	879.7	POL, OOPm	165	10.5
881.9	881.8	OOP	186	11.9
895.8	895.7	LnLnLn	110	7.0
897.8	897.7	LnLnL	114	7.3
899.8	899.7	LLLn, LnLnO	85	5.4
901.8	901.7	LLL, OLLn	40	2.5
903.9	903.7	OOLn, LLO	128	8.2
905.9	905.8	OOL	71	4.5
907.9	907.8	OOO	126	8.1

Table 5.8. The fatty acid component percentage of the fat body sample as calculated by the MALDI-TOF methodology.

Fatty acid	Composition percentage
16:0 (P)	17.0
16:1 (Pm)	7.1
18:1 (O)	34.9
18:2 (L)	20.0
18:3 (Ln)	21.0

5.4 Discussion and Conclusions

5.4.1 One-Step Method for Extraction and Derivatisation of Lipids

The one-step method for extraction and derivatisation was successful at determining the fatty acid composition of teratocytes. The analysis of teratocyte composition is very poorly covered in the literature, so the development of a successful method for analysis is significant, and it is possible that this method could be further developed to analyse teratocytes of other species.

Using this method it was found that the fatty acids present in teratocytes were not significantly different (at the 95% significance level) to the fatty acid composition of the CRWs presented in **Chapter 3**.

5.4.2 MALDI-TOF Mass Spectrometry

MALDI-TOF method measures intact TAGs, whereas the one-step method measures all fatty acids present, whether bound or free. The results for the TAGs in the teratocytes were largely dominated by the 16:0 fatty acid meaning that all of the other fatty acid percentages were very low. The results of the fat body analyses were very different; there was no dominance of the 16:0 fatty acid, and three out of the five fatty acids had percentage compositions of above 20%. It is possible that the TAGs within teratocytes are more similar to the TAGs within the

insect's haemolymph, however analysis of the haemolymph is beyond our capabilities. It is apparent that the MALDI-TOF methodology can clearly distinguish the TAGs of teratocytes from those of the fat body of the host; however there is a shortage of literature with which to compare this result. There are no reports in the literature describing TAGs in teratocytes.

Cvacka *et al*[74] compared the TAGs of several bumblebee species using the GC analysis of the fatty acids as well HPLC-MS to analyse the intact TAGs. Although the authors concluded that the acyls of the TAGs corresponded well with the fatty acids identified by GC, no attempt was made to reconcile the two sets of results quantitatively. MALDI-TOF has been utilised when a fingerprint is needed i.e. when comparing insects from different species.[212] The fatty acid composition of different classes of lipids (i.e. the free fatty acids versus the TAGs or phospholipids) are often reported in the literature, although most accounts simply determine the fatty acid composition of each class rather than comparing the intact TAGs composition to the free fatty acids,[334; 335] so comparison with the current findings is not possible. Although the MALDI-TOF has shown potential as an alternative method which is easy, rapid and sensitive with detection limits reported in the mid femtomole range, more work that is beyond the scope of this investigation is needed before this method can be used with any certainty. Other techniques such as HPLC have been discussed in the literature[79-82] and may be more useful at quantifying TAG profiles for better comparison with MALDI-TOF of the TAGs, however, time did not permit the acquisition of data using these techniques.

There are two possible reasons for the differences seen between the two methods of analysing teratocytes. Insect TAG results from MALDI-TOF are only semi-quantitative given the interference of isotopes and the differences in signal intensities for TAGs of different molecular weights. There is a significant decrease in signal intensity for higher molecular weight TAGs and for unsaturated TAGs.[212; 330],[333] This could contribute to the high result obtained for the 16:0 fatty acid but cannot be the sole factor since this dominance was not observed in

the fat body. Another possibility for the difference seen in the MALDI-TOF results is that only the TAGs have been taken into account and it is possible that DAGs (either naturally occurring or products of fragmentation), monoacylglycerols and fatty acids (not bonded to glycerol) themselves could alter the fatty acid composition.[212] With the one-step method, all fatty acids regardless of how they are bonded are quantified by the analysis. The two methods produce data that are fundamentally different (fatty acids *versus* TAGs) and the many assumptions that are made in the MALDI-TOF method render it at best semi-quantitative so statistical analysis between the results from each method is not possible. The comparison that is presented here is an attempt to summarise the differences of each method and explain the results obtained.

5.4.3 Biological Significance

The results from the one-step method indicate that the teratocytes of *M. aethiopoides* may simply obtain nutrients from absorbing lipids from the body fluid of its CRW host. This result is different to that reported by Cohen and Debolt.[326] Those authors were unable to determine whether the growing teratocytes themselves were differentially selective of some of the fatty acids or if the host tissue profiles differ with different types of parasite; therefore, it is possible that the different species of parasite in their investigation caused biochemical changes within the same host and in fact the teratocytes simply absorbed different fatty acids because of this. As teratocytes have been reported to have different functions,[59; 298] it is possible that teratocytes from different species have different mechanisms to obtain nutrients. It thus appears that the lipid requirements for developing *M. aethiopoides* larvae match the profile of those produced by the host and the teratocytes simply unselectively absorbs the available lipids from the haemolymph of the CRW.

Research into the genetic variation of *M. aethiopoides* has shown that within the species, there are clearly distinct biotypes associated with different hosts and even the food that these hosts feed on.[336; 337] Given that selection pressure is high for these quite specific host: parasitoid relationships (life or death for either), this

close match between Irish *M. aethiopoides* and its preferred host is perhaps not surprising. Possibly the same analyses would show different profiles for Irish *M. aethiopoides* in a non-preferred host.

Analysis of the *M. aethiopoides* larvae found that the larval fatty acid profile differed from that of the CRW (and hence that of the teratocyte samples). There were no significant differences (at the 95% confidence level) between the larvae and the CRW for six of the fatty acids (12:0, 14:0, 16:0, 18:0, 18:1 and 18:2 fatty acids); however, the larvae had significantly less 16:1 and significantly more 18:3 fatty acid than the CRW samples. This indicates that the larva may obtain some lipid from the CRW host but may also receive some from the egg from which it hatched. The ability of the larvae to obtain some lipid from its host is biologically significant as it guarantees a source of the majority of main fatty acids for the developing larvae. Larvae of the cabbage looper (*Trichoplusia ni*) reared on a synthetic diet were unable to synthesize 18:3 fatty acid from ^{14}C-acetate.[(221)] It is possible that *M. aethiopoides* larvae are also unable to synthesize 18:3 and therefore accumulate 18:3 from the egg prior to hatching. The biological significance of 16:1 in insects has been related to the need for accumulation of sufficient energy and a carbon reservoir in the developing new vitellum.[(277)] It is possible that the *M. aethiopoides* larvae utilises the 16:1 fatty acid present in its egg before hatching and therefore emerges with a 16:1 fatty acid deficit.

6 Juvenile Hormones in the Clover Root Weevil

6.1 Introduction

The juvenile hormones (JHs) are the predominant secretory products of the insect corpora allata (a pair of epithelial glands) located behind the insect brain.[338-340] They are sesquiterpenoids characterised by an epoxide group near one end and a methyl ester at the other end.[341] These hormones regulate important stages in the insect lifecycle. They inhibit metamorphosis during the larval stage and control ovarian development and vitellogenin synthesis in adults. They also play a role in caste determination in social insects such as termites and in the regulation of behaviour in honeybee colonies. JHs have also been implicated in the polyphenisms of aphids and locusts as well as in diapause regulation in larvae and adults.[340]

6.1.1 Discovery of Juvenile Hormones

Wigglesworth first reported the existence of a juvenilizing factor in insects in the 1930s and was the first person to use the term "juvenile hormone."[340] JHs were first extracted from insects in 1956 when Williams[342] was able to extract sufficient quantities of JH active lipid from the abdominal tissues of the male *Hyalophora cecropia* moth. This work led to the chemical characterisation of what is now termed JH I in 1967 using mass spectral data.[340] After the characterisation of JH I, the characterisation of JHs II and III followed. Discovery of the three remaining JHs (JH 0, JHB_3, JH I, 4-methyl JH I) occurred later and less is known about these.

6.1.2 Types of Juvenile Hormones

Six homologues of JH (**Figure 6.1**) (JH 0, JHB_3, JH I, 4-methyl JH I, JH II and JH III) have been identified in insects,[343] although JH III is by far the most common[341] and is often the only JH present in insects.[344; 345] Lepidopterans (butterflies and moths) are an exception and often only have JH I and/or JH II.[346]

The JHs differ in the number of methyl side chains and therefore the number of carbon atoms. JHs 0–III have 19, 18, 17 and 16 carbon atoms respectively.[346]

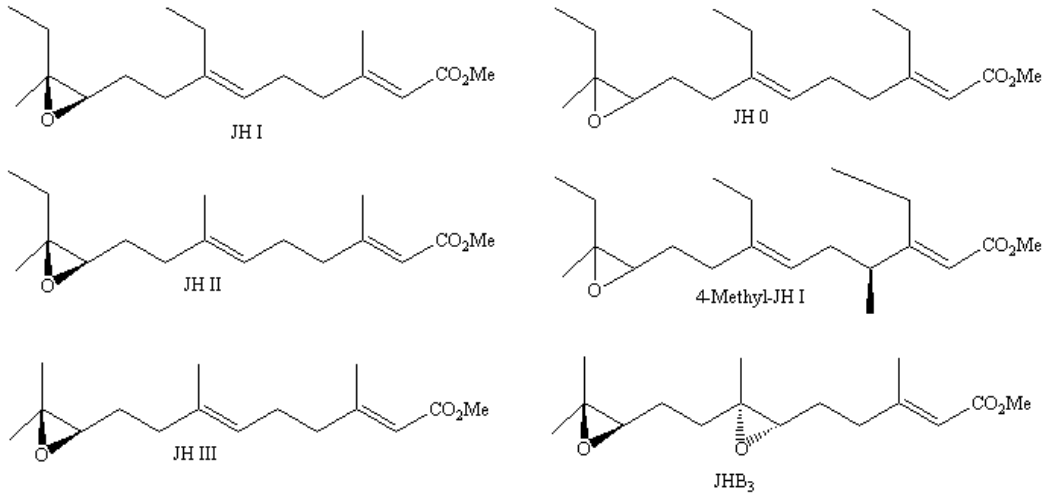


Figure 6.1. The chemical structures of the six homologues of JH that have been identified in insects.[347]

6.1.3 Biosynthesis of Juvenile Hormone III

JH III is structurally similar to the sesquiterpene farnesol and therefore its biosynthesis follows the normal terpenoid pathway, with the formation of isoprenoid units from acetate followed by the head to tail condensation to form farnesyl pyrophosphate (**Figure 6.2**). Farnesyl pyrophosphate is cleaved to farnesol and then oxidised to form farnesoic acid. There are two possible pathways for the final steps and this depends upon insect order. The farnesoic acid can be converted to an epoxy acid, which is then methylated, or methylation can occur first and is followed by epoxidation. The biosynthesis of JH III was first described by Schooley and Baker.[348; 349]

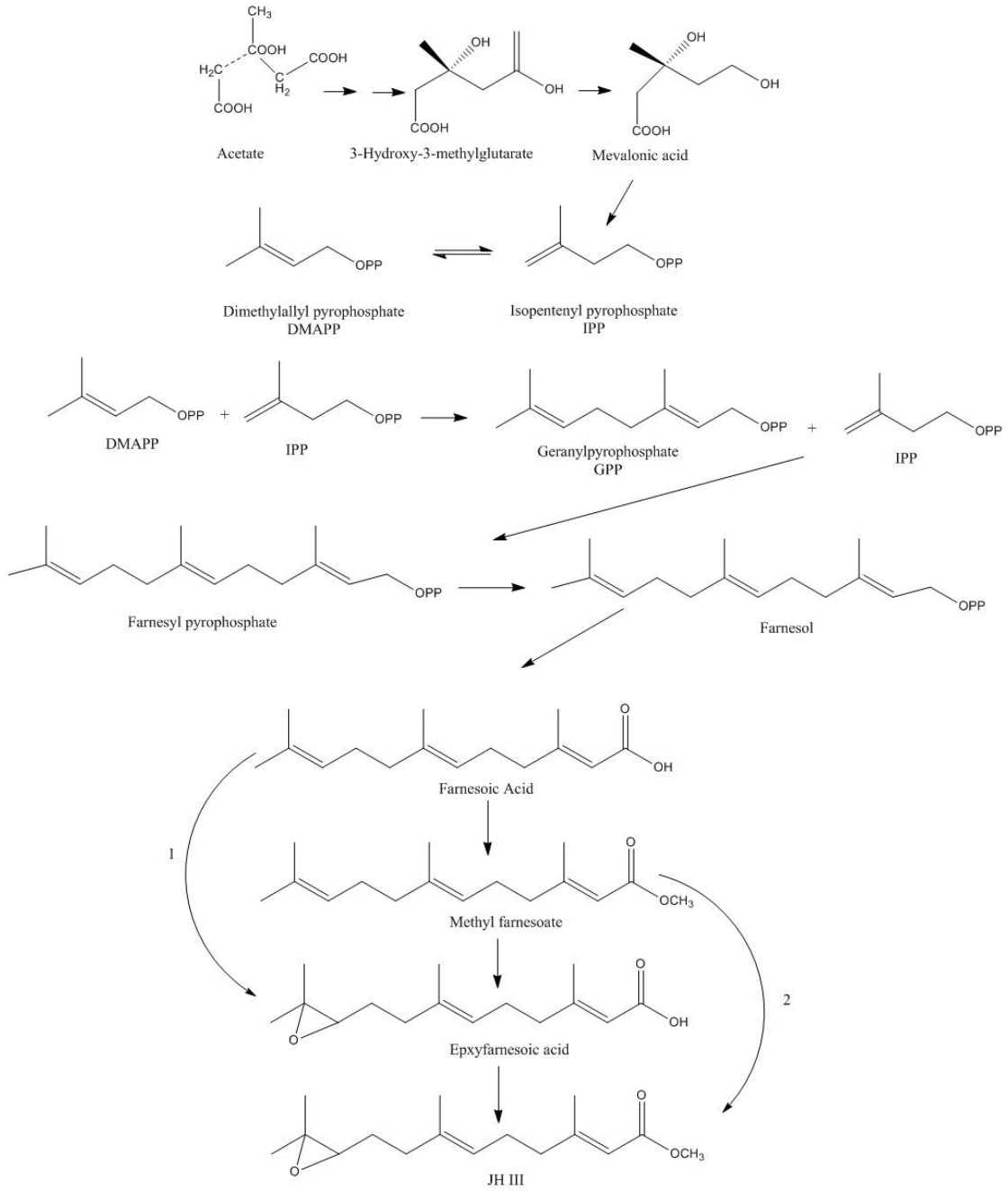


Figure 6.2. The biosynthesis of JH III.[348]

6.1.4 Regulation of Larval Moulting and Metamorphosis

JH is a key hormone in regulation of the insect's life cycle. It is important in both maintaining the larval state during moults and in directing reproductive maturation.[350-354] In most insects JH prevents metamorphosis at each of the larval moults until the insect has reached its species-specific size. Then JH disappears and this allows metamorphosis to proceed.[350; 351] A synthetic analogue of JH was used to investigate the proposal that, during the sexual maturation of *Drosophila*

melanogaster (fruit fly), JH provides a signal to the ovary that leads to the production of several maternally inherited mRNAs.[355] In some species JH is also involved in controlling other tissues, such as brain, fat body, flight muscle and accessory glands.[351]

6.1.5 Juvenile Hormones and Embryological Development of Insects

The exact role of JH in embryological development remains unclear despite decades of research.[348] It is likely that differences in JH titres during embryological development are correlated to events in organogenesis. In tobacco hornworm (*Manduca sexta*) embryo, the highest levels of JH during development were found to be correlated to the appearance of a true larval cuticle.[356] Similar results were reported for the cabbage looper (*Trichoplusia ni)*[357] and the African cotton leafworm (*Spodoptera littoralis*).[358]

6.1.6 Juvenile Hormones and Sexual Maturation of Insects

Juvenile hormones play a critical but not fully understood role in the sexual maturation of insects. In adults of most insect species, JHs coordinate reproductive processes. All tissues that are directly or indirectly involved in reproduction can be targets for JHs. However, the particular roles that JHs play in reproduction vary with insect species.[359] Ovaries in abdomens isolated from female mosquitoes, (*Aedes aegypti*) did not grow or become responsive. However, when a topical application of JH or S-methoprene (a JH analogue) was used on the isolated abdomens, induced growth and responsiveness in the ovaries occurred. S-Methoprene had a greater effect than JH, and ovarian growth and responsiveness were functions of the dose applied to abdomens.[360] This was similar to other results found in mosquitoes. Decapitation (therefore removal of the corpora allata) prevented normal development of the previtellogenic follicles. However, previtellogenic ovarian development could be induced to the same stage seen in non-decapitated insects by topically applying a JH analogue.[361] There was a positive correlation between JH titre and ovarian development of bumble bees (*Bombus terrestris*),[362] while a single, topical application of JH III accelerated the ovarian development of female wasps (*Ropalidia marginata*).[363]

JH involvement in male reproduction is poorly understood, although it is believed to be linked to protein synthesis.[364]

6.1.7 Juvenile Hormone Metabolism

The maintenance of effective JH concentrations in an insect is a precise balancing act among the various stages of synthesis, delivery, metabolism and cellular uptake.[348] Although nonspecific or general esterases have been reported as being capable of JH hydrolysis, later work has identified that the JH-specifiic esterase (JHE) is far more important in JH metabolism than any non-specific esterases.[339; 365] JHs are hydrolysed by JHE to yield JH acid which is hydrolyzed by epoxide hydrolase to produce the JH acid diol.[340] JHE is most commonly found in the insect's haemolymph and is synthesised in the fat body.[348]

6.1.8 Juvenile Hormone Analogues as Insecticides

During insect development, JHs are expressed at certain specific times in the process of metamorphosis. However, if JHs are present at other times, this can lead to morphogenetic abnormalities and this is the basic theory behind the use of S-methoprene and other JH analogues (JHAs) as insecticides.[366; 367]

Synthetic analogues of the juvenile hormone have been used for many years as insecticides.[368] The JHA most commonly used as an insecticide is S-methoprene, which is usually used against larvae or nymphs, generally for dipterans such as mosquitoes. It is a larvicide and kills by disrupting metamorphosis, therefore, most mortality occurs during the larval and pupal moults. However, as with other JHAs, its exact mode of action is not completely understood. Although S-methoprene is toxic to a range of insects from 12 orders, including Coleoptera, it is most toxic against Diptera. A report by Glare and O'Callaghan[366; 367] for the New Zealand Ministry of Health on the environmental and health impacts associated with the use of S-methoprene for control and eradication of mosquitoes in New Zealand, found it to be effective with very little risk to humans or the environment. S-Methoprene is also effective at preventing the development of immature insects in other species such as the red flour beetles (*Tribolium castaneum*)[369] and the lesser grain borer (*Rhyzopertha dominica*).[194]

There have been reports of successful use of S-methoprene against adults, although in some cases effects were not seen until the occurrence of abnormal egg laying by females.[366] In female mosquitoes (*Anopheles stephensi*) that were fed S-methoprene before a blood meal, over 80% of the eggs laid after this meal were small, white, deformed and fragile, and no larvae hatched from them.[370] The incorporation of S-methoprene into the diet of adult insects caused substantial reductions in oviposition of two species of Coleoptera, the red flour beetle (*Tribolium castaneum*) and the confused flour beetle (*T. confusum*).[371]

In some species of Coleoptera, the JHA fenoxycarb has been an effective insecticide. Fenoxycarb was one hundred times more effective than S-methoprene at preventing the formation of adult insects when used on larval stages of the lesser mealworm (*Alphitobius diaperinus*). These results suggested the potential use of fenoxycarb as a control of the lesser mealworm in intensive poultry production units.[372] Fenoxycarb was also found to been an effective JHA on the larvae of the bark beetle (*Ips paraconfusus*).[373] Another JHA, pyriproxyfen, proved to be an effective insecticide against the larvae of the mealworm (*Tenebrio molitor*).[374]

6.1.9 Methods used to Measure Juvenile Hormones

Juvenile hormones are particularly difficult to measure. Their lipophilic nature, low concentrations, lability and tendency to bind to nonspecific substrates all contribute to making them one of the most difficult hormones to measure.[348] The first investigations of JHs used bioassays but these are not widely used today.[348] Instead GC-MS,[344; 345; 375] radioimmunoassay (RIA) and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods are more common.[341; 376] While the majority of reports, especially the earlier work, use GC,[377] GC-MS analysis of JH involves extensive sample preparation. As JH is only present in minute concentrations, clean up procedures are required before GC-MS analysis. Common procedures include column chromatography, separatory cartridge purification and HPLC.[348] Improvements have been made to the GC-MS methods published. The use of capillary GC-chemical ionisation (CI)-MS allowed

the direct hexane extraction of the JH of the Caribbean fruit fly without the extensive sample preparation usually associated with this type of analysis. However, this work used haemolymph extraction, which gives a much less complex sample than whole body extracts.[282]

RIA involves a competitive protein-binding assay that sees JH from a sample compete with a fixed and known number of radiolabeled JH for a limited number of binding sites on JH-directed antibodies. Although RIAs are a sensitive and inexpensive way to determine JH titers, they do require extensive sample preparation and specific materials as well as a dedicated laboratory set up.[378-380]

LC-MS/MS has become more popular than the other techniques due to its ease of sample preparation, reduced solvent use and lack of sample degradation.[338; 341; 381-383] An optimised LC-MS method was also found to have higher sensitivity than a corresponding GC-MS method when the JH concentrations of termites were investigated.[384] A direct comparison of RIA and LC-MS/MS found that LC-MS/MS gave a significantly lower detection limit and a far easier sample preparation than RIA.[341] When insects are too small to collect enough haemolymph, it is useful to be able to analyse whole body extracts. The majority of whole body analyses of JH have been accomplished by GC-MS:[346; 384] however, these involve extensive sample preparation. Whole body extracts from nymphs and adults of the brown planthopper (*Nilaparvata lugens*) were analysed by GC-MS but prior to analysis JH was separated successively by solvent extraction, chromatography on an aluminium oxide column and HPLC.[345] Rembold and Lackner[385] used two extractions followed by adsorption onto alumina and a complex series of structural modifications and subsequent adsorptions, to produce the 10-dimethyl(nonafluorohexyl)siloxy-11-methoxy-JH derivative which was analysed using GC-MS.

Westerlund and Hoffmann[338; 381] developed a simple, fast and sensitive method to determine the concentration of JHs in insect haemolymph using LC-MS/MS. This reduced the need for the complex work up of samples needed for whole body analysis via GC-MS. The high abundance of Na^+ in the haemolymph meant that the $[M+Na]^+$ pseudomolecular ion was primarily formed. Miyazaki *et al*[384]

developed an optimised LC-MS method for determining the JH III concentration in whole body extracts of termites (Formosan subterranean termites; *Coptotermes formosanus*). Their method allowed for the quantification of JH levels in individual termites. An advantage of their method over previously reported LC-MS methods was that the ion fragmentation pattern of JH III was the same in authentic JH III as in the insect samples. The use of a pentafluorophenyl (PFP) column instead of a C18 column improved the separation of JH III in the samples from other compounds. The synthetic JH analogue S-methoprene, (**Figure 6.3**) is often used as an internal standard.[355; 381; 384; 386]

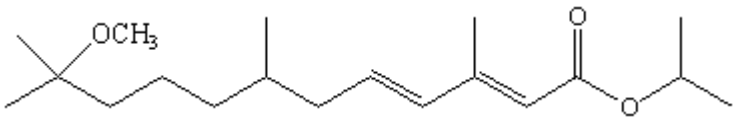


Figure 6.3. The chemical structure of S-methoprene.

6.1.10 Juvenile Hormones and Parasitism

Although host-parasitoid relationships have received significant coverage in the literature, parasitoid endocrinology is relatively unreported. A common theme in what has been reported is that the JH concentration in the host's haemolymph rises after parasitism.[343] It has been reported that teratocytes may be involved in decreasing the activity of JHE in the haemolymph of insects. JHE activity was significantly reduced in the haemolymph of the tobacco budworm (*Heliothis virescens)* larvae parasitised by the wasp (*Microplitis croceipes*); injecting the larvae with teratocytes had a similar effect,[352] which indicated that it was likely that the teratocytes were responsible for the inhibition of JHE release.[387; 388] In the *Microplitis demolitor:* soybean looper *(Pseudoplusia includes*) system it was concluded that the elevation in the JH concentration of the haemolymph after parasitism was due to a decrease in the activity of JHE rather than the release of JH from the parasitoid, teratocytes or the host.[305] A proteinaceous factor that was found to repress the JHE activity in the parasitised insect was isolated from the

last instar[††††] larvae of the armyworm (*Pseudaletia separata*) that had been parasitised by the wasp *Apantales kariyai*.[(317)] Parasitism of the tobacco hornworm (*M. sexta)* by the parasitic wasp *Cotesia congregata* caused almost ten times the concentration of total JH in the haemolymph. This prevented the host from pupating as well as controlling the moult cycle of the growing parasitoid. It appeared that the JH was synthesized and released by the parasitoid larva rather than from the teratocyte. The parasitoid also controlled the host's production of JH.[(343)] Teratocytes have also been reported as providing a source of non-host JH or JH-type compounds.[(343)] JHE activity in the haemolymph of 5th-instar tobacco budworm was inversely related to the number of teratocytes injected.[(387)]

6.1.11 Juvenile Hormones in Coleoptera

In Coleoptera, the effects of JH are in line with those reported for insects generally. JH III regulates the production of egg yolk protein and oocyte maturation in the red flour beetle (*Tribolium castaneum*).[(369)] The control of pheromone production in bark beetles (*Ips spp.*) is by JH,[(373)] while reproductive systems in female burying beetles (*Nicrophorus spp.*) are also controlled by JH.[(389)] No work has been reported that compares JH III titres with state of parasitism for Coleoptera.

There are very few reports concerning JHs in weevils and those that do occur are focussed on the use of JHAs as possible pesticides rather than intrinsic JH III concentrations of the weevils. JHAs were successfully used as insect growth-regulator pesticides against the rice weevil (*Sitophilus oryzae*), the boll weevil (*Anthonomus grandis* Boheman) and the southern cowpea weevil (*Callosobruchus maculatus*).[(390-392)] All of these reports used JHAs against the larval stage of the insect. Applying a JHA to the eggs of the white pine weevil (*Pissodes strobi*) prevented embryonic development.[(393)]

[††††] Developmental stage between moults

6.1.12 Context of the Present Research

Parasitism has been reported to alter the JH titres of insect hosts after parasitism,[343] therefore, by determining the JH III titres of non-parasitised and parasitised CRW (both of which are previously unreported) it would be possible to deduce any effect on JH III levels resulting from parasitism. An increase in the JH titre of the host has been linked to the presence of teratocytes[387; 388] and as teratocytes are seen within parasitised CRW, any change in JH titre could be a result of the presence of teratocytes.

CRW are substantially smaller than the insects that are commonly investigated in JH analysis (such as tobacco budworm). Therefore, the development of a suitable method of JH analysis for CRW would not only be significant in reporting the JH concentrations of this insect but may provide a suitable method for the analysis of other small insects.

6.2 Methods and Materials

6.2.1 General Methods

Liquid Chromatography-Mass Spectrometry: The LC-MS system was a Dionex Ultimate 3000™ LC (including an Ultimate 3000™ pump and autosampler) connected to a Bruker AmaZon X™ MS.
Sonication: An Astrason Ultrasonic™ sonicator was used.

6.2.2 Chemicals and Materials

Synthetic JH III (found to comprise 57% of the naturally occurring structural isomer by LC-MS, as previously reported[297]) and synthetic S-methoprene were purchased from Sigma.

Acetonitrile (HPLC grade, greater than 99.99% purity) was purchased from Honeywell Burdick and Jackson. Methanol (HPLC grade, greater than 99.7% purity), chloroform (HPLC grade, greater than 99.8% purity), *n*-hexane (HPLC grade, greater than 99.5% purity) were all purchased from Ajax Finechem Pty Ltd.

Sodium chloride (greater than 97% purity) was purchased from BDH. Millipore water was Barnstead E-pure system 18.2 MΩcm.

Strata™50 um Mono-Func C18-M 300 m^2/g, Un-end-cap, 500 mg/3 mL reversed phase solid phase extraction cartridges were purchased from Phenomenex. A reversed phase PFP LC column (Pursuit® PFP, 2.0 mm i.d. x 250 mm) and a PFP guard column (MetaGuard 2.0 mm Pursuit PFP) were purchased from Varian.

6.2.3 Clover Root Weevil Samples

Two sets of analyses were conducted – trials with less than 50 CRW samples and trials with more than 50 CRW samples. All of the CRWs used in the first trials (less than 50 CRW) were surplus field samples that had been stored in controlled laboratory conditions. They had not been exposed to *M. aethiopoides.* The first two samples used in the trials of 50 CRW (Non-parasitised 1a and Non-parasitised 2a) were also surplus field samples that had been stored in controlled conditions and not exposed to *M. aethiopoides*. They were taken from the same site at the same day; therefore, they should have been relatively similar to one another. In order to obtain enough samples for analysis, the remaining samples were all taken from a batch of CRWs that were collected from the field in Invermay during April 2011. They were split into two groups; one was exposed to the wasp (Parasitised 1 and 2) and one was not (Non-parasitised 1b-3b). Both groups were kept under controlled conditions for five days after the parasitised group had been exposed to *M. aethiopoides.* Laboratory parasitism rates after 5 days are usually in the range of 50-65% .[288]

Reported JH analyses frequently suffer from poor recoveries of JH due to degradation, volatility and adsorption of JH,[394-396] Therefore it was crucial that potential losses of JH were minimised. It was decided not to dissect the parasitised CRW samples to ensure that no JH was lost as part of this process. In aqueous solutions, JH has an increased ability to bind to plastic ware and glassware,[397] therefore it is likely that the dissection process would have caused significant JH loss.

6.2.4 Juvenile Hormone Extraction Method

The method of Miyazaki *et al*[384] was modified. For the first trial 100 mg of CRWs (*ca* 15 individuals) were homogenised in a vial with hexane (1 mL), acetonitrile (0.5 mL) and 2% sodium chloride (0.5 mL). The mixture was sonicated on ice (15 minutes). The hexane layer was collected and two more extractions of hexane (1 mL) were completed. The hexane layers were combined and the sample was dried gently under a stream of nitrogen. S-methoprene was added as an internal standard at the start of the extraction (**Section 6.3.1)**.

The sample was dissolved in chloroform:methanol (100 µL, 1:1, v/v) and applied to a C18 SPE cartridge that had been pre-washed with methanol (4 mL). Methanol (2.5 mL) was used to elute the JH III and S-methoprene. A further wash (5 mL, methanol) of the SPE cartridge was carried out to ensure that all of the JH III and S-methoprene had been extracted from the cartridge. Both samples were evaporated under a stream of dry nitrogen and then each was dissolved in methanol (300 µL) for analysis by LC-MS. For the samples with 50 CRWs (*ca* 300 mg) the solvent volumes were tripled.

6.2.5 Liquid Chromatography-Mass Spectrometry

(a) Liquid Chromatography-Mass Spectrometry Method

A system similar to that of Miyazaki *et al*[384] was used. Chromatography was achieved with a reversed phase, PFP LC column (2.0 mm i.d. x 250 mm) with a guard column. Solvent A (0.2% acetonitrile in water containing 1 mML^{-1} ammonium acetate) and solvent B (95% acetonitrile in water containing 1 mML^{-1} ammonium acetate) were used with a programmed gradient (50% B for 8 minutes, 50-100% B for 15 minutes, 100% B for 5 minutes and 100-50% B for 7 minutes). The flow rate was 200 $\mu Lmin^{-1}$ and the column was held at 40 °C. The autosampler was used with 20 µL injections.

MS and MS^2 analyses were carried out using electrospray ionisation (ESI) in positive ion mode under the following conditions: capillary voltage, -4500 V; end plate offset voltage -500 V in positive mode. The spray was nebulised with

nitrogen gas at 1.5 bar , and the drying gas pressure was 10 litres/min heated to 250°C. Mass ranges of *m/z* 50-1000 were scanned while MS^2 was carried out for the ion at *m/z* 235 and at *m/z* 279 for S-methoprene (internal standard). MS/MS scanning had a width of two *m/z* units and fragmentation amplitude of 0.85 V.

(b) Calibration Curve of Standards

A calibration curve of concentration (ppm, mg/L) versus peak area was plotted This calibration curve used only the peak detected at 15.8 minutes, as this was the naturally occurring structural isomer. The 57% purity was included in the concentration calculations.

(c) Purity of Synthetic Juvenile Hormone III

Synthetic JH III is a mixture of structural isomers[384; 388; 398] and therefore, it is critical that the composition of the standard JH III was determined. The naturally occurring structural isomer of JH III was found to elute at 14.9 minutes by Miyazaki *et al* (no retention time is reported for the other structural isomer) and to make up 75% of the synthetic JH III purchased from Sigma.[384] To determine the purity of the standard, standards of different concentrations were analysed on the LC-MS system and the average relative concentration of the structural isomers was calculated using peak areas.

(d) Detection Limit

The most dilute standard of JH III (*ca* 0.01 mg/L, 0.01 ppm), was further diluted to determine the detection limit of the LC-MS/MS system. Although dilution is usually less accurate than weighing when preparing standards, the tiny amounts of standard required for determination of the detection limit meant that weighing was not a viable option.

(e) S-Methoprene as the Internal Standard

A S-methoprene standard was accurately weighed to give a solution (in methanol of *ca* 0.01 mg/L (0.01 ppm). This was analysed in triplicate and the average peak area found. This standard (300 µL) was added to each sample at the start of the analysis. As the final volume of the sample was 300 µL, the resulting

concentration of S-methoprene in the sample should have remained the same as that of the standard solution if 100% recovery was achieved. The recovery percentage was calculated by comparing the peak area of the S-methoprene in the sample to that of the standard. Recovery was taken into account when calculating the JH III concentrations of the samples.

6.2.6 Juvenile Hormone III in Clover Root Weevil Samples

The JH III peak was integrated in each of the samples and the calibration curve was used to calculate the concentration of JH III in each sample. The recovery percentage was taken into account. The weight of each sample was then used to calculate the concentration of JH III per mg of sample. The mean JH III concentrations were calculated for both parasitised and non-parasitised samples and the standard deviations and coefficients of variation were also calculated.

6.2.7 Statistical Analysis of Parasitised *versus* Non-Parasitised Clover Root Weevil Samples

As five of the samples (Non-parasitised 1b-3b and Parasitised 1-2) had come from the same batch of samples it would have been possible to carry out a paired t-test. However, the sample sizes were different (three *versus* two) meaning that a paired t-test was not valid. Due to the limited availability of samples, it was not possible to get enough CRW to do three samples of parasitised. However, to overcome the issue of different sample sizes, two of the non-parasitised 1b-3b samples were picked at random to be used for a paired t-test with the Parasitised 1-2 samples. To confirm any difference and eliminate any error introduced by randomly selecting two non-parasitised samples, a two-sampled t-test was also conducted. The two-sample t-test incorporated all the non-parasitised samples as well as the two parasitised samples. For both t-tests a significance of 95% was used.

A two-sample t-test is a special case of an one-way ANOVA. It is the case where the single predictor factor has only two levels (parasitised and non-parasitised). It is appropriate to use t-tests in this situation.[155] T-tests were used by Chen *et al*[341] when comparing the JH titre in two insect species.

6.3 Results

6.3.1 Liquid Chromatography-Mass Spectrometry

(a) Purity of Synthetic Juvenile Hormone III

Analysis of synthetic JH III yielded two peaks at 11.5 minutes and 15.8 minutes (**Figure 6.4**). Both peaks produced MS spectra consistent with that of JH III.[384] The peak at 15.8 minutes comprised an average of 57%[‡‡‡‡] of the sample. It was assumed that this was the naturally occurring structural isomer. Ions found were consistent with those observed by Miyazaki *et al*[384] These were: *m/z* 235 $[M\text{-}CH_3OH+H]^+$ (base peak), *m/z* 289 $[M+Na]^+$, *m/z* 267 $[M+H]^+$, *m/z* 249 $[M\text{-}H_2O+H]^+$ and *m/z* 217 $[M\text{-}CH_3OH\text{-}H_2O+H]^+$.

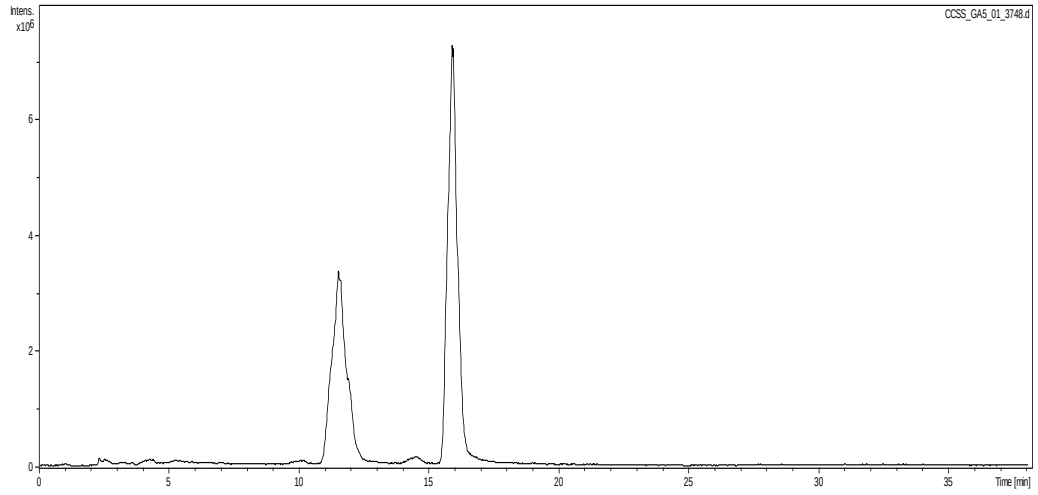


Figure 6.4. Total ion chromatogram of synthetic JH III. Two peaks are seen (11.5 and 15.8 minutes).

JH III was the only JH considered because it is commonly the only JH found in insects.

[‡‡‡‡] At the time of analysis the highest purity JH III available for purchase was an isomeric mixture with a purity (both isomers) of *ca* 80%. This is the likely cause of the low content of the naturally occurring isomer in the current work. Although Miyazaki *et al* make no comment regarding the stated purity of the JH III that they purchased, it is possible that it was higher than the JH III used for this work.

(b) Calibration Curve of Standards

A calibration curve of concentration (ppm, mg/L) *versus* peak area was plotted (**Figure 6.5).**

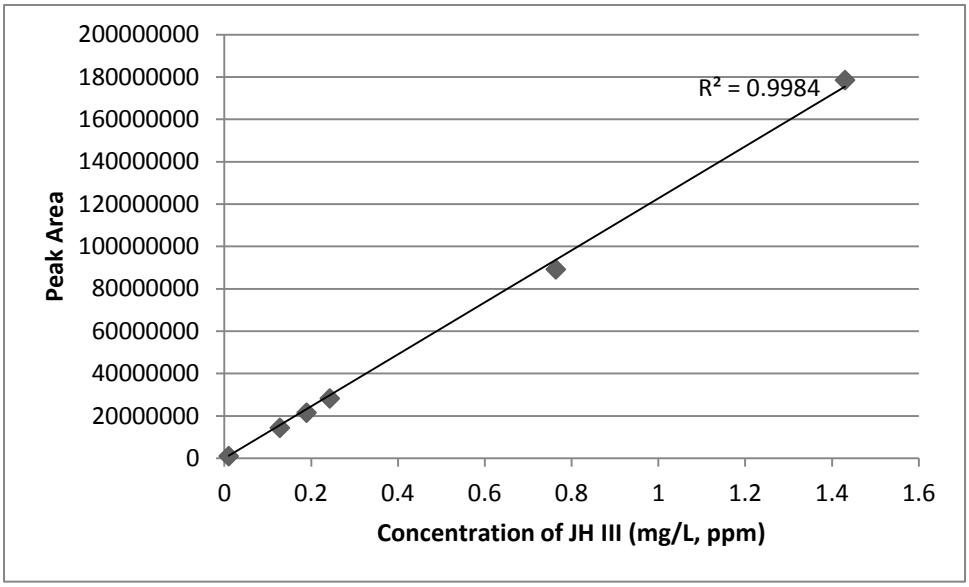


Figure 6.5. The calibration curve for JH III standards, with peak area (15.8 minutes) plotted against JH III concentration (mg/L, ppm, 0.57). Linear regression line equation is y = 122,000,000x. R^2 and linear regression line calculated using Microsoft Excel 2007.

(c) Detection Limit

A ten-fold dilution of the most dilute standard produced a detectable peak with a S/N of 34 (*ca* 0.001 mg/L, 0.001 ppm). A further ten-fold dilution (*ca* 0.0001 mg/L, ppm) produced a peak that was just detectable over the baseline noise (S/N of 5), but which would not be amenable to accurate integration. A solution of *ca* 0.0005 mg/L produced a peak with a S/N ratio of 17. It was concluded that this was the detection limit of JH III for this system.

(d) S-Methoprene as the Internal Standard

When S-methoprene was analysed by the LC-MS system described, a peak was detected at 21.6 minutes. The *m/z* 279 $[M\text{-}CH_3OH+H]^+$ (base peak), *m/z* 237 $[M\text{-}CH_3OH\text{-}CH_3CH{=}CH_2+H]^+$, *m/z* 219 $[M\text{-}CH_3OH\text{-}CH_3CH{=}CH_2\text{-}H_2O+H]^+$ and *m/z* 191 $[M\text{-}CH_3OH\text{-}CH_3CH{=}CH_2\text{-}H_2O\text{-}CO+H]^+$ ions found for S-methoprene (**Figure 6.6**) were consistent with those observed by Miyazaki *et al*[304]

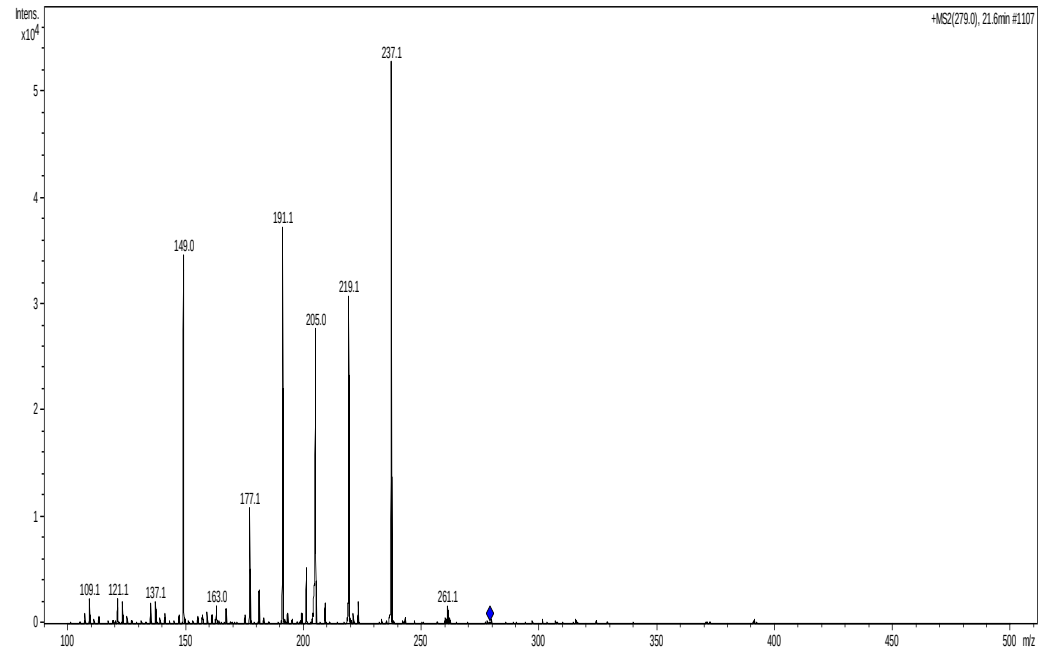


Figure 6.6. MS^2 spectrum from the ion at *m/z* 279 detected at 21.6 minutes (S-methoprene).

6.3.2 Juvenile Hormone III in Clover Root Weevil Samples

a) Samples with Less than 50 Clover Root Weevils

The first trials used 100 mg (*ca* 15 individuals) or less of CRWs. As the goal was to detect changes of JH III concentration between CRWs in different physiological states, detection of JH III in individual CRWs would have been useful. However, all of the tested samples showed no traces of JH III.

b) Samples with More than 50 Clover Root Weevils

As the smaller sample sizes did not have detectable levels of JH III, the sample size was increased to 50 CRWs. Seven samples of 50 CRWs were analysed, (**Table 6.1**). JH III was detected in all seven samples. Confirmation of its presence was by a peak at 15.8 minutes (**Figure 6.7**) and the matching mass spectra showing fragmentation of *m/z* 235 in the samples and in authentic JH III (**Figure 6.8** and **Figure 6.9**). The weight of each sample was used to calculate the concentration of JH III per mg of sample (**Table 6.1**).The mean JH III concentrations were calculated for both parasitised and non-parasitised samples and the standard deviations and coefficients of variation were also calculated

(**Table 6.2**). The levels of JH III in the two parasitised samples were considerably higher (at least 40%) than those in the non-parasitised samples.

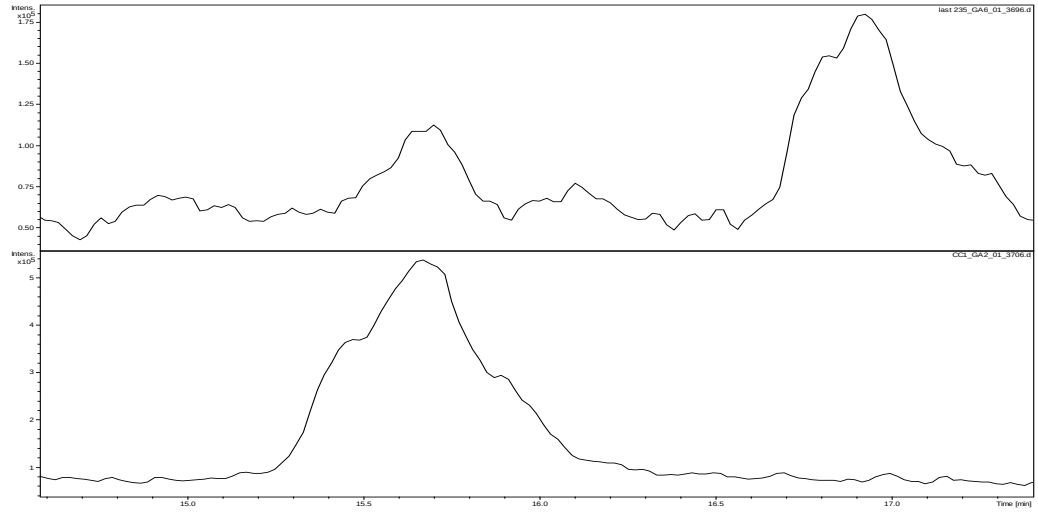


Figure 6.7. The LC-MS/MS chromatogram of *m/z* 235 of a sample of 50 parasitised CRWs (top panel) and authentic JH III (bottom panel).

Table 6.1. The seven samples of 50 CRWs that were analysed for JH III by LC-MS and their results.

Sample name	Description	Weight of sample (mg)	Concentration of JH III (pg/mg of sample weight)
Non-parasitised 1a	50 CRWs kept in controlled lab environment and not exposed to wasp.	299.86	4.34
Non-parasitised 2a	Duplicate of non-parasitised 1a.	300.86	4.75
Non-parasitised 1b	50 CRWs collected from pasture in Invermay and not exposed to wasp.	350.27	4.02
Non-parasitised 2b	Duplicate of non-parasitised 1b.	327.44	3.89
Non-parasitised 3b	Triplicate of non-parasitised1b.	302.60	4.18
Parasitised 1	50 CRWs collected from pasture in Invermay and exposed to wasp in lab conditions; analysed after five days of exposure.	361.02	6.62
Parasitised 2	Duplicate of Parasitised 1.	320.45	6.96

Table 6.2. The mean JH III concentrations, standard deviations and coefficients of variation for non-parasitised and parasitised CRW samples.

Sample type	Mean concentration (pg/mg)	Standard deviation	Coefficient of variation %
Non-parasitised	4.10	0.11	2.68
Parasitised	6.79	0.24	3.53

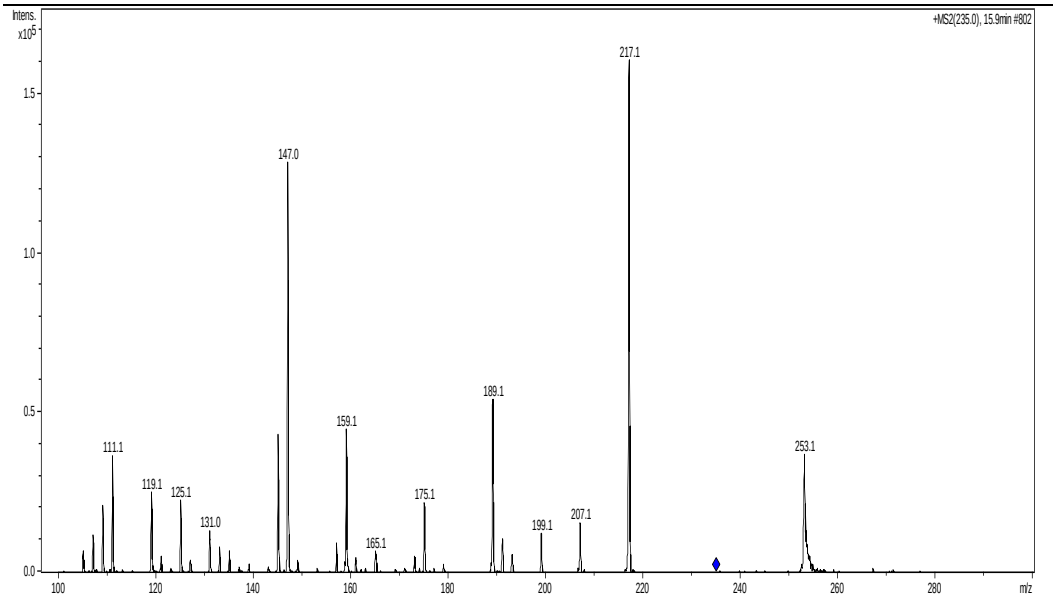


Figure 6.8. MS^2 spectrum from the ion at *m/z* 235 detected at 15.8 minutes from authentic JH III.

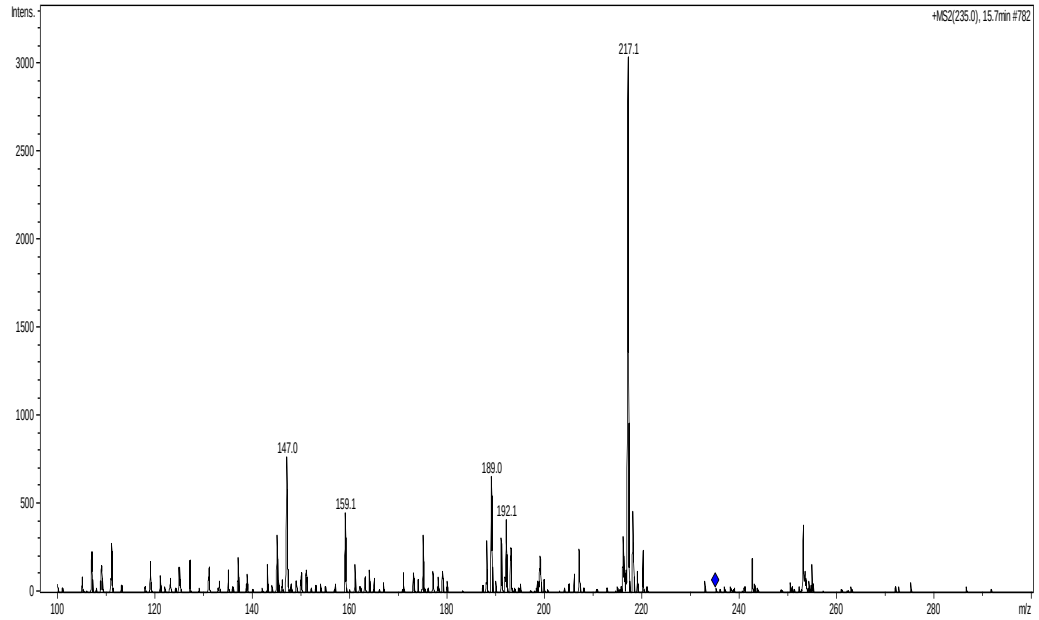


Figure 6.9. MS^2 spectrum from the ion at *m/z* 235 detected at 15.8 minutes from a CRW sample.

6.3.3 Statistical Analysis of Parasitised *versus* Non-Parasitised Clover Root Weevil Samples

(a) Paired T-Test

To overcome the issue of different sample sizes, two of the non-parasitised 1b-3b samples were picked at random to be used for a paired t-test with the parasitised 1-2 samples (**Table 6.3**). Raw data is in **Appendix 9.8.1**. This resulted in significant differences (at the 95% confidence level) being found between the means of the parasitised samples and the non-parasitised samples. Thus parasitised CRW have significantly (at the 95% confidence level) higher JH III levels than non-parasitised.

(b) Two-Sample T-Test

The two-sample t-test incorporated all the non-parasitised samples as well as the two parasitised samples (

Table 6.4). Raw data is in **Appendix 9.8.2**. This resulted in significant differences (at the 95% confidence level) being found between the means of the parasitised samples and the non-parasitised samples. This analysis confirms that parasitised CRWs (mean concentration of 6.79 pg/mg of sample weight) have significantly more JH III than non-parasitised CRWs (mean concentration of 4.24 pg/ mg of sample weight).

Table 6.3. A paired t-test was used to compare the mean JH concentrations of the parasitised and non-parasitised samples. This also calculated the estimate for the difference (between means), as well as the 95% confidence interval (for difference between means) and the p-value (at the 95% confidence level).

	N	Mean concentration (pg/mg of sample s weight)	Standard deviation
Non-Parasitised	2	4.10	0.11
Parasitised	2	6.79	0.24
Estimate for difference = -2.69			
95% Confidence interval for mean difference: (-3.82, -1.56)			
T-Test of mean difference = 0 (*vs* ≠0): T-Value = -30.24 P-Value = 0.021			

Table 6.4. A two-sample t-test was used to compare the mean JH concentrations of the parasitised and non-parasitised samples. This also calculated the estimate for the difference (between means), as well as the 95% confidence interval (for difference between means) and the p- value (at the 95% confidence level).

	N	Mean concentration (pg/mg of sample weight)	Standard deviation
Non-Parasitised	5	4.24	0.33
Parasitised	2	6.79	0.24

Estimate for difference = -2.55
95% Confidence interval for mean difference: (-3.53, -1.58)
T-Test of mean difference = 0 (*vs* ≠0): T-Value = -11.30 P-Value = 0.008

6.4 Discussion and Conclusions

6.4.1 Development of a Liquid Chromatography-Mass Spectrometry Method

The development of a method to analyse whole body insect samples is significant. Whole body samples are much more complex than haemolymph samples and it is likely that this method could be extended to analyse other insect species.

Synthetic JH III and S-methoprene were detected at retention times very similar to those reported by Miyazaki *et al*[384], and mass spectral data were also used to confirm the identity of the peaks. Their method was able to detect JH III in single termites but no JH III was detected in samples of up to 15 CRWs. This was to be expected however as it was likely that termites contained higher levels of JH III than CRWs (see below) and also the currently used autosampler system required a higher volume of sample meaning that the sample was more diluted, even with the use of vial inserts. Manual injections were not possible on the current system. The detection limit achieved in this study was *ca* ten-fold higher than that which was achieved by Miyazaki *et al*[384] but close inspection of their chromatograms revealed that their baseline was a lot less noisy than that achieved in the current study.

6.4.2 Juvenile Hormone III in Clover Root Weevil Samples

Most reported JH analyses have been on termite samples and as termites have a distinct caste life cycle, they have relatively high amounts of JH III.[338; 381; 384] Therefore, it was not surprising that the JH III concentration in both the parasitised and non-parasitised CRW samples was less than that reported for termites of between 10.2-13 pg/mg of body weight.[384] Also, as it was not possible to determine the percentage of parasitised samples that were actually parasitised for reasons previously discussed, this effectively diluted the sample. As the majority of other reports focus on the concentration of JH III in haemolymph extracts, [310; 344; 381-383; 399] it is difficult to compare them to the current results.

The detection limit of JH III for the current system was compared to the concentration of JH III that would be estimated to be found in 15 CRW (calculated by using the average JH III concentration found in the samples of 50 non-parasitised CRW multiplied by the weight of sample). It was found that the estimated concentration of JH III was *ca* two-fold higher than the detection limit, indicating that JH III should have been detected in the samples containing 15 CRW. However, this calculation is only an estimate as there is natural variability between JH III concentrations of different CRW samples. It would have been beneficial to have more replicates but due to the large number (50) required per sample, the number of samples was limited.

6.4.3 Statistical Analysis of Parasitised *versus* Non-Parasitised Clover Root Weevil Samples

The increased JH III concentration in the parasitised samples agreed with what has been observed in other insects. Parasitism of the tobacco hornworn by the parasitoid wasp *Cotesia congregata*, caused almost ten times the concentration of total JH in the haemolymph.[343] Similar effects were seen when the gypsy moth (*Lymantria dispar)* was parasitised by the endoparasitoid *Glyptapantels liparidis*.[399] As indicated previously it is frequently reported that parasitism may reduce the activity of JHE hence increasing the concentration of JH.[305; 310; 388; 400; 401]

6.4.4 Biological Significance of Results

In Coleoptera, JHs have been reported to influence reproduction, especially in female insects.[290] Parasitised female CRW absorb their reproductive organs as soon as the parasitoid egg is laid. Those that contain diapausing parasitoid larvae during the winter accumulate fat in a similar manner to males. It is likely that this fat accumulation both prolongs host longevity and provides resources for the growing parasitoid in spring. From the current results and what has been reported about the role of JHs, [352-354] it is likely that the increase in JH III as a result of parasitism contributes to these processes.

This work represents the first comparison of JH III titres in Coleoptera with differing states of parasitism. However, these results concur with what has been reported in other insects. A rise in JH III levels after parasitism has been reported for numerous other insect species and teratocytes have also been implicated in some of these reports.[343; 387; 388] Although the parasitised CRWs were not dissected, it can be assumed that they contained teratocytes.[299] Teratocytes have been implicated in decreasing the activity of JHE in insects.[387; 388] JHE activity was significantly reduced in the haemolymph of tobacco budworm larvae parasitised by the parasitoid wasp *Microplitis croceipes* while injecting the larvae with teratocytes had a similar effect.[352] It is possible that if the parasitised CRWs were exposed for a longer time before analysis that the JHE activity could have been further reduced hence increasing the JH III concentration further.

The current results indicate that there may be potential for using a JHA as a pesticide for CRW. If an increase in JH III concentration is caused by parasitism and parasitised female CRW are no longer reproductive, then it is possible that artificially increasing the JH III concentration of a female CRW may also prevent it from being reproductive. JHAs have been successfully used as pesticides against the rice weevil (*Sitophilus oryzae*), the boll weevil (*Anthonomus grandis* Boheman), southern cowpea weevil (*Callosobruchus maculates*) [390-392] and white pine weevil (*Pissodes strobe*).[393] S-Methoprene has been a successful pesticide against wheat weevil (*Sitophilus granaries*) and merchant grain beetle (*Oryzaephilus mercator*) when sprayed directly onto their food.[402] Although in

these cases JHA was used against the larval stage of the insects, the incorporation of S-methoprene into the diet of adult Coleoptera insects has caused substantial reductions in oviposition,[371] and this suggests the potential for the use of S-methoprene as a pesticide against adult CRW. The use of such a pesticide for CRW would be significant as although *M. aethiopoides* has been successful at controlling CRW, as a biological control agent it is unlikely to ever completely eradicate its host. If a JHA could be successfully used against adult CRW, it would provide a tool for farmers when parasitoid populations have been compromised e.g. following cultivation, severe drought or pesticide application for other pasture pests. Although the use of a general insecticide against *Sitona discoideus* was unsuccessful in Australia,[25] the use of a JHA could represent a much more environmentally friendly and less toxic insecticidal option. In this context it should be noted that S-methoprene has an oral LD_{50} (rat) of >34,000mg/kg, is not carcinogenic and is rapidly broken down by sun and in the soil. It is however very toxic to aquatic organisms.[367]

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7 Final Discussion and Suggestions for Future Work

7.1 Final Discussion

Clover root weevil is a serious pest to agriculture in New Zealand that has spread throughout the country. This research is an investigation into previously unreported aspects of the CRW, with a focus on the internal chemistry of the species, and how this is affected by parasitism.

Visual observations by AgResearch scientists led to the hypothesis that the parasitism of CRW by *M. aethiopoides* alters the lipids of the host, reinforced by reports of alterations in lipid levels in other insect hosts.[57; 58] In particular, AgResearch Ruakura, scientists observed that the appearance of the abdominal fat body and lipids present in adult CRW varied with season, sex, insect age and parasitism. However, the chemical composition of lipids present in parasitised and non-parasitised CRW adults and how these lipids change with physiological state and parasitoid development has not been previously reported. The focus of the present research was the fat changes that occurred within the CRW once parasitism occurred. By also investigating the composition of teratocytes that are seen in parasitised CRW and any effects on the juvenile hormone titer that parasitism caused, a greater understanding of the changes parasitism causes to CRW was reached.

The development of a one-step method to analyse the whole body fatty acid profile of individual CRW is significant and the success of this method alone is a scientific advance. There are no other examples in the literature where a one-step method for fatty acid extraction and derivatisation for samples less than 10 mg in size has been developed, and this method has demonstrated superior sensitivity over traditional two-step methods. Previously successful one-step methods all used much larger sample weights of up to 100 mg.[142; 144; 403] The success of the one-step method at such a small scale allows it to have potential for analyses of other small samples. Being able to analyse the profile of individual small

organisms or life stages gives researchers the opportunity to study more precisely how lipids and physiological state are interconnected and the variation that may exist within a population. Many organisms, not only insects, have distinct physiological stages within their lifecycle and this method offers an opportunity for researchers to be able to accurately and rapidly analyse the whole body fatty acid profile of these individual stages. The advantage of whole body analysis of individual insects in this study was that the researcher had the potential to directly compare lipid profile with the visual differences that had been observed in the lipids of dissected CRW that were in different physiological states. There is potential for this method to be used to analyse lipids of any type of biological sample of this scale. The success of this method in analysing the fatty acids of teratocytes shows it can be used with samples weighing less than 5 mg.

The chemical composition of the CRW lipids was determined and compared to other insect species (objective 1) and found to be very similar to that commonly found in insects,[73; 75] including for example other weevils.[215; 231; 232] As expected, the fatty acid profile of the CRW was related to that for white clover foliage,[170] the preferred diet for adult CRW, apart from the predominance of 18:1. The latter is possibly due to extensive biohydrogenation of linoleic acid (18:2) by gut bacteria but it is also likely, based on these results, that the CRW preferentially utilises 18:2 fatty acid while storing 18:1 fatty acid, and that the CRW obtains at least some of its fatty acids via *de novo* synthesis. The range for total percentage of fatty acid was similar to that which had been reported for other insects [101; 103; 104; 255] and in line with what had been reported for the lucerne weevil (*S. discoideus*).[248] As expected, individual CRW with higher body weights had higher amounts of total fatty acids present.

Multivariate analysis of the Gisborne subset revealed significant differences in fatty acid composition between parasitised and non-parasitised weevils. Parasitised samples from the Gisborne sample set had less 12:0 and 18:0 fatty acids than the corresponding non-parasitised samples. Although it is difficult to assign the biological significance of specific fatty acids, [296] it is likely that the reduction in levels of 12:0 and 18:0 fatty acids in parasitised CRW makes the host environment more favourable for the parasitoid. Crawford *et.al* [404] sequenced the

three main proteins found in the venom of the parasitic wasps *M. hyperodae* and *M. aethiopoides*, and found that the dominant protein in the venom of these *Microctonus spp.* to be a lipase. It is possible that this lipase in the venom that is injected into the CRW by *M. aethiopoides* during parasitisation, contributes to the change in fatty acid profile.

When the results obtained from the Gisborne subset and the entire sample set are compared it is clear that site effects could have been a large source of variation when comparing different physiological states. Therefore, this section of work would have been seriously inhibited by using samples from multiple sites and dates. Although some changes in the lipids that occur with differing physiological states and parasitism were identified (objective 2), much more carefully designed sampling protocols would be needed to help explore and explain the visual observations that have been made by AgResearch researchers. The CRW lifecycle is such that individuals within the weevil population are not synchronised with each other and this also affected results. Grapes *et al*[111] reported that the coefficient of variation of up to 50% seen in the analysis of house cricket (*Acheta domesticus)* was due to the variation between insects when samples consisted of unsynchronised insects. This was similar to that reported by Nowosielski and Patton.[405]

The results from the analysis of the teratocytes were not significantly different from the results for the fatty acid profile of the CRW. This means that teratocytes of *M. aethiopoides* within the CRW host probably obtain all of their lipids from absorbing fat from the body fluid of the CRW (objective 3). In contrast, the only other report regarding the fatty acid composition of teratocytes found differences in teratocytes from two different parasitoids growing in the same species of host, suggesting that absorption varies with species. [326] This difference is plausible, as teratocytes from different species have different functions.[59; 298]

The larval fatty acid profile of the parasitoid differed from that of the adult CRW (and hence that of the teratocyte samples). The larvae had significantly less 16:1 fatty acid and significantly more 18:3 fatty acid than the CRW samples. Insect eggs have been reported to contain higher amounts of 16:1 fatty acid than other

life stages, due to the need to provide extra energy and structural materials for embryonic development.[199; 200] Parasitoid larvae have very rapid growth, reaching maturity in around 2 weeks. Therefore, it is probable that the parasitoid larvae has utilised a lot of 16:1 during this phase and has less 16:1 fatty acid when compared to the adult CRW. A main function of unsaturated fatty acids is to serve as a structural component of membranes, maintaining proper fluidity and permeability.[244] The increased amount of 18:3 fatty acid in the parasitoid larvae may be a reflection of the greater need of maintaining fluidity and permeability in a larva compared to an adult insect. The ability of the larva to obtain lipid from its host is biologically significant as it is a parasitoid and totally dependent on the host for nutrition.

Larvae of the cabbage looper (*Trichoplusia ni*) reared on a synthetic diet were unable to synthesize 18:3 fatty acid from ^{14}C-acetate, and therefore normally obtain 18:3 fatty acid from the egg from which it hatches.[221] The size of the egg of the cabbage looper is significantly bigger than the egg of the *M. aethiopoides*, which is too small to carry any significant fat reserves. Therefore, the differences between the larval parasitoid fatty acid profile and the adult CRW host's profile may be due to different utilisation and storage of fatty acids by the larval parasitoid rather than fat reserves from the parasitoid egg.

Most reported analyses of JH III in insects have used haemolymph which is a lot less complex in composition than whole body extracts.[384] The modification of the method developed by Miyazaki *et al*,[384] to analyse whole body samples of CRW indicates an achievement and this method could be further developed to analyse other insects. The comparison between parasitised and non-parasitised samples found that parasitised samples had significantly higher levels of JH III than their non-parasitised counterparts did (objective 4). This agrees with what has been reported for parasitism of larval Lepidoptera[343],[399] but represents the first report of parasitism affecting the JH titre in a parasitised adult host. The results of this work show that parasitism by *M. aethiopoides* causes the JH III levels in its CRW host to rise (objective 4). This rise in JH III is likely to be the cause of the cessation of egg production and commencement of fat storage in parasitised female CRW. Although a parasitism-induced reduction of the activity

of JHE has been suggested as the cause of a rise in the haemolymph JH III levels after parasitism,[399] it was beyond the scope of this study to investigate enzyme activity. Teratocytes have been implicated in decreasing the activity of JHE in insects,[387; 388] therefore, it is possible that the teratocytes of *M. aethiopoides* reduce the JHE activity in the host CRW's haemolymph. However, the parasitoid's effect on the parasitised female CRW begins before teratocytes appear and therefore may be caused by the venom that is injected at the same time that the parasitoid egg is. Crawford *et al*'s [404] work found that the second most frequent transcript code in the venom of *Microctonus spp*. is for a vitellogen receptor and they postulate that this receptor may disrupt host egg development. The third most common transcript code in the venom interferes with *Drosophila spp*. larval development, causing a juvenilising affect, and this effect may be related to the increase in JH III found in this study. Both of these proteins could switch off host egg production, ensuring plenty of resources for the parasitoid larvae.

7.2 Suggestions for Further Work

The one-step method of extraction and derivatisation of fatty acids has major potential in the field of lipid analysis where quantity is restricted.

As in this study, the one-step method is particularly useful for invertebrates and other small animals as they are vulnerable to losses during sample preparation. AgResearch scientists undertake research on many different insect species, and in some, lipids are drivers of pest status, for example, black beetle (*Heteronychus arator*) fecundity and life span is highly influenced by lipids stored the previous autumn.[406] Determining the fatty acid profile of such a pest and how it changes with factors such as disease and pasture composition would give researchers a greater understanding of its lifecycle and potential control options.

This method of analysis could be of value to researchers working trying to understand the physiological changes associated with insect parasitism, especially in the type and amount of lipids in hosts and teratocytes. The majority of reports concerning teratocytes involve the tobacco budworm (*Heliothis virescens*),[303] and the composition of teratocytes is very rarely reported. Although the MALDI-

TOF method needs further development for the analysis of teratocytes, the one-step analysis method could be extended to analyse the teratocytes for parasitoids attacking a range of international pests.

International researchers are also investigating insects as possible environmentally sustainable nutrient sources of polyunsaturated fatty acids, including the edible black ant (*Polyrhachis vicina*), the edible red ant (*Oecophylla smaragdina*) and the mole cricket (*Gryllotalpa africana)*.[108; 407]

Other lipid researchers could use this method for a range of purposes, from profiling of lipids and their oxidation products in human hair [408] to investigating the fatty acid profile of milk.[409] Recent work by Plant and Food Research scientists have investigated oils from hoki (*Macruronus novaezelandiae)* and Greenshell™ mussel (*Perna canaliculus)* for their lipid content and fatty acid profile.[410] The small quantities of oils involved in their work are ideal candidates for the one-step method. Minute samples of lubricant vegetable oils on forensic swabs might be analysed by this method.

The modified method of Miyazaki *et al*[384] for JH III could be optimised for the analysis of JH III concentration in other insects. As this method does not need haemolymph, it is very accessible to the general laboratory. It would be worthwhile to determine whether the JH III concentration of other pest species such as the lucerne weevil or the Argentine stem weevil, increased after parasitism by their respective parasitoids. Juvenile hormone analogues have been successfully used as pesticides against other weevils[373; 402; 411] and although further work would be needed the results from this thesis suggest that there is potential for the development of a JHA pesticide against CRW. Also, since in this thesis the assumption was made that only JH III was present, an additional project could investigate the possibility that other JHs are present. The reasons for the accumulation of JH III in parasitised CRW might be also investigated, that is whether it arises from inhibition of JH esterase or by production from the parasitoid larva or teratocytes.

The results from this study illustrate the difficulties that arise when using field collected specimens for physiological studies. Although a single species that feeds exclusively on a single host plant was investigated, it is possible the influences such as season, climate, soils, management etc influenced both the composition of the white clover and the CRW within and between sites. It would be better to rear CRW under controlled conditions; however, CRW will not tolerate an artificial diet.[288] However, the results from the Gisborne subset show that distinctions are possible if other major sources of variation are eliminated. Therefore if field-collected insects are to be used, the sampling protocol must take into account the possible site and seasonal variables and ensure sufficient replication for any statistically valid differences to be proven.

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9 Appendix

9.1 Reproducibility

9.1.1 Peak Areas for Halves

- CRW samples are labelled 1-20 with each halve labelled a or b.

Table 9.1. Peak areas for samples 1 and 2.

Fatty acid	Half 1a	Half 1b	Half 2a	Half 2b
12:0	1989484	2194428	1343191	1456489
13:0	471771027	486718899	481970991	519536766
14:0	6956866	6420571	3996829	4293137
16:0	252708707	266043756	159423176	165119969
16:1	243818071	255982105	199762557	205350013
17:0	291483149	300721926	285995702	309861016
18:0	119950851	119307792	99204250	102295463
18:1	557594627	599645902	702164525	738288631
18:2	145940244	140860517	115311582	142029621
18:3	597234701	632907742	495044967	528120310

Table 9.2. Peak areas for samples 3 and 4.

Fatty acid	Half 3a	Half 3b	Half 4a	Half 4b
12:0	3928627	3441245	2838339	3164238
13:0	485290752	448182426	463303380	439874179
14:0	11055755	9951539	8642530	9625887
16:0	221247108	226020627	165550385	178819046
16:1	69687994	74530597	99899737	103445190
17:0	319702138	311419008	202616796	207914483
18:0	132436478	136454083	79403102	86722214
18:1	638928496	612704294	742174613	771848781
18:2	276473170	272170588	170553776	184209721
18:3	707934672	688503649	458053771	447654605

Table 9.3. Peak areas for samples 5 and 6.

Fatty acid	Half 5a	Half 5b	Half 6a	Half 6b
12:0	1532292	1396309	1967260	1738781
13:0	396518248	422787216	509309158	490374249
14:0	5607693	5013720	3713817	3968222
16:0	94383277	104099850	203957063	180473977
16:1	35615901	35873436	105349197	103968899
17:0	9752127	10077425	301304780	277011811
18:0	297337209	298706627	100544374	93007155
18:1	75802397	76661003	503120376	488030374
18:2	296518925	293466378	134069107	124743715
18:3	133638133	143680892	619653195	598970236

Table 9.4. Peak areas for samples 7 and 8.

Fatty acid	Half 7a	Half 7b	Half 8a	Half 8b
12:0	2228703	1923766	1601347	1791135
13:0	474106742	444471067	451403804	424190098
14:0	3194203	2907479	4203954	4546823
16:0	72928010	66072335	195523636	176262764
16:1	20231695	18135983	3240262	2608766
17:0	276467150	249678457	163111762	146553116
18:0	51699208	50927235	313388600	293433377
18:1	228127132	203075025	83796931	74578570
18:2	136501626	128608626	481686412	435970801
18:3	204846445	181337909	111364517	98199480

Table 9.5. Peak areas for samples 9 and 10.

Fatty acid	Half 9a	Half 9b	Half 10a	Half 10b
12:0	3477489	3888781	3637290	3174445
13:0	751702258	731585498	540992094	500658697
14:0	32993893	28666054	8196083	8263743
16:0	750542680	741996881	54142952	48453937
16:1	326658772	319026539	15661506	14236055
17:0	396760148	401302024	313056796	288991482
18:0	773704632	799592333	48398667	42871738
18:1	1.416E+09	1.457E+09	135287814	123133047
18:2	760244926	786479294	60294821	56511144
18:3	766870912	789935647	132827517	117734777

Table 9.6. Peak areas for samples 11 and 12.

Fatty acid	Half 11a	Half 11b	Half 21a	Half 12b
12:0	9040535	9870052	1529094	1388221
13:0	503927089	528240433	520625215	469789985
14:0	19108005	20667553	3844354	3470047
16:0	309072401	321298933	144620062	127638308
16:1	166486231	173818260	168600151	150398187
17:0	332916538	342053128	309579696	277282501
18:0	149400910	154110350	81955734	72006991
18:1	1.04E+09	1.056E+09	572974008	509520438
18:2	273527992	281349053	86718578	77810798
18:3	755459457	772898941	402030654	361336052

Table 9.7. Peak areas for samples 13 and 14.

Fatty acid	Half 13a	Half 13b	Half 14a	Half 14b
12:0	5660578	5085587	4587585	3739852
13:0	451814593	429997283	506090582	487662873
14:0	20553015	18516790	30226915	33517781
16:0	304387935	297800343	719531120	704766825
16:1	406404366	381159214	334225939	323772371
17:0	284656757	256160810	335397394	313606312
18:0	146453173	139416264	169142341	163570481
18:1	1.276E+09	1.198E+09	1.359E+09	1.323E+09
18:2	288030593	262720939	236864569	227877396
18:3	815373652	788294326	994758074	941259335

Table 9.8. Peak areas for samples 15 and 16.

Fatty acid	Half 15a	Half 15b	Half 16a	Half 16b
12:0	2599632	2390239	2515331	2231267
13:0	505462221	480609633	558037891	537574786
14:0	18202709	16408444	12139900	13110822
16:0	96814995	83219263	224654988	241471714
16:1	51618880	49246704	250626252	273896602
17:0	269551094	254563831	298502820	335035456
18:0	111969028	100528451	131015360	142905600
18:1	303305902	270633585	797167309	852574183
18:2	89809634	83316249	164200723	168561574
18:3	166593937	158461606	571329307	565342249

Table 9.9. Peak areas for samples 17 and 18.

Fatty acid	Half 17a	Half 17b	Half 18a	Half 18b
12:0	1115319	1181297	2791321	2556794
13:0	506408161	502114525	401846709	399295922
14:0	2172818	2321073	3443178	3101153
16:0	45173999	46176373	81663624	82587895
16:1	18092438	16691978	34784430	34684064
17:0	282076620	274786177	244009942	231752871
18:0	60052571	56810123	54264507	57137910
18:1	187282470	196965659	330015686	336780956
18:2	45234234	45861286	110538035	113350601
18:3	140412948	144628306	166866388	169964182

Table 9.10. Peak areas for samples 19 and 20.

Fatty Acid	Half 19a	Half 19b	Half 20a	Half 20b
12:0	974891	1060910	1388051	1343021
13:0	457512094	467124870	491271968	492670325
14:0	1804317	1992356	4304380	4772081
16:0	73038207	77807543	143254076	137292478
16:1	31430734	31361449	201851118	190980061
17:0	294211928	289865412	299613633	291410603
18:0	59686408	62257532	700053372	710447190
18:1	198659254	205907609	556005599	576992225
18:2	56751421	58442878	88053576	94931645
18:3	191695312	199489111	298559000	311987969

9.1.2 Nested ANOVA Results

a) *Nested ANOVA raw data for 12:0 (Peak A)*

Nested ANOVA: peak_A versus weevil, rep

Analysis of Variance for peak_A

Source	DF	SS	MS	F	P
weevil	19	1.43360E+14	7.54526E+12	1 02.929	0.000
rep	20	1.46610E+12	7.33052E+10		
Total	39	1.44826E+14			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	3.73598E+12	98.08	1932867.442
rep	7.33052E+10	1.92	270749.278
Total	3.80928E+12		1951738.128

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

b) Nested ANOVA raw data for 13:0 (Peak B)

Nested ANOVA: peak_B versus weevil, rep

Analysis of Variance for peak_B

Source	DF	SS	MS	F	P
weevil	19	1.84434E+17	9.70705E+15	28.963	0.000
rep	20	6.70306E+15	3.35153E+14		
Total	39	1.91137E+17			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	4.68595E+15	96.33	6.84540E+07
rep	3.35153E+14	3.67	1.83072E+07
Total	502110E+15		7.08597E+07

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

c) Nested ANOVA raw data for 14:0 (Peak C)

Nested ANOVA: peak_C versus weevil, rep

Analysis of Variance for peak_C

Source	DF	SS	MS	F	P
weevil	19	3.18221E+15	1.67485E+14	152.199	0.000
rep	20	2.20086E+13	1 .10043E+12		
Total	39	3.20422E+15			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	8.31921E+13	98.69	9120969.660
rep	1.10043E+12	1.31	1049013.889
Total	8.42925E+13		9181095.669

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

d) Nested ANOVA raw data for 16:0 (Peak D)

Nested ANOVA: peak_D versus weevil, rep

Analysis of Variance for peak_D

Source	DF	SS	MS	F	P
weevil	19	1.41961E+18	7.47162E+16	1065.317	0.000
rep	20	1.40270E+15	7.01352E+13		
Total	39	1.42101E+18			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	3.73230E+16	99.81	1.93192E+08
rep	7.01352E+13	0.19	8374673.531
Total	3.73931E+16		1.93373E+08

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

e) Nested ANOVA raw data for 16:1 (Peak E)

Nested ANOVA: peak_E versus weevil, rep

Analysis of Variance for peak_E

Source	DF	SS	MS	F	P
weevil	19	5.64159E+17	2.96926E+16	570.679	0.000
rep	20	1.04060E+15	5.20302E+13		
Total	39	5.65199E+17			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	1.48203E+16	99.65	1.21738E+08
rep	5.20302E+13	0.35	7213194.575
Total	1.48723E+16		1.21952E+08

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

f) Nested ANOVA raw data for 17:0 (Peak F)

Nested ANOVA: peak_F versus weevil, rep

Analysis of Variance for peak_F

Source	DF	SS	MS	F	P
weevil	19	2.38288E+17	1.25415E+16	69.716	0.000
rep	20	3.59785E+15	1.79893E+14		
Total	39	2.41886E+17			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	6.18079E+15	97.17	7 . 86180E+07
rep	1.79893E+14	2.83	1.34124E+07
Total	6.36068E+15		7.97539E+07

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

g) Nested ANOVA raw data for 18:0 (Peak G)

Nested ANOVA: peak_G versus weevil, rep

Analysis of Variance for peak_G

Source	DF	SS	MS	F	P
weevil	19	1.60067E+18	8.42459E+16	1826.184	0.000
rep	20	9.22644E+14	4.61322E+13		
Total	39	1.60159E+18			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	4.20999E+16	99.89	2.05183E+08
rep	4.61322E+13	0.11	6792069.277
Total	4.21460E+16		2.05295E+08

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

h) Nested ANOVA raw data for 18:1 (Peak H)

Nested ANOVA: peak_H versus weevil, rep

Analysis of Variance for peak_H

Source	DF	SS	MS	F	P
weevil	19	6.73130E+18	3.54279E+17	593.775	0.000
rep	20	1.19331E+16	5.96656E+14		
Total	39	6.74324E+18			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	1.76841E+17	99.66	4.20525E+08
rep	5.96656E+14	0.34	2.44265E+07
Total	1.77438E+17		4.21234E+08

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

i) Nested ANOVA raw data for 18:2 (Peak I)

Nested ANOVA: peak_I versus weevil, rep

Analysis of Variance for peak_I

Source	DF	SS	MS	F	P
weevil	19	1.10583E+18	5.82017E+16	477.334	0.000
rep	20	2.43861E+15	1.21931E+14		
Total	39	1.10827E+18			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	2.90399E+16	99.58	1.70411E+08
rep	1.21931E+14	0.42	1.10422E+07
Total	2.91618E+16		1.70768E+08

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

j) Nested ANOVA raw data for 18:3 (Peak J)

Nested ANOVA: peak_J versus weevil, rep

Analysis of Variance for peak_J

Source	DF	SS	MS	F	P
weevil	19	3.01967E+18	1.58930E+17	594.	568 0.000
rep	20	5.34607E+15	2.67303E+14		
Total	39	3.02502E+18			

Variance Components

Source	Var Comp.	% of Total	StDev
weevil	.93314E+16	99.66	2.81658E+08
rep	2.67303E+14	0.34	1.63494E+07
Total	7.95987E+16		2.82132E+08

Expected Mean Squares

1 weevil 1.00(2) + 2.00(1)

2 rep 1.00(2)

9.2 Response Factor Graphs for Standard Fatty Acids

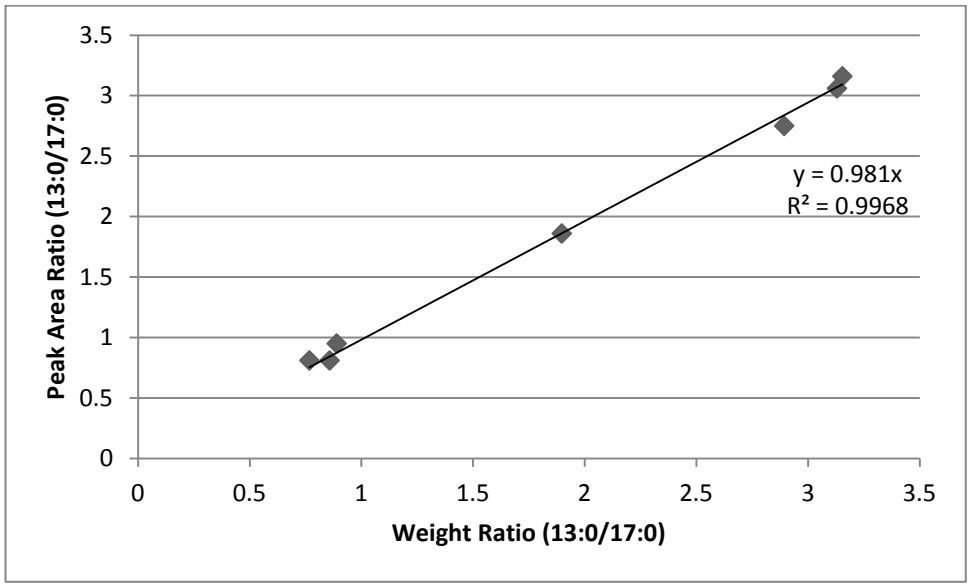

Figure 9.1. Response factor graph for the standard 13:0.

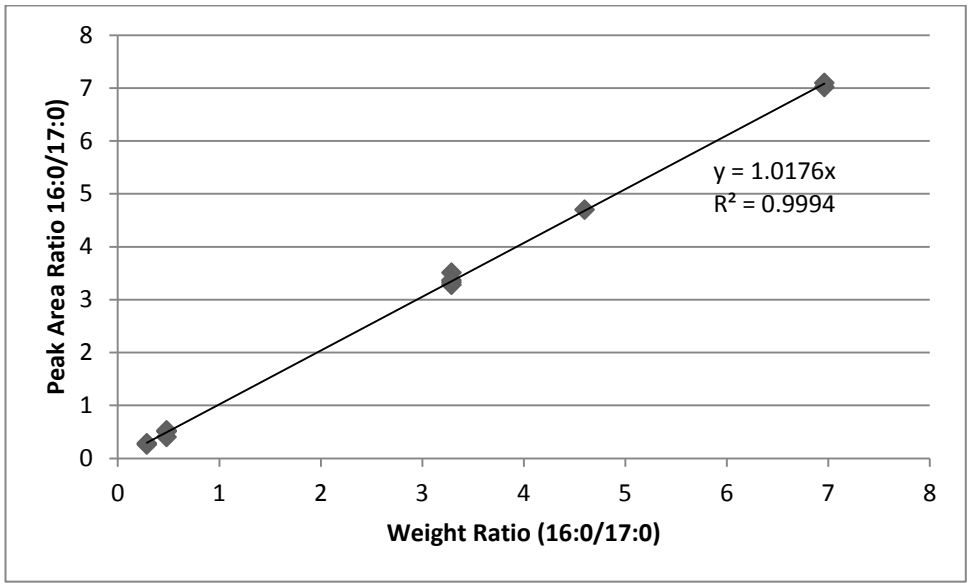

Figure 9.2. Response factor graph for the standard 16:0.

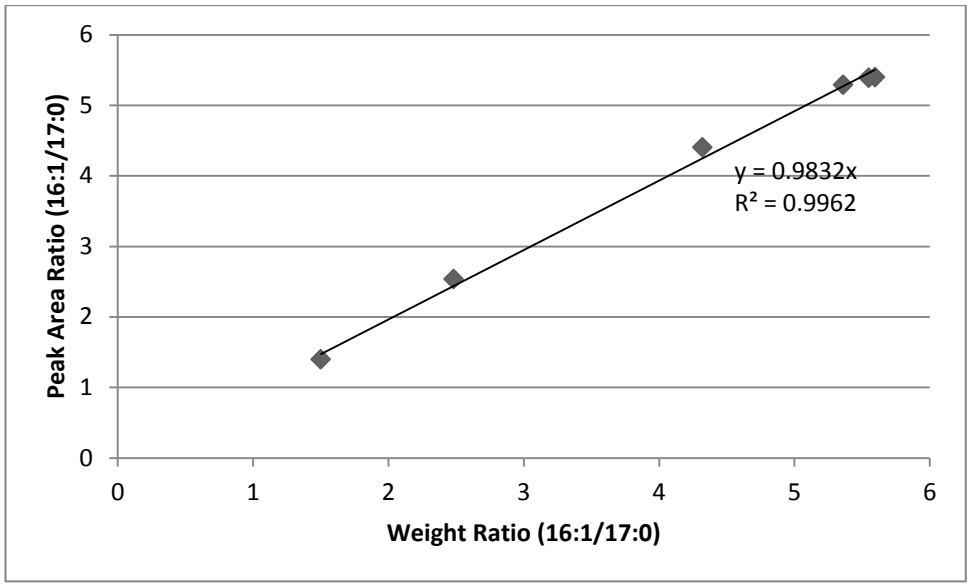

Figure 9.3. Response factor graph for the standard 16:1.

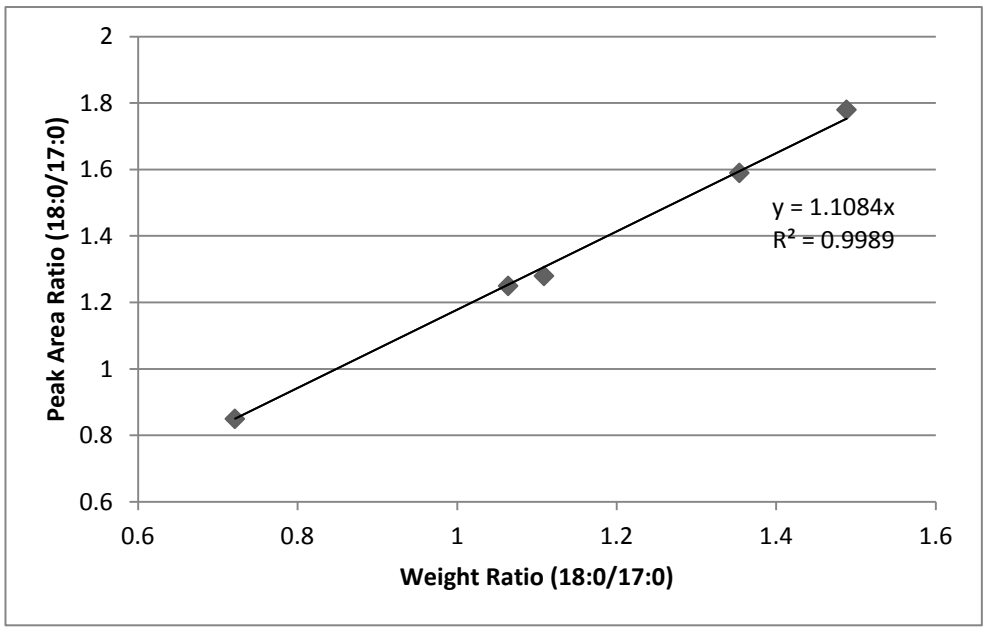

Figure 9.4. Response factor graph for the standard 18:0.

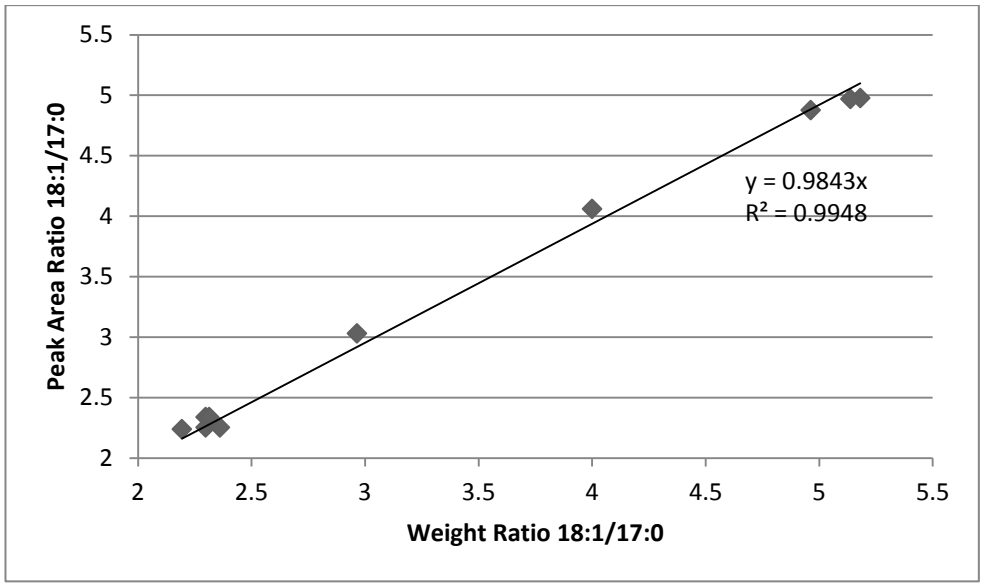

Figure 9.5. Response factor graph for the standard 18:1.

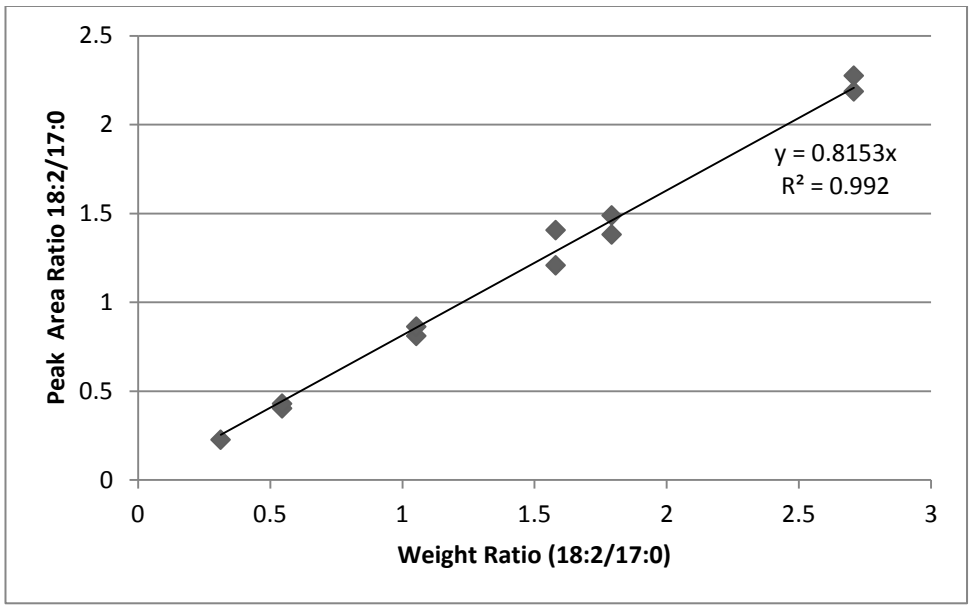

Figure 9.6. Response factor graph for the standard 18:2.

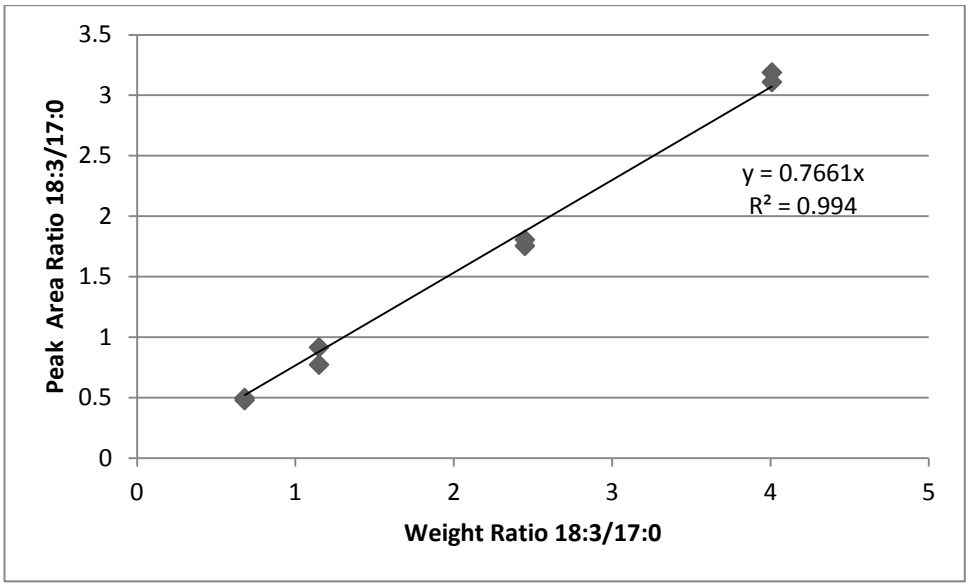

Figure 9.7. Response factor graph for the standard 18:3.

9.3 Multivariate Analysis

9.3.1 Multivariate Methods

(a) Principal component analysis (PCA) is a commonly used method to visualise data and find the true dimensions of a data set. The v-parameters (variables) measured for each sample describe each sample (object) in a v-dimensional space. PCA uses orthogonal transformation to produce a new set of variables which are linear combinations of the original ones.[(287)] Due to the nature of the orthogonal transformation, the first principal component has the highest variance possible (accounts for as much of the variability in the data set as possible) and each successive principal component has as much variance as possible while still being uncorrelated to the preceding components. As a result, the process concentrates the variance of the data set into a smaller number of variables than there were originally hence making succeeding statistical analysis easier. PCA requires centred log-ratio (clr) transformation of the compositional data. This is calculated (**Equation 9.1)**, by taking the logarithm of the ratio of each element to the geometric mean of the variables.[(412)]

$$clx = \left[ln\left(\frac{x_1}{g(x)}\right), ln\left(\frac{x_2}{g(x)}\right), \ldots, ln\left(\frac{x_p}{g(x)}\right)\right] \qquad \textbf{(9.1)}$$

Where the geometric mean is calculated by:

$$g(x) = \left(\prod_{i=1}^{\mathrm{p}} x_1 \right)^{\frac{1}{p}}$$

For this work an eight principal component (PC) model was calculated producing eight principal components. Appropriate scatterplots of the principal components were used to determine whether PCA was useful for predicting whether samples were parasitised or non-parasitised, or for distinguishing between different physiological states.

(b) Linear Discriminant Analysis (LDA) is related to PCA as both use linear combinations of variables to describe the data set; however, LDA is a probabilistic classification method and hence models the differences between the classes of data. LDA requires additive log-ratio (alr) transformation. This is calculated (**Equation 9.2)**, by taking the natural logarithm of each component over the *p*th component:[412] With LDA, classes are assumed to have multivariate normal distribution and similar variance–co-variance matrices. In all calculations of alr, the component of 18:3 was used as the pth component.

$$alx = \left[ln\left(\frac{x_1}{x_p}\right), ln\left(\frac{x_1}{x_p}\right), \dots, ln\left(\frac{x_{p-1}}{x_p}\right) \right]$$

Where: **(9.2)**

$$x = \left(x_1, x_2, \dots, x_p\right)^r$$

The compositional data presented in **Chapter 3** was transformed (as required for LDA)[156] to give the additive log-ratio (alr) data that were used to carry out LDA. LDA was used to investigate if it was possible to determine if a CRW sample was parasitised or non-parasitised. Therefore, the response used was parasitised, and the predictors were the alrs for the compositional data. LDA was calculated both with and without cross-validation. Without cross-validation the misclassification rate is that calculated directly from the sample data, while with cross-validation a better estimate of the misclassification rate actually achieved in practice is produced.

(c) Quadratic Discriminant Analysis (QDA) is similar to LDA however, the classes are assumed to have different variance–co-variance matrices. QDA was

also used to investigate if it was possible to determine if a CRW sample was parasitised or non-parasitised. Therefore, the response used was parasitised, and the predictors were the alrs for the compositional data. QDA was also calculated both with and without cross-validation.

9.3.2 Multivariate Analysis of Differences in Fatty Acid Composition Between Clover Root Weevils of Different Physiological States

i. Principal Component Analysis

(a) Full Sample Set

An eight principal component (PC) model was calculated producing eight principal components (**Table 9.11**). However, it was found that 75.9% of the variation (cumulative = 0.759) was described in the first three PCs and that the eighth principal component contributed nothing. Therefore, a three element PCA was used instead (**Table 9.12**).

Table 9.11. Eigenanalysis of the correlation matrix for PCA (eight element model).

Principal component number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Eigenvalue	3.21	1.59	1.28	0.69	0.52	0.38	0.33	0.00
Proportion	0.40	0.20	0.16	0.09	0.07	0.05	0.04	0.00
Cumulative	0.40	0.60	0.76	0.85	0.91	0.96	1.00	1.00

The PC values reveal that the component of 12:0 makes a large negative contribution to the first PC, while the components of 16:0 and 18:1 make a large positive contribution to the first PC. The second PC is largely made up of a positive contribution from the component of 16:1 and negative contributions from the components of 18:2 and 18:3. The third PC is largely made up of a positive contribution from the component of 14:0 and negative contributions from the components of 18:0 and 18:2. By plotting a scatterplot of PC1 *versus* PC2 with the data grouped into parasitised or non-parasitised (**Figure 9.8**) it is possible to determine whether the PCs are useful at predicting whether the data is parasitised or non-parasitised. Inspection of this plot reveals that there is no clear distinction

between parasitised and non-parasitised, with the data points of the two groups completely overlapping. This indicates that the PCs may not be more useful for distinguishing between the parasitised and non-parasitised than the original data. When PC1 (scor1) was plotted *versus* the component of 18:1 (**Figure 9.9**) with data also labelled by location to investigate any possible pattern), there was a strong positive correlation. This suggests that the variation within the component of 18:1 contributes largely to PC1. This is reinforced by the large positive contribution that the component of 18:1 makes to PC1 (**Table 9.12**). The grouping of data points, especially the samples from Invermay, in **Figure 9.9** indicated that PC1 (scor1) could be used to determine location. This led to further ANOVA of location in **Section 9.3.2 (iv)**.

Table 9.12. Contribution that each fatty acid component (percentage of body weight, via its clr) makes to the three PCs (three element model).

Variable	PC1	PC2	PC3
12:0clr	-0.465	0.016	0.313
14:0clr	-0.366	0.181	0.360
16:0clr	0.384	0.308	-0.225
16:1clr	0.453	0.141	-0.019
18:0clr	-0.218	0.477	-0.462
18:1clr	0.434	0.058	0.322
18:2clr	-0.195	-0.385	-0.633
18:3clr	0.153	-0.688	0.058

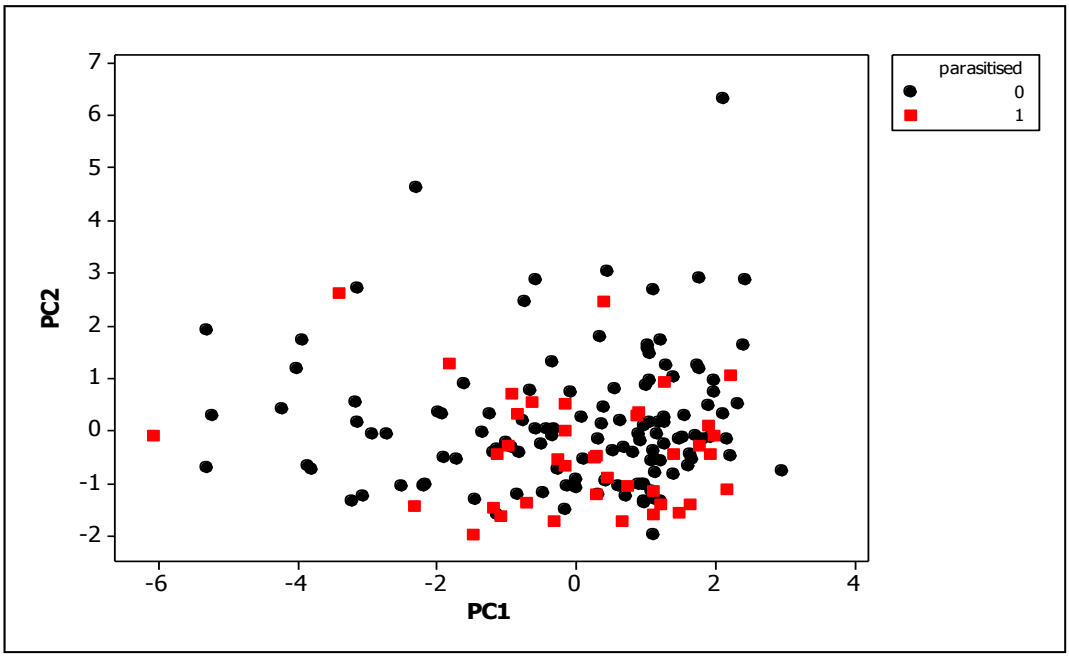

Figure 9.8. Scatterplot of PC1 (scor1) *versus* PC2 (scor2) with the data labelled according to whether it is parasitised (parasitised = 1) or non-parasitised (parasitised = 0).

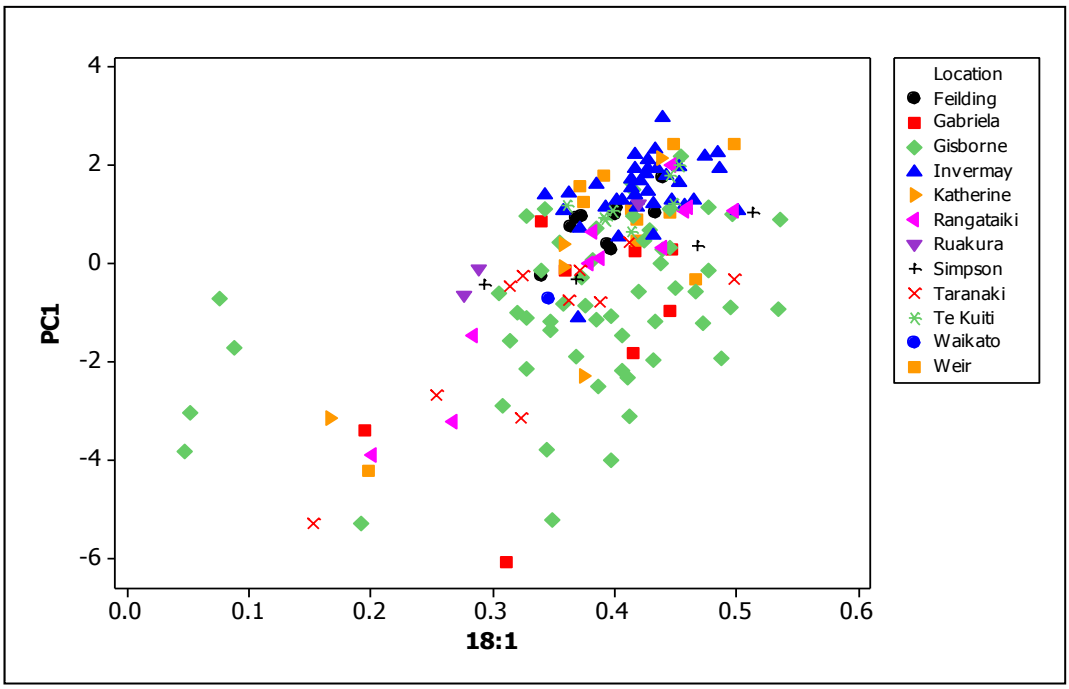

Figure 9.9. A scatterplot of the component of 18:1 *versus* PC1 (scor1) with the data labelled by location.

To investigate further the ability to distinguish between different physiological states using the PCs, scatterplots for each physiological state and each of the three PCs were plotted. The two panels on each graph represented parasitised and non-

parasitised and regression lines were added. **Figure 9.10**-**Figure 9.12** are the graphs for fat (0-5) *versus* each of the PCs. No obvious correlations between the physiological states and the PCs or differences between parasitised and non-parasitised were seen (**Table 9.13**). See **Appendix 9.4.4** for the regression equations and coefficients of determination of the principal components versus the other physiological states. Therefore, PCA was no more useful than the statistical analysis of the raw data at predicting whether the data was for parasitised or non-parasitised samples or distinguishing between different physiological states.

Table 9.13. Regression equations and coefficients of determination (R^2) for the regression lines of the first three principal components *versus* fat (0-5).

Parasitised	Regression equation	R^2
0	Scor1 = -0.9626+0.3760Fat	0.107
1	Scor1 = -2.153+0.7479Fat	0.204
0	Scor2 = 0.4410-0.1230Fat	0.024
1	Scor2 = 0.7023+0.1010Fat	0.008
0	Scor3 = -0.0913+0.02648Fat	0.001
1	Scor3 = -0.0955+0.0576Fat	0.003

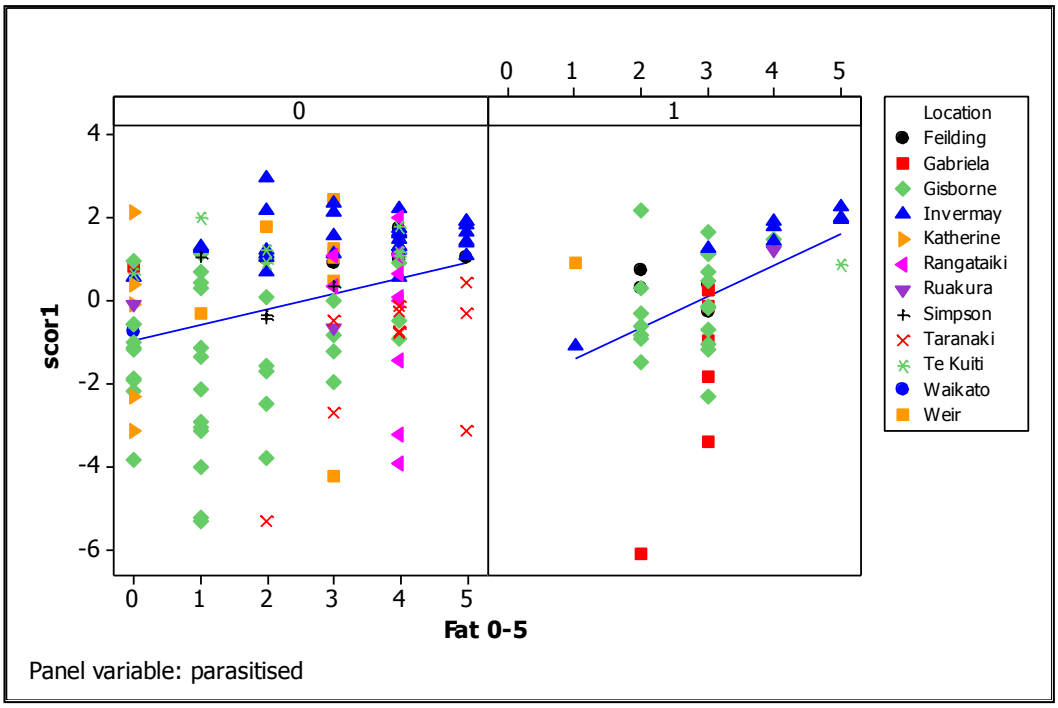

Figure 9.10. A scatterplot of fat (0-5) *versus* PC1 (scor1) split into non-parasitised (left panel; 0) and parasitised (right panel; 1). Linear egression lines have been added.

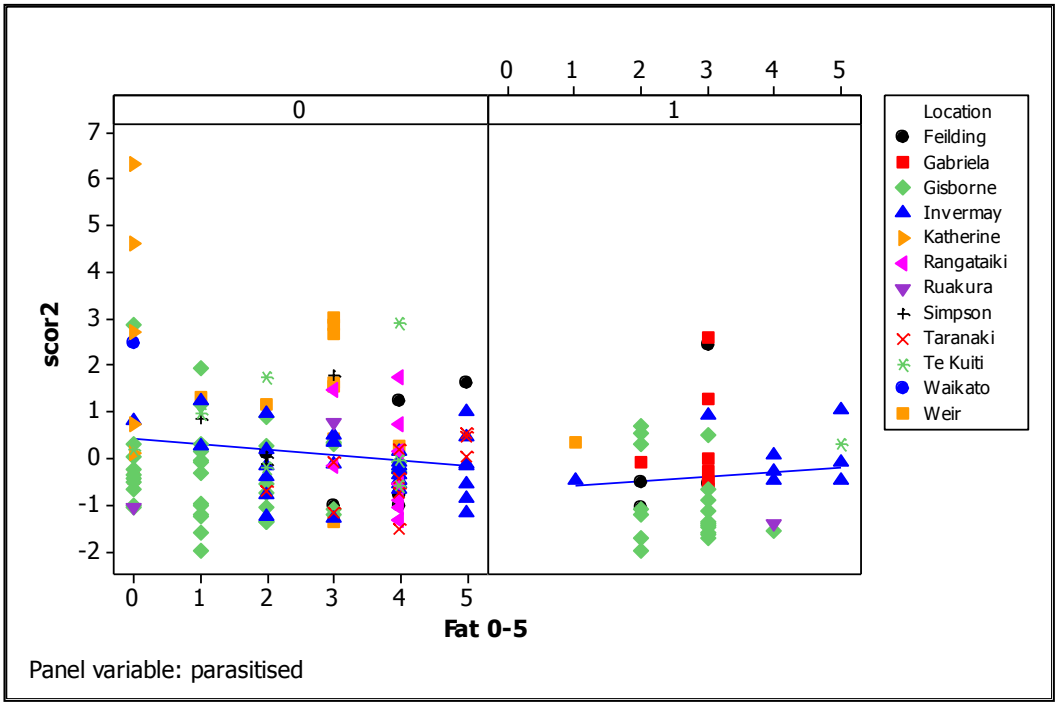

Figure 9.11. A scatterplot of fat (0-5) *versus* PC2 (scor2) split into non-parasitised (left panel; 0) and parasitised (right panel; 1). Linear regression lines have been added.

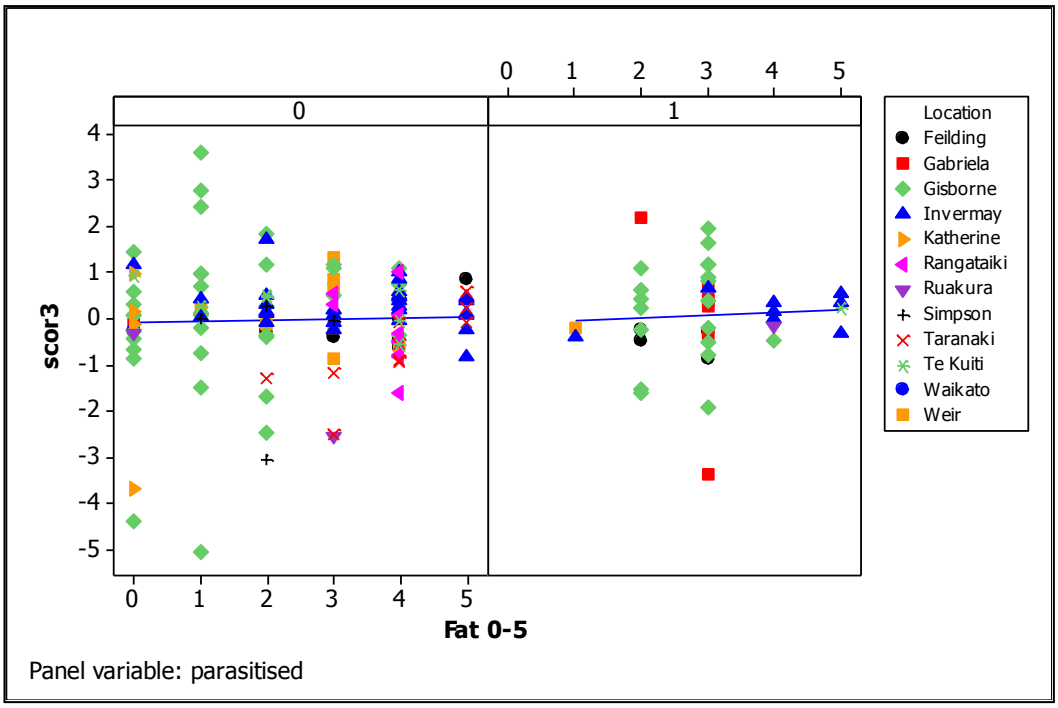

Figure 9.12. A scatterplot of fat (0-5) *versus* PC3 (scor3) split into non-parasitised (left panel; 0) and parasitised (right panel; 1). Linear egression lines have been added.

(b) Gisborne Sample Set

An eight component model was calculated producing eight principal components, (**Table 9.14**). However, it was also found that 77.7% of the variation (cumulative = 0.777) was described in the first three principal components and that the eighth principal component contributed nothing. Therefore, a three element PCA was used and the contribution that each fatty acid component made (*via* its clr) was calculated (**Table 9.15**). The PC values reveal that the components of 12:0 and 14:0 make a large negative contribution to the first PC, while the component of 16:1 makes a large positive contribution to the first PC. The second PC is largely made up of positive contributions from the components of 18:0 and 18:2 and a negative contribution from the component of 18:1. The third PC is largely made up of a positive contribution from the component of 18:3 and negative contributions from the components of 16:0 and 16:1.

Table 9.14. Eigenanalysis of the correlation matrix for PCA (eight element model) of the Gisborne sample set.

Principal	1	2	3	4	5	6	7	8
Eigenvalue	3.36	1.88	0.98	0.81	0.45	0.27	0.25	0.00
Proportion	0.42	0.24	0.12	0.10	0.06	0.03	0.03	0.00
Cumulative	0.42	0.66	0.78	0.88	0.94	0.97	1.00	1.00

Table 9.15. Contribution that each fatty acid component of the Gisborne sample set makes (*via* its clr) to the three PCs (three element model).

Variable	PC1	PC2	PC3
12:0clr	-0.509	-0.103	0.057
14:0clr	-0.439	0.100	-0.096
16:0clr	0.402	0.116	-0.402
16:1clr	0.442	-0.129	-0.311
18:0clr	-0.042	0.581	-0.142
18:1clr	0.129	-0.585	-0.175
18:2clr	0.302	0.493	0.263
18:3clr	0.285	-0.163	0.781

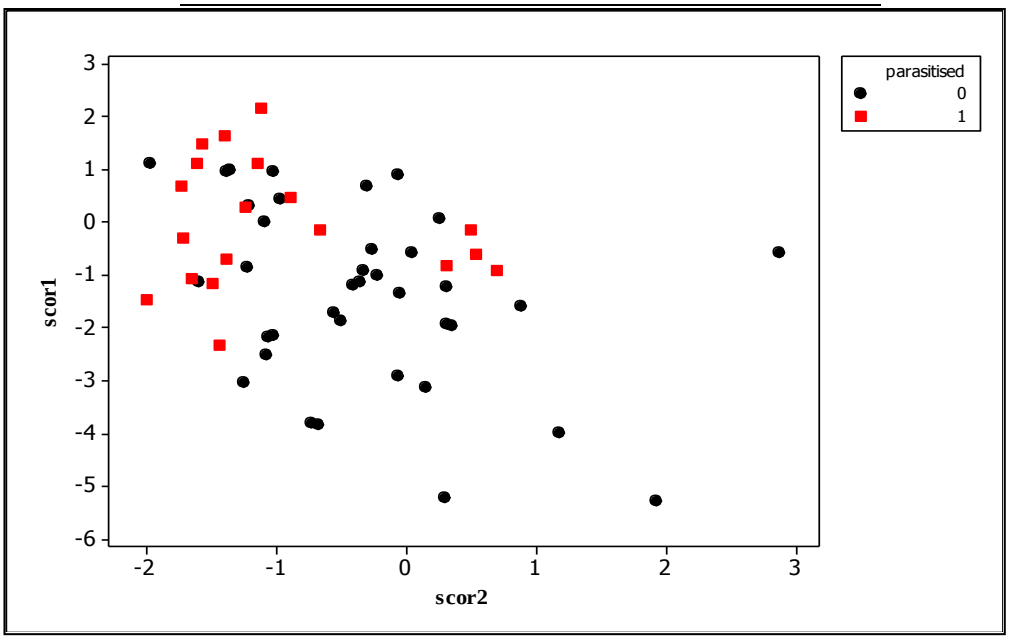

Figure 9.13. Scatterplot of PC1 (scor1) *versus* PC2 (scor2) for the Gisborne sample set with the data labelled according to whether it is parasitised (parasitised = 1) or non-parasitised (parasitised = 0).

A scatterplot of PC1 *versus* PC2 with the data grouped into parasitised or non-parasitised (**Figure 9.13**) was created to determine whether the PCs were useful at predicting whether the data describe parasitised or non-parasitised. Inspection of this plot reveals that the two groups (parasitised and non-parasitised) are somewhat separated from one another. The ratios of the first two PCs may be useful to distinguish between parasitised and non-parasitised CRW samples in the Gisborne sample set.

The four parasitised (parasitised = 1) that had a higher PC2 (scor2) value than the rest of the parasitised samples were investigated to determine whether they could be distinguished from the remaining parasitised samples. Apart from all having a colour score of three (but not being the only samples with this colour score) no other features that distinguished these four samples were apparent.

ii. Linear Discriminant Analysis

(a) *Full Sample Set*

LDA was calculated both without (**Table 9.16**) and with (**Table 9.17**) cross validation. Out of the 164 samples LDA was able to correctly assign 103 (62.8%) without cross validation or 100 (61.0%) with cross validation to their correct group (parasitised or non-parasitised). When this result is compared to the 50% success one would expect by simply predicting a binary outcome (i.e. parasitised or non-parasitised) by chance, these percentages were relatively low LDA. Therefore LDA could not be accurately used to predict whether a sample was parasitised or non-parasitised.

Table 9.16. LDA for all CRW samples without cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0	**1**
Put in group 0	76	14
Put into group 1	47	27
Total N	123	41
N correct	76	27
Proportion	0.62	0.66

Table 9.17. LDA for all CRW samples with cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0	**1**
Put in group 0	74	15
Put into group 1	49	26
Total N	123	41
N correct	74	26
Proportion	0.60	0.63

(b) Gisborne Sample Set

LDA was calculated for both without (**Table 9.18**) and with, (**Table 9.19**) cross validation. Out of the 56 samples LDA was able to correctly assign 41 (73.2%) without cross validation or 37 (66.1%) with cross validation to their correct group (parasitised or non-parasitised). These values are higher than their respective values for the full sample set; therefore, LDA is more effective at predicting whether a CRW sample is parasitised or non-parasitised in the Gisborne sample set than in the full sample set. However, to be effective at predicting parasitism, LDA values of at least 80% (with cross-validation) are desired.[155]

Table 9.18. LDA for Gisborne samples without cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0 (Non-parasitised)	**1 (Parasitised)**
Put in group 0	26	4
Put into group 1	11	15
Total N	37	19
N correct	26	15
Proportion	0.70	038

Table 9.19. LDA for Gisborne samples with cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0 (Non-parasitised)	**1 (Parasitised)**
Put in group 0	23	5
Put into group 1	14	14
Total N	37	19
N correct	23	14
Proportion	0.62	0.74

iii. Quadratic Discriminant Analysis

(a) Full Sample Set

QDA was calculated both without (**Table 9.20**) and with (**Table 9.21**) cross validation. Out of the 164 samples QDA was able to correctly assign 110 (67.1%) without cross validation or 98 (59.8%) with cross validation to their correct group (parasitised or non-parasitised). As with LDA, these percentages were relatively low, so QDA could not be accurately used to predict whether a sample was parasitised or not.

Table 9.20. QDA for all CRW samples without cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0	**1**
Put in group 0	77	8
Put into group 1	46	33
Total N	123	41
N correct	77	33
Proportion	0.63	0.81

Table 9.21. QDA for all CRW samples with cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0	**1**
Put in group 0	73	16
Put into group 1	50	25
Total N	123	41
N correct	73	25
Proportion	0.60	0.61

(b) Gisborne Sample Set

QDA was calculated for both without (**Table 9.22**) and with (**Table 9.23**) cross validation. Out of the 56 samples QDA was able to correctly assign 43 (76.8%) without cross validation or 36 (64.3%) with cross validation to their correct group (parasitised or non-parasitised). These values are also higher than their respective values for the full sample set, however, to be effective at predicting parasitism, QDA values of at least 80% (with cross-validation) are desired.[155]

Table 9.22. QDA for Gisborne samples without cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:1alr.

	True group	
	0 (Non-parasitised)	**1 (Parasitised)**
Put in group 0	26	2
Put into group 1	11	17
Total N	37	19
N correct	26	17
Proportion	0.70	0.90

Table 9.23. QDA for Gisborne samples with cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0 (Non-parasitised)	**1 (Parasitised)**
Put in group 0	23	6
Put into group 1	14	13
Total N	37	19
N correct	23	13
Proportion	0.62	0.68

iv. Differences Between Location

One-way ANOVA was used to investigate the differences between the PC1 values for each location (**Table 9.24**).The p-value for differences between these means was found to be less than 0.05 (0.00), therefore the null

hypothesis (that there was no difference between PC1 values between locations) had to be rejected. This suggested that PC1 values could be useful at predicting the location of a sample. However, when the 95% confidence intervals that were calculated as part of the one-way ANOVA calculation by Minitab 16 were investigated (**Table 9.25**), it was visually obvious that there was considerable overlap between the intervals for each location. This is also obvious in **Figure 9.9** as overlap can be seen. Part of the reason for this may be the small sample sizes at some of the locations which causes large interval sizes. Further experimentation using large sample sizes from each location would be required to determine the usefulness of PC1 values at predicting the location of the sample.

Table 9.24. One-way ANOVA results for PC1 differences between locations.

Source	DF	F Value	p Value
Location	11	7.96	0.00
Error	152		
Total	163		

Table 9.25. 95% confidence intervals produced by the one-way ANOVA of PC1 differences between locations.

```
Individual 95% CIs For Mean Based on Pooled StDev

Level      N   Mean  StDev ----+---------+---------+---------+----
Feilding   10  0.800 0.548                    (-----*-----)
Gabriela   8  -1.384 2.341          (------*------)
Gisborne   56  0.926 1.675                        (--*-)
Invermay   37  1.491 0.672                           (--*--)
Katherine  5  -0.603 2.122           (--------*--------)
Rangataiki 11 -0.206 1.880              (-----*-----)
Ruakura    3   0.156 0.964              (----------*----------)
Simpson    4   0.157 0.675               (---------*---------)
Taranaki   10 -1.348 1.794            (-----*-----)
Te Kuiti   8   1.214 0.458                      (------*-----)
Waikato    1  -0.729  * (-----------------*-----------------)
Weir     11 0.767 1.837                                (-----*-----)
                        ----+---------+---------+---------+----
                          -3.0     -1.5       0.0       1.5

Pooled StDev = 1.479
```

9.3.3 Multivariate Analysis of the Weighed Subset

i. Principal Component Analysis

The fatty acid concentration (as percentage of body weight) data presented in **Chapter 3** was transformed to give the centred log-ratio data. Again, an eight principal component (PC) model was calculated producing eight principal components (**Table 9.26**). As it was found that 84.3% of the variation (cumulative = 0.843) was described in the first three principal components and that the eighth principal component contributed only 0.3%, a three element PCA was used. The contribution that each fatty acid component made (*via* its clr) was calculated (**Table 9.27**).

Table 9.26. Eigenanalysis of the correlation matrix for PCA (eight element model) for the weighed subset.

Principal component number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Eigenvalue	4.03	1.76	0.95	0.66	0.33	0.19	0.06	0.02
Proportion	0.50	0.22	0.12	0.08	0.04	0.02	0.01	0.00
Cumulative	0.50	0.72	0.84	0.93	0.97	0.99	1.00	1.00

Table 9.27. Contribution that each fatty acid component (percentage of body weight, via its clr) makes to the three PCs (three element model) for the weighed subset.

Variable	**PC1**	**PC2**	**PC3**
12:0clr	0.045	-0.434	-0.792
14:0clr	0.435	0.058	-0.129
16:0clr	0.453	-0.063	0.261
16:1clr	0.245	0.571	-0.296
18:0clr	0.267	0.302	-0.374
18:1clr	0.397	0.283	0.161
18:2clr	0.401	-0.382	0.026
18:3clr	0.392	-0.401	0.183

The PC values reveal that the concentrations of 14:0, 16:0, 18:1 and 18:2 make large positive contributions to the first PC. The second PC is largely comprised of a positive contribution from the component of 16:1 and negative contributions from the components of 12:0, 18:2 and 18:3. The third PC is comprised of a positive contribution from the component of 16:0 and negative contributions from the components of 12:0, 16:1 and 18:3. To determine whether the PCs were useful at predicting whether the data was parasitised or non-parasitised, a matrix plot of the three PCAs (weightscor1, weightscor2 and weightscor3) was plotted (**Figure 9.14**). Inspection of this plot reveals that there is no clear distinction between parasitised and non-parasitised, with the data points of the two groups completely overlapping. To investigate further the ability to distinguish between different physiological states using the PCs, scatterplots for each physiological state and each of the three PCs were plotted. For example **Figure 9.15**-**Figure 9.17** are the graphs for fat (0-5) *versus* each of the PCs (weightscor1-weightsocr3) with the data split into parasitised and non-parasitised. For the regression equations and coefficients of determination graphs for the other physiological factors see **Appendix9.5.1**. No obvious correlations between the physiological states and the PCs were seen. It was concluded that the PCA of the weighed subset was unable to predict parasitised or non-parasitised or distinguish between different physiological states.

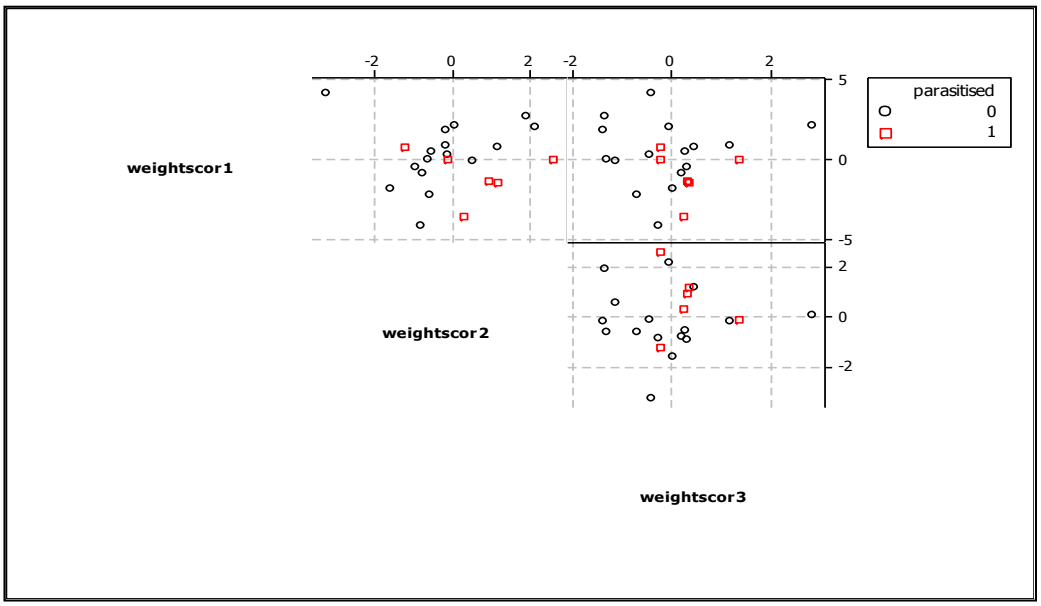

Figure 9.14. Matrix plot of the three PCAs for the CRW samples of the weighed subset.

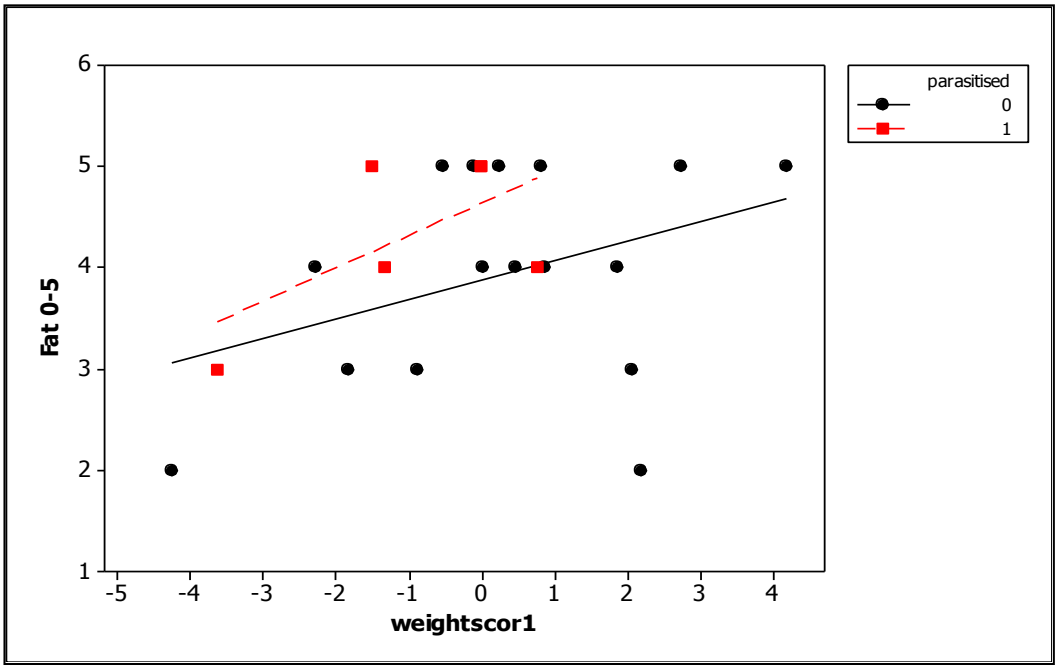

Figure 9.15. A scatterplot of fat (0-5) *versus* PC1 (weightscor1).

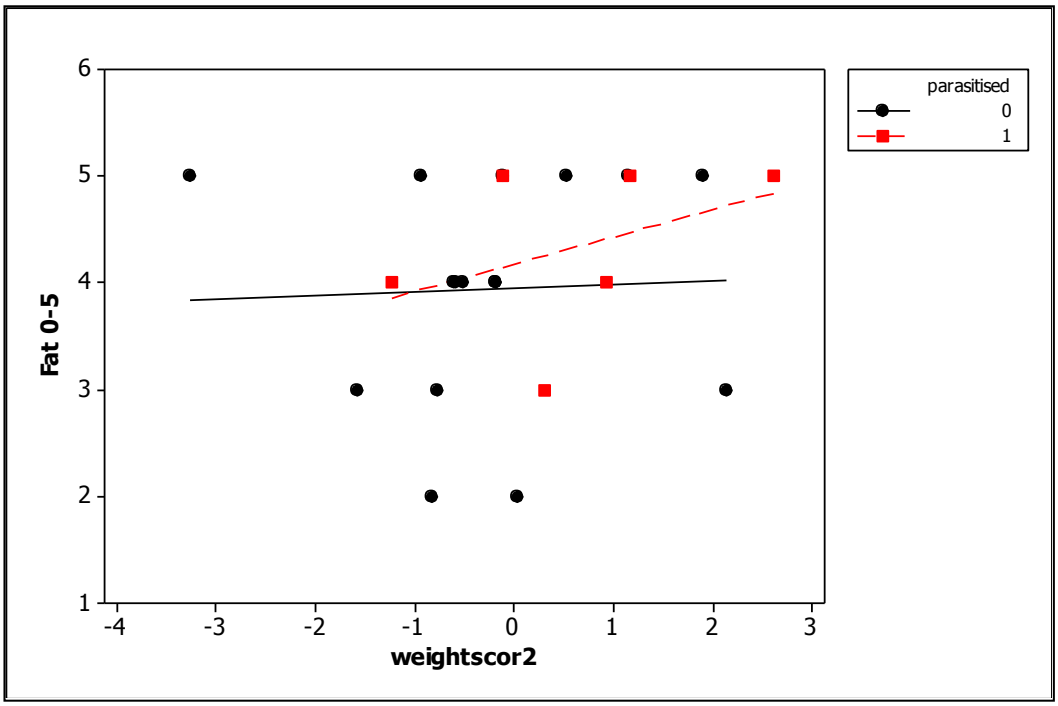

Figure 9.16. A scatterplot of fat (0-5) *versus* PC2 (weightscor2).

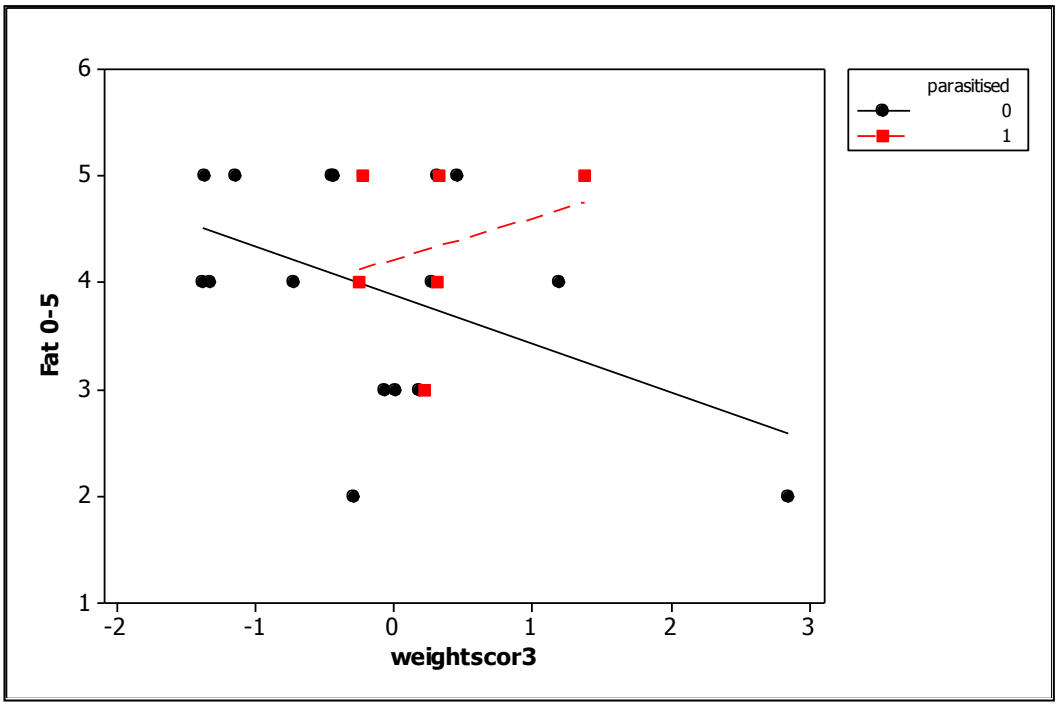

Figure 9.17. A scatterplot of fat (0-5) *versus* PC3 (weightscor3).

ii. Linear Discriminant Analysis

The fatty acid concentration (as percentage of body weight) data presented in **Chapter 3** was transformed to give the additive log-ratio (alr) data. LDA was calculated both without (**Table 9.28**) and with (**Table 9.29**) cross validation.

Table 9.28. LDA for the weighed subset without cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True group	
	0 (Non-parasitised)	**1 (Parasitised)**
Put in group 0	12	1
Put into group 1	4	5
Total N	16	6
N correct	12	5
Proportion	0.75	0.83

Table 9.29. LDA for the weighed subset with cross validation for the response = parasitised and the predictors 12:0alr, 14:0alr, 16:0alr, 16:1alr, 18:0alr, 18:1alr, 18:2alr.

	True Group	
	0 (Non-parasitised)	**1 (Parasitised)**
Put in Group 0	9	3
Put into Group 1	7	3
Total N	16	6
N Correct	9	3
Proportion	0.56	0.50

Out of the 22 samples LDA was able to correctly assign 17 (77.3%) without cross validation or 12 (54.5%) with cross validation to their correct group (parasitised or non-parasitised). These percentages were too low (especially with cross-validation) for LDA to be used to predict accurately whether a CRW sample was parasitised or not.

9.4 Entire Sample Set

9.4.1 Scatterplots of each Fatty Acid Composition *versus* each Physiological State

- The graphs are panelled with parasitised (bottom) and non-parasitised (top).

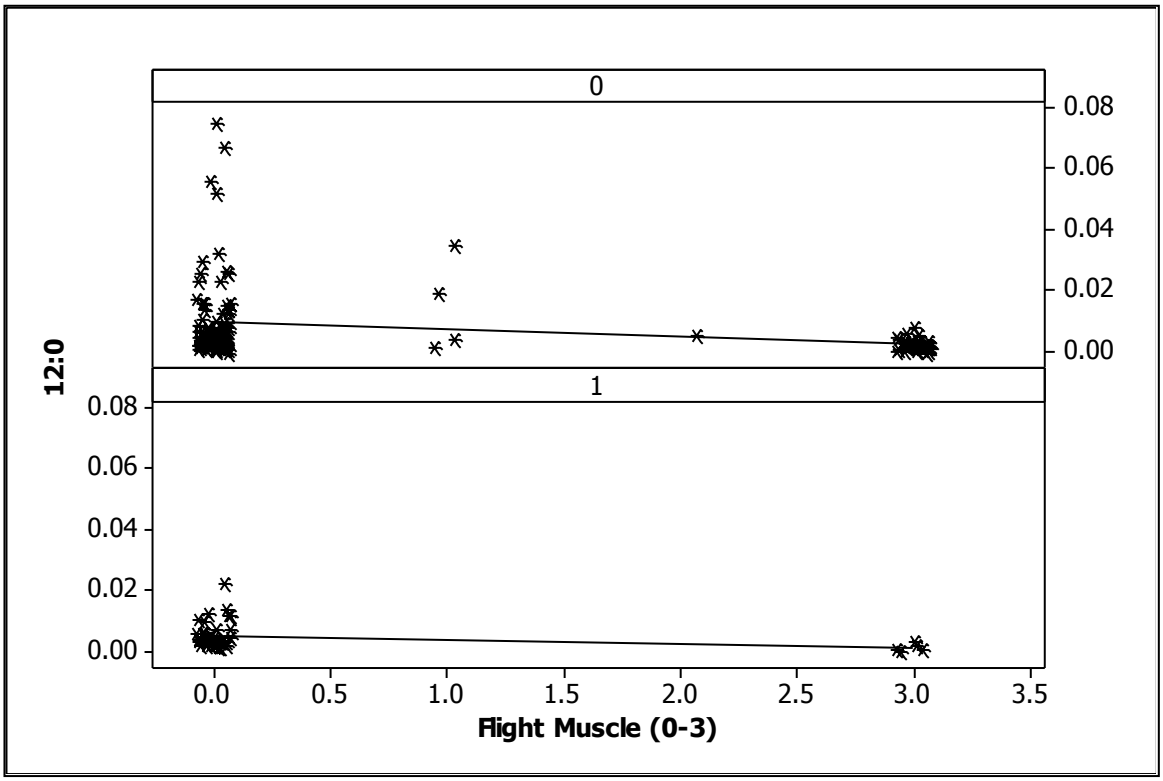

Figure 9.18. Scatterplot of flight muscle (0-3) *versus* 12:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

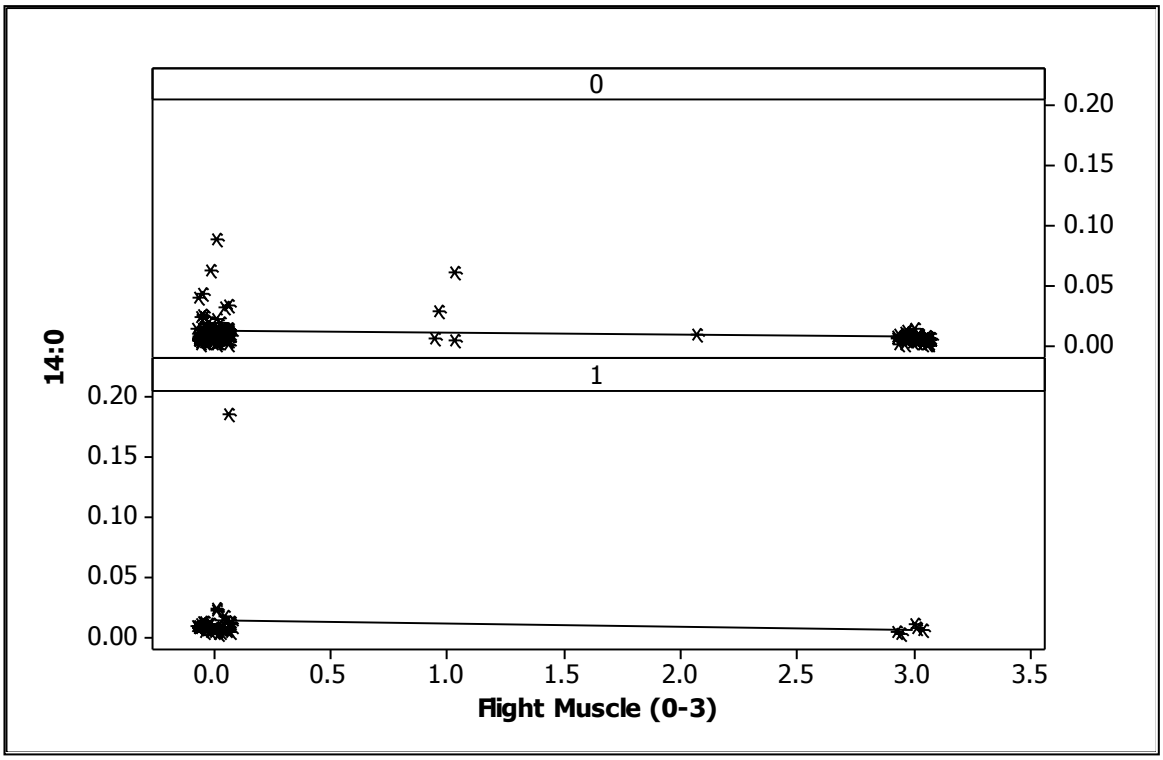

Figure 9.19. Scatterplot of flight muscle (0-3) *versus* 14:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

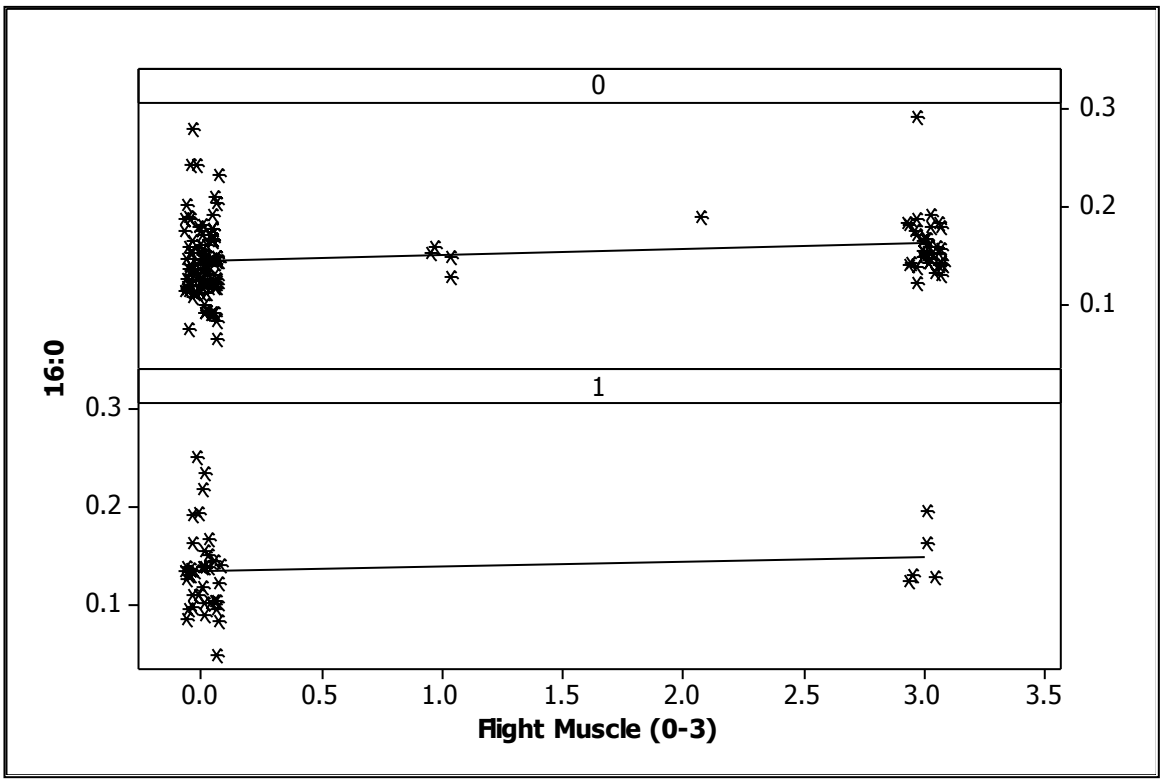

Figure 9.20. Scatterplot of flight muscle (0-3) *versus* 16:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

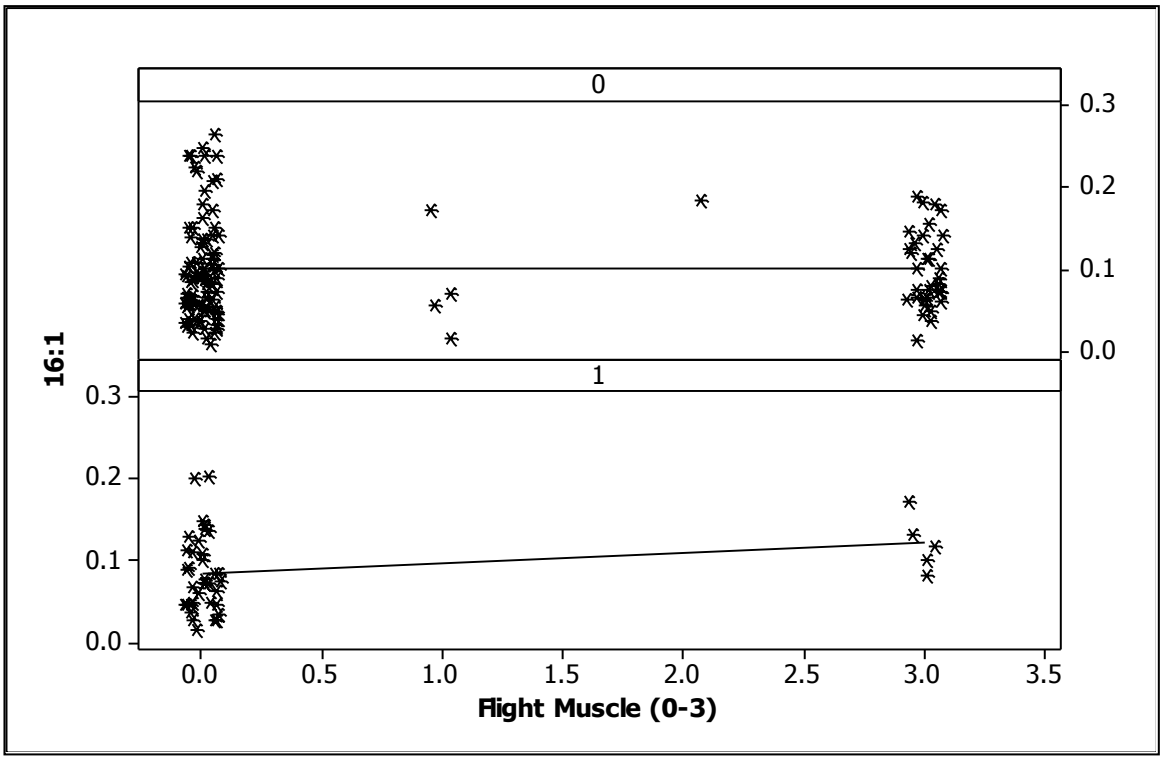

Figure 9.21. Scatterplot of flight muscle (0-3) *versus* 16:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

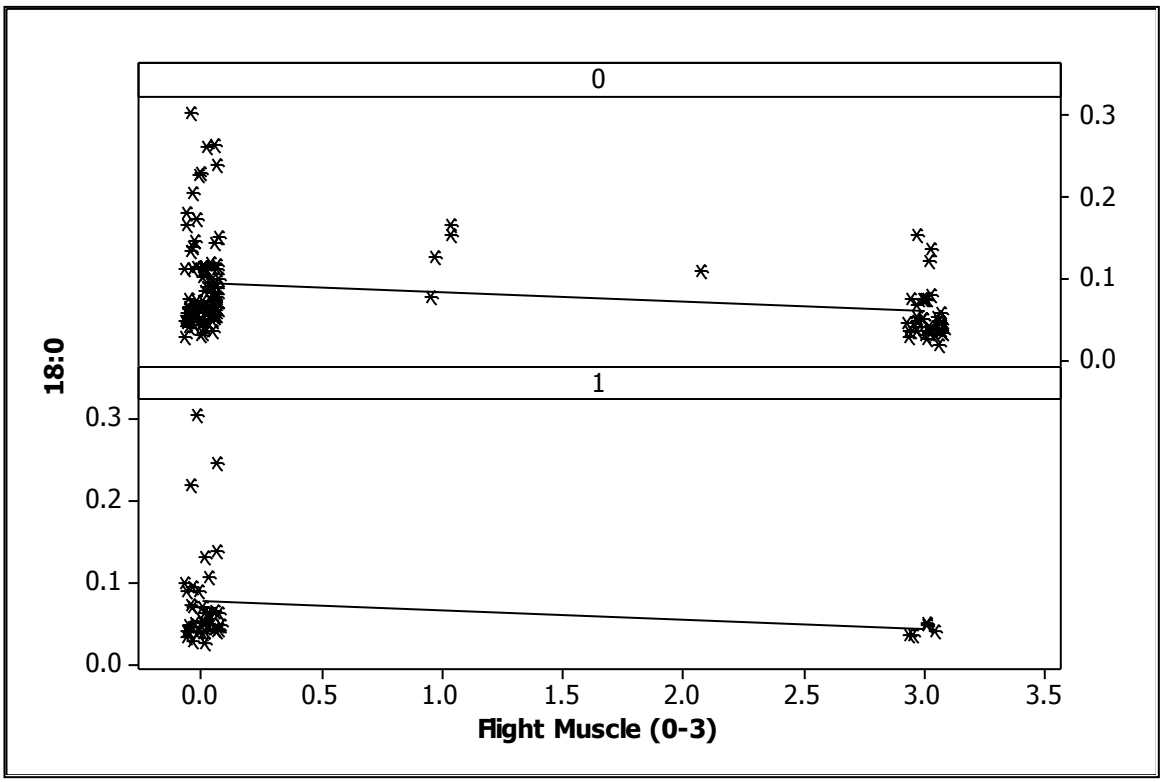

Figure 9.22. Scatterplot of flight muscle (0-3) *versus* 18:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

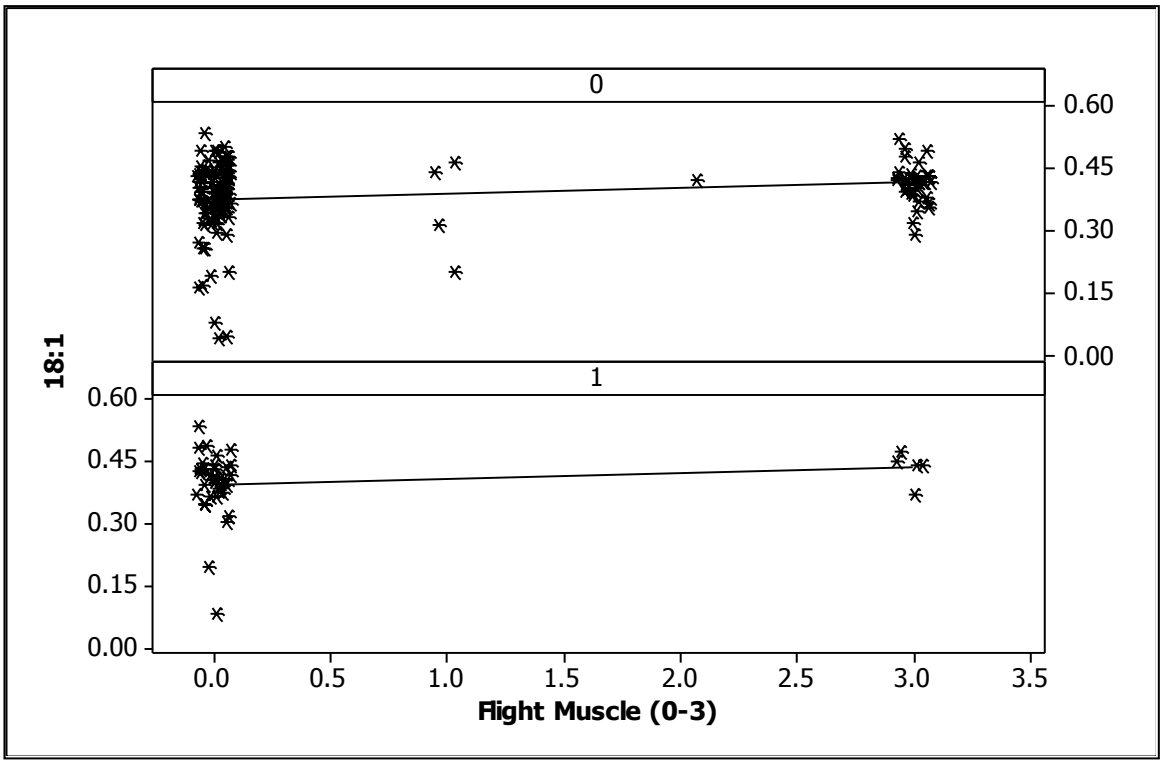

Figure 9.23. Scatterplot of flight muscle (0-3) *versus* 18:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

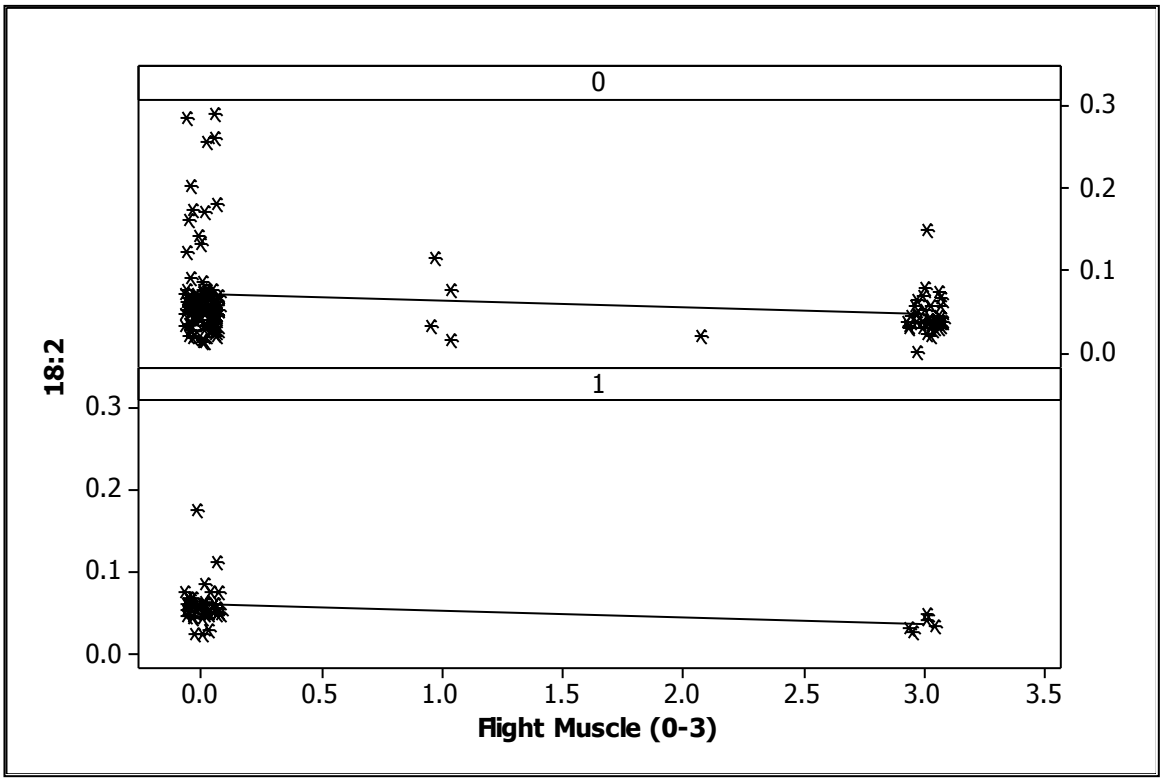

Figure 9.24. Scatterplot of flight muscle (0-3) *versus* 18:2 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

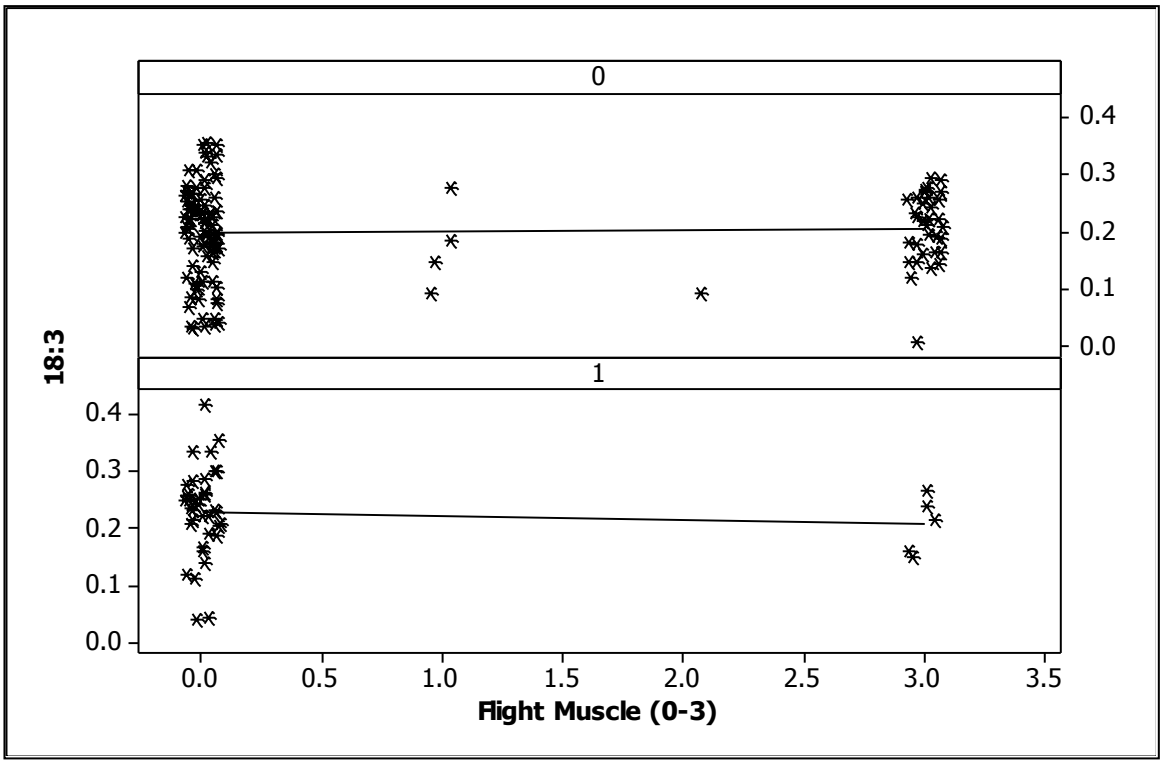

Figure 9.25. Scatterplot of flight muscle (0-3) *versus* 18:3 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

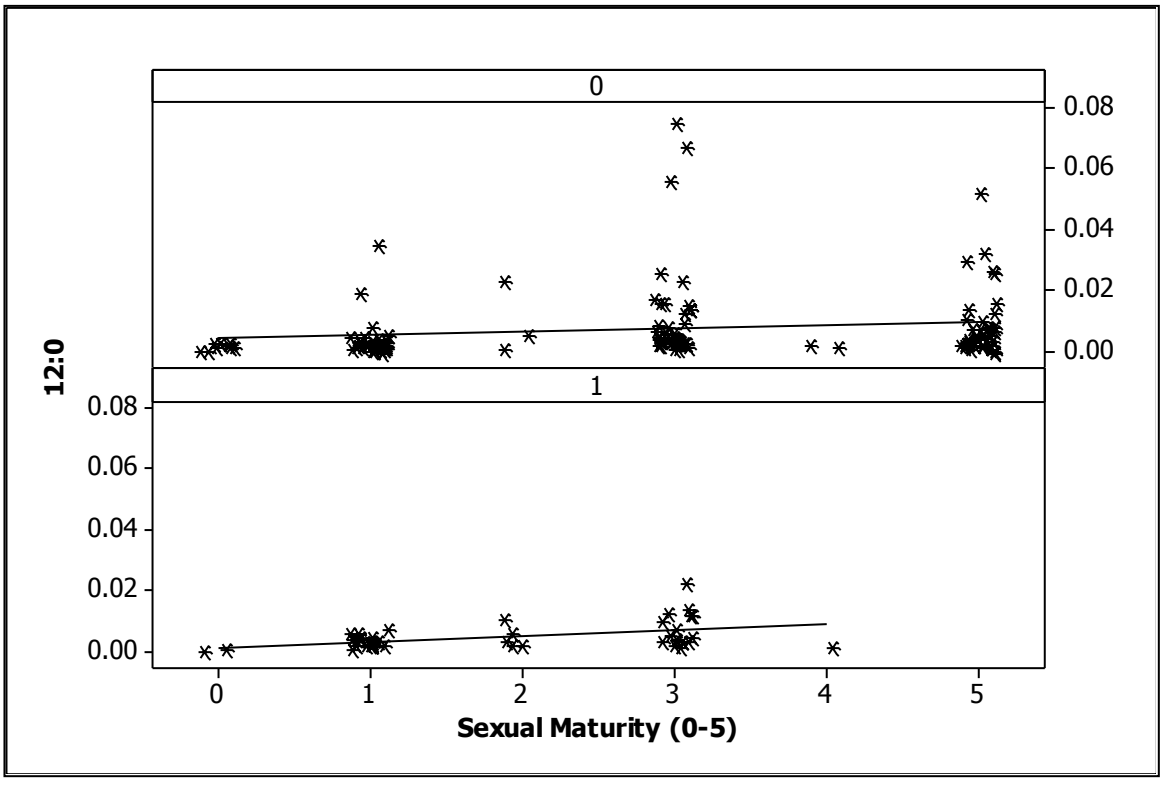

Figure 9.26. Scatterplot of sexual maturity (0-5) *versus* 12:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

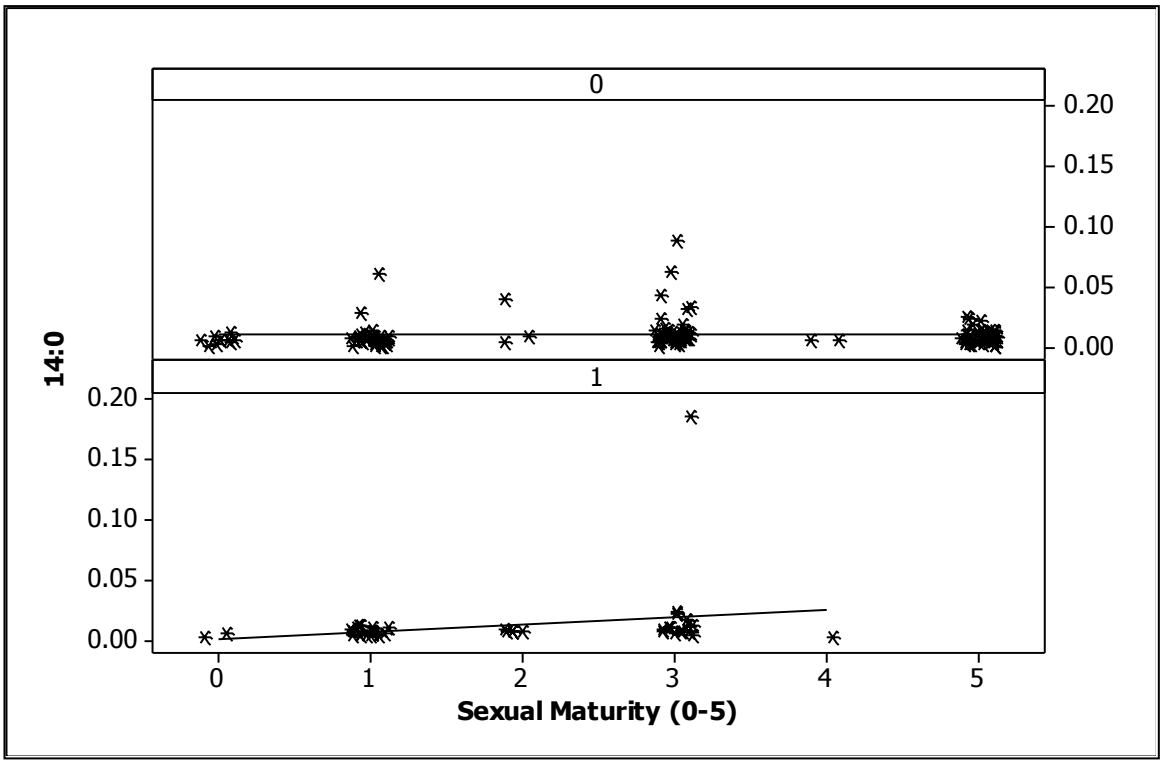

Figure 9.27. Scatterplot of sexual maturity (0-5) *versus* 14:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

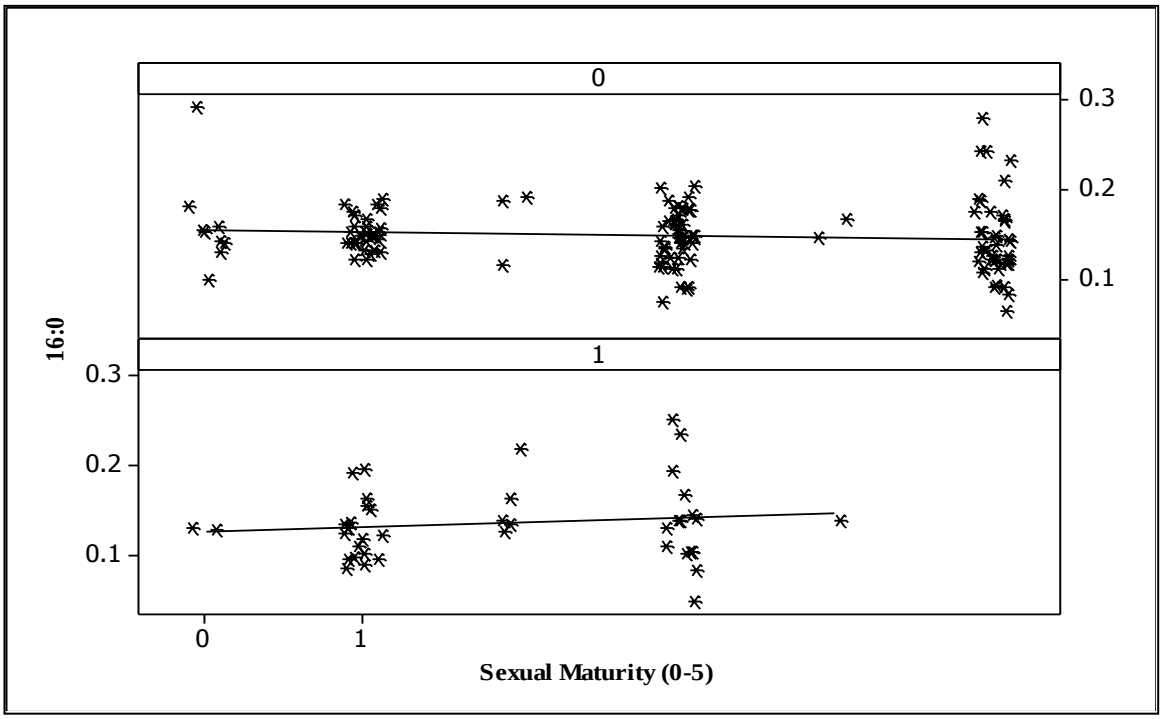

Figure 9.28. Scatterplot of sexual maturity (0-5) *versus* 16:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

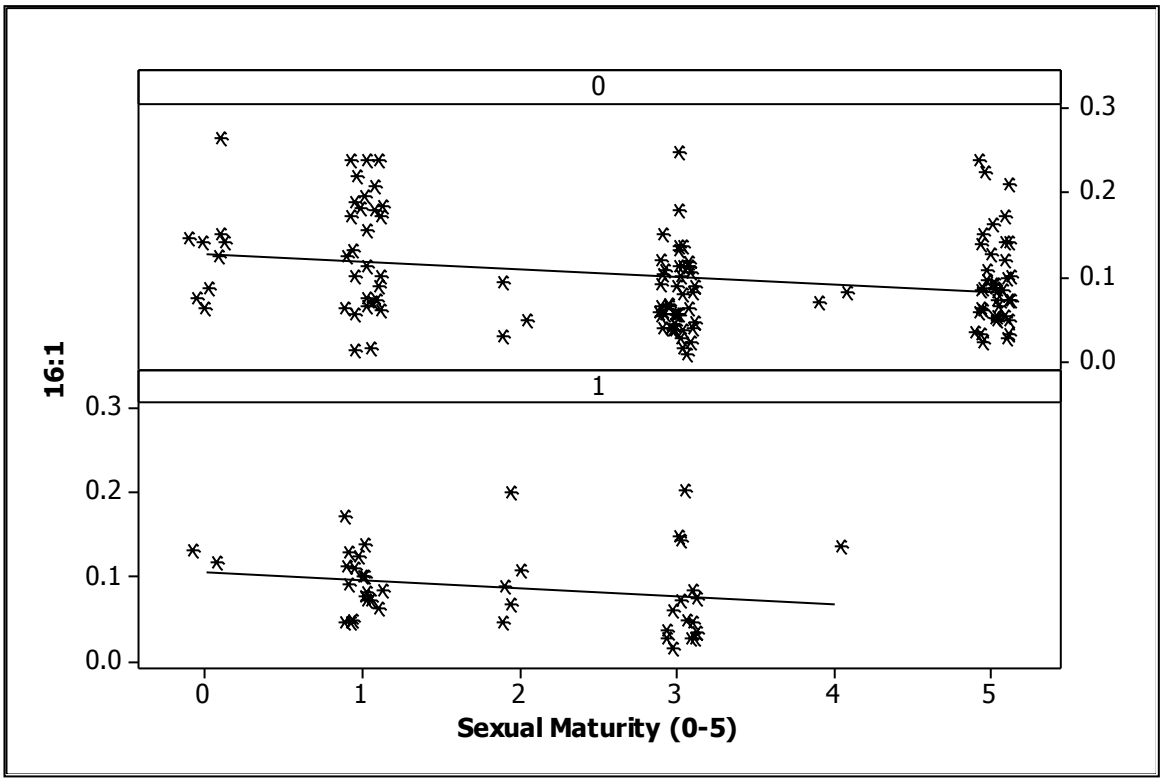

Figure 9.29. Scatterplot of sexual maturity (0-5) *versus* 16:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

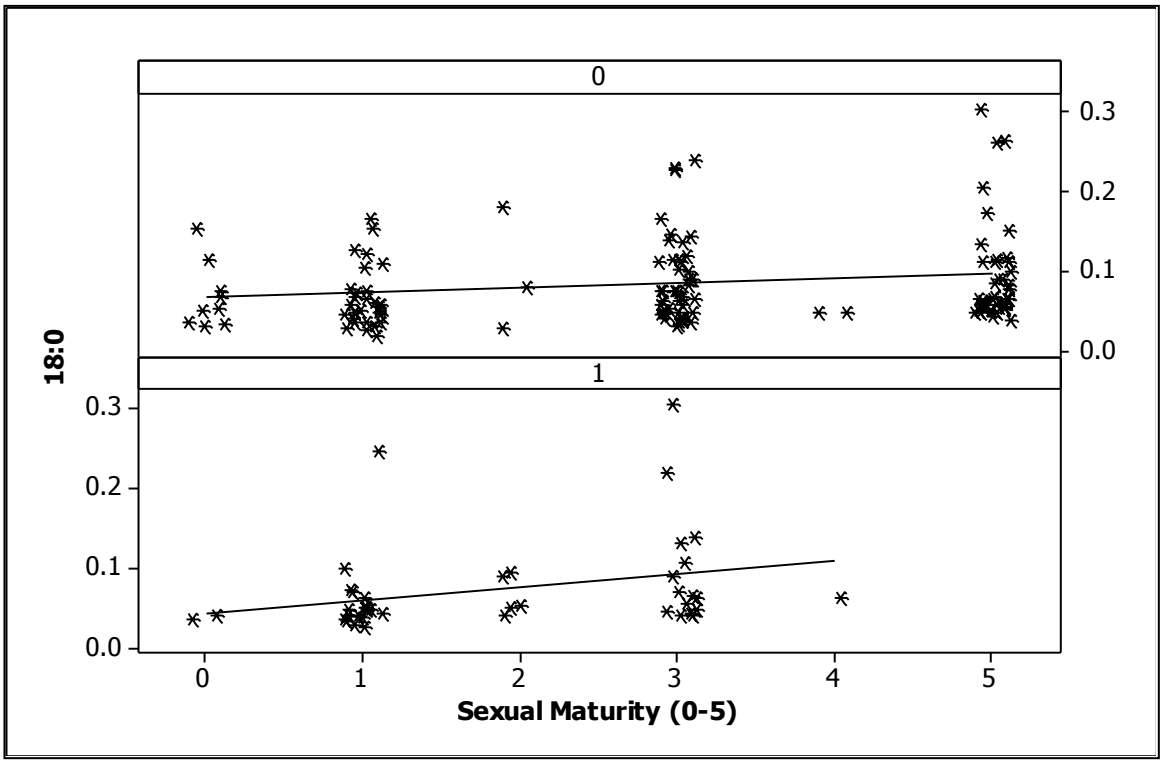

Figure 9.30. Scatterplot of sexual maturity (0-5) *versus* 18:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

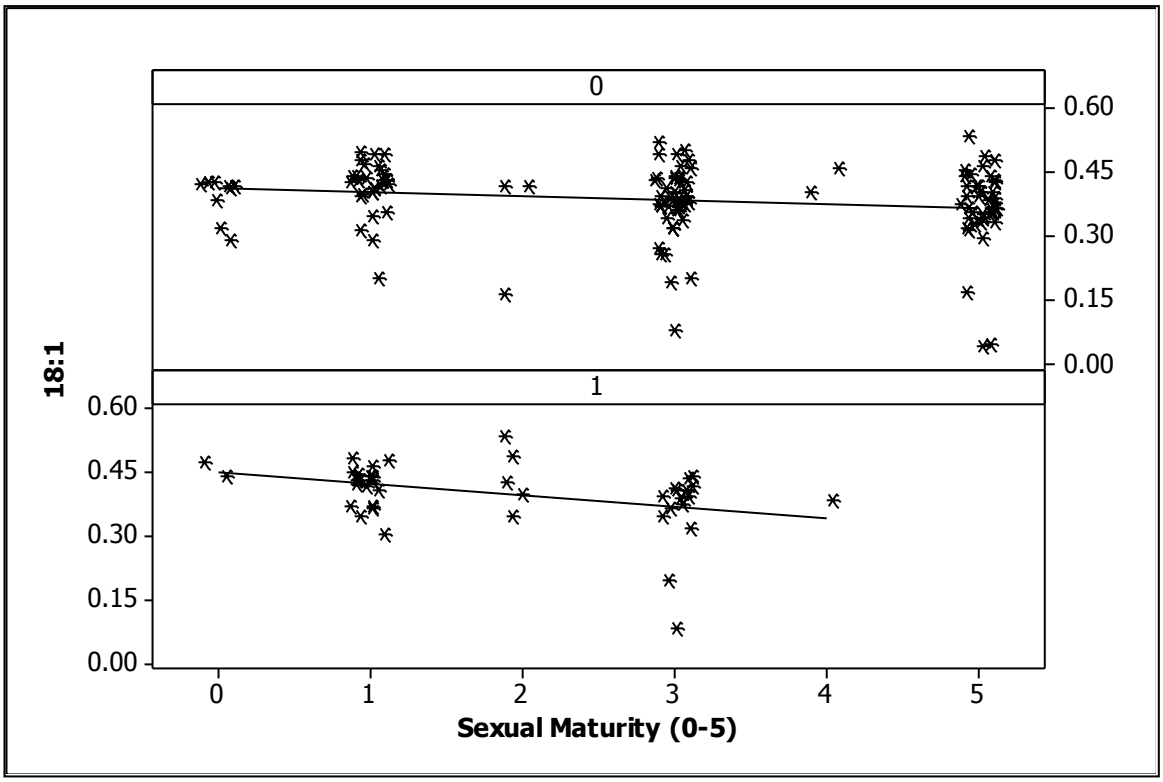

Figure 9.31. Scatterplot of sexual maturity (0-5) *versus* 18:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

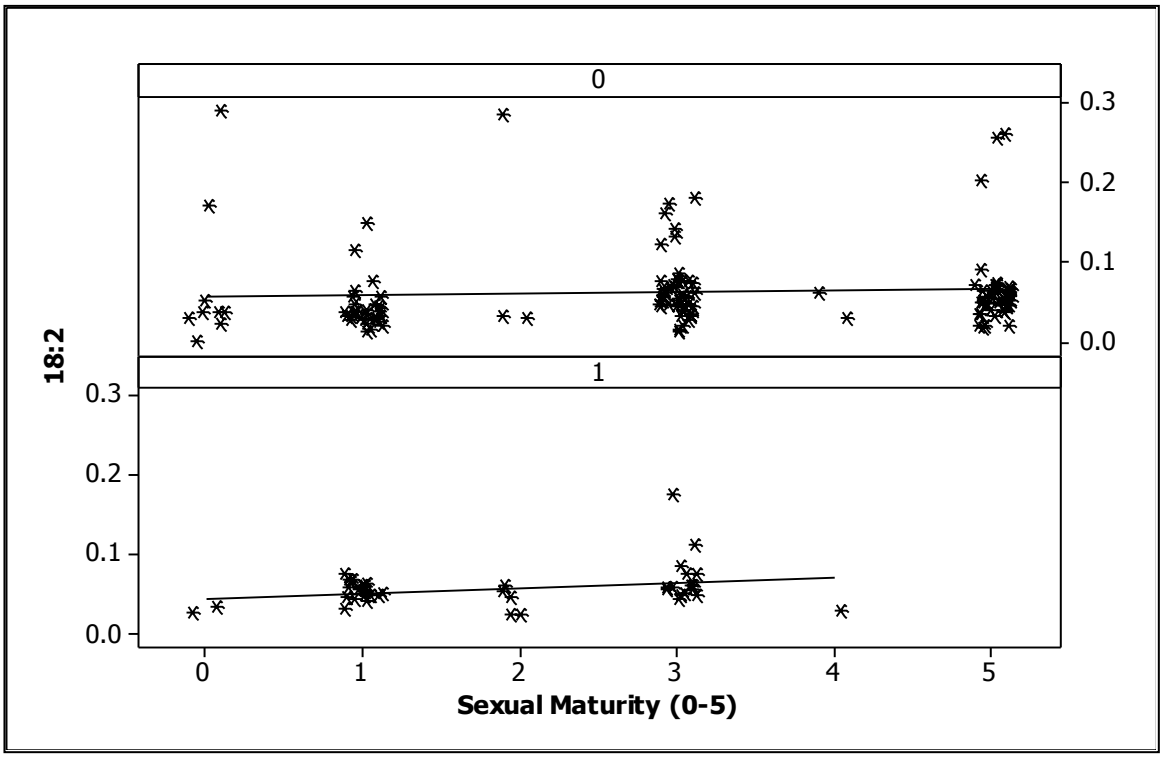

Figure 9.32. Scatterplot of sexual maturity (0-5) *versus* 18:2 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

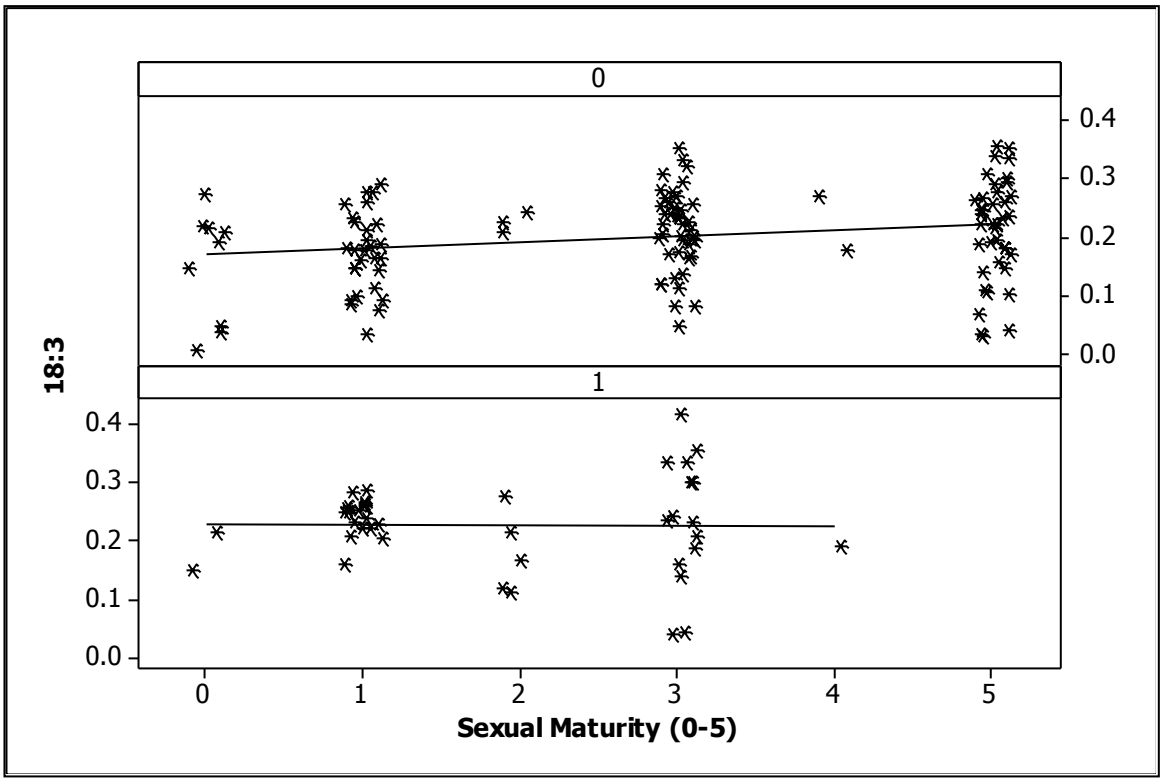

Figure 9.33. Scatterplot of sexual maturity (0-5) *versus* 18:3 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

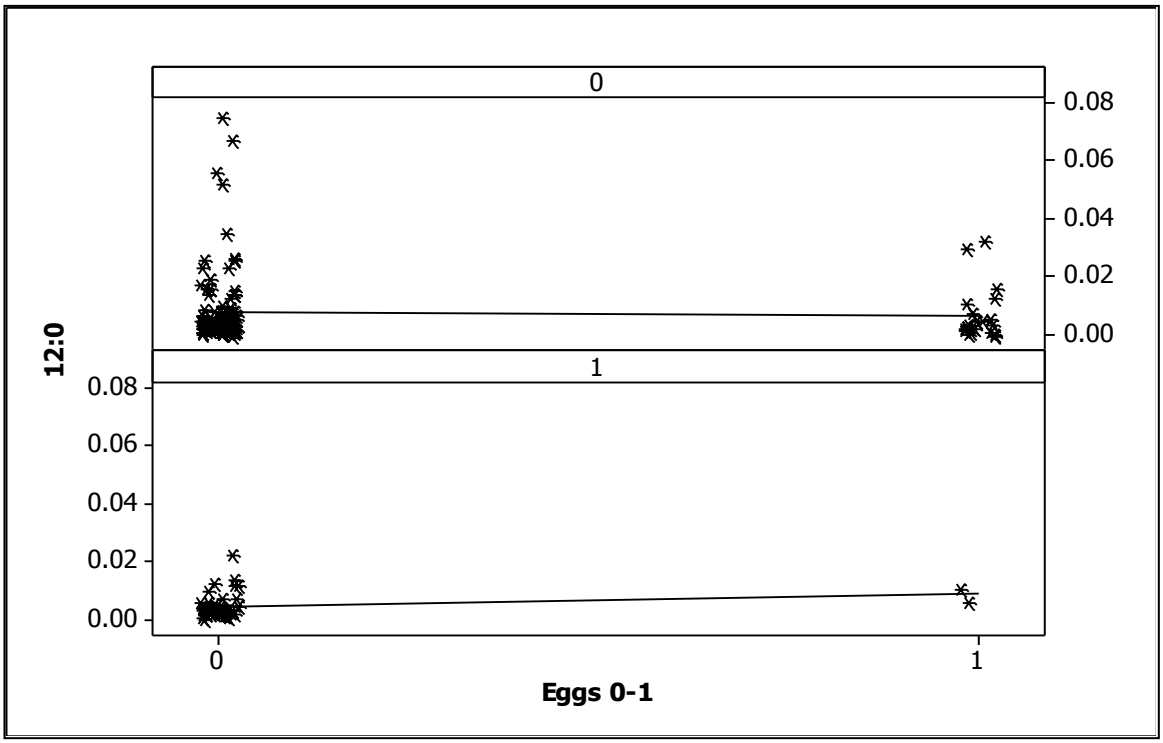

Figure 9.34. Scatterplot of eggs (0-1) *versus* 12:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

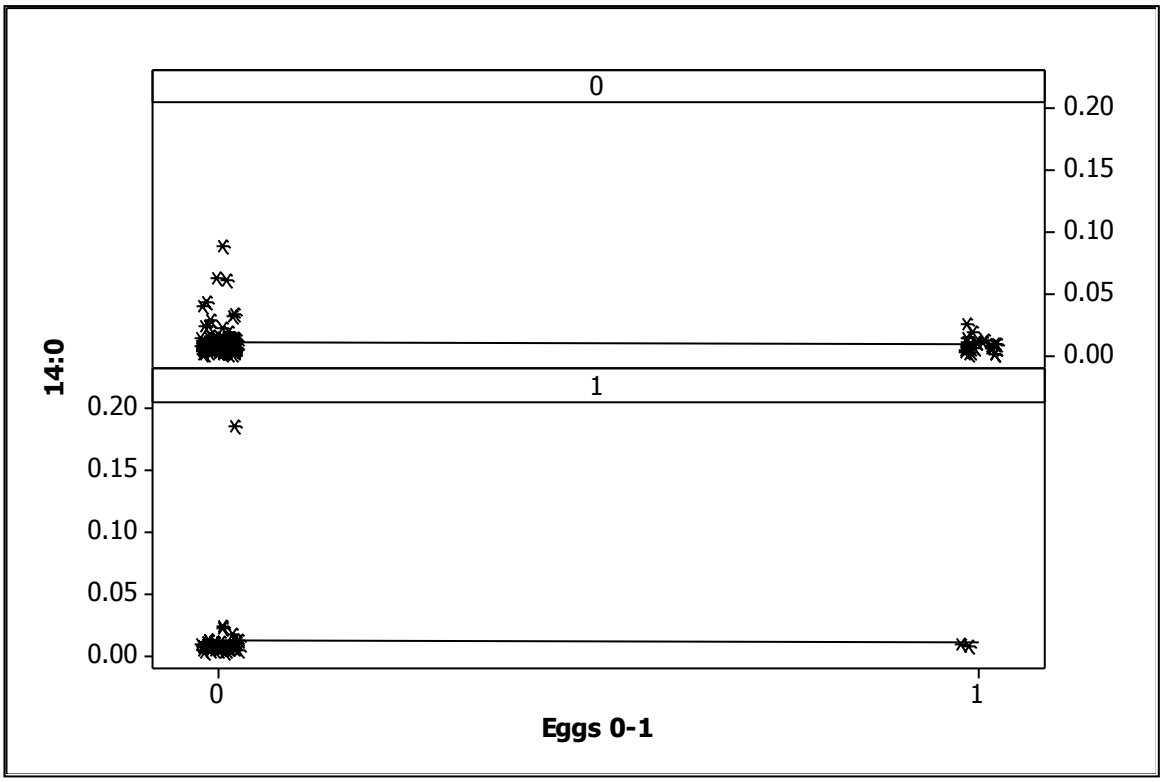

Figure 9.35. Scatterplot of eggs (0-1) *versus* 14:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

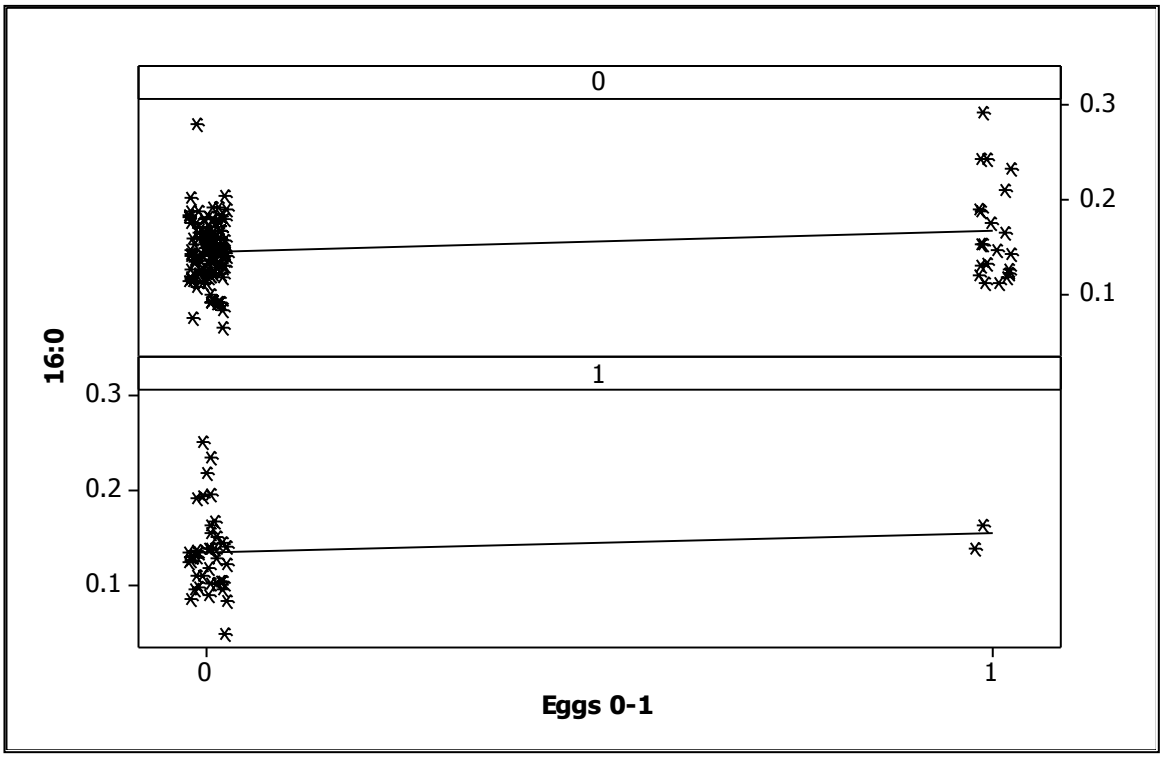

Figure 9.36. Scatterplot of eggs (0-1) *versus* 16:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

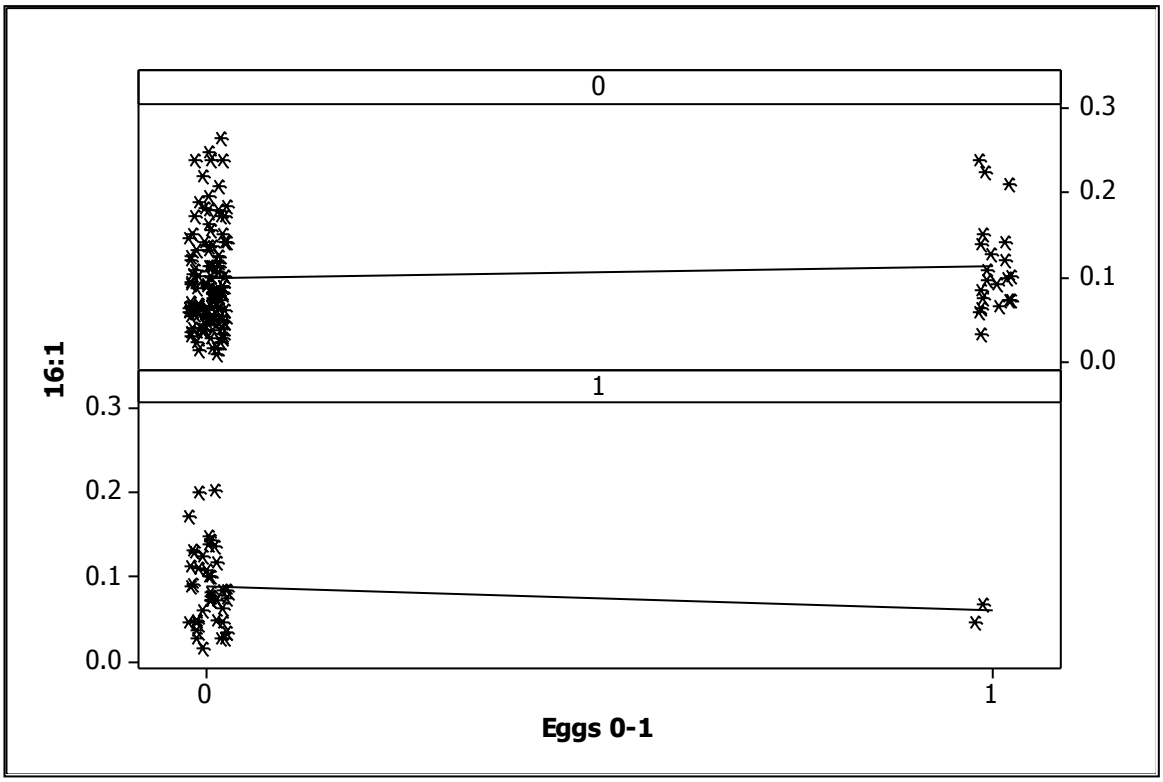

Figure 9.37. Scatterplot of eggs (0-1) *versus* 16:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

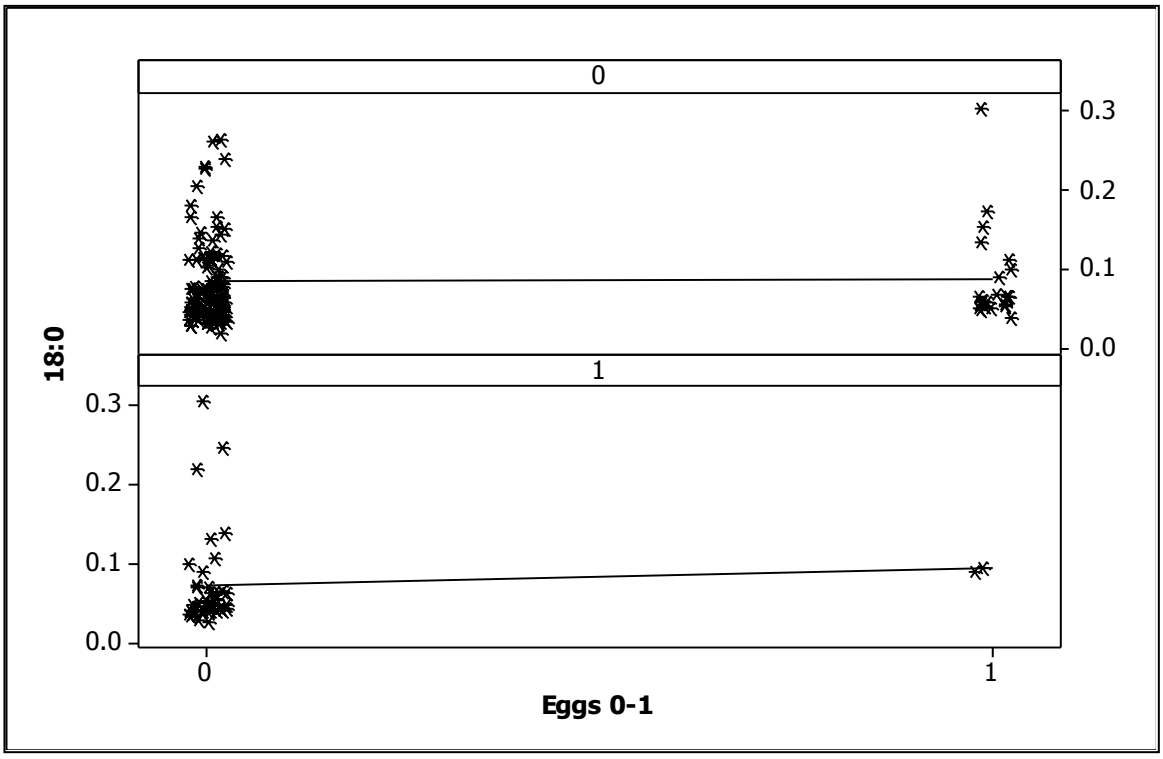

Figure 9.38. Scatterplot of eggs (0-1) *versus* 18:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

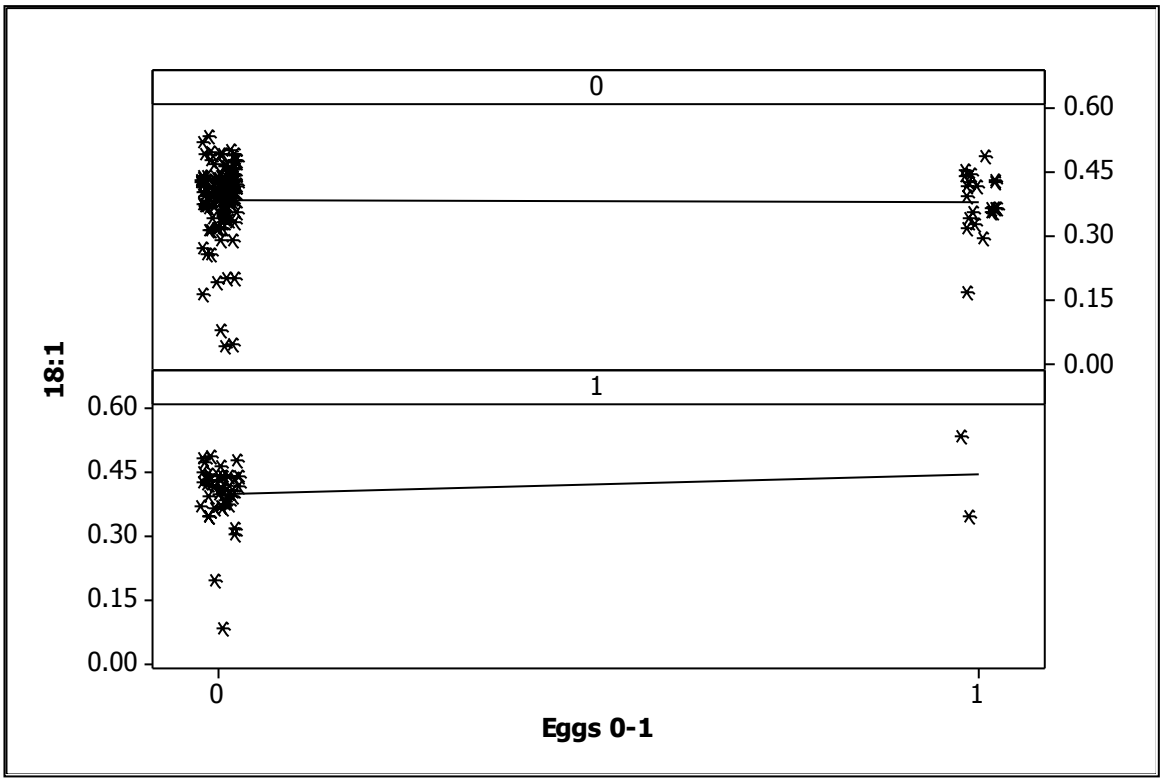

Figure 9.39. Scatterplot of eggs (0-1) *versus* 18:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

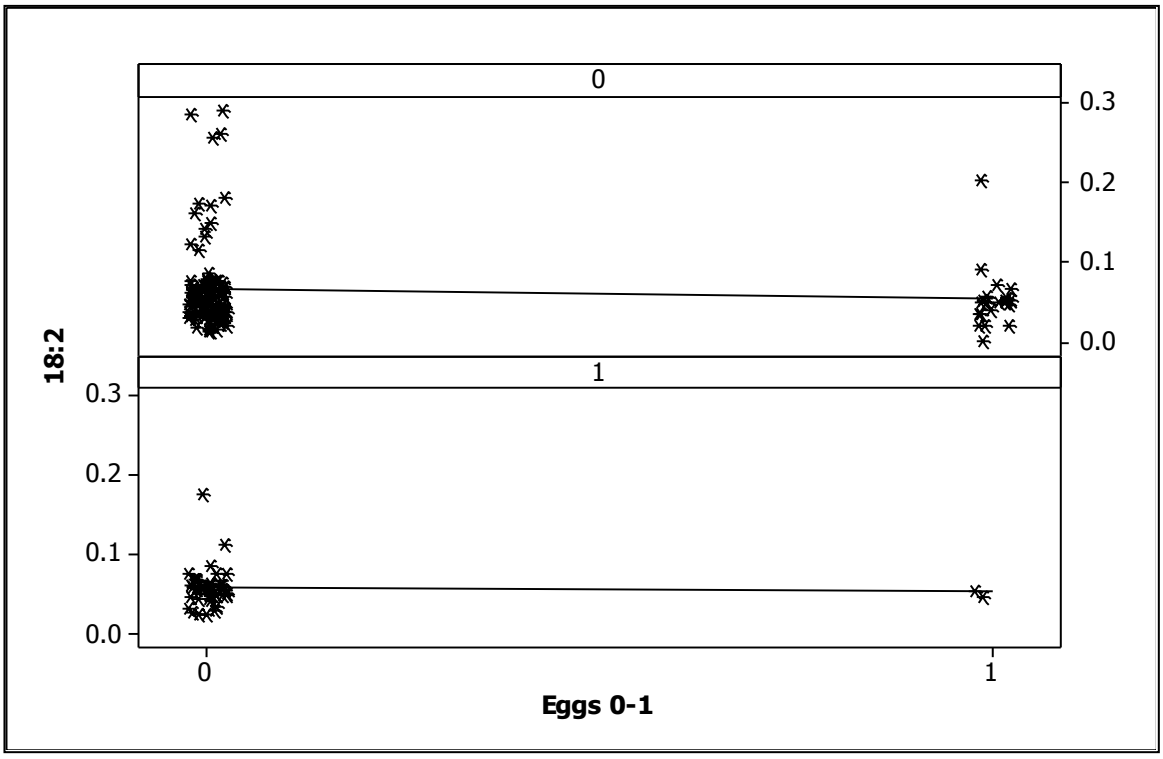

Figure 9.40. Scatterplot of eggs (0-1) *versus* 18:2 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

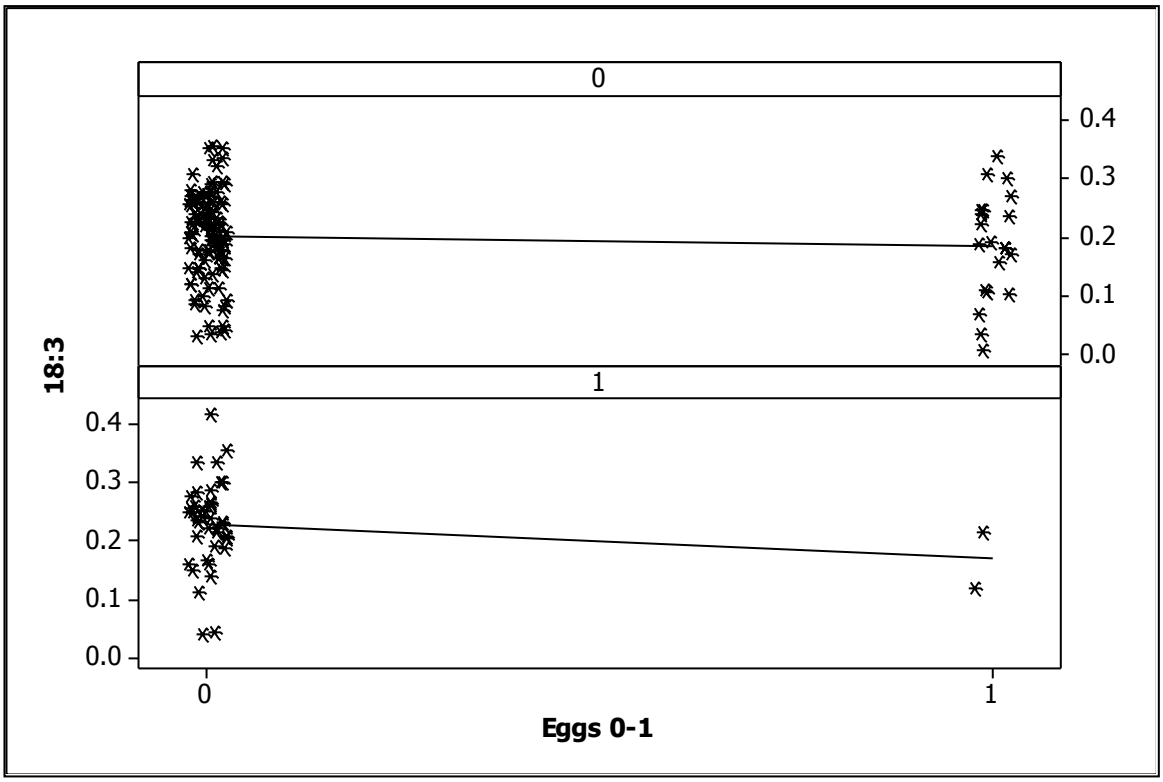

Figure 9.41. Scatterplot of eggs (0-1) *versus* 18:3 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

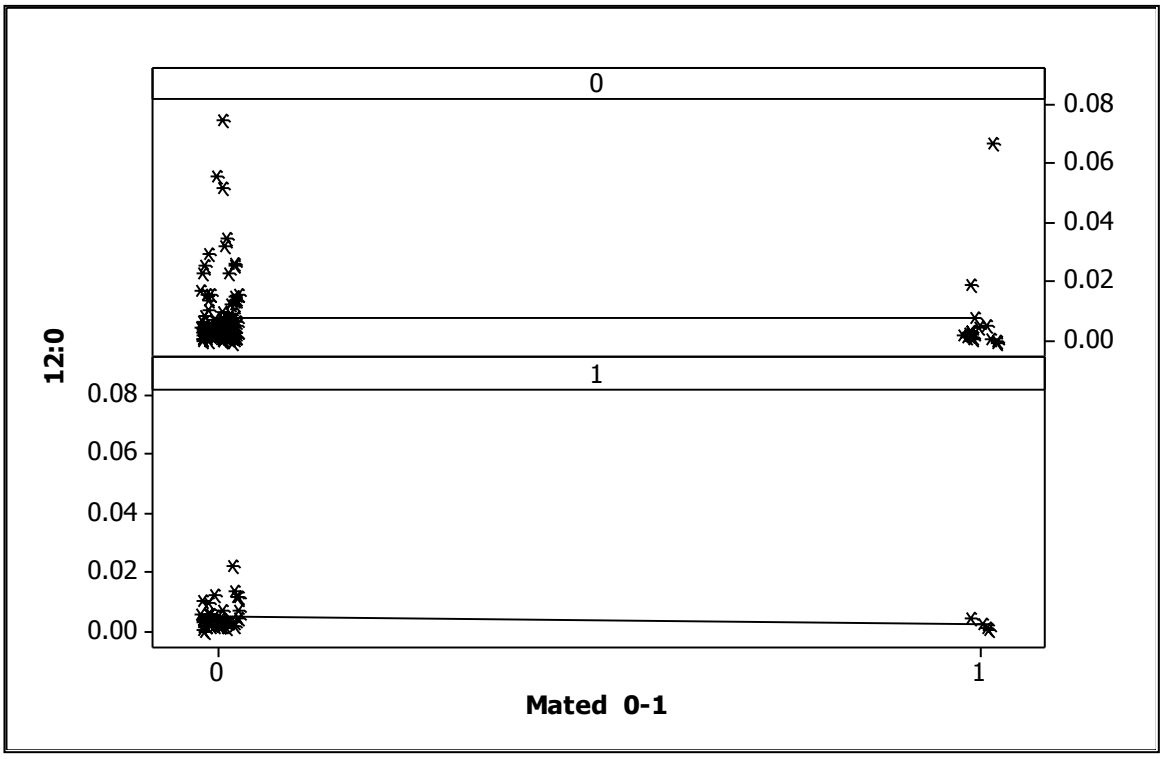

Figure 9.42. Scatterplot of mated (0-1) *versus* 12:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

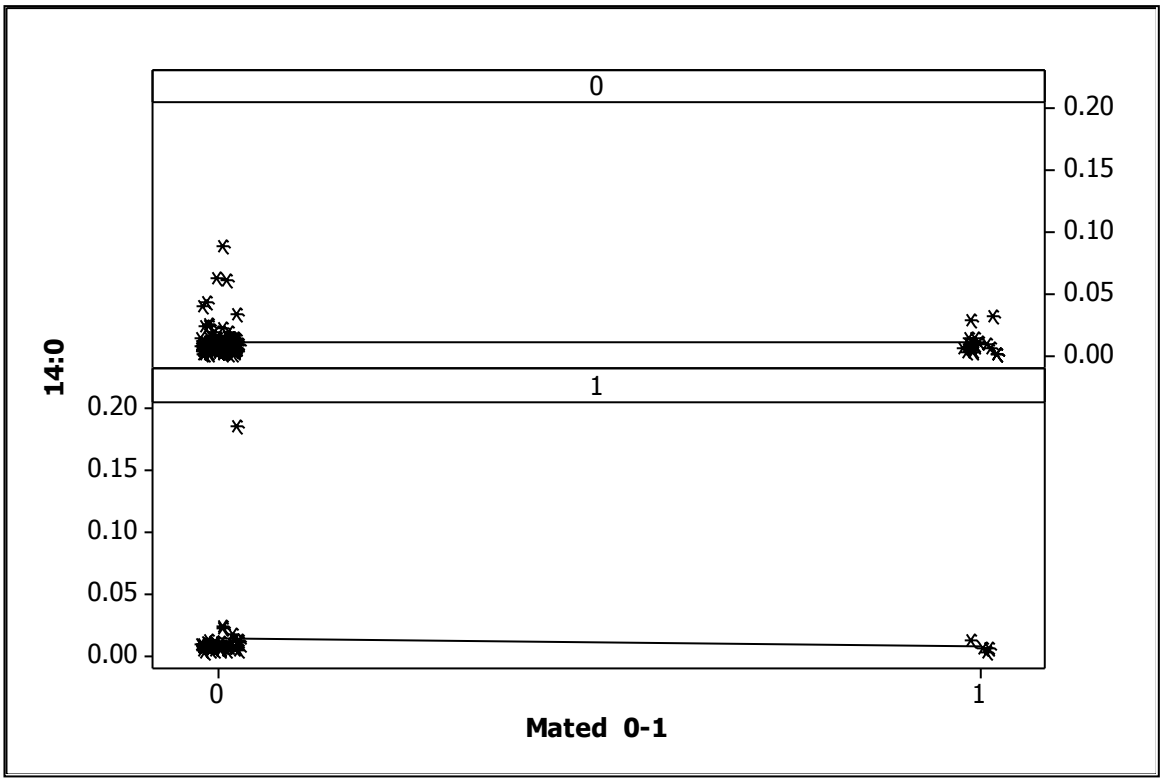

Figure 9.43. Scatterplot of mated (0-1) *versus* 14:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

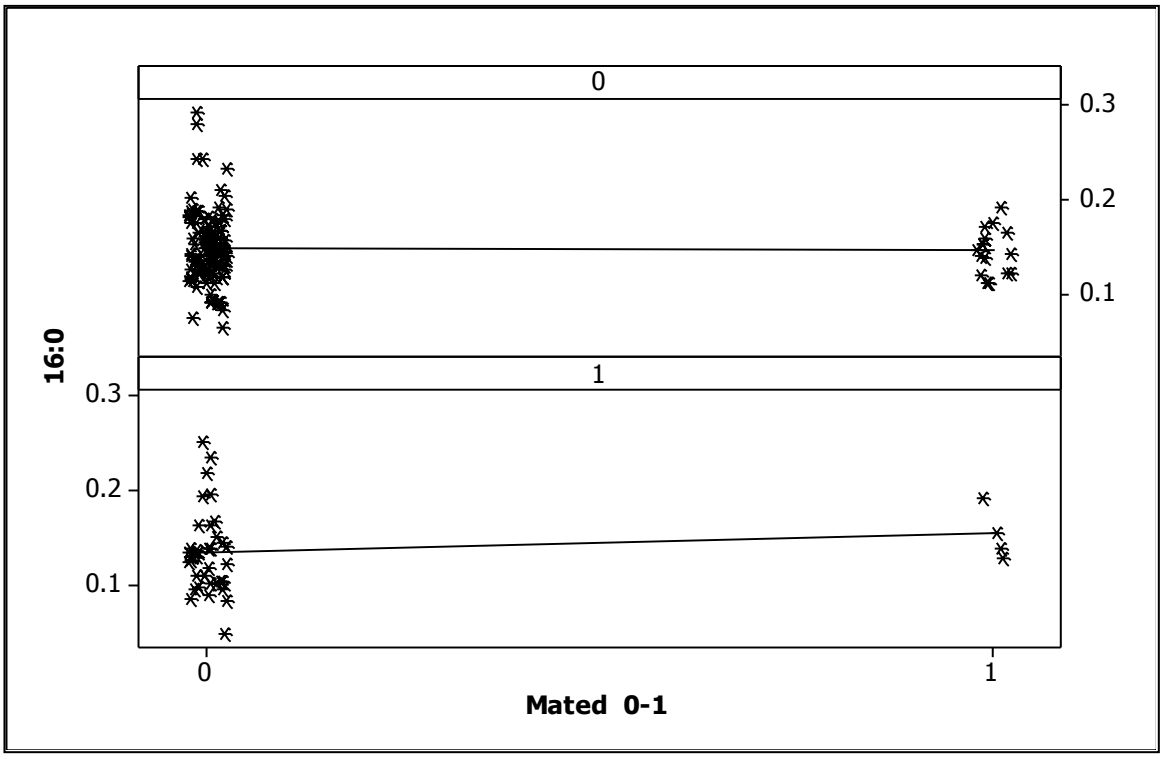

Figure 9.44. Scatterplot of mated (0-1) *versus* 16:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

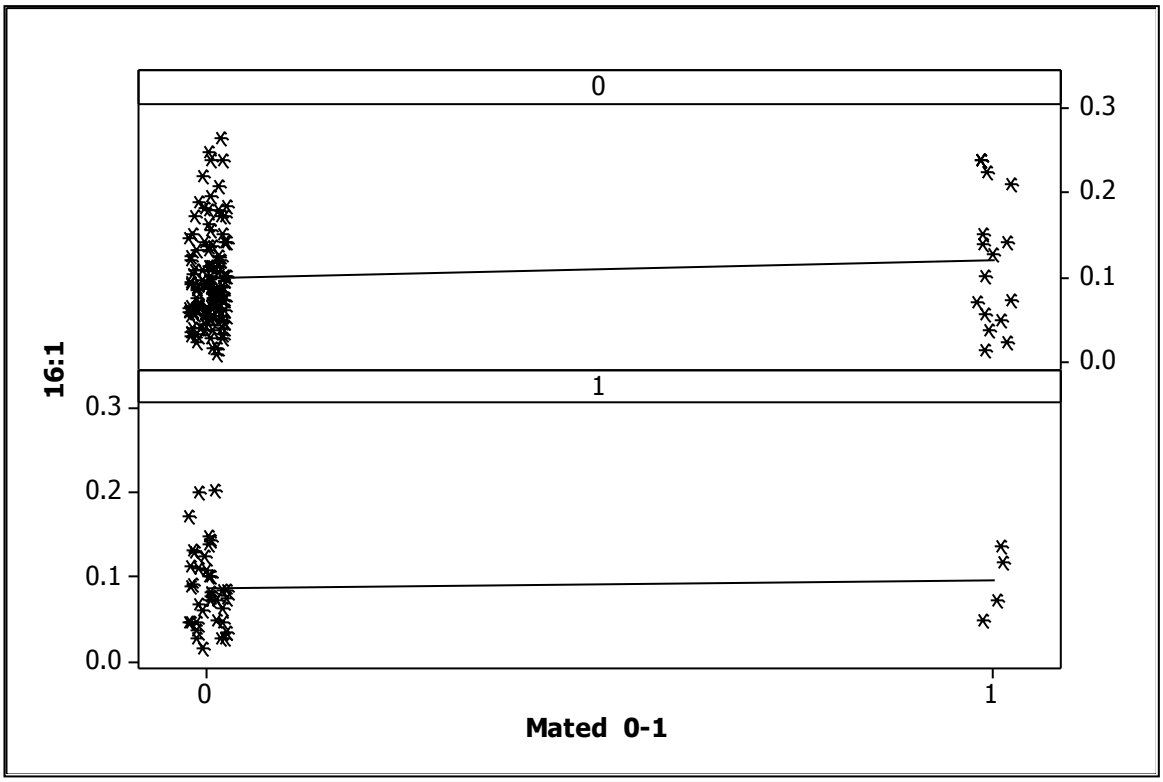

Figure 9.45. Scatterplot of mated (0-1) *versus* 16:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

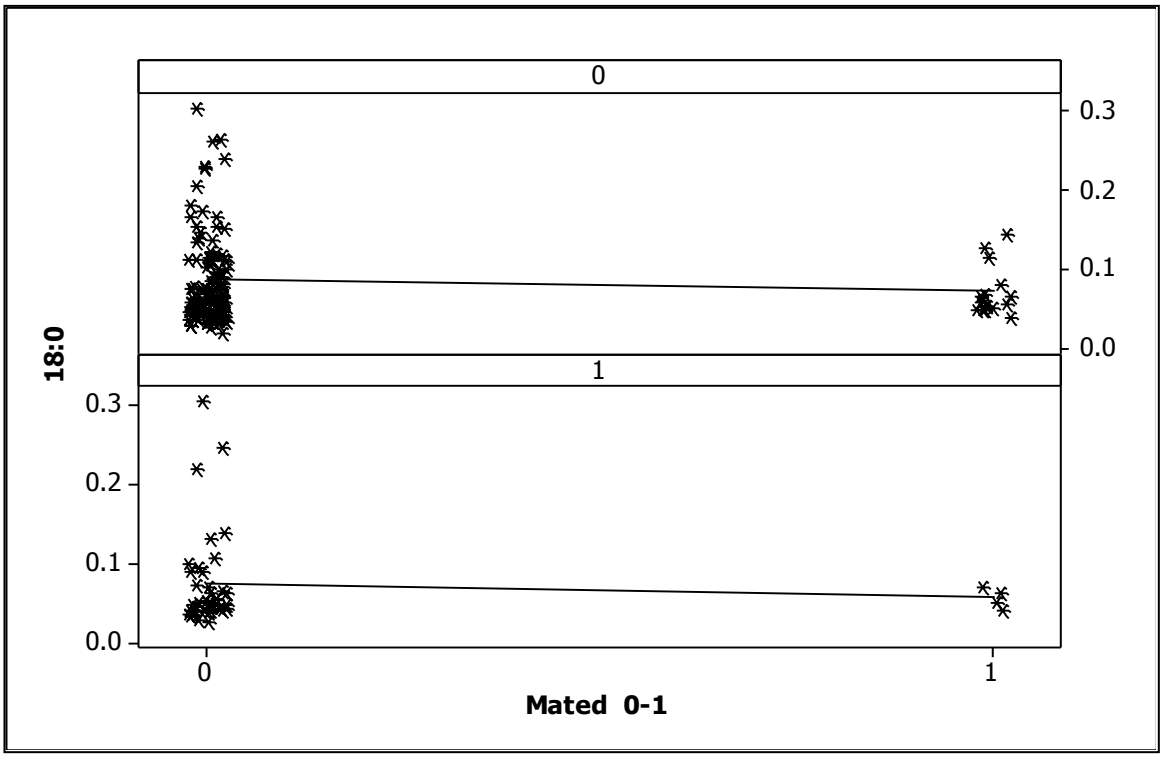

Figure 9.46. Scatterplot of mated (0-1) *versus* 18:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

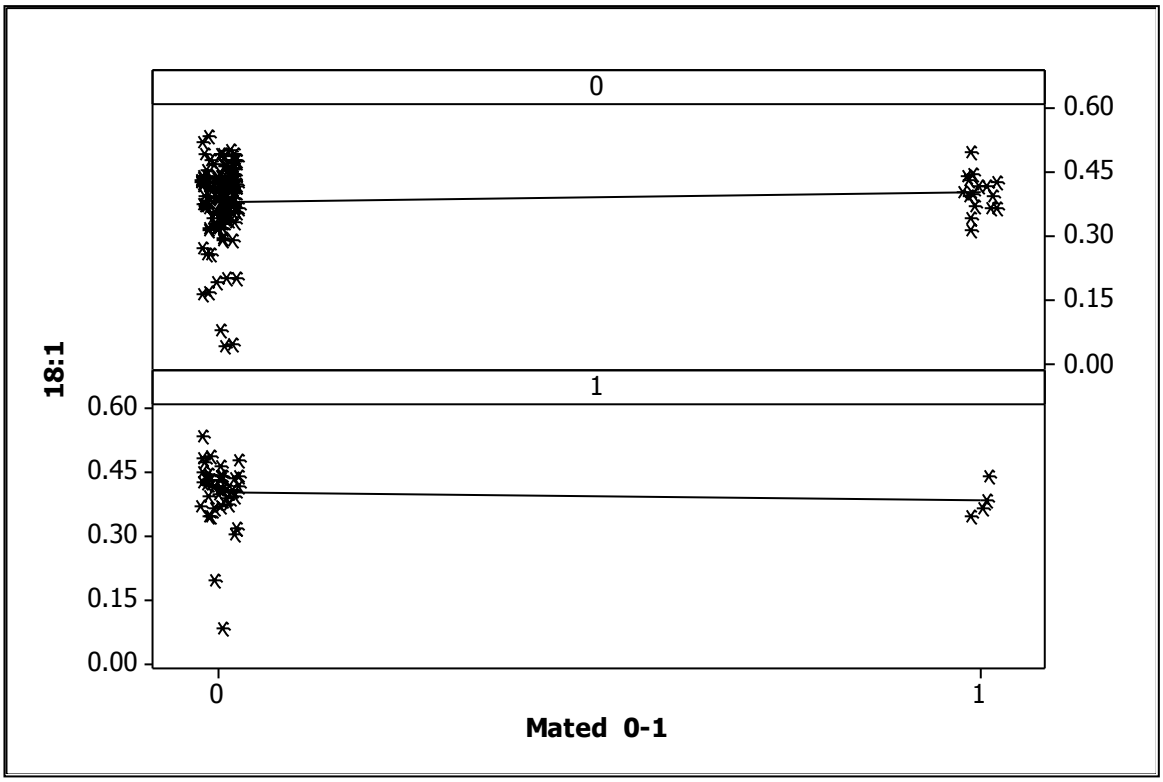

Figure 9.47. Scatterplot of mated (0-1) *versus* 18:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

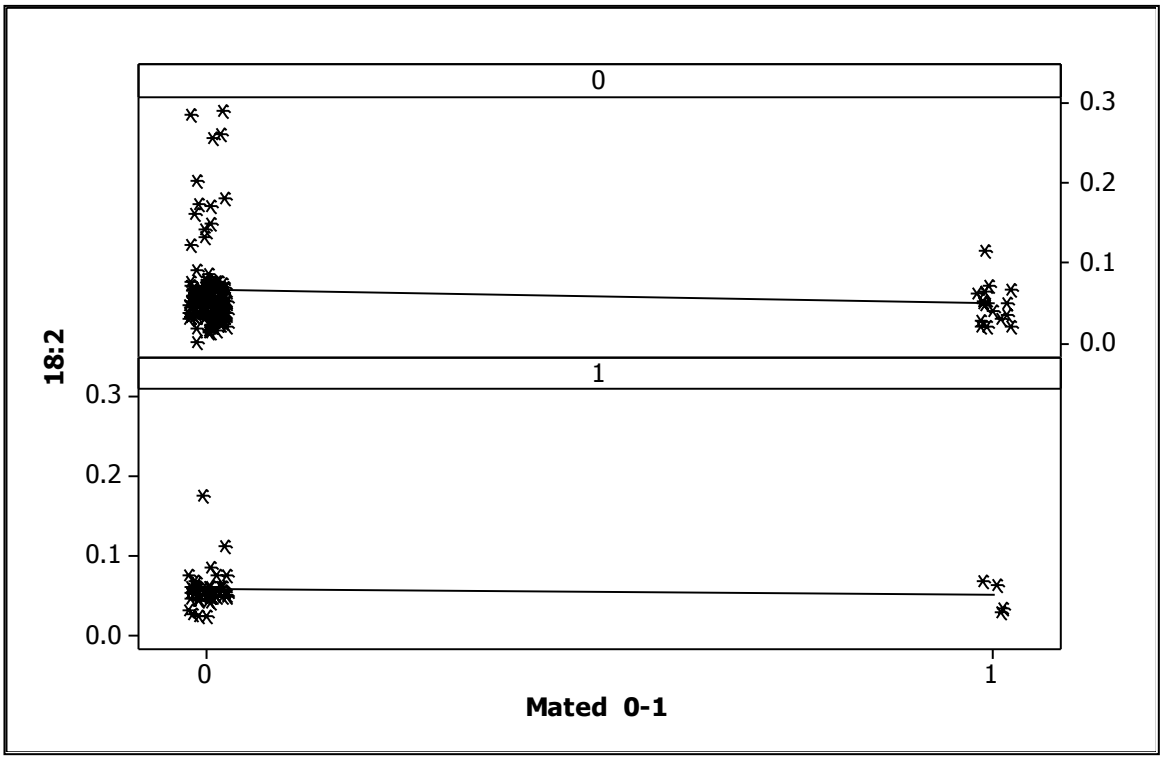

Figure 9.48. Scatterplot of mated (0-1) *versus* 18:2 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

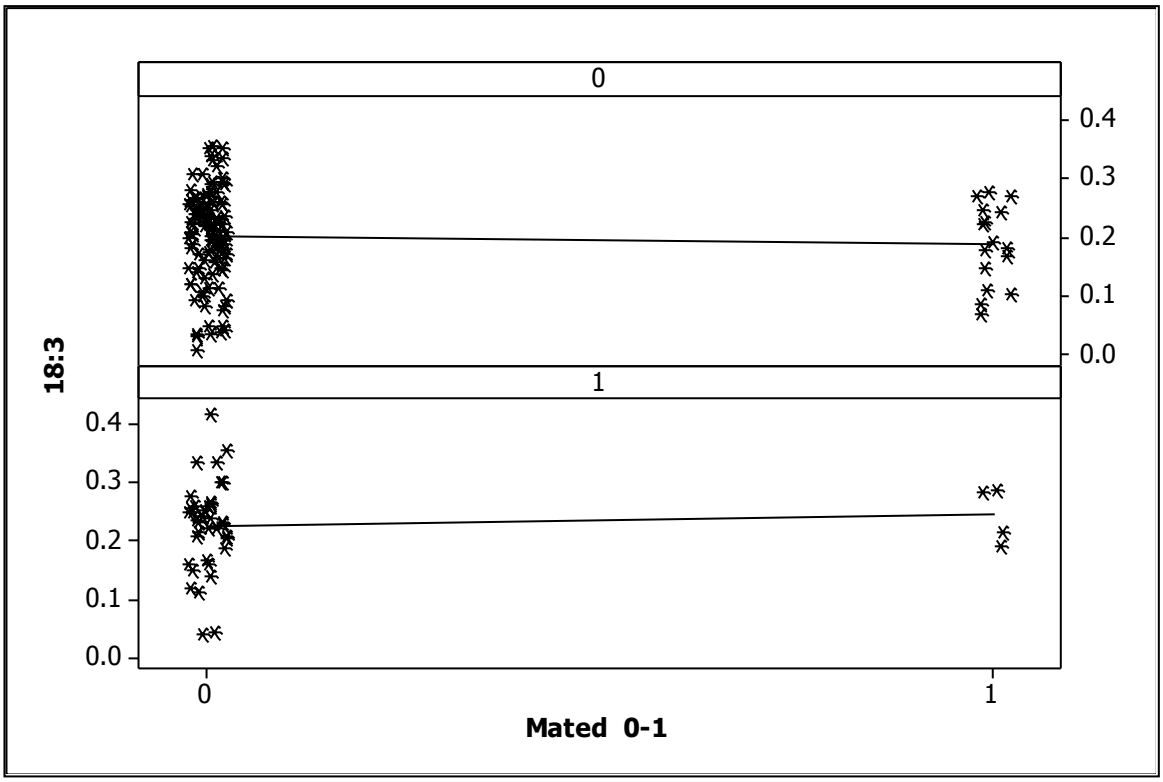

Figure 9.49. Scatterplot of mated (0-1) *versus* 18:3 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

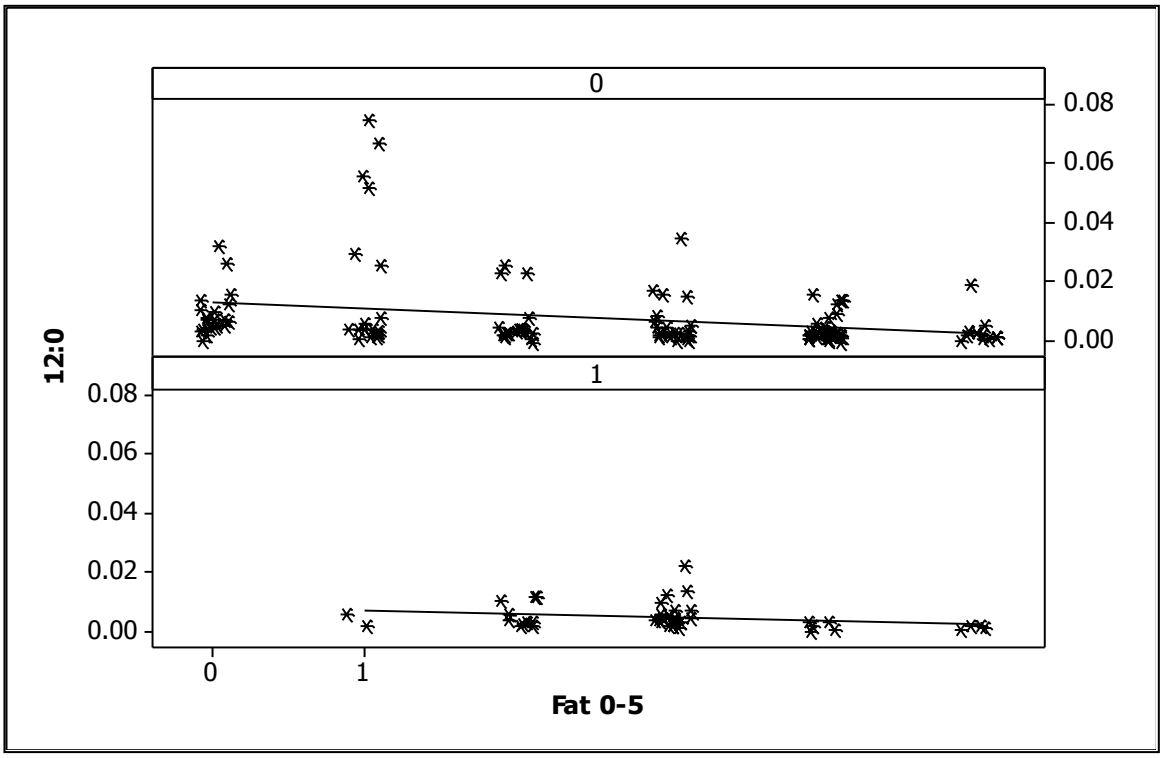

Figure 9.50. Scatterplot of fat (0-5) *versus* 12:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

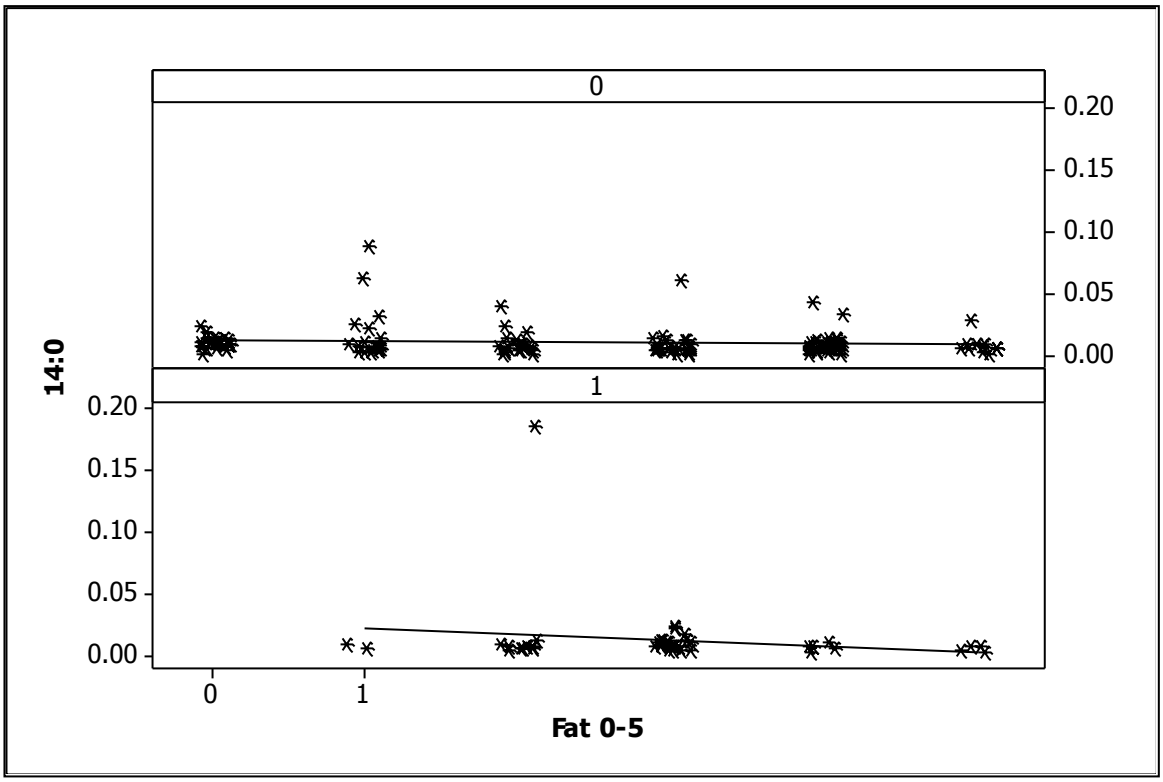

Figure 9.51. Scatterplot of fat (0-5) *versus* 14:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

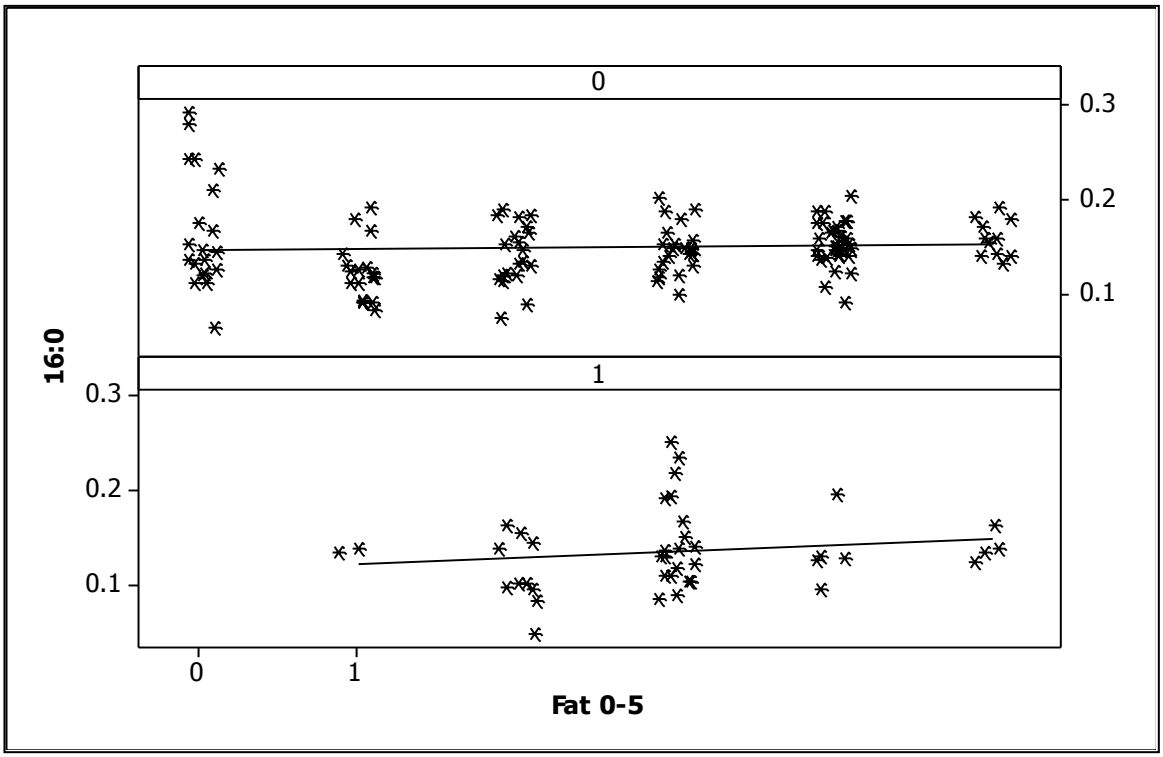

Figure 9.52. Scatterplot of fat (0-5) *versus* 16:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

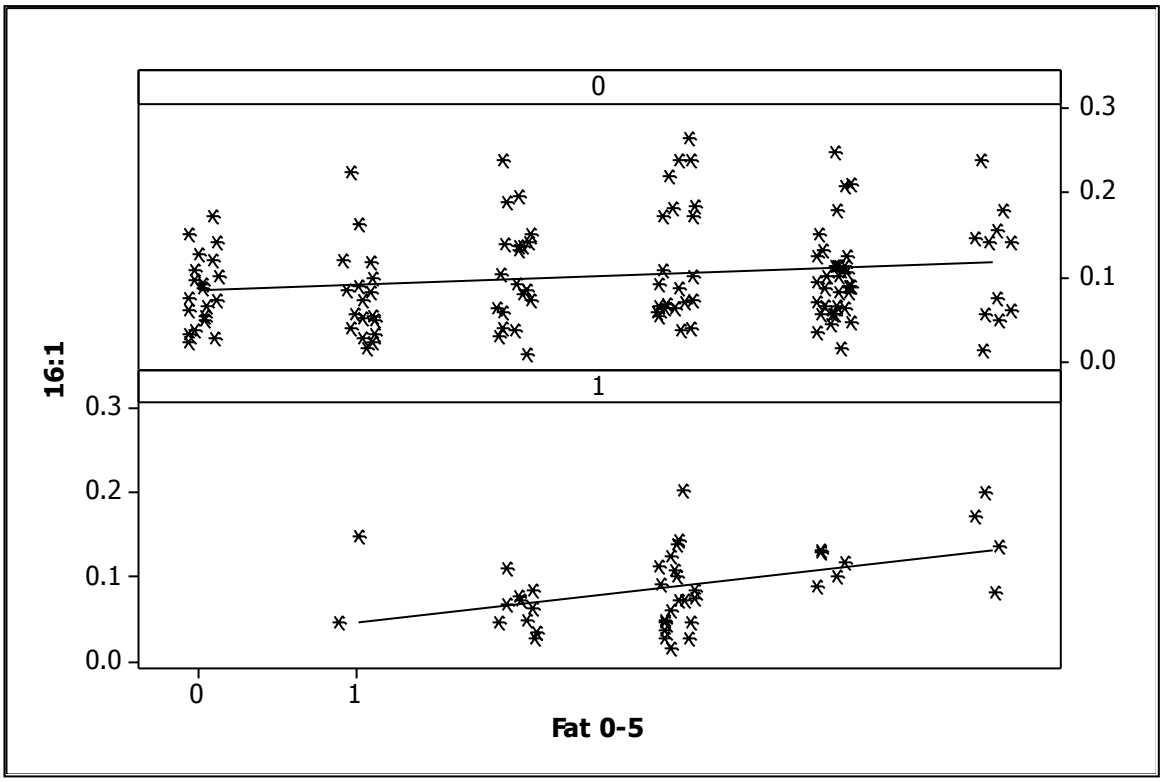

Figure 9.53. Scatterplot of fat (0-5) *versus* 16:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

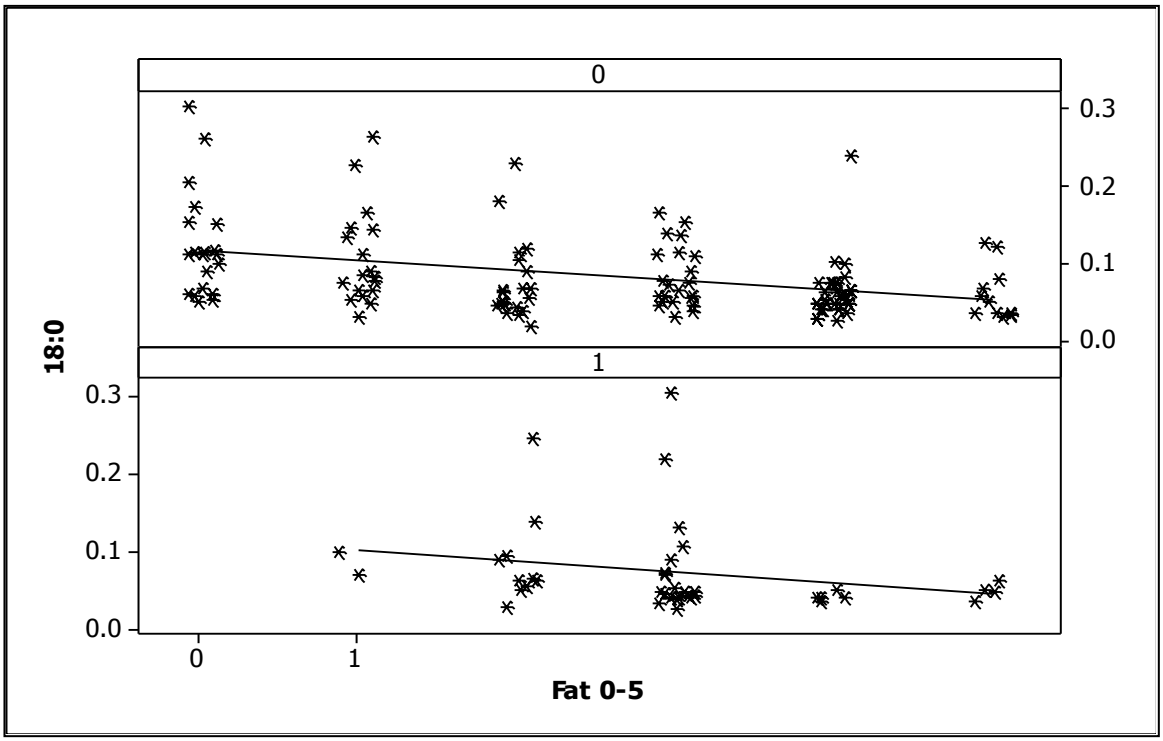

Figure 9.54. Scatterplot of fat (0-5) *versus* 18:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

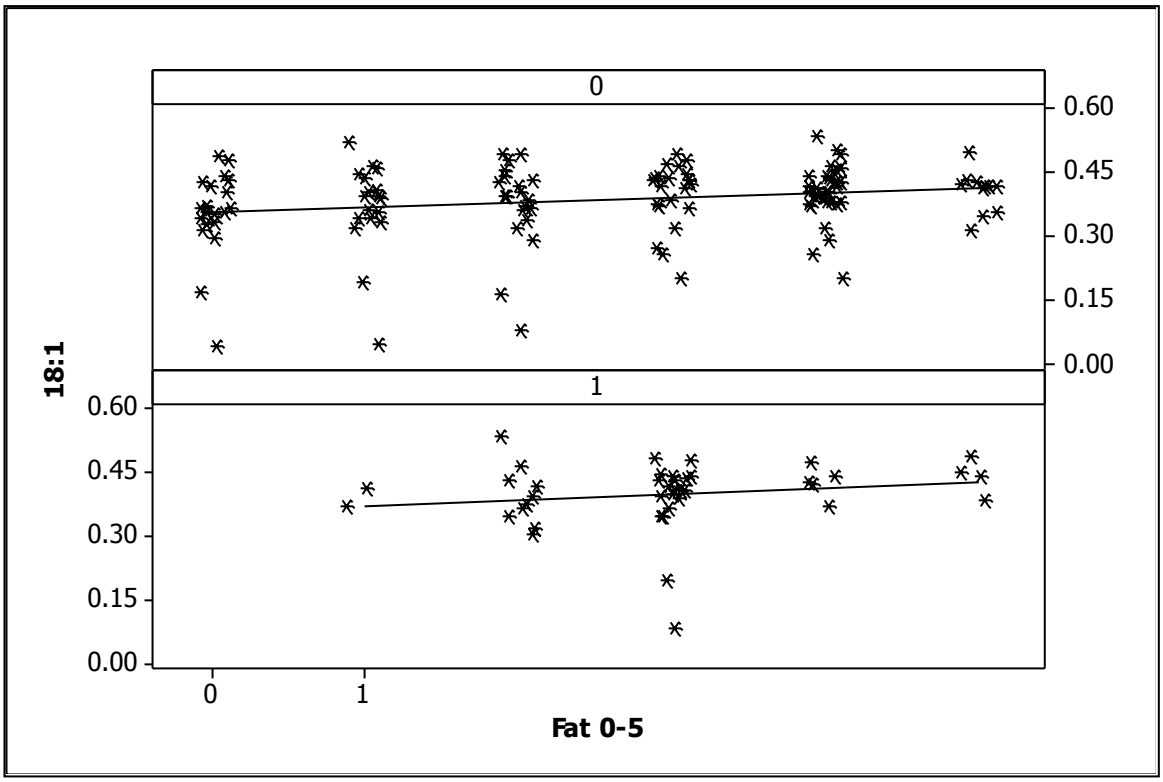

Figure 9.55. Scatterplot of fat (0-5) *versus* 18:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

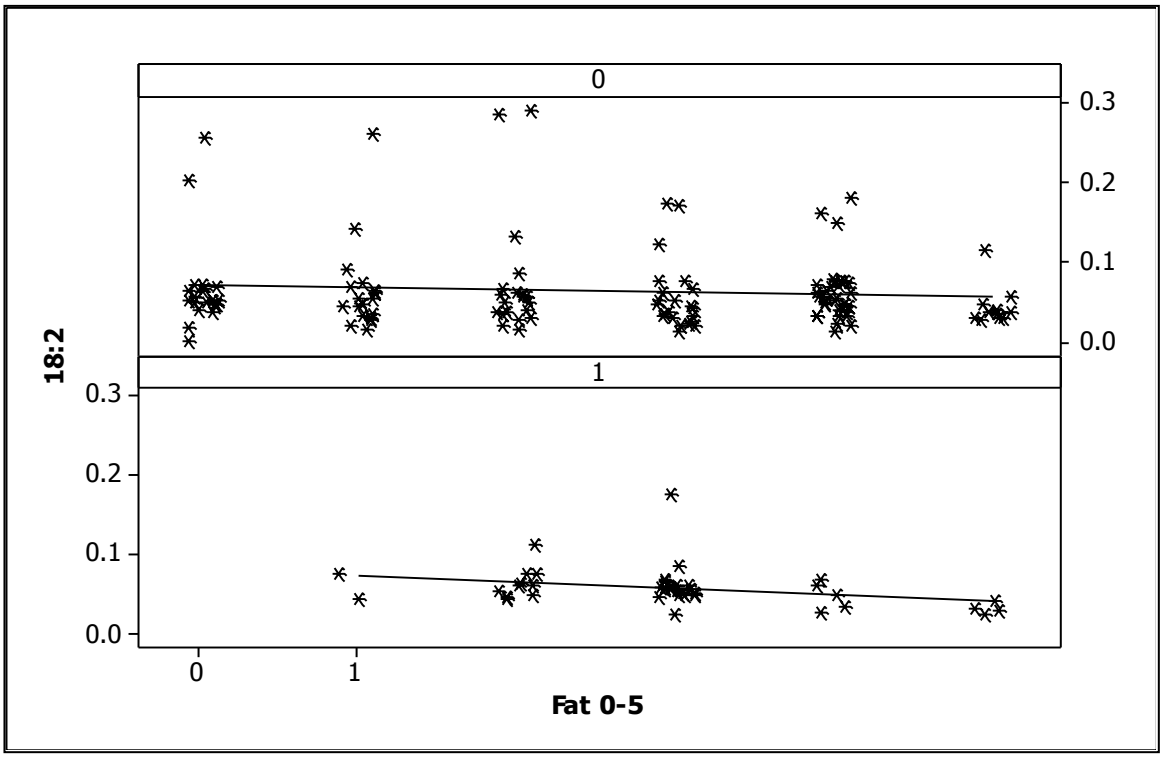

Figure 9.56. Scatterplot of fat (0-5) *versus* 18:2 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

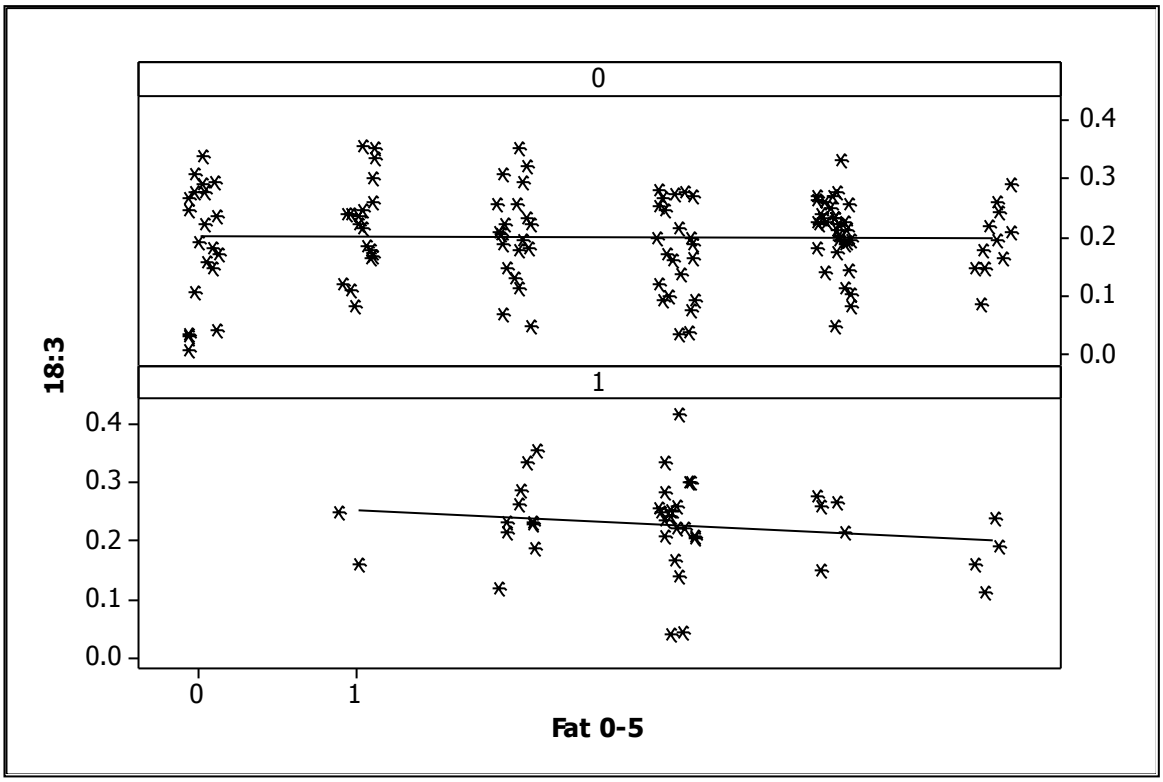

Figure 9.57. Scatterplot of fat (0-5) *versus* 18:3 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

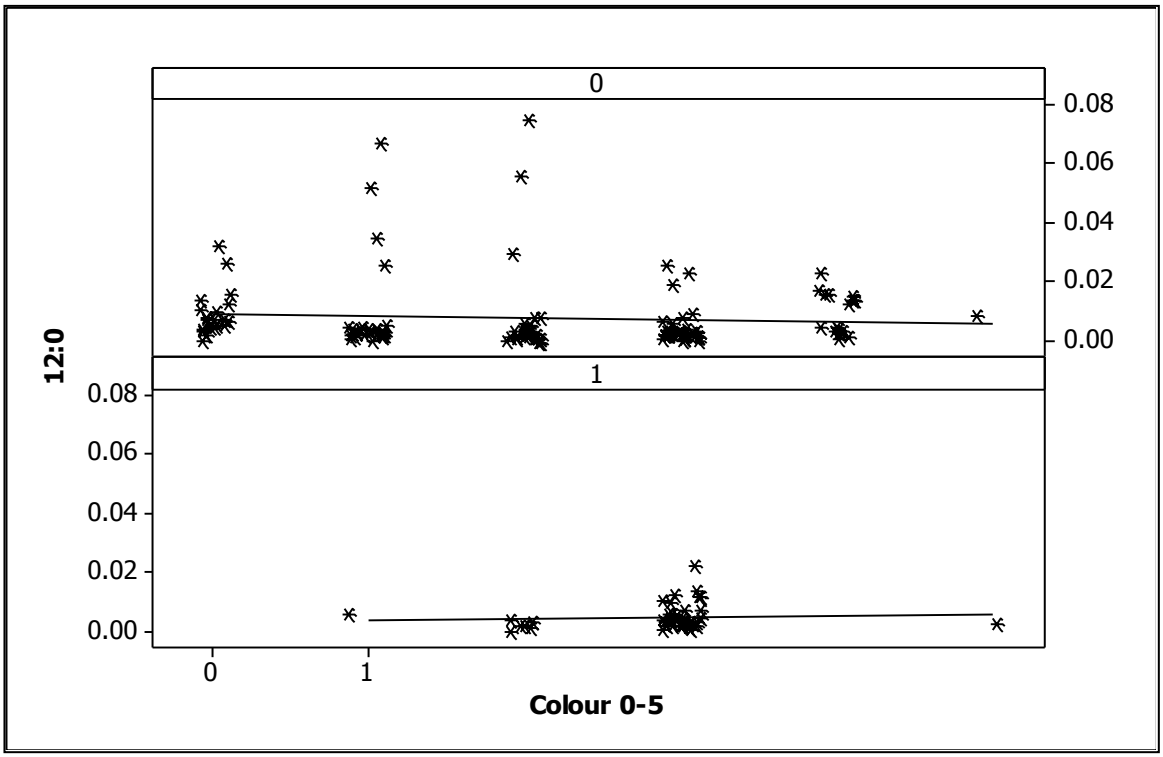

Figure 9.58. Scatterplot of colour (0-5) *versus* 12:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

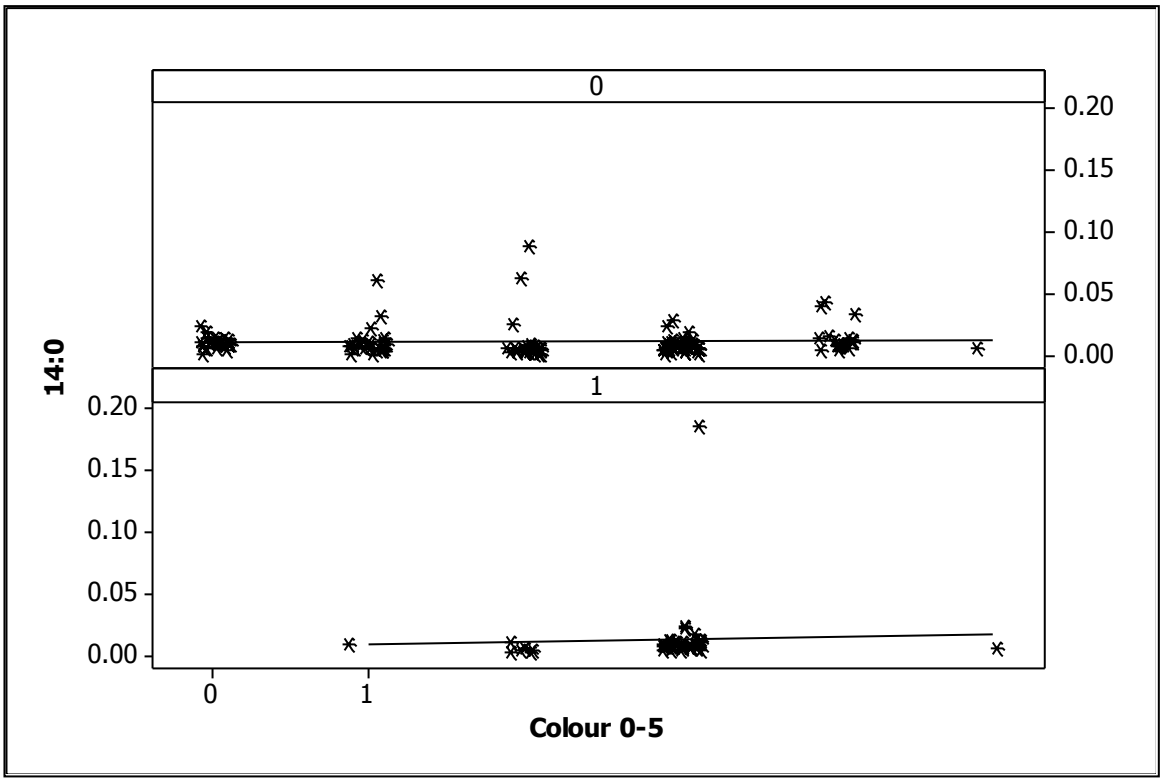

Figure 9.59. Scatterplot of colour (0-5) *versus* 14:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

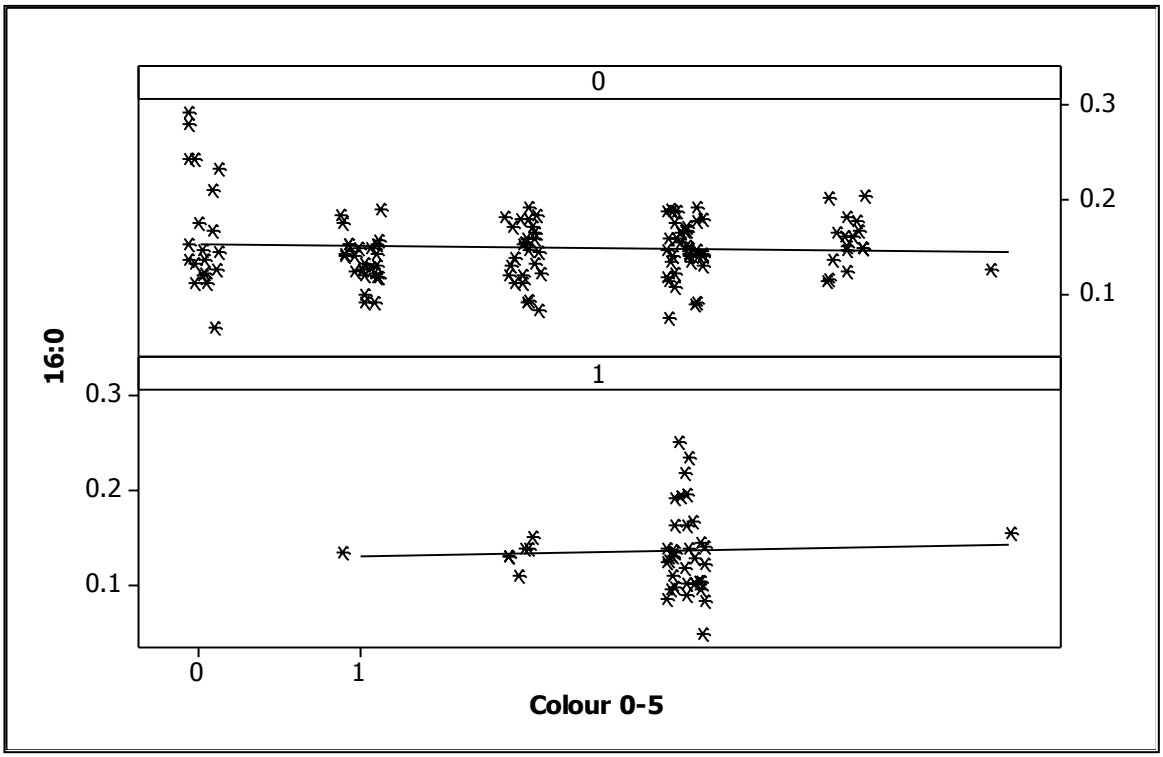

Figure 9.60. Scatterplot of colour (0-5) *versus* 16:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

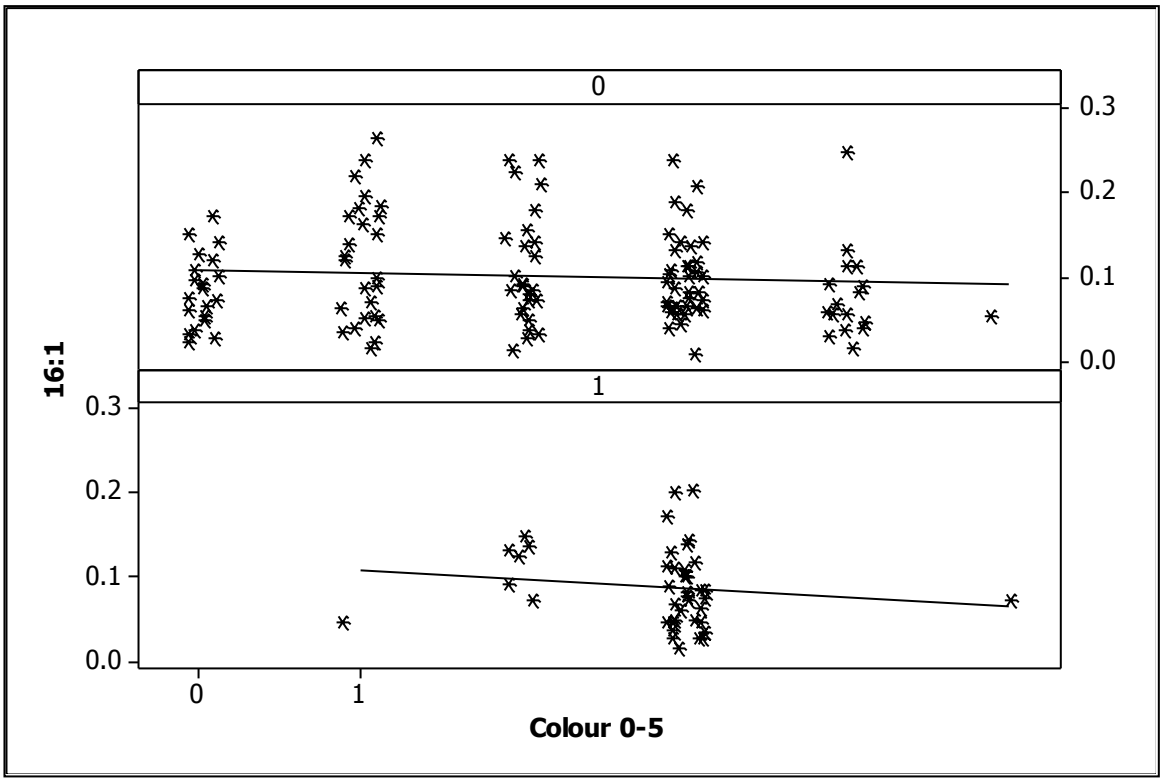

Figure 9.61. Scatterplot of colour (0-5) *versus* 16:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

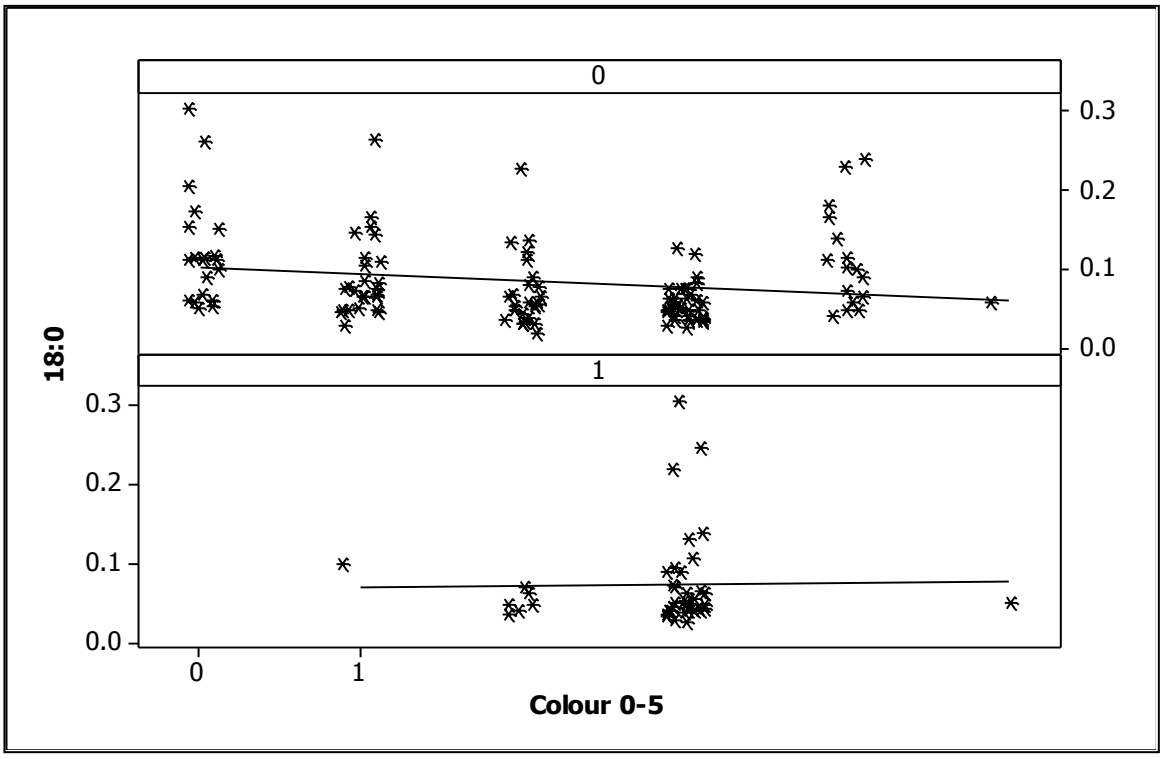

Figure 9.62. Scatterplot of colour (0-5) *versus* 18:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

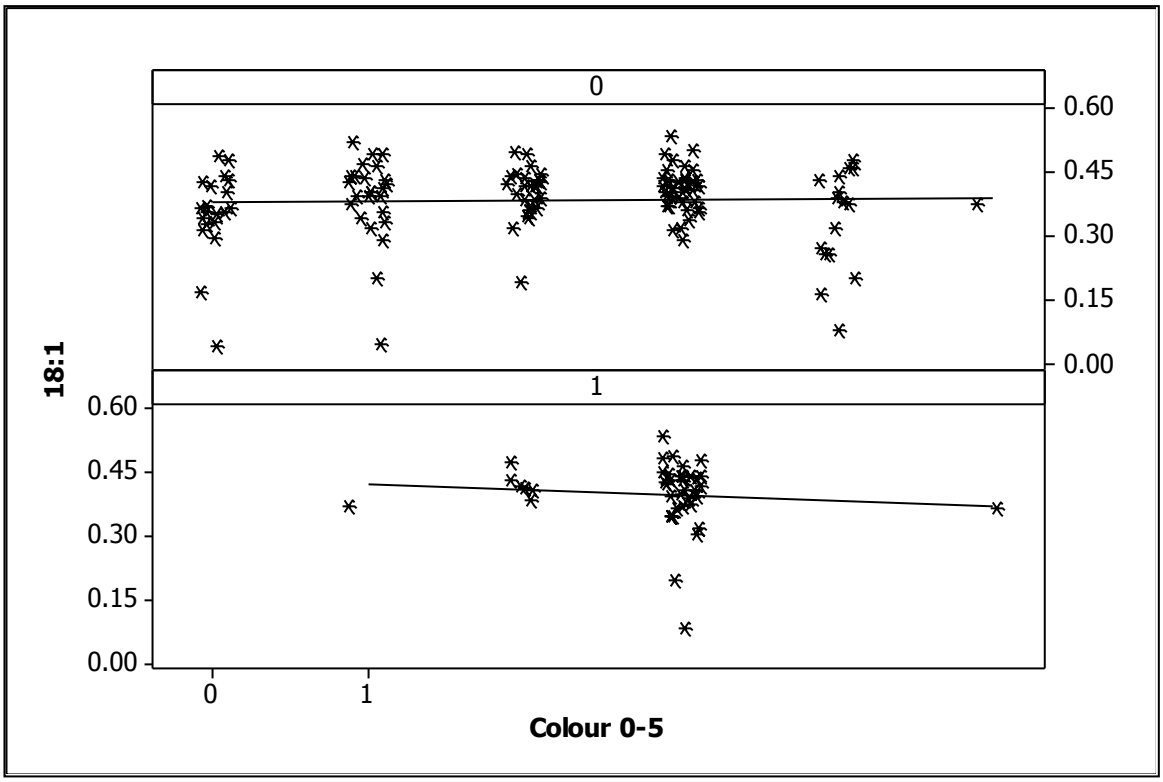

Figure 9.63. Scatterplot of colour (0-5) *versus* 18:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

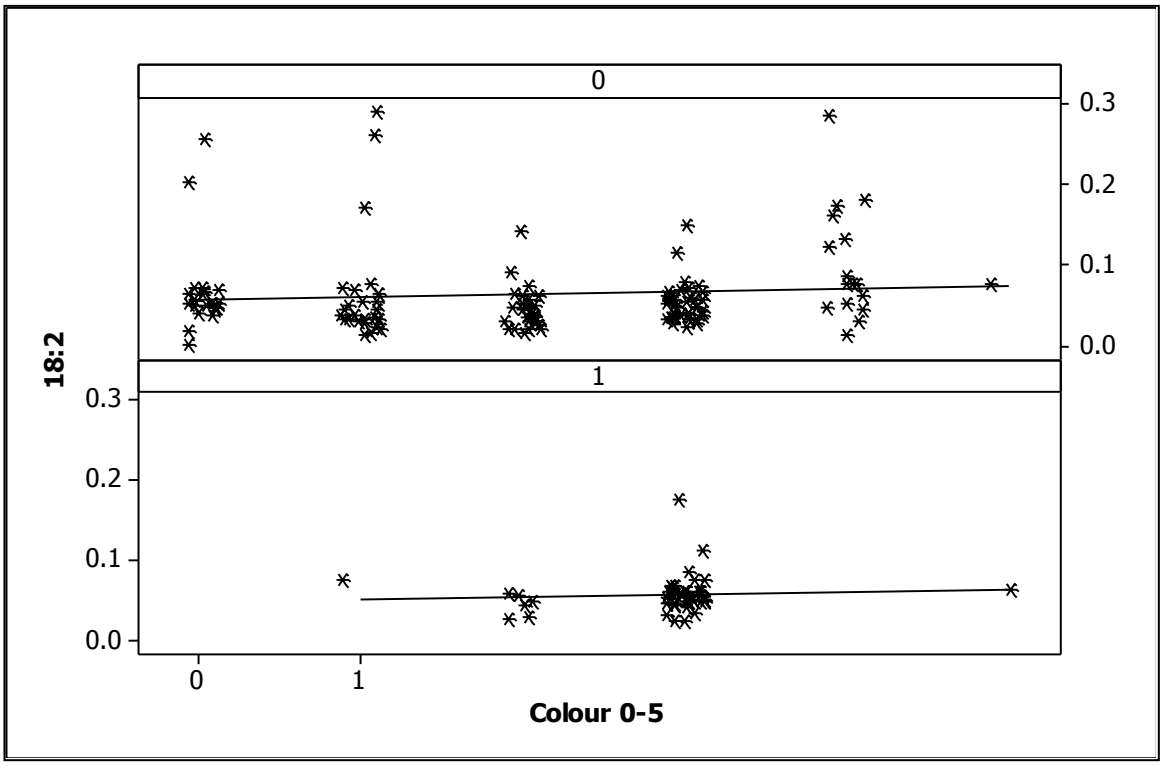

Figure 9.64. Scatterplot of colour (0-5) *versus* 18:2 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

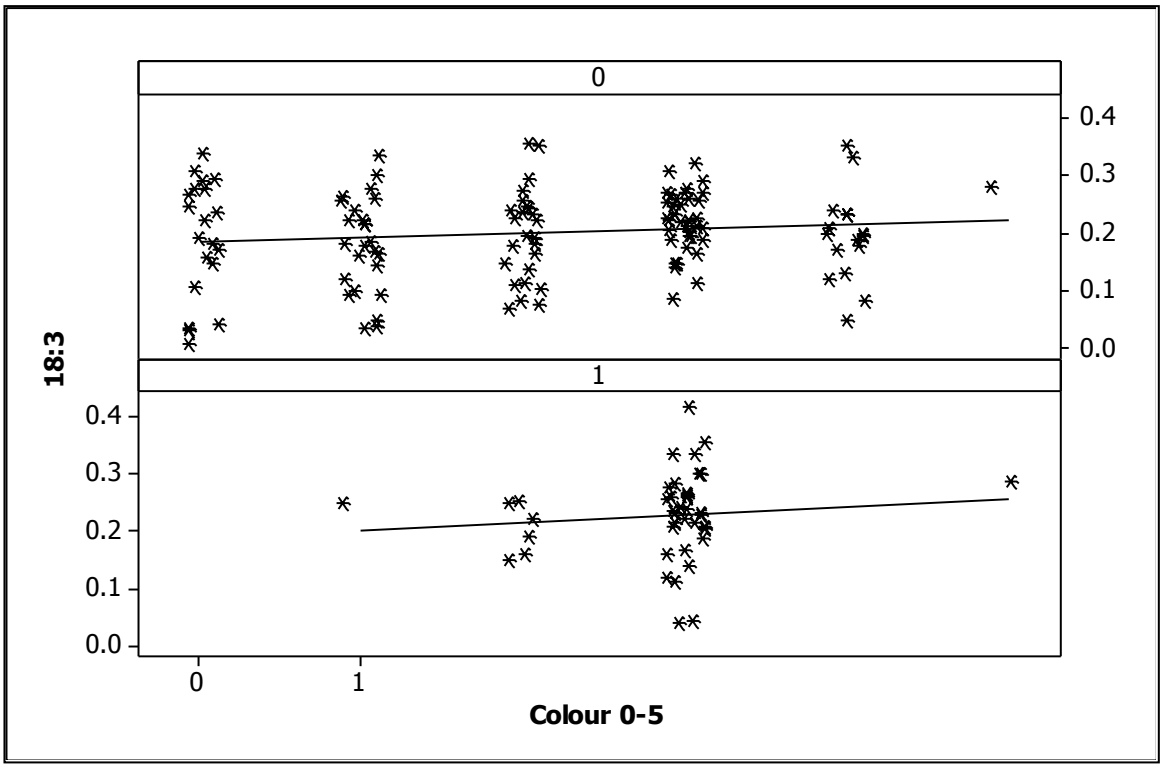

Figure 9.65. Scatterplot of colour (0-5) *versus* 18:3 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

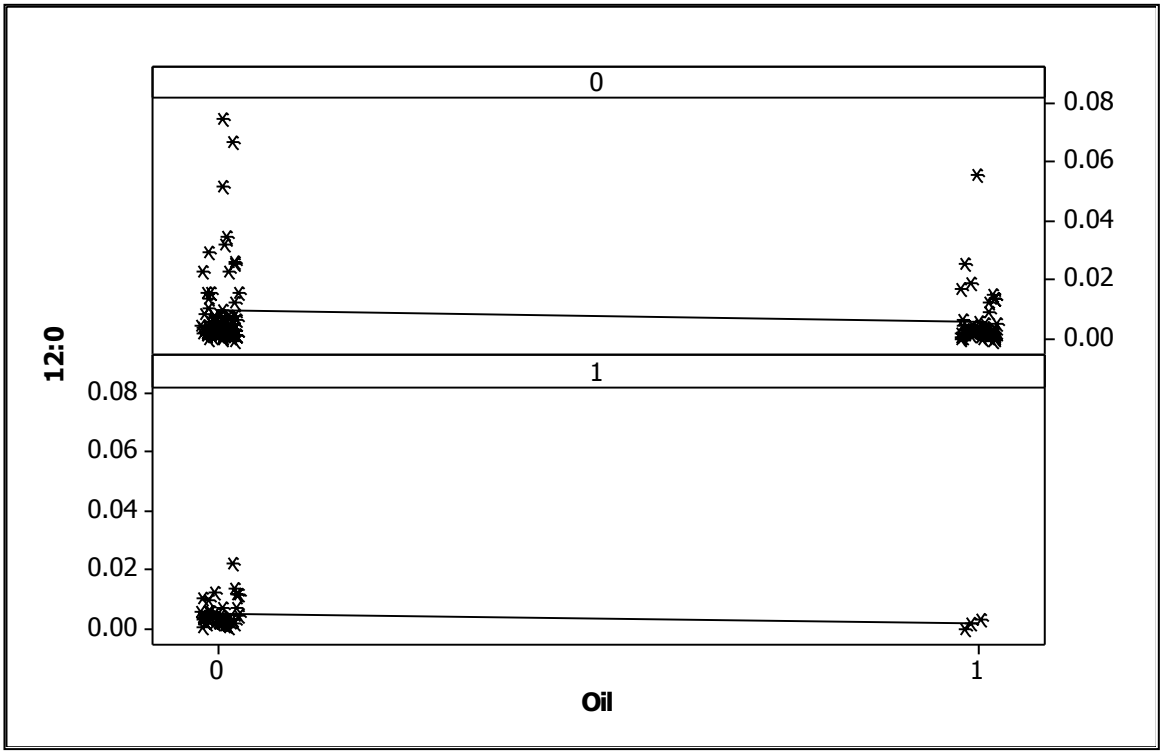

Figure 9.66. Scatterplot of oil (0-1) *versus* 12:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

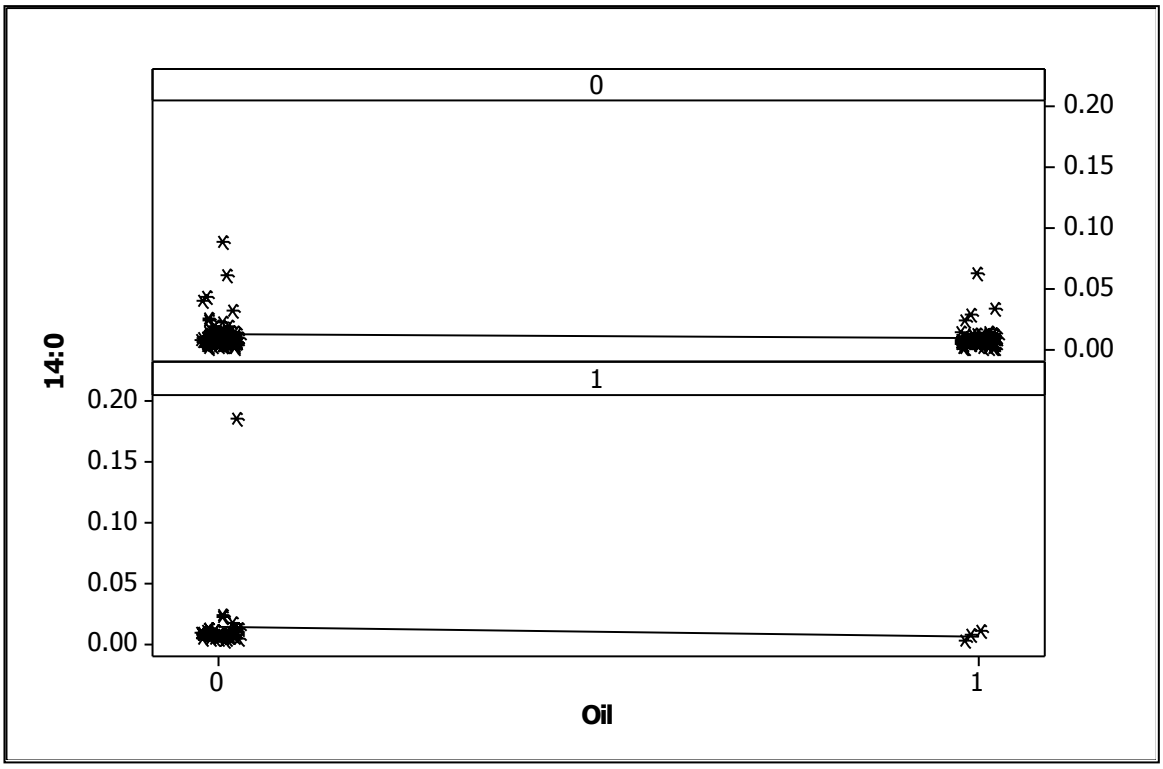

Figure 9.67. Scatterplot of oil (0-1) *versus* 14:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

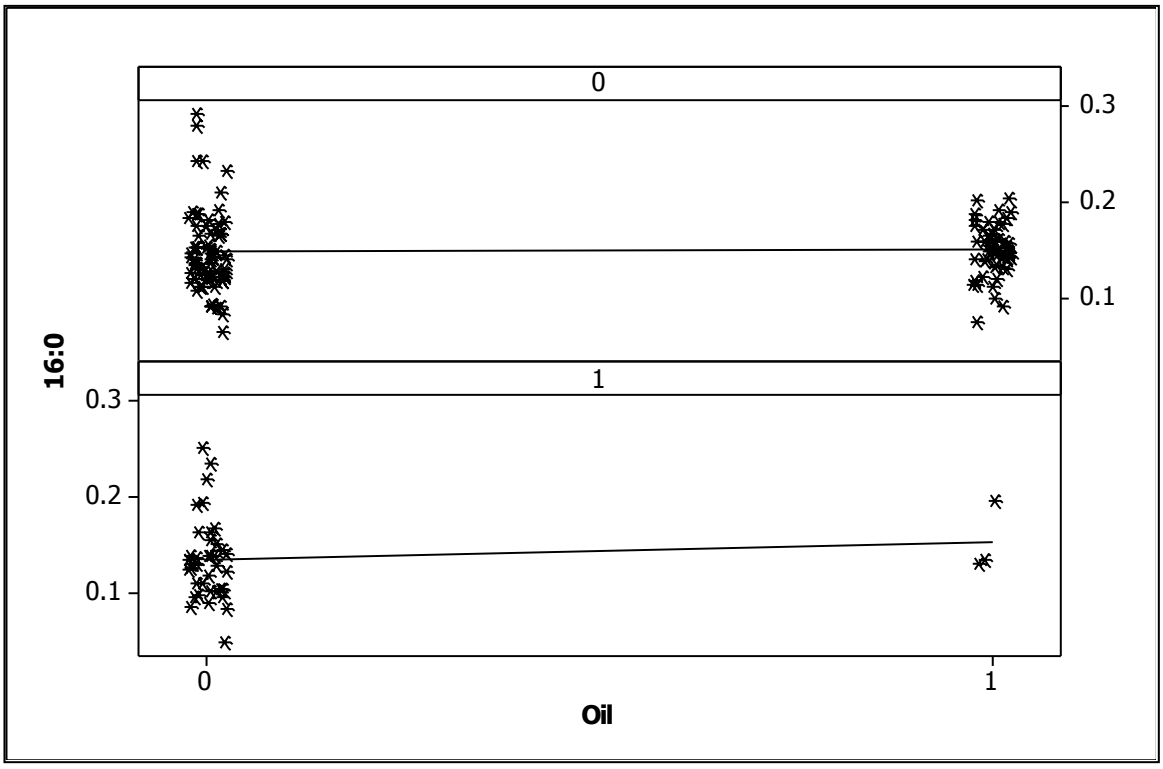

Figure 9.68. Scatterplot of oil (0-1) *versus* 16:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

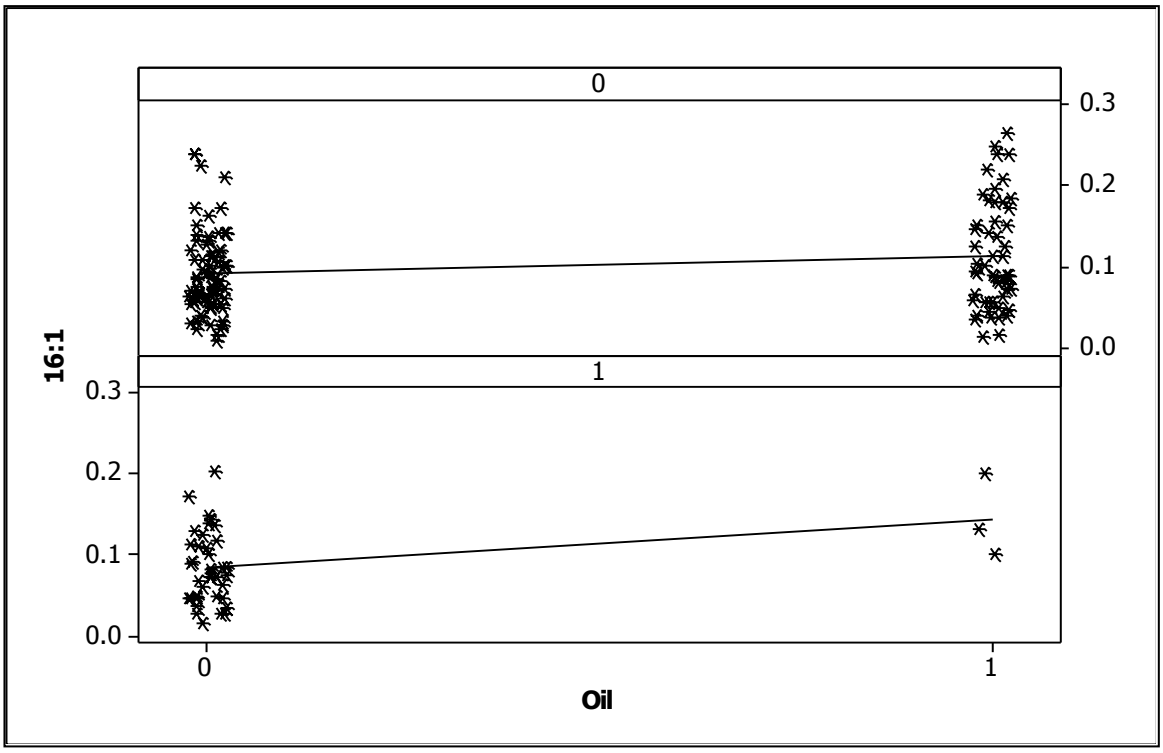

Figure 9.69. Scatterplot of oil (0-1) *versus* 16:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

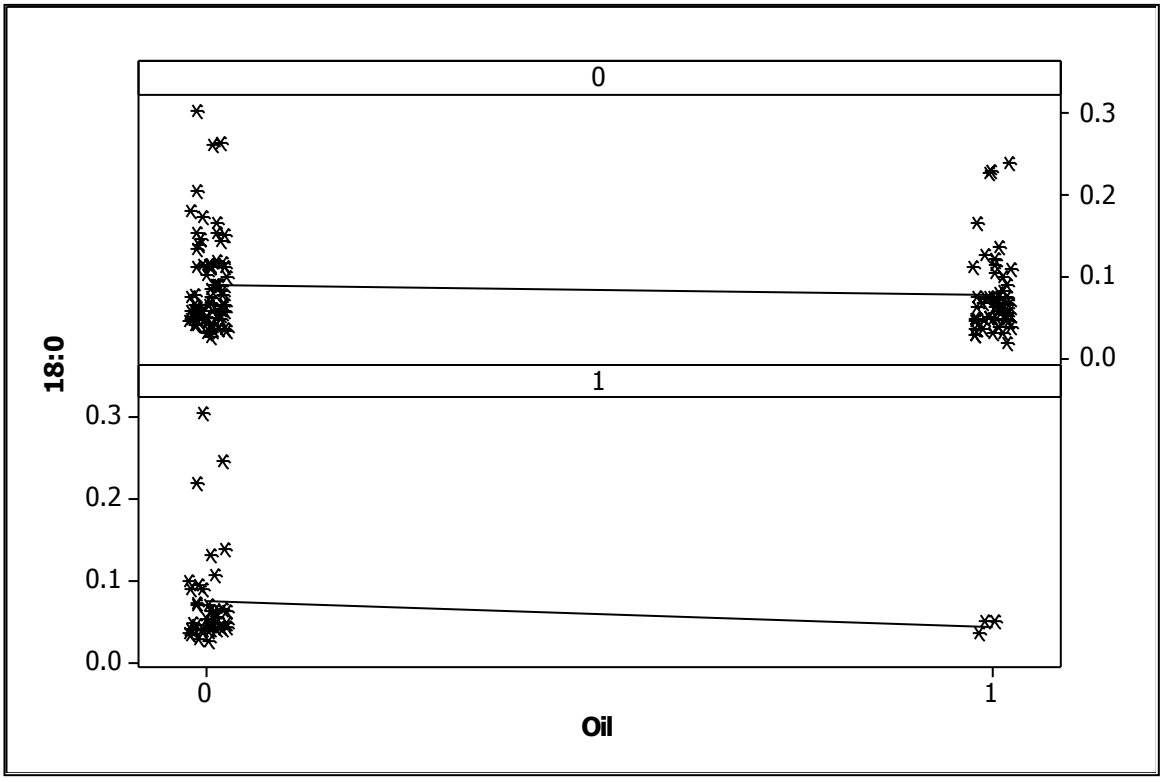

Figure 9.70. Scatterplot of oil (0-1) *versus* 18:0 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

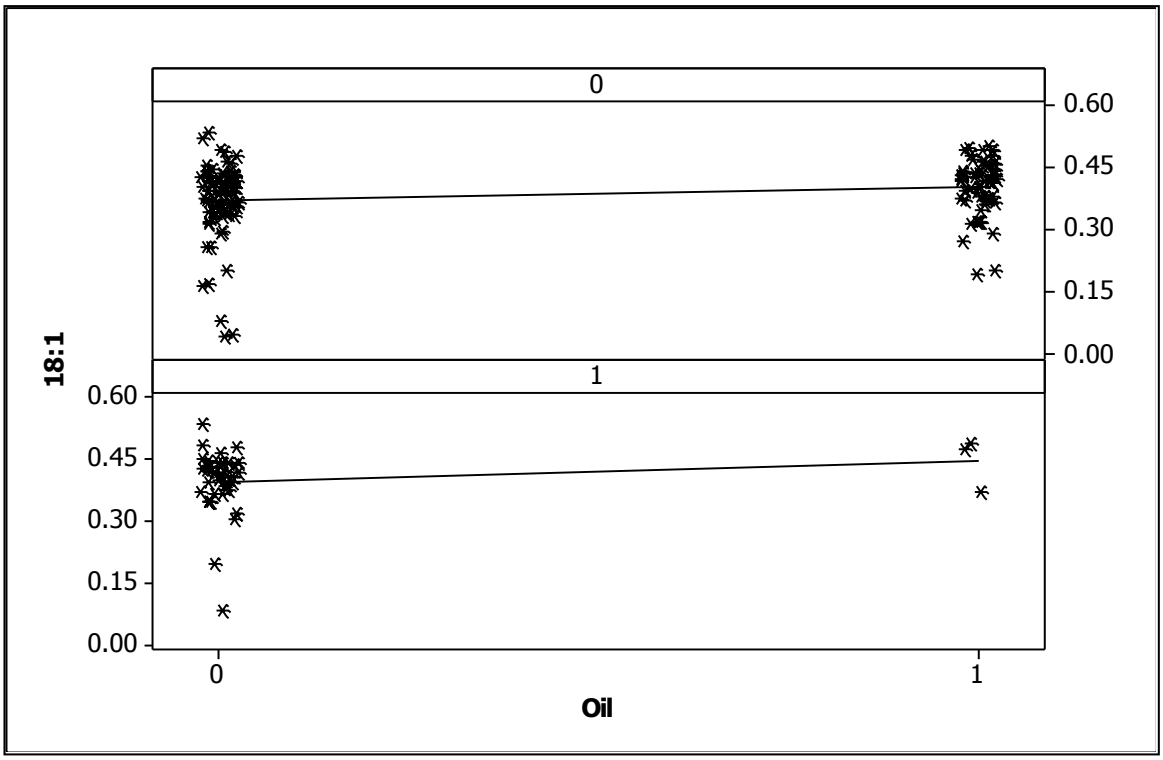

Figure 9.71. Scatterplot of oil (0-1) *versus* 18:1 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

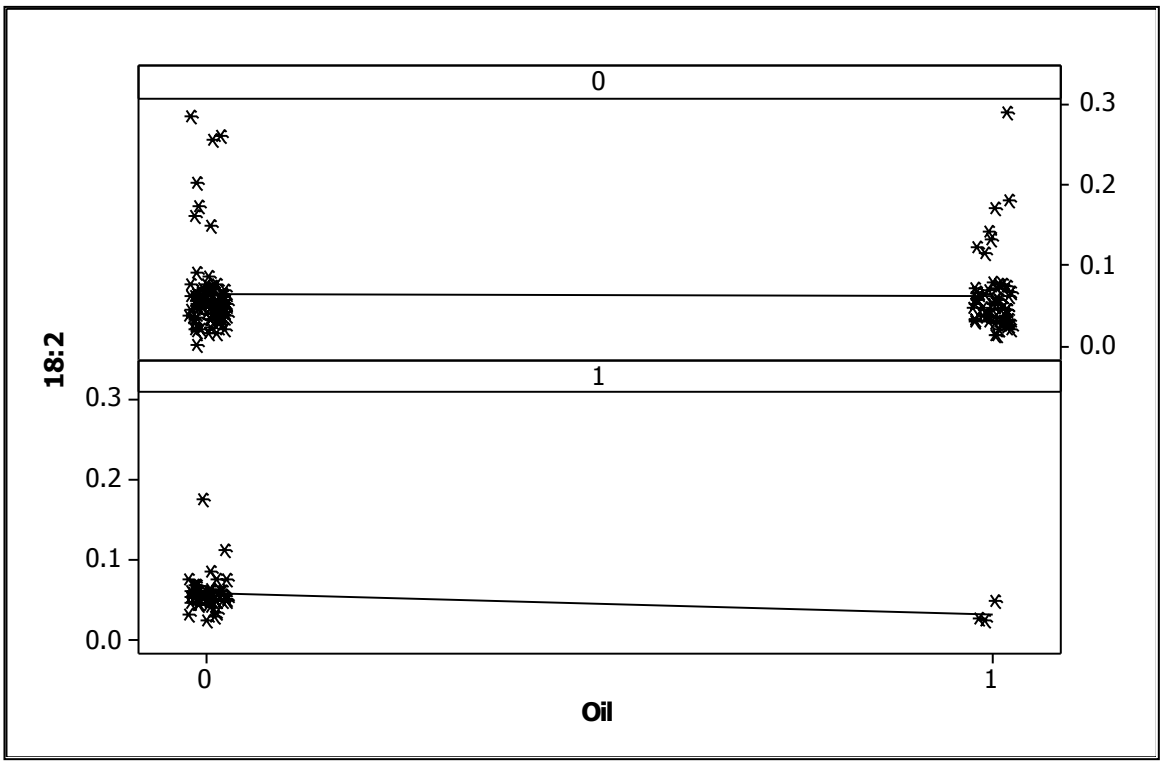

Figure 9.72. Scatterplot of oil (0-1) *versus* 18:2 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

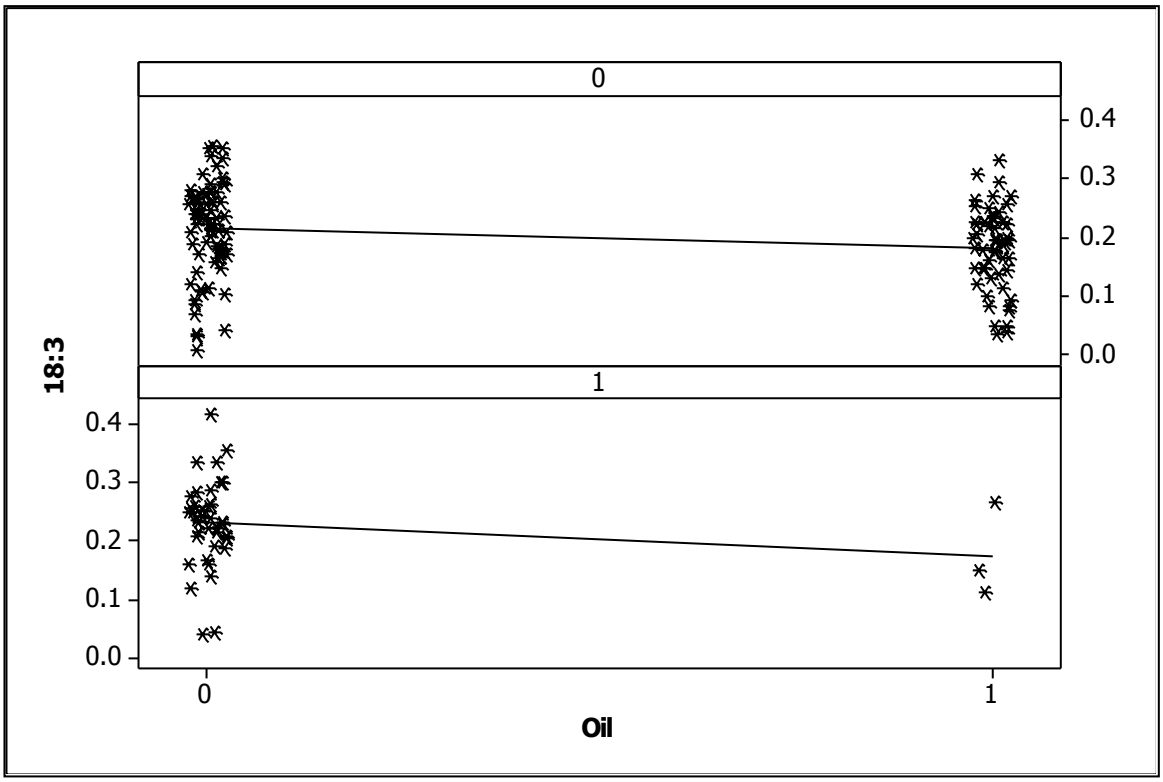

Figure 9.73. Scatterplot of oil (0-1) *versus* 18:3 with data split into parasitised (bottom panel) and non-parasitised (top). Linear regression lines have been added.

9.4.2 Regression Equations and Coefficients of Determination (R^2) for the Linear Regression Lines on the Scatterplots

- Sp = 12:0, Tp =14:0, Up = 16:0, Vp = 16:1, Wp = 18:0, Xp = 18:1, Yp = 18:2, Zp = 18:3.

Table 9.30. Regression equations and coefficients of determination (R^2) for the linear regression lines on the oil scatterplots.

Parasitised/Non-parasitised	Regression Equation	R^2
Non-parasitised	Sp = 0.009203-0.003955Oil	0.025
Parasitised	Sp = 0.004910-0.003544Oil	0.043
Non-parasitised	Tp = 0.01197-0.002484Oil	0.011
Parasitised	Tp = 0.01333-0.00724Oil	0.005
Non-parasitised	Up = 0.1484+0.001966Oil	0.001
Parasitised	Up = 0.1353+0.01813Oil	0.013
Non-parasitised	Vp = 0.09115+0.02198Oil	0.034
Parasitised	Vp = 0.08382+0.05960Oil	0.110
Non-parasitised	Wp = 0.09081-0.01372Oil	0.016
Parasitised	Wp = 0.07628-0.03199Oil	0.020
Non-parasitised	Xp = 0.3707+0.03169Oil	0.033
Parasitised	Xp =0.3954+0.04848Oil	0.027
Non-parasitised	Yp = 0.06412-0.002560Oil	0.001
Parasitised	Yp = 0.05972-0.02723Oil	0.088
Non-parasitised	Zp = 0.2137-0.03292Oil	0.044
Parasitised	Zp = 0.2312-0.05621Oil	0.041

Table 9.31. Regression equations and coefficients of determination (R^2) for the linear regression lines on the flight muscle (F.M.) scatterplots.

Parasitised/Non-parasitised	Regression Equation	R^2
Non-parasitised	Sp = 0.009527-0.002338F.M.	0.064
Parasitised	Sp = 0.005135-0.001324F.M.	0.085
Non-parasitised	Tp = 0.01234-0.001665F.M.	0.036
Parasitised	Tp = 0.01370-0.002461F.M.	0.008
Non-parasitised	Up = 0.1437+0.006372F.M.	0.054
Parasitised	Up = 0.1348+0.004867F.M.	0.013
Non-parasitised	Vp = 0.1000+0.000476F.M.	0.000
Parasitised	Vp = 0.08360+0.01250F.M.	0.069
Non-parasitised	Wp = 0.09509-0.01181F.M.	0.084
Parasitised	Wp = 0.07826-0.01180F.M.	0.039
Non-parasitised	Xp = 0.3721+0.01404F.M.	0.046
Parasitised	Xp =0.3939+0.01378F.M.	0.031
Non-parasitised	Yp = 0.06944+-0.008086F.M.	0.042
Parasitised	Yp = 0.06057-0.007755F.M.	0.101
Non-parasitised	Zp = 0.1973+0.003010F.M.	0.003
Parasitised	Zp = 0.2300-0.00781F.M.	0.011

Table 9.32. Regression equations and coefficients of determination (R^2) for the linear regression lines on the colour (C.) scatterplots.

Parasitised/Non-parasitised	Regression Equation	R^2
Non-parasitised	Sp = 0.009011-0.000722C.	0.006
Parasitised	Sp = 0.003366+0.000450C.	0.003
Non-parasitised	Tp = 0.01026+0.000320C.	0.001
Parasitised	Tp = 0.00657+0.002181C.	0.002
Non-parasitised	Up = 0.1519-0.001331C.	0.002
Parasitised	Up = 0.1280+0.00302C.	0.002
Non-parasitised	Vp = 0.1068-0.003127C.	0.005
Parasitised	Vp = 0.1185-0.01063	0.017
Non-parasitised	Wp = 0.1206-0.008598C.	0.044
Parasitised	Wp = 0.07030+0.00127C.	0.000
Non-parasitised	Xp = 0.3797+0.002138C.	0.001
Parasitised	Xp =0.4564-0.01309C.	0.009
Non-parasitised	Yp = 0.05614+0.003363C.	0.007
Parasitised	Yp = 0.04946+0.002899C.	0.005
Non-parasitised	Zp = 0.1835+0.007957C.	0.018
Parasitised	Zp = 0.1875+0.01389C.	0.012

Table 9.33. Regression equations and coefficients of determination (R^2) for the linear regression lines on the fat (F.) scatterplots.

Parasitised/Non-parasitised	Regression Equation	R^2
Non-parasitised	Sp = 0.01271-0.002074F.	0.074
Parasitised	Sp = 0.008311-0.001230F.	0.073
Non-parasitised	Tp = 0.01285-0.000776F.	0.011
Parasitised	Tp = 0.02742-0.004914F.	0.031
Non-parasitised	Up = 0.1462+0.001210F.	0.003
Parasitised	Up = 0.1171+0.006530F.	0.024
Non-parasitised	Vp = 0.08438+0.006437F.	0.031
Parasitised	Vp = 0.02599+0.02090F.	0.190
Non-parasitised	Wp = 0.1173-0.01295F.	0.149
Parasitised	Wp = 0.1169-0.01442F.	0.058
Non-parasitised	Xp = 0.3542+0.01198F.	0.049
Parasitised	Xp =0.3583+0.01369F.	0.030
Non-parasitised	Yp = 0.07084-0.003129F.	0.009
Parasitised	Yp = 0.08302-0.008499F.	0.120
Non-parasitised	Zp = 0.2016-0.000705F.	0.000
Parasitised	Zp = 0.2630-0.001205F	0.026

Table 9.34. Regression equations and coefficients of determination (R^2) for the linear regression lines on the mated (M.)scatterplots.

Parasitised/Non-parasitised	Regression Equation	R^2
Non-parasitised	Sp = 0.007500+0.000237M.	0.000
Parasitised	Sp = 0.004916-0.002711M.	0.033
Non-parasitised	Tp = 0.1088+0.000273M.	0.000
Parasitised	Tp = 0.01331-0.00521M.	0.003
Non-parasitised	Up = 0.1495-0.002417M.	0.001
Parasitised	Up = 0.1345+0.02114M.	0.0023
Non-parasitised	Vp = 0.09758+0.02198M.	0.016
Parasitised	Vp = 0.08746+0.00736M.	0.002
Non-parasitised	Wp = 0.008693+0.01481M.	0.009
Parasitised	Wp = 0.07564-0.01741M.	0.008
Non-parasitised	Xp = 0.3810+0.02325M.	0.008
Parasitised	Xp =0.4005-0.01564M.	0.004
Non-parasitised	Yp = 0.06506-0.01559M.	0.010
Parasitised	Yp = 0.05848-0.00771M.	0.009
Non-parasitised	Zp = 0.2015-0.01293M.	0.003
Parasitised	Zp = 0.2252+0.02019M.	0.007

Table 9.35. Regression equations and coefficients of determination (R^2) for the linear regression lines on the Eggs (E.) scatterplots.

Parasitised/Non-parasitised	Regression Equation	R^2
Non-parasitised	Sp = 0.007777-0.001440E.	0.002
Parasitised	Sp = 0.004444+0.004241E.	0.042
Non-parasitised	Tp = 0.01143-0.003016E.	0.001
Parasitised	Tp = 0.01292-0.00247E.	0.000
Non-parasitised	Up = 0.1456+0.02125E.	0.048
Parasitised	Up = 0.1356+0.01910E.	0.001
Non-parasitised	Vp = 0.09790+0.01489E.	0.009
Parasitised	Vp = 0.08964-0.02995E.	0.019
Non-parasitised	Wp = 0.08428+0.00427E.	0.001
Parasitised	Wp = 0.07283+0.02276E.	0.007
Non-parasitised	Xp = 0.3851-0.00630E.	0.001
Parasitised	Xp =0.3966+0.04979E.	0.019
Non-parasitised	Yp = 0.06513-0.01227E.	0.008
Parasitised	Yp = 0.05799-0.00521E.	0.002
Non-parasitised	Zp = 0.2028-0.01738E.	0.007
Parasitised	Zp = 0.2300-0.05832E.	0.030

Table 9.36. Regression equations and coefficients of determination (R^2) for the linear regression lines on the sexual maturity (S. M.)scatterplots.

Parasitised/Non-parasitised	Regression Equation	R^2
Non-parasitised	Sp = 0.004266+0.001106S. M.	0.023
Parasitised	Sp = 0.001198+0.001839S. M.	0.185
Non-parasitised	Tp = 0.01070+0.000073S. M.	0.000
Parasitised	Tp = 0.001869+0.005819S. M.	0.049
Non-parasitised	Up = 0.1557-0.002204S. M.	0.011
Parasitised	Up = 0.1265+0.005349S. M.	0.018
Non-parasitised	Vp = 0.1263-0.008753S. M.	0.063
Parasitised	Vp = 0.1056-0.009268S. M.	0.043
Non-parasitised	Wp = 0.06689+0.006140S. M.	0.037
Parasitised	Wp = 0.04338+0.01627S. M.	0.084
Non-parasitised	Xp = 0.4096-0.008667S. M.	0.029
Parasitised	Xp =0.4484-0.02630S. M.	0.126
Non-parasitised	Yp = 0.05709+0.002013S. M.	0.004
Parasitised	Yp = 0.04524+0.006653S. M.	0.084
Non-parasitised	Zp = 0.1694+0.01029S. M.	0.051
Parasitised	Zp = 0.2278-0.00036S. M.	0.000

9.4.3 General Linear Model Investigating Interactions Between Sex and Parasitism

a) ANOVA Raw Data for 12:0 (Sp)

General Linear Model: Sp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
Parasitised	fixed	2	0, 1

Analysis of Variance for Sp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.0004421	0.0004165	0.0004421	3.77	0.054
parasitised	1	0.0002925	0.0002449	0.0002925	2.49	0.116
Sex*parasitised	1	0.0000051	0.0000051	0.0000051	0.04	0.835
Error	160	0.0187804	0.0187804	0.0001174		
Total	163	0.0195201				

S = 0.0108341 R-Sq = 3.79% R-Sq(adj) = 1.99%

Unusual Observations for Sp

Obs	Sp	Fit	SE Fit	Residual	St Resid
11	0.033654	0.006470	0.001175	0.027184	2.52 R
119	0.031423	0.006470	0.001175	0.024952	2.32 R
133	0.067212	0.006470	0.001175	0.060742	5.64 R
134	0.073316	0.009904	0.001758	0.063412	5.93 R
137	0.053519	0.006470	0.001175	0.047048	4.37 R
164	0.055970	0.009904	0.001758	0.046066	4.31 R

Least Squares Means for Sp

Sex		Mean	SE Mean
F		0.004776	0.001214
M		0.008638	0.001652
parasitised			
0		0.008187	0.001057
1		0.005226	0.001756
Sex*parasitised			
F	0	0.006470	0.001175
F	1	0.003082	0.002125
M	0	0.009904	0.001758
M	1	0.007371	0.002797

b) ANOVA Raw Data for 14:0 (Tp)

General Linear Model: Tp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Tp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.0018802	0.0026035	0.0018802	6.78	0.010
parasitised	1	0.0000666	0.0002614	0.0000666	0.24	0.625
Sex*parasitised	1	0.0007879	0.0007879	0.0007879	2.84	0.094
Error	160	0.0443871	0.0443871	0.0002774		
Total	163	0.0471218				

S = 0.0166559 R-Sq = 5.80% R-Sq(adj) = 4.04%

Unusual Observations for Tp

Obs	Tp	Fit	SE Fit	Residual	St Resid
11	0.058768	0.009575	0.001807	0.049193	2.97 R
26	0.047428	0.013918	0.002702	0.033510	2.04 R
96	0.183155	0.022289	0.004301	0.160867	10.00 R
134	0.084471	0.013918	0.002702	0.070553	4.29 R
164	0.063029	0.013918	0.002702	0.049111	2.99 R

Least Squares Means for Tp

Sex		Mean	SE Mean
F		0.008449	0.001866
M		0.018103	0.002539
parasitised			
0		0.011747	0.001625
1		0.014806	0.002700
Sex*parasitised			
F	0	0.009575	0.001807
F	1	0.007323	0.003266
M	0	0.013918	0.002702

M	1	0.022289	0.004301

c) ANOVA Raw Data for 16:0 (Up)

General Linear Model: Up versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Up, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.001242	0.000107	0.001242	0.86	0.355
parasitised	1	0.004640	0.002895	0.004640	3.22	0.075
Sex*parasitised	1	0.001409	0.001409	0.001409	0.98	0.325
Error	160	0.230812	0.230812	0.001443		
Total	163	0.238103				

S = 0.0379812 R-Sq = 3.06% R-Sq(adj) = 1.24%

Unusual Observations for Up

Obs	Up	Fit	SE Fit	Residual	St Resid
53	0.221834	0.134699	0.007449	0.087135	2.34 R
92	0.236550	0.151982	0.004120	0.084569	2.24 R
94	0.296314	0.151982	0.004120	0.144332	3.82 R
96	0.046198	0.139841	0.009807	-0.093643	-2.55 R
99	0.241348	0.151982	0.004120	0.089366	2.37 R
101	0.283981	0.151982	0.004120	0.132000	3.50 R
102	0.251216	0.139841	0.009807	0.111374	3.04 R
131	0.066758	0.151982	0.004120	-0.085224	-2.26 R
148	0.232360	0.139841	0.009807	0.092519	2.52 R

Least Squares Means for Up

Sex	Mean	SE Mean
F	0.1433	0.004256

M		0.1414	0.005791
parasitised			
0		0.1475	0.003706
1		0.1373	0.006157
Sex*parasitised			
F	0	0.1520	0.004120
F	1	0.1347	0.007449
M	0	0.1429	0.006161
M	1	0.1398	0.009807

d) ANOVA Raw Data for 16:1 (Vp)

General Linear Model: Vp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Vp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.019286	0.017237	0.019286	6.20	0.014
parasitised	1	0.003695	0.004276	0.003695	1.19	0.277
Sex*parasitised	1	0.000612	0.000612	0.000612	0.20	0.658
Error	160	0.497510	0.497510	0.003109		
Total	163	0.521102				

Unusual Observations for Vp

Obs	Vp	Fit	SE Fit	Residual	St Resid
10	0.242043	0.106673	0.006048	0.135370	2.44 R
13	0.238368	0.106673	0.006048	0.131694	2.38 R
14	0.268807	0.106673	0.006048	0.162134	2.92 R
25	0.212670	0.086510	0.009046	0.126159	2.29 R
76	0.243655	0.106673	0.006048	0.136981	2.47 R
77	0.227780	0.106673	0.006048	0.121107	2.18 R
78	0.251510	0.086510	0.009046	0.164999	3.00 R
85	0.239695	0.106673	0.006048	0.133022	2.40 R
87	0.205115	0.069458	0.014398	0.135658	2.52 R

Least Squares Means for Vp

Sex	Mean	SE Mean
F	0.10283	0.006249

M		0.07798	0.008502
parasitised			
0		0.09659	0.005441
1		0.08422	0.009040
Sex*parasitised			
F	0	0.10667	0.006048
F	1	0.09898	0.010936
M	0	0.08651	0.009046
M	1	0.06946	0.014398

e) ANOVA Raw Data for 18:0 (Wp)

General Linear Model: Wp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Wp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.013804	0.018313	0.013804	4.62	0.033
parasitised	1	0.004580	0.002154	0.004580	1.53	0.218
Sex*parasitised	1	0.003706	0.003706	0.003706	1.24	0.267
Error	160	0.478328	0.478328	0.002990		
Total	163	0.500417				

Unusual Observations for Wp

Obs	Wp	Fit	SE Fit	Residual	St Resid
24	0.240748	0.094743	0.008870	0.146005	2.71 R
99	0.303984	0.080656	0.005931	0.223328	4.11 R
101	0.210407	0.080656	0.005931	0.129751	2.39 R
102	0.305092	0.097479	0.014117	0.207614	.93 R
110	0.226774	0.094743	0.008870	0.132031	2.45 R
144	0.216294	0.097479	0.014117	0.118815	2.25 R
147	0.247865	0.060355	0.010723	0.187510	3.50 R
154	0.266206	0.080656	0.005931	0.185551	3.41 R
155	0.265759	0.080656	0.005931	0.185103	3.41 R
164	0.228811	0.094743	0.008870	0.134068	2.48 R

Least Squares Means for Wp

Sex	Mean	SE Mean

F	0.07051	0.006127
M	0.09611	0.008336
parasitised		
0	0.08770	0.005335
1	0.07892	0.008864
Sex*parasitised		
F 0	0.08066	0.005931
F 1	0.06035	0.010723
M 0	0.09474	0.008870
M 1	0.09748	0.014117

f) ANOVA Raw Data for 18:0 (Xp)

General Linear Model: Xp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Xp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.022806	0.040872	0.022806	3.25	0.073
parasitised	1	0.008255	0.002067	0.008255	1.18	0.279
Sex*parasitised	1	0.019086	0.019086	0.019086	2.72	0.101
Error	160	1.121714	121714	0.007011		
Total	163	1.171861				

Unusual Observations for Xp

Obs	Xp	Fit	SE Fit	Residual	St Resid
11	0.198030	0.387805	0.009082	-0.189775	-2.28 R
24	0.201912	0.375692	0.013583	-0.173780	-2.10 R
30	0.152943	0.387805	0.009082	-0.234862	-2.82 R
99	0.166554	0.387805	0.009082	-0.221251	-2.66 R
102	0.195447	0.358155	0.021619	-0.162708	-2.01 R
148	0.076669	0.358155	0.021619	-0.281486	-3.48 R
154	0.047866	0.387805	0.009082	-0.339939	-4.08 R
155	0.051655	0.387805	0.009082	-0.336150	-4.04 R
161	0.088600	0.375692	0.013583	-0.287092	-3.47 R
164	0.191862	0.375692	0.013583	-0.183831	-2.22 R

Least Squares Means for Xp

Sex		Mean	SE Mean
F		0.4052	0.009382
M		0.3669	0.012766
parasitised			
0		0.3817	0.008170
1		0.3904	0.013574
Sex*parasitised			
F	0	0.3878	0.009082
F	1	0.4225	0.016421
M	0	0.3757	0.013583
M	1	0.3582	0.021619

g) ANOVA Raw Data for18:2 (Yp)

General Linear Model: Yp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Yp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.004847	0.006029	0.004847	2.20	0.140
parasitised	1	0.001097	0.000495	0.001097	0.50	0.481
Sex*parasitised	1	0.000982	0.000982	0.000982	0.45	0.505
Error	160	0.352297	0.352297	0.002202		
Total	163	0.359223				

Unusual Observations for Yp

Obs	Yp	Fit	SE Fit	Residual	St Resid
1	0.293586	0.060328	0.005090	0.233258	5.00 R
24	0.181476	0.069089	0.007612	0.112387	2.43 R
26	0.168024	0.069089	0.007612	0.098935	2.14 R
30	0.279651	0.060328	0.005090	0.219323	4.70 R
35	0.170446	0.060328	0.005090	0.110119	2.36 R
36	0.172280	0.069089	0.007612	0.103191	2.23 R
99	0.203293	0.060328	0.005090	0.142966	3.06 R
102	0.174169	0.070810	0.012116	0.103359	2.28 R
154	0.261384	0.060328	0.005090	0.201056	4.31 R
155	0.263293	0.060328	0.005090	0.202965	4.35 R

Least Squares Means for Yp

Sex		Mean	SE Mean
F		0.05526	0.005258
M		0.06995	0.007154
parasitised			
0		0.06471	0.004578
1		0.06050	0.007607
Sex*parasitised			
F	0	0.06033	0.005090
F	1	0.05019	0.009203
M	0	0.06909	0.007612
M	1	0.07081	0.012116

h) ANOVA Raw Data for 18:3 (Zp)

General Linear Model: Zp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Zp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.005424	0.003530	0.005424	0.92	0.340
parasitised	1	0.021839	0.020128	0.021839	3.69	0.057
Sex*parasitised	1	0.000008	0.000008	0.000008	0.00	0.971
Error	160	0.948190	0.948190	0.005926		
Total	163	0.975460				

Unusual Observations for Zp

Obs	Zp	Fit	SE Fit	Residual	St Resid
10	0.041528	0.196511	0.008350	-0.154983	-2.03 R
14	0.042889	0.196511	0.008350	-0.153622	-2.01 R
78	0.053195	0.207224	0.012488	-0.154029	-2.03 R
87	0.050144	0.234598	0.019877	-0.184453	-2.48 R
94	0.015204	0.196511	0.008350	-0.181307	-2.37 R
99	0.033331	0.196511	0.008350	-0.163180	-2.13 R
101	0.037249	0.196511	0.008350	-0.159262	-2.08 R
102	0.038714	0.234598	0.019877	-0.195883	-2.63 R
127	0.354747	0.196511	0.008350	0.158235	2.07 R

128	0.351774	0.196511	0.008350	0.155263	2.03 R
145	0.033284	0.234598	0.019877	0.176281	2.37 R
161	0.359222	0.207224	0.012488	0.151999	2.00 R

Least Squares Means for Zp

Sex		Mean	SE Mean
F		0.2097	0.008626
M		0.2209	0.011737
parasitised			
0		0.2019	0.007511
1		0.2287	0.012480
Sex*parasitised			
F	0	0.1965	0.008350
F	1	0.2228	0.015097
M	0	0.2072	0.012488
M	1	0.2346	0.019877

9.4.4 Linear Regression Equations and Coefficients of Determination (R^2) for Principal Components v*ersus* Physiological Factors

Table 9.37. Linear regression line equations and coefficients of determination (R^2) for Fat.

Factor	Regression Equation	R^2
Scor1	=-2.153+0.7479Fat (Parasitised)	0.206
Scor1	=-0.9626+0.3760Fat (Non-parasitised)	0.107
Scor2	=-0.7023+0.1010Fat (Parasitised)	0.008
Scor2	=0.4410-0.1230Fat (Non-parasitised)	0.024
Scor3	=-0.0955+0.0576Fat (Parasitised)	0.003
Scor3	=-0.0913+0.02648Fat (Non-parasitised)	0.001

Table 9.38. Linear regression line equations and coefficients of determination (R^2) for Flight Muscle (F. M.)

Factor	Regression equation	R^2
Scor1	=-0.1683+0.6587F.M. (Parasitised)	0.161
Scor1	=-0.4712+0.5236F.M. (Non-parasitised)	0.142
Scor2	=-0.4237+0.0598F.M. (Parasitised)	0.003
Scor2	=0.1848-0.05953F.M. (Non-parasitised)	0.004
Scor3	=0.0714+0.0118F.M. (Parasitised)	0.000
Scor3	=-0.0569+0.03710F.M. (Non-parasitised)	0.002

Table 9.39. Linear regression line equations and coefficients of determination (R^2) for Sexual Maturity (S. M.)

Factor	Regression equation	R^2
Scor1	=1.525-0.7737S.M. (Parasitised)	0.249
Scor1	=0.8267-0.2883S.M. (Non-parasitised)	0.071
Scor2	=-0.8292+0.2276S.M. (Parasitised)	0.049
Scor2	=0.4436-0.01049S.M. (Non-parasitised)	0.019
Scor3	=0.0606+0.0081S.M. (Parasitised)	0.000
Scor3	=0.0907-0.03928S.M. (Non-parasitised)	0.003

Table 9.40. Linear regression line equations and coefficients of determination (R^2) for Eggs.

Factor	Regression equation	R^2
Scor1	=0.1212-0.9950Eggs (Parasitised)	0.018
Scor1	=-0.0953+0.4165Eggs (Non-parasitised)	0.070
Scor2	=-0.4482+0.9497Eggs (Parasitised)	0.036
Scor2	=0.0438+0.5279Eggs (Non-parasitised)	0.024
Scor3	=0.0623+0.2757Eggs (Parasitised)	0.003
Scor3	=-0.0128-0.0729Eggs (Non-parasitised)	0.001

Table 9.41. Linear regression line equations and coefficients of determination (R^2) for Mated (M.)

Factor	Regression equation	R^2
Scor1	=-0.0049+0.7947M. (Parasitised)	0.021
Scor1	=-0.0638+0.3040M. (Non-parasitised)	0.003
Scor2	=-0.4021+0.0023M. (Parasitised)	0.000
Scor2	=0.1183+0.1204M. (Non-parasitised)	0.001
Scor3	=0.0935-0.1819M. (Parasitised)	0.003
Scor3	=-0.0680+0.3280M. (Non-parasitised)	0.090

Table 9.42. Linear regression line equations and coefficients of determination (R^2) for Colour (C.)

Factor	Regression equation	R^2
Scor1	=0.4880-0.1457C. (Parasitised)	0.003
Scor1	=-0.1200+0.0468C. (Non-parasitised)	0.001
Scor2	=-0.0595-0.1200C. (Parasitised)	0.004
Scor2	=0.6730-0.2632C. (Non-parasitised)	0.073
Scor3	=0.1459-0.0246C. (Parasitised)	0.000
Scor3	=-0.0272+0.00094C. (Non-parasitised)	0.000

Table 9.43. Linear regression line equations and coefficients of determination (R^2) for Oil.

Factor	Regression equation	R^2
Scor1	=-0.0683+1.926Oil (Parasitised)	0.097
Scor1	=-0.3574+0.7880Oil (Non-parasitised)	0.045
Scor2	=-0.4516+0.6795Oil (Parasitised)	0.027
Scor2	=0.0845+0.1170Oil (Non-parasitised)	0.002
Scor3	=0.0575+0.2498Oil (Parasitised)	0.004
Scor3	=0.0496-0.1750Oil (Non-parasitised)	0.106

9.5 Weighed Subset

One-way ANOVA: Sp versus parasitised

```
Source       DF         SS         MS     F      P
parasitised   1  0.0000003  0.0000003  0.59  0.453
Error        20  0.0000088  0.0000004
Total        21  0.0000091

S = 0.0006641   R-Sq = 2.84%   R-Sq(adj) = 0.00%

                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level   N       Mean      StDev --+---------+---------+---------+-------
0      16  0.0016483  0.0007076               (-----------*----------)
1       6  0.0014051  0.0005119 (------------------*------------------)
                                --+---------+---------+---------+-------
                                0.00090   0.00120   0.00150   0.00180

Pooled StDev = 0.0006641
```

One-way ANOVA: Tp versus parasitised

```
Source       DF         SS         MS     F      P
parasitised   1  0.0000003  0.0000003  0.23  0.636
Error        20  0.0000284  0.0000014
Total        21  0.0000287

S = 0.001191   R-Sq = 1.14%   R-Sq(adj) = 0.00%


Level   N      Mean     StDev
0      16  0.006210  0.001024
1       6  0.006484  0.001592

       Individual 95% CIs For Mean Based on Pooled StDev
Level  ---------+---------+---------+---------+
0        (----------*---------)
1      (----------------*----------------)
       ---------+---------+---------+---------+
              0.00600   0.00660   0.00720   0.00780
```

```
Pooled StDev = 0.001191
```

One-way ANOVA: Up versus parasitised

```
Source       DF        SS        MS     F      P
parasitised   1  0.000064  0.000064  0.11  0.748
Error        20  0.012125  0.000606
Total        21  0.012189

S = 0.02462   R-Sq = 0.53%   R-Sq(adj) = 0.00%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev  ---+---------+---------+---------+------
0      16  0.16561  0.01828           (----------*----------)
1       6  0.16177  0.03772  (-----------------*-----------------)
                             ---+---------+---------+---------+------
                              0.144     0.156     0.168     0.180

Pooled StDev = 0.02462
```

One-way ANOVA: Vp versus parasitised

```
Source       DF       SS       MS     F      P
parasitised   1  0.00171  0.00171  0.91  0.350
Error        20  0.03733  0.00187
Total        21  0.03904

S = 0.04320   R-Sq = 4.37%   R-Sq(adj) = 0.00%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev  -----+---------+---------+---------+----
0      16  0.11255  0.04212  (----------*----------)
1       6  0.13233  0.04630     (-----------------*------------------)
                             -----+---------+---------+---------+----
                                0.100     0.120     0.140     0.160

Pooled StDev = 0.04320
```

One-way ANOVA: Wp versus parasitised

```
Source       DF        SS        MS     F      P
parasitised   1  0.000004  0.000004  0.01  0.920
Error        20  0.007157  0.000358
Total        21  0.007161

S = 0.01892   R-Sq = 0.05%   R-Sq(adj) = 0.00%


                             Individual 95% CIs For Mean Based on Pooled
StDev
Level   N     Mean    StDev    +---------+---------+---------+---------
0      16  0.04470  0.02156         (---------*---------)
1       6  0.04562  0.00602    (---------------*---------------)
                               +---------+---------+---------+---------
                             0.030     0.040     0.050     0.060

Pooled StDev = 0.01892
```

One-way ANOVA: Xp versus parasitised

```
Source       DF       SS       MS     F      P
parasitised   1  0.00268  0.00268  2.21  0.153
Error        20  0.02423  0.00121
Total        21  0.02691

S = 0.03480   R-Sq = 9.96%   R-Sq(adj) = 5.45%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev  ---+---------+---------+---------+------
0      16  0.41197  0.02889  (--------*--------)
1       6  0.43675  0.04839         (-------------*--------------)
                            ---+---------+---------+---------+------
                             0.400     0.420     0.440     0.460

Pooled StDev = 0.03480
```

One-way ANOVA: Yp versus parasitised

```
Source       DF         SS         MS     F      P
parasitised   1  0.0001302  0.0001302  1.67  0.211
Error        20  0.0015577  0.0000779
Total        21  0.0016879

S = 0.008825   R-Sq = 7.71%   R-Sq(adj) = 3.10%


Level   N      Mean     StDev
0      16  0.038591  0.009271
1       6  0.033129  0.007326

       Individual 95% CIs For Mean Based on Pooled StDev
Level  ---------+---------+---------+---------+
0                    (--------*--------)
1      (--------------*--------------)
       ---------+---------+---------+---------+
              0.0300    0.0350    0.0400    0.0450

Pooled StDev = 0.008825
```

One-way ANOVA: Zp versus parasitised

```
Source       DF       SS       MS     F      P
parasitised   1  0.00572  0.00572  2.81  0.109
Error        20  0.04066  0.00203
Total        21  0.04637

S = 0.04509   R-Sq = 12.33%   R-Sq(adj) = 7.95%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev  --+---------+---------+---------+-------
0      16  0.21872  0.04151                     (--------*---------)
1       6  0.18252  0.05442  (--------------*--------------)
                            --+---------+---------+---------+-------
                            0.150     0.175     0.200     0.225

Pooled StDev = 0.04509
```

One-way ANOVA: tot%S-Z versus parasitised

```
Source       DF     SS    MS     F      P
parasitised   1   3.39  3.39  1.21  0.285
Error        20  56.06  2.80
Total        21  59.44

S = 1.674   R-Sq = 5.70%   R-Sq(adj) = 0.98%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  -------+---------+---------+---------+--
0      16  7.359  1.719                    (----------*----------)
1       6  6.478  1.531  (------------------*-----------------)
                          -------+---------+---------+---------+--
                               5.60      6.40      7.20      8.00

Pooled StDev = 1.674
```

9.5.1 Linear Regression Values for Principal Components v*ersus* Physiological Factors

Table 9.44. Linear regression line equations and coefficients of determination (R^2) for Fat.

Factor	Regression equation	R^2
Fat =	4.640+0.3230WS1 (Parasitised)	0.388
	3.869+0.1927WS1 (Non-parasitised)	0.142
	4.176+0.2541WS2 (Parasitised)	0.163
	0.3945+0.0337WS2 (Non-parasitised)	0.002
	4.217+0.3890WS3 (Parasitised)	0.079
	3.887-0.4545WS3 (Non-parasitised)	0.214

Table 9.45. Linear regression line equations and coefficients of determination (R^2) for Flight Muscle.

Factor	Regression equation	R^2
Flight Muscle =	2.398+0.4194WS1 (Parasitised)	0.182
	2.382+0.1549WS1 (Non-parasitised)	0.071
	2.374-0.06029WS2 (Parasitised)	0.255
	2.461+0.0994WS2 (Non-parasitised)	0.011
	1.698+1.012WS3 (Parasitised)	0.148
	2.148-0.1724WS3 (Non-parasitised)	0.024

Table 9.46. Linear regression line equations and coefficients of determination (R^2) for Sexual Maturity.

Factor	Regression equation	R^2
Sexual Maturity =	1.062-0.1099WS1 (Parasitised)	0.053
	1.239-0.1454WS1 (Non-parasitised)	0.056
	1.065+0.1640WS2 (Parasitised)	0.080
	1.160-0.1191WS2 (Non-parasitised)	0.015
	1.270-0.3466WS3 (Parasitised)	0.074
	1.202+0.1256WS3 (Non-parasitised)	0.011

Table 9.47. Linear regression line equations and coefficients of determination (R^2) for Colour.

Factor	Regression equation	R^2
Colour =	2.862+0.0299WS1 (Parasitised)	0.013
	2.235+0.0436WS1 (Non-parasitised)	0.146
	2.85-0.0370WS2 (Parasitised)	0.014
	2.194-0.2421WS2 (Non-parasitised)	0.165
	2.836-0.0093WS3 (Parasitised)	0.000
	2.266+0.1441WS3 (Non-parasitised)	0.034

Table 9.48. Linear regression line equations and coefficients of determination (R^2) for Oil.

Factor	Regression equation	R^2
Oil =	0.6762+0.1858WS1 (Parasitised)	0.281
	0.4579+0.1184WS1 (Non-parasitised)	0.227
	0.4664+0.0541WS2 (Parasitised)	0.016
	0.5616+0.2648WS2 (Non-parasitised)	0.443
	0.6800-0.6024WS3 (Parasitised)	0.421
	0.5010+0.0087WS3 (Non-parasitised)	0.000

9.6 Gisborne Subset

9.6.1 General Linear Model Investigating Interactions Between Sex and Parasitism

a) ANOVA Raw Data for 12:0 (Sp)

General Linear Model: Sp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Sp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.0003097	0.0002587	0.0003097	1.25	0.268
parasitised	1	0.0011936	0.0010929	0.0011936	4.83	0.032
Sex*parasitised	1	0.0000042	0.0000042	0.0000042	0.02	0.896
Error	52	0.0128514	0.0128514	0.0002471		
Total	55	0.0143589				

S = 0.0157207 R-Sq = 10.50% R-Sq(adj) = 5.34%

Unusual Observations for Sp

Obs	Sp	Fit	SE Fit	Residual	St Resid
25	0.067212	0.013940	0.003352	0.053272	3.47 R
26	0.073316	0.018019	0.004059	0.055297	3.64 R
29	0.053519	0.013940	0.003352	0.039579	2.58 R

56	0.055970	0.018019	0.004059	0.037952	2.50 R

Least Squares Means for Sp

Sex	Mean	SE Mean
F	0.008833	0.002821
M	0.013511	0.003598
parasitised		
0	0.015979	0.002632
1	0.006365	0.003738
Sex*parasitised		
F 0	0.013940	0.003352
F 1	0.003726	0.004538
M 0	0.018019	0.004059
M 1	0.009004	0.005942

b) ANOVA Raw Data for 14:0 (Tp)

General Linear Model: Tp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Tp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.0007234	0.0004762	0.0007234	4.20	0.045
parasitised	1	0.0003489	0.0004064	0.0003489	2.03	0.161
Sex*parasitised	1	0.0000716	0.0000716	0.0000716	0.42	0.522
Error	52	0.0089570	0.0089570	0.0001723		
Total	55	0.0101009				

S = 0.0131244 R-Sq = 11.32% R-Sq(adj) = 6.21%

Unusual Observations for Tp

Obs	Tp	Fit	SE Fit	Residual	St Resid
26	0.084471	0.019156	0.003389	0.065315	5.15 R
56	0.063029	0.019156	0.003389	0.043873	3.46 R

Least Squares Means for Tp

Sex	Mean	SE Mean
F	0.008648	0.002355

M	0.014995	0.003004
parasitised		
0	0.014753	0.002197
1	0.008890	0.003121
Sex*parasitised		
F 0	0.010349	0.002798
F 1	0.006947	0.003789
M 0	0.019156	0.003389
M 1	0.010833	0.004961

c) ANOVA Raw Data for 16:0 (Up)

General Linear Model: Up versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Up, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.0011790	0.0010601	0.0011790	1.30	0.260
parasitised	1	0.0001498	0.0001302	0.0001498	0.17	0.686
Sex*parasitised	1	0.0000039	0.0000039	0.0000039	0.00	0.948
Error	52	0.0471826	0.0471826	0.0009074		
Total	55	0.0485153				

S = 0.0301224 R-Sq = 2.75% R-Sq(adj) = 0.00%

Unusual Observations for Up

Obs	Up	Fit	SE Fit	Residual	St Resid
40	0.232360	0.122920	0.011385	0.109440	3.92 R
53	0.186591	0.125664	0.007778	0.060927	2.09 R

Least Squares Means for Up

Sex	Mean	SE Mean
F	0.1148	0.005405
M	0.1243	0.006894
parasitised		
0	0.1212	0.005043
1	0.1179	0.007163
Sex*parasitised		

F	0	0.1168 0.006422
F	1	0.1129 0.008696
M	0	0.1257 0.007778
M	1	0.1229 0.011385

d) ANOVA Raw Data for 16:1 (Vp)

General Linear Model: Vp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Vp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.007095	0.009460	0.007095	6.32	0.015
parasitised	1	0.001531	0.000591	0.001531	1.36	0.248
Sex*parasitised	1	0.003288	0.003288	0.003288	2.93	0.093
Error	52	0.058364	0.058364	0.001122		
Total	55	0.070277				

S = 0.0335020 R-Sq = 16.95% R-Sq(adj) = 12.16%

Unusual Observations for Vp

Obs	Vp	Fit	SE Fit	Residual	St Resid
40	0.140127	0.050293	0.012663	0.089834	2.90 R
41	0.158077	0.071510	0.007143	0.086567	2.64 R
53	0.136071	0.059900	0.008650	0.076172	2.35 R

Least Squares Means for Vp

Sex	Mean	SE Mean
F	0.08338	0.006011
M	0.05510	0.007668
parasitised		
0	0.06570	0.005609
1	0.07277	0.007967
Sex*parasitised		
F 0	0.07151	0.007143
F 1	0.09526	0.009671
M 0	0.05990	0.008650
M 1	0.05029	0.012663

e) ANOVA Raw Data for 18:0 (Wp)

General Linear Model: Wp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Wp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.000231	0.000100	0.000231	0.06	0.802
parasitised	1	0.019323	0.016550	0.019323	5.34	0.025
Sex*parasitised	1	0.000707	0.000707	.000707	0.20	0.660
Error	52	0.188132	0.188132	0.003618		
Total	55	0.208392				

S = 0.0601491 R-Sq = 9.72% R-Sq(adj) = 4.51%

Unusual Observations for Wp

Obs	Wp	Fit	SE Fit	Residual	St Resid
2	0.226774	0.100661	0.015530	0.126113	2.17 R
36	0.216294	0.070981	0.022734	0.145313	2.61 R
39	0.247865	0.066160	0.017364	0.181705	3.16 R
46	0.266206	0.111306	0.012824	0.154901	2.64 R
47	0.265759	0.111306	0.012824	0.154453	2.63 R
56	0.228811	0.100661	0.015530	0.128150	2.21 R

Least Squares Means for Wp

Sex	Mean	SE Mean
F	0.08873	0.01079
M	0.08582	0.01377
parasitised		
0	0.10598	0.01007
1	0.06857	0.01430
Sex*parasitised		
F 0	0.11131	0.01282
F 1	0.06616	0.01736
M 0	0.10066	0.01553
M 1	0.07098	0.02273

f) ANOVA Raw Data for 18:1 (Xp)

General Linear Model: Xp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Xp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.00325	0.01188	0.00325	0.29	0.591
parasitised	1	0.01678	0.00698	0.01678	1.50	0.225
Sex*parasitised	1	0.03137	0.03137	0.03137	2.81	0.100
Error	52	0.58004	0.58004	0.01115		
Total	55	0.63145				

S = 0.105615 R-Sq = 8.14% R-Sq(adj) = 2.84%

Unusual Observations for Xp

Obs	Xp	Fit	SE Fit	Residual	St Resid
40	0.076669	0.348021	0.039919	-0.271352	-2.78 R
46	0.047866	0.355431	0.022517	-0.307565	-2.98 R
47	0.051655	0.355431	0.022517	-0.303776	-2.94 R
53	0.088600	0.375237	0.027270	-0.286637	-2.81 R

Least Squares Means for Xp

Sex	Mean	SE Mean
F	0.3933	0.01895
M	0.3616	0.02417
parasitised		
0	0.3653	0.01768
1	0.3896	0.02511
Sex*parasitised		
F 0	0.3554	0.02252
F 1	0.4312	0.03049
M 0	0.3752	0.02727
M 1	0.3480	0.03992

g) ANOVA Raw Data for 18:2 (Yp)

General Linear Model: Yp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Yp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.000009	0.000039	0.000009	0.00	0.944
parasitised	1	0.002575	0.001734	0.002575	1.42	0.240
Sex*parasitised	1	0.001025	0.001025	0.001025	0.56	0.456
Error	52	0.094579	0.094579	0.001819		
Total	55	0.098188				

S = 0.0426477 R-Sq = 3.68% R-Sq(adj) = 0.00%

Unusual Observations for Yp

Obs	Yp	Fit	SE Fit	Residual	St Resid
46	0.261384	0.075434	0.009093	0.185950	4.46 R
47	0.263293	0.075434	0.009093 0	.187859	4.51 R

Least Squares Means for Yp

Sex	Mean	SE Mean
F	0.06472	0.007653
M	0.06654	0.009761
parasitised		
0	0.07169	0.007140
1	0.05958	0.010142
Sex*parasitised		
F 0	0.07543	0.009093
F 1	0.05401	0.012311
M 0	0.06794	0.011012
M 1	0.06515	0.016119

h) ANOVA Raw Data for 18:3 (Zp)

General Linear Model: Zp versus Sex, parasitised

Factor	Type	Levels	Values
Sex	fixed	2	F, M
parasitised	fixed	2	0, 1

Analysis of Variance for Zp, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Seq MS	F	P
Sex	1	0.006501	0.019478	0.006501	1.50	0.226
parasitised	1	0.007490	0.016146	0.007490	1.73	0.194
Sex*parasitised	1	0.032500	0.032500	0.032500	7.50	0.008
Error	52	0.225332	0.225332	0.004333		
Total	55	0.271824				

S = 0.0658279 R-Sq = 17.10% R-Sq(adj) = 12.32%

Unusual Observations for Zp

Obs	Zp	Fit	SE Fit	Residual	St Resid
37	0.033284	0.245261	0.014035	-0.211976	-3.30 R
56	0.083472	0.233420	0.016997	-0.149949	-2.36 R

Least Squares Means for Zp

Sex	Mean	SE Mean
F	0.2375	0.01181
M	0.2781	0.01507
parasitised		
0	0.2393	0.01102
1	0.2763	0.01565
Sex*parasitised		
F 0	0.2453	0.01403
F 1	0.2298	0.01900
M 0	0.2334	0.01700
M 1	0.3228	0.02488

9.7 Raw Data for Teratocytes

9.7.1 T-Test Between Fatty Acid Composition of Teratocytes and CRW

a) T-Test Raw Data for 12:0 (S)

Two-Sample T-Test and CI: Sp, 12

Two-sample T for Sp vs 12

	N	Mean	StDev	SE Mean
Sp	164	0.0068	0.0109	0.00085
12	10	0.0127	0.0106	0.0032

Difference = mu (Sp) - mu (12)

Estimate for difference: -0.00588

95% CI for difference: (-0.01314, 0.00138)

T-Test of difference = 0 (vs not =): T-Value = -1.78 P-Value = 0.102 DF = 11

b) T-Test Raw Data for 14:0 (T)

Two-Sample T-Test and CI: Tp, 14

Two-sample T for Tp vs 14

	N	Mean	StDev	SE Mean
Tp	164	0.0114	0.0170	0.0013
14	10	0.0181	0.0169	0.0051

Difference = mu (Tp) - mu (14)

Estimate for difference: -0.00671

95% CI for difference: (-0.01832, 0.00489)

T-Test of difference = 0 (vs not =): T-Value = -1.27 P-Value = 0.229 DF = 11

c) T-Test Raw Data for 16:0 (U)

Two-Sample T-Test and CI: Up, 16

Two-sample T for Up vs 16

	N	Mean	StDev	SE Mean
Up	164	0.1460	0.0382	0.0030
16	10	0.1544	0.0577	0.017

Difference = mu (Up) - mu (16)

Estimate for difference: -0.0084

95% CI for difference: (-0.0477, 0.0309)

T-Test of difference = 0 (vs not =): T-Value = -0.48 P-Value = 0.644 DF = 10

d) T-Test Raw Data for 16:1 (V)

Two-Sample T-Test and CI: Vp, 16 1

Two-sample T for Vp vs 16 1

	N	Mean	StDev	SE Mean
Vp	164	0.0974	0.0565	0.0044
16 1	10	0.0888	0.0242	0.0073

Difference = mu (Vp) - mu (16 1)

Estimate for difference: 0.00853

95% CI for difference: (-0.00937, 0.02643)

T-Test of difference = 0 (vs not =): T-Value = 1.00 P-Value = 0.330 DF = 18

e) T-Test Raw Data for 18:0 (W)

Two-Sample T-Test and CI: Wp, 18

Two-sample T for Wp vs 18

	N	Mean	StDev	SE Mean
Wp	164	0.0822	0.0554	0.0043
18	10	0.0534	0.0440	0.013

Difference = mu (Wp) - mu (18)

Estimate for difference: 0.0289

95% CI for difference: (-0.0015, 0.0593)

T-Test of difference = 0 (vs not =): T-Value = 2.07 P-Value = 0.061 DF = 12

f) T-Test Raw Data for 18:1 (X)

Two-Sample T-Test and CI: Xp, 18 1

Two-sample T for Xp vs 18 1

	N	Mean	StDev	SE Mean
Xp	164	0.3878	0.0848	0.0066
18 1	10	0.371	0.101	0.031

Difference = mu (Xp) - mu (18 1)

Estimate for difference: 0.0170

95% CI for difference: (-0.0528, 0.0867)

T-Test of difference = 0 (vs not =): T-Value = 0.54 P-Value = 0.599 DF = 10

g) T-Test Raw Data for 18:2 (Y)

Two-Sample T-Test and CI: Yp, 18 2

Two-sample T for Yp vs 18 2

	N	Mean	StDev	SE Mean
Yp	164	0.0617	0.0469	0.0037
18 2	10	0.0585	0.0234	0.0071

Difference = mu (Yp) - mu (18 2)

Estimate for difference: 0.00325

95% CI for difference: (-0.01360, 0.02011)

T-Test of difference = 0 (vs not =): T-Value = 0.41 P-Value = 0.688 DF = 16

h) T-Test Raw Data for 18:3 (Z)

Two-Sample T-Test and CI: Zp, 18 3

Two-sample T for Zp vs 18 3

	N	Mean	StDev	SE Mean
Zp	164	0.2066	0.0774	0.0060
18 3	10	0.243	0.125	0.038

Difference = mu (Zp) - mu (18 3)

Estimate for difference: -0.0366

95% CI for difference: (-0.1220, 0.0487) T-Test of difference = 0 (vs not =): T-Value = -0.96 P-Value = 0.361 DF = 10

9.7.2 T-Test Between Fatty Acid Composition of Larvae and CRW

a) T-Test Raw Data for 12:0 (S)

Two-Sample T-Test and CI: 12:0, Sp

Two-sample T for 12:0 vs Sp

	N	Mean	StDev	SE Mean
12:0	7	0.00910	0.00773	0.0029
Sp	164	0.0068	0.0109	0.00085

Difference = mu (12:0) - mu (Sp)

Estimate for difference: 0.00229

95% CI for difference: (-0.00491, 0.00949)

T-Test of difference = 0 (vs not =): T-Value = 0.75 P-Value = 0.476 DF = 7

b) T-Test Raw Data for 14:0 (T)

Two-Sample T-Test and CI: 14:0, Tp

Two-sample T for 14:0 vs Tp

	N	Mean	StDev		SE Mean
14:0	7	0.0181	0.0149	.	0056
Tp	164	0.0114	0.0170		0.0013

Difference = mu (14:0) - mu (Tp)

Estimate for difference: 0.00673

95% CI for difference: (-0.00740, 0.02086)

T-Test of difference = 0 (vs not =): T-Value = 1.17 P-Value = 0.288 DF = 6

c) T-Test Raw Data for 16:0 (U)

Two-Sample T-Test and CI: 16:0, Up

Two-sample T for 16:0 vs Up

	N	Mean	StDev		SE Mean
16:0	7	0.1578	0.0400		0.015
Up	164	.1460	.0382	0.0030	

Difference = mu (16:0) - mu (Up)

Estimate for difference: 0.0118

95% CI for difference: (-0.0259, 0.0495)T-Test of difference = 0 (vs not =): T-Value = 0.76 P-Value = 0.473 DF = 6

d) T-Test Raw Data for 16:1 (V)

Two-Sample T-Test and CI: 16:1, Vp

Two-sample T for 16:1 vs Vp

	N	Mean	StDev	SE Mean
16:1	7	0.0748	0.0147	0.0055
Vp	164	0.0974	0.0565	0.0044

Difference = mu (16:1) - mu (Vp)

Estimate for difference: -0.02253

95% CI for difference: (-0.03765, -0.00742) T-Test of difference = 0 (vs not =): T-Value = -3.18 P-Value = 0.006 DF = 15

e) T-Test Raw Data for 18:0 (W)

Two-Sample T-Test and CI: 18:0, Wp

Two-sample T for 18:0 vs Wp

	N	Mean	StDev	SE Mean
18:0	7	0.0561	0.0441	0.017
Wp	164	0.0822	0.0554	0.0043

Difference = mu (18:0) - mu (Wp)

Estimate for difference: -0.0261

95% CI for difference: (-0.0683, 0.0161) T-Test of difference = 0 (vs not =): T-Value = -1.52 P-Value = 0.181 DF = 6

f) T-Test Raw Data for 18:1 (X)

Two-Sample T-Test and CI: 18:1, Xp

Two-sample T for 18:1 vs Xp

	N	Mean	StDev	SE Mean
18:1	7	0.3225	0.0781	0.030
Xp	164	0.3878	0.0848	0.0066

Difference = mu (18:1) - mu (Xp)

Estimate for difference: -0.0653

95% CI for difference: (-0.1393, 0.0087) T-Test of difference = 0 (vs not =): T-Value = -2.16 P-Value = 0.074 DF = 6

g) T-Test Raw Data for 18:2 (Y

Two-Sample T-Test and CI: 18:2, Yp

Two-sample T for 18:2 vs Yp

	N	Mean	StDev	SE Mean
18:2	7	0.0982	0.0540	0.020
Yp	164	0.0617	0.0469	0.0037

Difference = mu (18:2) - mu (Yp)

Estimate for difference: 0.0365

95% CI for difference: (-0.0142, 0.0872) T-Test of difference = 0 (vs not =): T-Value = 1.76 P-Value = 0.129 DF = 6

h) T-Test Raw Data for 18:3 (Z)

Two-Sample T-Test and CI: 18:3, Zp

Two-sample T for 18:3 vs Zp

	N	Mean	StDev	SE Mean
18:3	7	0.2633	0.0554	0.021
Zp	164	0.2066	0.0774	0.0060

Difference = mu (18:3) - mu (Zp)

Estimate for difference: 0.0567

95% CI for difference: (0.0051, 0.1082) T-Test of difference = 0 (vs not =): T-Value = 2.60 P-Value = 0.035 DF = 7

9.8 Raw Data for Juvenile Hormone III

9.8.1 Paired T-Test Between Non-Parasitised and Parasitised

Paired T-Test and CI: UP, P

Paired T for UP - P

	N	Mean	StDev	SE Mean
UP	2	4.097	0.112	0.079
P	2	6.788	0.238	0.168
Difference	2	-2.6918	0.1259	0.0890

95% CI for mean difference: (-3.8229, -1.5607)

T-Test of mean difference = 0 (vs not = 0): T-Value = -30.24 P-Value = 0.021

9.8.2 Two Sample T-Test Between Non-Parasitised and Parasitised

Two-Sample T-Test and CI: Parasitised, Unparasitised

Two-sample T for Parasitised vs Unparasitised

	N	Mean	StDev	SE Mean
Parasitised	5	4.236	0.333	0.15
Unparasitised	2	6.790	0.240	0.17

Difference = mu (Parasitised) - mu (Unparasitised)

Estimate for difference: -2.554

95% CI for difference: (-3.527, -1.581)

T-Test of difference = 0 (vs not =): T-Value = -11.30 P-Value = 0.008 DF = 2