Behavioural Endocrinology of Breeding Adelie Penguins (*Pygoscelis adeliae*)

A thesis
Submitted in partial fulfilment for the degree
of
Master of Science in Biological Sciences
at
The University of Waikato

by

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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2008

Abstract

Measuring hormonal changes is vital for understanding how the social and physical environment influences behaviour, reproduction and survival. Various methods of hormone measurement exist, potentially explaining variation in results across studies; methods should be cross validated to ensure they correlate. I directly compare faecal and plasma hormone measurements (Chapter 2), and use the most suitable endocrine measure to test the Darling hypothesis (Chapter 3) - that breeding is hastened and synchronized in larger colonies due to increased social stimulation (mediated by the endocrine system).

Blood and faecal samples were simultaneously collected from individual Adelie penguins (*Pygoscelis adeliae*) for comparison, and assayed for testosterone and corticosterone (or their metabolites). Sex differences and variability within each measure, and correlation of values across measures were compared. For both hormones, plasma samples showed greater variation than faecal samples. Males had higher corticosterone levels than females, but the difference was only significant in faecal samples. Plasma testosterone, but not faecal testosterone, was significantly higher in males than females. Correlation between sample types was poor overall, and weaker in females than in males; perhaps because measures from plasma represent hormones that are both free and bound to globulins, whereas measures from faeces represent only the free portion. Faecal samples also represent a cumulative measure of hormones over time, as opposed to a plasma 'snapshot' concentration. Faecal sampling appears more suitable for assessing baseline hormone levels.

In the second study I examined, over two seasons, whether the timing of breeding varied with colony size; larger colonies present occupants with higher levels of social stimulation and are predicted to show earlier, more synchronous breeding. Baseline faecal hormone levels throughout the breeding season, and survival, were measured to investigate possible proximate and ultimate mechanisms for the results. The influence of environmental variability was examined, by relating the timing of breeding, survival, and endocrine changes to sea ice conditions. Colony size did not influence the timing or synchrony of breeding, survival, or hormone

levels within years; perhaps because colonies in an Adelie rookery are not independent from the 'social environment' of adjacent colonies. Across years, synchrony in the smaller rookery was higher than in the larger rookery. The scale of these comparisons may exceed the applicability of the Darling hypothesis. Therefore, no support was found for the Darling hypothesis, at the colony or rookery level, in this species. Higher corticosterone metabolite and lower sex hormone levels in the first season correlated to later breeding and lower survival compared to the second season. This is likely due to the persistence of extensive sea ice conditions late into the first season.

Researchers should take care in selecting the most appropriate method of hormone measurement for their question. Future studies testing the Darling hypothesis must carefully select their definition of a colony (i.e. a truly isolated social unit) and the scale at which the hypothesis is tested. Combining endocrine measurements with behavioural, survival, and environmental information allows for a more comprehensive interpretation of animal ecology.

Acknowledgements

Many people have helped me along the way in undertaking this thesis, and indeed I could have never of done it without them.

Firstly, I would like to give my most sincere and absolute thanks to my supervisor Joe Waas. My appreciation for the opportunities you have given me is immense. As an undergraduate student, I could only listen in awe as lecturers described their research in Antarctica, and wonder if it was even possible for me to do such things. I have learned so much through the course of this masters, due to the opportunities and support which you have given me, and the various people that you have referred me to, that I now feel that I can achieve anything. I could not have wished for a better supervisor, and will be forever grateful.

I would like to thank Sue McCurdy, who gave me the great opportunity of working for a summer with the Kakapo on Codfish Island during my undergraduate degree, and persuaded Joe to be the supervisor of the report I produced. Had it not been for Sue facilitating this field experience and connecting me with Joe, I may never have had the opportunity to undertake this thesis.

Thanks to Quanah Hudson, Jess Wallace, Nick Ling, and Dudley Bell for being great companions in the field. Although we were in a challenging physical environment, you ensured that we were in a comfortable and enjoyable social environment, which made a great experience into an amazing one. Thanks also to our American neighbours at Cape Crozier, who gave us some additional good company and tasty treats on several occasions.

Big thanks to Erich Möstl and all the staff at the Institute for Biochemistry at the University of Veterinary Medicine, Vienna, Austria, for being wonderfully hospitable and patient with me. Particularly, thanks to Alexandra Kuchar whose patience in training me to undertake the hormonal analysis (being a behaviour student with no knowledge of biochemistry) was astounding. Also huge thanks to

Jonathan Banks, who laboured through the analysis of hundreds of samples with me, and was a friendly kiwi companion to share a beer with after long days in the lab, as well as to seek advice from.

Dick Wilkins and Raewyn Towers gave me guidance with sexing blood samples, and Karen Nutt provided me with the necessary primers, which was much appreciated.

Huge thanks to Shinichi Nakagawa, who gave me advice and direction on statistical methods from the other side of the world, via email and multiple long skype conversations. I would have been lost in the dataset without you, and have developed a much better understanding of statistics, which I am grateful for.

Erich Möstl, Shinichi Nakagawa, Nick Ling, Jonathan Banks, and of course Joe Waas all provided helpful comments on drafts, with special thanks to Joe for the after hours time you invested to read countless drafts, which has improved my writing skills considerably. Thanks to Cheryl Ward for helpful advice on referencing and formatting techniques, and all those librarians behind the scenes that got me countless interloan requests.

Dai Morgan shared an office space with me, endured my endless random questions, and even made me cups of tea. I have been lucky to have your company and wisdom in the office, at conferences, and socially.

I must acknowledge The Perry Foundation, The Ministry of Education, The Waikato Students Union, The University of Waikato and the Department of Biological Sciences (from which thanks to Bruce Clarkson and Joe Waas), all of whom have provided financial assistance which was essential to undertaking this thesis, and allowed me to present my findings at an international conference. I am greatly appreciative of this financial support, without which this thesis would not have been possible!

I would like to acknowledge the various other people who contributed to this project over the years, before I came along and took it on as my baby. Although I

did not meet many of you, I hope I have done your work justice by what I have produced in this thesis.

Finally, I would like to give huge thanks to my parents, who have always supported me in my studies. They have enabled and encouraged me to grasp every opportunity that has come my way, and shared great interest in my studies. Sometimes I wonder if mum is as enthusiastic about what I am doing as I am! Much love and thanks to you for your endless support in all its forms.

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Chapter 1

General Introduction

Behavioural endocrinology is an exciting field which is increasingly being applied to the study of breeding birds. It enables one to investigate the physiological underpinning of animal behaviour and ecology, greatly increasing our understanding of biological processes. Various methods of measuring hormones have rapidly developed, and been widely applied in a range of studies. In this general introduction I will start with a brief account of the field of behavioural endocrinology. I will then consider the physiology of the hormonal processes that are central to concepts explored in this thesis, as well as the importance of measuring hormones in studies of behaviour and ecology. Finally, I will review relevant aspects of the biology of my study animal, the Adelie penguin (*Pygoscelis adeliae*), before outlining the aims and format of this thesis.

Behavioural endocrinology

The first formal experiments in behavioural endocrinology, the study of how hormones influence an animal's behaviour, were conducted in 1849 by the Swedish scientist A. A. Berthold, who discovered that the removal of the testes from a rooster caused it to cease displaying sexual or aggressive behaviours (Berthold, 1849). When a testis was re-implanted into the body cavity, the rooster again displayed sexual and aggressive behaviour. Berthold concluded that the testes produced some chemical(s) which influenced the rooster's behaviour, as nerve connections had not been re-established (Becker & Breedlove, 1992). These chemicals were a group of steroid hormones called androgens, which influence an animal's behaviour, particularly male sexual behaviour, by acting directly on the brain as well as acting indirectly on androgen-sensitive tissues (e.g. prostate gland, seminal vesicles) (Baum, 1992). Estrogens (and other hormones) are important in influencing female sexual behaviour and similarly to androgens, function at multiple levels (Carter, 1992). Glucocorticoids, which form part of an

animal's response to stress (Archer, 1979), are also an important and commonly measured group of hormones (Millspaugh & Washburn, 2004). The measurement of sex and stress hormones can compliment each other, as well as behavioural measurements; for example stressful events appear to influence the pituitary-gonadal system, suppressing testicular and ovarian functions, and hence reproductive success (Levine, 1985). Behavioural endocrinology thus examines hormonal influences on behaviour, and indeed behavioural influences on the endocrine system. This can provide a more comprehensive understanding of the state and responses of an animal under various conditions than can be achieved by measuring these parameters in isolation (Koren et al., 2002).

The neuroendocrinology of the sex hormones

The main vertebrate sex hormones in both sexes are produced and released from the gonads following a cascade of neuroendocrine signals. The hypothalamus, located at the base of the brain just above the brain stem, secretes gonadatropin releasing hormone (GnRH), which stimulates the anterior pituitary gland to release the gonadatropins: luteinizing hormone (LH), and follicle stimulating hormone (FSH) (Archer, 1979; Buckle, 1983). In birds, LH stimulates steroidogenesis of estrogens, progestagens, and androgens in the ovaries of females, and androgens in the testes of males (Buckle, 1983; Harvey et al., 1987). The chief hormone in each of the estrogen, progestagen, and androgen groups is estradiol-17β, progesterone, and testosterone, respectively (Harvey et al., 1987).

The main gonadal hormones then have a negative feedback effect, inhibiting the release on GnRH, decreasing pituitary sensitivity to GnRH, and suppressing basal pituitary LH release, creating a dynamic equilibrium between gonadatropin secretion and gonadal steroid production; under some circumstances other forms of gonadal steroids may have positive feedback effects (Harvey et al., 1987). FSH is important for maturation and yolk deposition of eggs in females, and for spermatogenesis in males (Harvey et al., 1987).

Alone, neither progesterone nor androgens have a major effect on the reproductive physiology of female birds, but they act synergistically with estrogens to affect

oviduct development and egg white production (Harvey et al., 1987). The testes can also produce female sex steroids (estrogens and progestagens), but these may not be released into circulation (Harvey et al., 1987), although estradiol has been shown to be an essential component for expression of copulatory behaviour in male Japanese quails (*Coturnix japonica*) (Massa, 1984).

The major gonadal steroids cause the development of secondary sex organs, and secondary sexual characteristics (Harvey et al., 1987). In birds, elevated levels of sex hormones usually elicit territorial, aggressive, courtship, and copulatory behaviours in males, and receptive and parental behaviours in females (Wingfield et al., 1990; Schlinger et al., 2001).

The neuroendocrinology of the stress response

Glucocorticoids and catecholamines are the most important hormones released during the stress response (Sapolsky, 1992). Catecholamines are released from the adrenal medulla by direct innervation from the sympathetic nervous system, entering into circulation very rapidly (several seconds) (Sapolsky, 1992). A hormonal cascade produces the release of glucocorticoids. When the brain perceives a stressor, corticotrophin releasing hormone (CRH) is released from the hypothalamus, which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), stimulating the adrenal cortex to release glucocorticoids (see Chester-Jones, 1957 for the definitive work detailing the adrenal cortex and its hormones; for more recent work see Ringer, 1976, Schulster et al., 1976, Harvey et al., 1984, and Sapolsky, 1992). Hence glucocorticoids are slower to enter circulation (several minutes) than catecholamines. This hormonal cascade is known as the hypothalamic-pituitary-adrenal (HPA) axis, and is stimulated in all vertebrates in response to a vast array of different noxious stimuli (Wingfield, 1994), leading Selye (1956) to term the response the 'general adaptation syndrome'. The main glucocorticoids released during this response are cortisol and corticosterone, the latter dominating in birds (Archer, 1979; Wingfield, 1994). Elevated glucocorticoids put an animal into an 'emergency life-history stage' (Wingfield et al., 1998), in which energy is mobilized by catabolising fat stores, and functions which are not essential during times of challenged

homeostasis, such as growth, reproduction, and immune defence are suppressed (Siegel, 1980; Pickering & Fryer, 1994; Silverin, 1998). Behaviour is also redirected away from reproduction towards survival, by stimulating dispersal and/or foraging behaviour (Silverin, 1998). Thus, short elevations can help an animal escape a dangerous situation, whereas chronically elevated levels can have significant deleterious effects to the animal (Wingfield, 1994; Wingfield et al., 1998).

Measuring hormone levels

Before the mid 20th century, the only way to measure hormone concentrations in blood or tissues was by biological assay, or bioassay (Becker & Breedlove, 1992). This method usually involved injecting the sample of unknown hormone concentration into a laboratory animal, and examining consequent physiological changes in that animal (e.g. injecting bull plasma into a castrated male rat, then using prostate weight of the rat to infer bull testosterone levels) (Schulster et al., 1976; Becker & Breedlove, 1992). However, bioassays were not very sensitive or convenient, and became outdated as more rapid and precise methodologies were developed.

In the 1950s, Rosalyn Yalow and Seymour Berson developed the radioimmunoassay (RIA) (Yalow & Berson, 1960), for which they later received a Nobel prize (Becker & Breedlove, 1992). This involved the competitive binding of a sample hormone (unknown concentration) against a radiolabelled hormone (known concentration) to an antibody; the greater the amount of hormone in the sample, the less radioactive hormone will be bound by the antibodies. The antibodies (and bound hormones) could then be isolated and radioactivity quantified. By assaying samples of various known concentrations to create a standard curve of sample concentration to radioactivity, the concentrations of unknown samples (but known radioactivity) could be determined (Yalow & Berson, 1960).

The enzymeimmunoassay (EIA) was later developed (Engvall & Perlman, 1971; Van Weemen & Schuurs, 1971), which was faster, required simpler equipment,

did not require licences, and for which labels were stable and longer-lasting than for RIAs (Engvall & Perlman, 1971; Adkins-Regan, 2005). The EIA functioned in a similar way to the RIA, but utilized an enzyme labelled hormone instead of one which was labelled radioactively. The enzyme would react with a substrate to produce a colour reaction. Thus the magnitude of the colour change, representing the amount of bound label, could be measured instead of measuring radioactivity. Despite this, however, RIAs continue to be used in recent studies (e.g. Astheimer et al., 1995; Tyrrell & Cree, 1998; Cockrem & Silverin, 2002; Blas et al., 2005; Angelier et al., 2006; Walker et al., 2006), possibly because some laboratories are already set up for undertaking RIAs, and in some cases an RIA may have already been biologically validated in the study species.

Recently the emphasis has shifted away from which assay type to use, and onto what medium to measure the hormone from. Traditionally, blood plasma has been the obvious and most used sample type to assay for hormones (Washburn et al., 2003), as hormones are blood-borne messengers (Buckle, 1983). However, there are limitations associated with blood collection, as (1) the stress of collection consequently influences hormone levels, (2) the amount that can be taken may be limited, and (3) various safety and ethical issues are associated with the invasive nature of the capture and restraint process (Koren et al., 2002). Additionally, due to the pulsatile fashion of secretion of some hormones, plasma hormone levels can change quickly (Palme et al., 2005). Lately there has been substantial growth in the use of noninvasive methods (Buchanan & Goldsmith, 2004). Studies now use hair (Koren et al., 2002), urine (Creel et al., 1992; Sannen et al., 2004), saliva (von Engelhardt et al., 2000; Cook, 2002), sweat (Cook, 2002), and faeces (Wasser et al., 2000; Pfeffer et al., 2002) to quantify hormone levels in animals. Obviously some of these methods will be restricted to certain taxa as, for example, birds do not produce some of these media. Faeces, however, are produced from all vertebrates, and have become a very popular sample type used to noninvasively measure hormones across many taxa (Millspaugh & Washburn, 2004).

Faecal measurements of hormones are noninvasive, usually easy to collect, and avoid the problems of quickly fluctuating hormone levels associated with plasma

measurements (Palme et al., 1996; Palme et al., 2005). However, recent literature has clearly outlined that faecal hormone assays must be carefully chosen and validated, as metabolism and excretion patterns can vary from species to species (Buchanan & Goldsmith, 2004; Möstl et al., 2005; Palme, 2005; Palme et al., 2005). It is notable that different methods for measuring hormones (e.g. using blood or faeces) appear to have both been used in similar studies (e.g. assessing baseline glucocorticoids). However, no study has simultaneously used both plasma and faecal measures to formally examine differences in the results that may be obtained.

Why measure hormones?

The full array of uses for hormone measurement are too broad to describe here. Therefore, I will focus on uses that pertain to the main topics of this thesis: animal behaviour and ecology.

Hormones as a mechanism to explain behavioural phenomena

Because hormones influence behaviour, and vice versa, monitoring hormone levels can provide insights into the mechanistic aspects of behaviour (Koren et al., 2002). For example, Creel et al. (1992) have described that reproductive suppression in dwarf mongooses (*Helogale parvula*) is achieved by both behavioural and endocrine mechanisms, and Pfeffer et al. (2002) have correlated the ability to perform operant tasks with increased corticosterone in greylag geese (*Anser anser*). Sexual, parental, aggressive and feeding behaviours have all been associated with hormones, thus hormonal analysis can provide insights into evolutionary theories that are based on behaviour (Koren et al., 2002). The 'challenge hypothesis' (Wingfield et al., 1990), is one such evolutionary theory, which postulates a relationship between breeding strategy (i.e polygamy or monogamy) and male paternal and aggressive behaviour. Testosterone is postulated to mediate this relationship, with testosterone levels also influenced by behavioural interactions. The predictions generated by the challenge hypothesis have been supported in many studies on birds (Wingfield et al., 1990), however, findings in dwarf mongooses differ from expectations (Creel et al., 1993).

Another evolutionary concept involving hormonal processes is the 'Darling effect' (Darling, 1938). Darling hypothesized that the timing and synchrony of reproduction in birds is altered by the amount of social stimulation received from conspecifics. Hormonal measurement is essential to explore and validate this hypothesis by providing a mechanism for the phenomenon, and to invalidate alternative explanations. It has been recently demonstrated in a free-living species, the yellow-eyed penguin (*Megadyptes antipodes*), that hormonal changes are associated with behavioural responses to social stimuli (Setiawan et al., 2007). Such studies in behavioural endocrinology have also emphasized the applicability of many theories across taxa (e.g. Creel et al., 1993), allowing them to be further refined (Creel et al., 1993). Additionally, it has been long noted that valid inferences cannot always be made from laboratory studies, necessitating field studies for meaningful tests of such theories (Darling, 1964).

Endocrine measurements can also reveal information that would not otherwise be obtained through other measures, such as behaviour, allowing us to better understand the effects on animals of various abiotic, biotic, and anthropogenic perturbations. Measuring hormones, such as those described above, can provide information on an animal's internal state, which may not be detected by behaviour alone (Wikelski & Cooke, 2006).

Assessing the effects of stress on wildlife

Stress can be caused by physical and social changes in an animal's environment, which produce a behavioural and/or physiological response (Sapolsky, 1990; Sapolsky, 1992; Broom & Johnson, 1993). Moberg (2000, p.1) defines stress as "the biological response elicited when an individual perceives a threat to its homeostasis". Stress can also inhibit reproductive hormones (Rabin et al., 1988; Sapolsky, 1992). Thus, measuring stress is useful in determining the effects of human disturbance, or environmental conditions on wildlife, as well as in studies of reproduction. Measuring hormones can provide a reliable objective measurement of stress, which is critical because without information about the

physiological state of an animal, the debate on stress will not advance beyond the subjective level (Moberg, 1985a).

Humans have encroached, and continue to encroach on the habitats of wildlife. The most intensive impacts, such as habitat destruction, have obvious deleterious effects on wildlife. However, for more subtle forms of human disturbance, like the recreational use of wildlife habitat by humans, or ecotourism, the effects on wildlife may be less explicit. Because many wild animals perceive humans as predators (Roberts & Evans, 1993), it is likely that human activity in proximity to wild animals can disturb them. This has been a popular field of study in recent times, and many authors have assessed the effects of human disturbance on birds (e.g. Burger, 1994; Giese, 1996; Fowler, 1999; McClung et al., 2004; Walker et al., 2005; Ellenberg et al., 2006; Walker et al., 2006; Arlettaz et al., 2007; Carlini et al., 2007; Ellenberg et al., 2007). Hormonal measurements have been crucial in revealing stress in some of these studies, as it is common for many animals to freeze in response to a perceived threat (Manser, 1992), hence showing no obvious behavioural signs of stress. This may be the case for Adelie penguins in Antarctica (Wilson et al., 1991; see also Giese, 1996), where tourism is rapidly increasing (Stewart et al., 2005; Spennemann, 2007).

Environmental conditions can also cause stress to animals, having subsequent influences on behaviour. Humidity and temperature can influence glucocorticoid levels (Marple et al., 1972), and reduced food abundance produced elevated corticosterone levels in the common murre (*Uria aalge*), which predicted reduced reproductive performance (Kitaysky et al., 2007). The measurement of stress hormones in the study by Kitaysky et al. (2007) allowed them to provide the first unequivocal evidence for corticosterone secretion as a mechanism by which fluctuations in food abundance influence population processes in a seabird.

It has been suggested that scientists should deemphasize measuring discrete physiological responses to stress and instead examine the effects stress has on reproduction (Moberg, 1985a). Even adopting this suggestion, measuring hormones may still be crucial because of the necessity to identify components of the reproductive process that are easy to monitor (Moberg, 1985b). However,

ultimately, measuring single parameters to assess stress may prove inadequate. Progressive studies will need to examine breeding success as a function of glucocorticoid levels (Tarlow & Blumstein, 2007), as Mullner et al. (2004) has done for hoatzin chicks (*Opisthocomus hoazin*)

Ecological endocrinology

The ecology of an animal is a complicated matrix of interactions between physiology, behaviour, predation, and the social and physical environment. For example, coloniality in birds may function to reduce an individual's chance of predation through predator swamping, whereas the social environment in colonies may also affect physiology, which may influence breeding behaviour and predation (reviewed by Wittenberger & Hunt jr, 1985). The Darling effect (Darling, 1938), as discussed earlier, was the first comprehensive sociobiological theory to integrate a complex blend of physiology, behaviour, ecology and evolution (Gochfeld, 1979). The Darling effect predicted that higher levels of social stimulation will hasten and synchronize breeding, via endocrine pathways, reducing predation by having vulnerable young present over a shorter temporal range (Darling, 1938). Such complex theories are difficult to test, as they require the measurement of multiple ecological parameters (Gochfeld, 1979). No study of which I am aware has managed to give a comprehensive test of this theory, accounting for all of the ecological aspects which comprise it. Hernandez-Matias et al. (2003) found that nest predation was lower in larger colonies, and higher for individuals breeding before the colony average breeding date in the common tern (*Sterna hirundo*), and Burger (1979) found that breeding synchrony increased with colony size (and hence social stimulation) in herring gulls (*Larus argentatus*), while Weatherhead and Sommerer (2001) addressed the relationship between breeding synchrony and predation in red-winged blackbirds (*Agelaius phoeniceus*).

Other explanations for colonial behaviour include the influence of the physical environment, such as spatiotemporally clumped food resources or suitable nesting habitat (Wittenberger & Hunt jr, 1985), and other social factors such as improved

food finding ability from social foraging (Emlen & Demong, 1975). The different ecological reasons do not necessarily need to be mutually exclusive.

The physical environment can also influence behaviour and survival rates. Sea ice extent has been found to affect survival (Wilson et al., 2001), locomotion and diving behaviour (Rodary et al., 2000; Yoda & Ropert-Coudert, 2007), foraging behaviour (Ainley et al., 1998; Rombola et al., 2003), physical condition (Ainley et al., 1998; Yoda & Ropert-Coudert, 2007), and diet (Ainley et al., 1998) in Adelie penguins. Rainfall has been shown to influence timing of breeding in the zebra finch (*Taeniopygia guttata*) in arid Australia (Zann et al., 1995). Smith et al. (1994) found corticosterone levels of diving petrels (*Pelecanoides urinatrix*) were higher, and body mass was lower, following storm conditions; corticosterone levels may have been at their physiological maximum, as they did not rise in response to capture and restraint post-storm, when they had after calm conditions. The many effects that these climatic influences have are likely to affect other aspects of the ecology of animals, such as predation rates and reproduction, complicating tests on theories such as the Darling hypothesis.

Hormones may be a useful additional parameter to include in such ecological studies. The effects that sex and stress hormones can have on behaviour have already been described in this Introduction. Additionally, elevated corticosterone was related to colony size, parasite load, and bad weather in cliff swallows (*Petrochelidon pyrrhonota*), which probably caused increased allocation of time and energy to foraging and increased energy assimilation (Raouf et al., 2006). Marra and Holberton (1998) found that corticosterone levels could be used to indicate habitat quality in the American redstart (*Serophaga ruticilla*), which had subsequent effects on physiological condition and their annual cycle.

Some species may also show temporally changing behaviour or survival, whilst other factors appear relatively constant. Hormonal responses were correlated with poor survival at the juvenile stage in hoatzin chicks (*Opisthocomus hoazin*) in response to human visitation, whereas adults and nestlings appeared relatively insensitive (Mullner et al., 2004). Grey-faced petrels (*Pterodroma macroptera gouldi*) were found to exhibit higher stress responses during late incubation than

at other periods (Adams et al., 2005). Romero et al. (1997) found baseline and stress-induced corticosterone levels to differ with season in white-crowned sparrows (*Zonotrichia leucophrys gambelii*), independent of weather conditions. It has also been proposed that the levels and effects of glucocorticoids and testosterone have an ecological basis, changing with season and the life-history stage of an animal (Wingfield et al., 1998; Wingfield et al., 2001). Accounting for such endocrine changes could be important for the interpretation of the effects of other ecological variables.

Given the relationships between hormones, behaviour, reproduction, and the social and physical environment of animals, hormonal measurements are useful for more than just providing a mechanism for behaviour, but are another aspect to be considered when examining the general ecology of an animal.

The Adelie penguin

The Adelie penguin is a good species for studies of behavioural endocrinology, as they are abundant colonial breeders. Their habitat and nests are relatively exposed, making it easy to observe their breeding behaviour, reproductive success, and sample. Adelie penguins are also part of a very simple ecosystem (in terms of species composition, and food webs), increasing the possibility of quantifying various ecological factors completely.

Classification and status

All penguins belong to the subfamily Spheniscinae (order Sphenisciformes, family Spheniscidae), within which the Adelie penguin (*Pygoscelis adeliae*) shares its genus with two other extant species: the chinstrap penguin (*Pygoscelis antarctica*) and the gentoo penguin (*Pygoscelis papua*) (Marchant & Higgins, 1990). The population of Adelie penguins has been estimated at 2.5 to 2.6 million breeding pairs (Marchant & Higgins, 1990; Ainley, 2002), and their conservation status is currently 'least concern'. However fluctuations in population numbers have been reported, with many of the monitored populations showing increases until the late 1980s, then declining through the 1990s, especially around the

Antarctic Peninsula region; however, Adelie populations of the East Antarctica region seem to have steadily increased (Woehler & Croxall, 1997; Woehler et al., 2001). Some authors have indicated that these fluctuations may in part be due to sensitivity of breeding Adelie penguins to environmental variability (e.g. sea ice conditions, Croxall et al., 2002) and human disturbance (Reid, 1968; Woehler & Croxall, 1997).

Distribution and habitat

Adelies have a circumpolar continental distribution (Trivelpiece & Trivelpiece, 1995). They nest around the coast of continental Antarctica, as well as on coasts of islands up to 1500 km off the continental shore; they exploit any accessible site surrounded by pack ice during winter and early spring, that has low gradient (<45º) exposed rocky terrain, and where gravel is present for nest construction (Ainley, 2002). At these sites they congregate in groups ranging from dozens to hundreds of thousands of pairs (Marchant & Higgins, 1990). The larger congregations are dispersed into smaller groups throughout the general area, forming a patchwork of colonies; the most favourable sites within a general breeding area are knolls and ridges that will avoid melt water later in the season (Ainley, 2002). Associated with Adelie colonies are breeding south polar skua (*Catharacta maccormicki*), which also nest on exposed rocky substrate. The skua are scavengers and predators of Adelie eggs and young chicks (Ainley, 2002).

Breeding biology

Adelie penguins are monogamous colonial breeders, with closely spaced nests which abut at least one, but usually more, adjacent territories (Williams, 1995; Ainley, 2002). Birds arrive at the breeding grounds in late September to mid October (Marchant & Higgins, 1990), with males arriving several days before females to establish territories and build nests (Spurr, 1975; Müller-Schwarze, 1984; Ainley, 2002). Some latitudinal variation in arrival date occurs, with birds arriving earlier at northern than southern rookeries (Marchant & Higgins, 1990; Ainley, 2002). Pairing and copulation occurs quickly following female arrival, especially in experienced breeders (Ainley, 2002). This period will be referred to

as the courtship period throughout this thesis. Egg laying also occurs later in the more southern sites than in those further north, ranging from late October to late November (Spurr, 1975; Ainley, 2002). Total incubation time of eggs averages 35 days (Müller-Schwarze, 1984). The male undertakes the early incubation duties for approximately 2 weeks, while the female returns to sea to feed (Ainley, 2002). The female then returns for the late incubation period (2-3 weeks), while the male, having fasted for 5-6 weeks (during nest establishment, courtship, and early incubation), goes to sea to feed (Ainley & LeResche, 1973). Sometimes, upon return of the male, there may be time for shorter secondary foraging trips before the eggs hatch (Müller-Schwarze, 1984; Davis, 1988), but return of the foraging parent from sea usually corresponds well with hatching (Davis, 1988), and the start of the guard stage. Two distinct phases comprise chick rearing: the guard stage, lasting about 3 weeks, and the crèche stage, in which chicks leave their nests and huddle together with neighbouring chicks while both parents forage at sea, returning every 1-2 days to feed chicks (Müller-Schwarze, 1984; Williams, 1995). During the guard stage parents change roles every one to three days, with males being on the nest slightly more often (52-55% of days) than females (Ainley, 2002). Chicks then enter crèches, defined as three or more chicks closer to one another than half the inter-nest distance (Davis, 1982), at an average age of 22 days (at Cape Bird, Davis, 1982) to 23 days (at Cape Crozier, Ainley et al., 1983) after hatching, once they are able to thermoregulate on their own (Goldsmith & Sladen, 1961). This allows both parents to forage simultaneously, which is essential to sustain the chick(s) until they fledge, at 50 to 60 days of age (Ainley, 2002).

Aims and format of thesis

In this thesis I address a current gap of information by investigating and directly comparing methods of hormone measurement, which have up till now been used interchangeably in similar types of ecological studies. This will reveal whether information from the different methods is comparable. After setting this essential platform, I then use these methods in an ecological study testing the social stimulation hypothesis of Fraser Darling. This is the first study to incorporate information on timing of breeding, endocrinology, survival, and environmental

conditions. Examining multiple ecological aspects in concert is essential for testing evolutionary theories, and could provide new information on how these factors interact in this species, progressing our understanding of their breeding ecology.

Chapter 2 directly compares the results obtained from measuring hormones from both faecal and plasma samples taken from the same individuals. In this chapter I analyze each method individually, inspecting variation and differences between sexes. I then directly correlate the two measures, to compare the similarity of values and patterns obtained from each sample type for each bird. This is important because, if large differences occur across the methods, trends that have been reported as absent or insignificant in the literature may be an artefact of sampling methodology, and hence unduly neglected. Values and limitations of each method are discussed, and recommendations made on the context in which each method may be most appropriately used. This study will provide essential knowledge of how results may differ between these methods, allowing more educated comparisons to be made between studies using differing methods, and allowing me to proceed to Chapter 3 with methodological confidence.

In Chapter 3, I conduct an ecological study testing the Darling hypothesis as applied to colony size - that the breeding schedule is hastened and synchronized in larger colonies due to greater levels of social stimulation relative to smaller colonies, producing greater reproductive success. Building from the findings of Chapter 2, I use the most suitable methodologies to obtain robust endocrine measurements at five stages throughout the breeding season from colonies of four size classes. I then compare these data with data on the timing of breeding, survival, and environmental variability, to address, for the first time, both the proximate and ultimate mechanisms hypothesized in Darling's theory. This progresses the debate on the applicability of Darling's theory, and sets the stage for more comprehensive studies in the future that incorporate multiple ecological factors, as is necessary to truly address complex evolutionary concepts, and obtain more meaningful information on the ecology of species.

Chapter four summarizes and synthesizes the findings of the previous two chapters. Significant conclusions and theoretical contributions are discussed, as well as suggestions for future research.

This thesis is formatted as a pair of scientific papers, to facilitate their publication in scientific journals. As each chapter stands as an autonomous unit, some repetition will be evident. The reader has, however, been provided with a General Introduction and a Conclusions chapter in order to integrate the document as a thesis.

This thesis represents part of a three year study instigated by Professor Joe Waas (University of Waikato), and involved contributions from many other people. I would like to briefly detail my level of contribution. I undertook eight weeks of sample and data collection (along with others) in Antarctica. I performed the EIA analyses over six weeks, with support from Jonathan Banks (University of Waikato), at the University for Veterinary Medicine in Vienna, under the guidance of Professor Erich Möstl and his staff. Upon return to the University of Waikato, I conducted DNA tests on blood samples to determine the sex of the animals that generated the samples, under the guidance of Professor Dick Wilkins. After learning statistical methodologies, and with advice from Dr Shinichi Nakagawa (University of Sheffield), I formatted and statistically analyzed all the data. Finally, I undertook the literature research and writing of the papers that comprise this thesis, under the supervision of Professor Joe Waas.

Plates

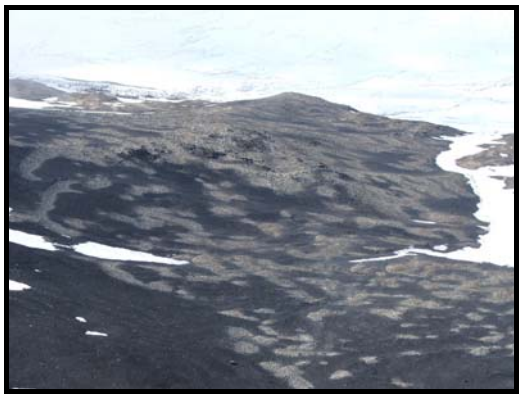


Plate 1: Cape Crozier western rookery. Lighter coloured patches are Adelie penguin colonies.

Plate 2: A pair of Adelie penguins copulating.

Plate 3: An incubating Adelie penguin on a very large, well built nest.

Plate 4: An Adelie penguin with it's chicks during the guard period.

Plate 5: Adelie chicks in a crèche (Photo: Quanah Hudson).

Plate 6: A south polar skua eating the intestines of a chick it has just killed.

Chapter 2

Venous or Anus? Comparing plasma and faecal measures of steroid hormones in Adelie penguins *Pygoscelis adeliae*

Introduction

Assessing stress in animals, particularly chronic stress, is crucial in fields like animal welfare and conservation biology (Vleck et al., 2000; Möstl & Palme, 2002). Physiological measurements of stress are increasingly being used because legislators and managers require easily quantifiable information on, for example, the effects of human disturbance on wildlife, or species decline (Wikelski & Cooke, 2006). Recently researchers have found that physiological measurements do not always correlate with other measurements of stress, such as behaviour (Walker et al., 2005a), and that these physiological measurements can reveal additional information that has remained masked in more conventional behavioural studies (Wikelski & Cooke, 2006).

Activity of the sympathetic-adrenal and hypothalamic-pituitary-adrenal systems, which release catecholamines and glucocorticoids respectively, are amongst the most useful physiological parameters to measure in the context of stress assessment, because they are triggered (though not exclusively) by stimuli perceived by an animal to be aversive (Broom & Johnson, 1993). The most important of these hormones in relation to chronic stress are the two main glucocorticoids, cortisol and corticosterone, as they function over a longer time period (i.e. they have a slower release and longer half-life) than catecholamines, and have longer term effects, thus making them more biologically relevant for chronic stress assessment (Broom & Johnson, 1993; von Holst, 1998; Palme et al., 2005). Corticosterone is the main avian glucocorticoid (Ringer, 1976), and has been traditionally measured, as have most steroid hormones, from blood plasma using RIAs (radio immunoassays) or EIAs (enzyme immunoassays) (Adkins-Regan, 2005). More recently, however, the measurement of corticosterone (and

its metabolites), as well as other steroid hormones, from faecal samples has become increasingly popular (Adkins-Regan, 2005); for example, the method has been used extensively in recent studies measuring steroid hormones in birds (e.g. Washburn et al., 2003; Sorato & Kotrschal, 2006; Arlettaz et al., 2007; Kralj-Fišer et al., 2007).

One major benefit of faecal sampling is that it is relatively non-invasive; thus samples can be collected with minimal disturbance to the animal, enabling new possibilities for re-sampling the same individuals without bias from sampling disturbance. Blood and faecal measures differ, however, in that whilst plasma samples represent a 'snapshot' of circulating hormone levels at a specific point in time, faecal measures represent a summation of the total amount of circulating hormone over a species-specific time period, representing the intestinal time passage from duodenum to rectum (Palme et al., 1996; von Holst, 1998; Möstl & Palme, 2002). There are now several papers reviewing how to interpret faecal hormone (or hormone metabolite) measures with respect to blood measures (e.g. Möstl & Palme, 2002; Millspaugh & Washburn, 2004; Möstl et al., 2005; Palme et al., 2005; Touma & Palme, 2005); a primary difference being the time scale of circulating concentration that each measure represents. Ultimately, blood and faecal measures appear suited for application to quite different questions. Despite this, there seems to be an assumption that the two measures should reflect similar results, as each measure has been used in different studies to examine baseline hormone levels in birds (e.g. Groscolas et al., 1986; Angelier et al., 2006; Arlettaz et al., 2007; Kralj-Fišer et al., 2007), with emphasis being placed on the non-invasiveness of faecal sampling as the primary difference between the two methods.

The aim of this study was to investigate, for the first time in a wild free-living species, the variation within and correlations between baseline levels of blood plasma measurements and faecal measurements of three commonly measured steroid hormones: the stress hormone corticosterone (and metabolites in faeces), and the sex hormones testosterone and estrogen, in Adelie penguins (*Pygoscelis adeliae*). Sex hormones are often quantified in conjunction with corticosterone (e.g. Wingfield et al., 1982; Klukowski et al., 1997; Knapp & Moore, 1997;

McQueen et al., 1999; Wada et al., 1999; Sorato & Kotrschal, 2006; Kralj-Fišer et al., 2007; Roberts et al., 2007) to investigate interactions between corticosterone and sex hormones across a breeding season. Differences across the sexes within each measure will also be investigated. Penguins are commonly used as model species for hormonal and stress studies in birds (e.g. Holberton et al., 1996; McQueen et al., 1998; Fowler, 1999; Walker et al., 2005b; Walker et al., 2006), so my results will be particularly valuable for the interpretation of previous studies, and for the undertaking of future studies.

Methods

Study site

This study was undertaken at the southern colonies of Cape Bird, Ross Island, Antarctica (ca 77°14'S, 166°28'E), from early November to mid January during the austral summer of 2004/05. The southern colonies are distributed over ice-free rocky substrate at altitudes from 30-70 m above mean sea level, and within 500 m of the McMurdo Sound shoreline. Sixteen sub-colonies were used and marked within the population. The colonies differed in size (ranging from c. 50 pairs to >1000 pairs) and in the amount of human disturbance they were exposed to (i.e. ranging from isolated, relatively undisturbed colonies to those that were visited up to two times a day by researchers). These differences ensured that the sampled birds varied widely in terms of the amount of stress they experienced. This level of variation was necessary to effectively compare plasma and faecal measures and to ensure that any relationships identified were robust across birds experiencing different social conditions and levels of disturbance.

Sample collection

Samples were collected at five periods throughout the breeding season (corresponding to courtship, early incubation (male's shift), late incubation (female's shift), guard (post hatching), and pre-crèche (just before both parents leave the nest and chicks form crèches)) to account for temporal variation. At each sampling period, two individuals were sampled from each of the sixteen sub-colonies (where possible, one suspected male and one suspected female, based on

observed copulations, occurrence of tread marks on the female's back, and bill size comparisons (Ainley & Emison, 1972; Marchant & Higgins, 1990)). This was done by monitoring a sub-colony from a distance (>5 m) until a bird was observed defecating. At this point the nest was slowly approached by two observers; one observer removed the bird and carried it a short distance away from the colony for blood sample collection and banding (the bird's head was covered to calm the subject). Simultaneously the other observer would collect the expelled faeces, and cover the eggs/chicks with a wool cap to protect them from predation and the cold. As unrinal and faecal excretion are combined in birds, I attempted to collect only the faecal portion, which was distinguishable by colour. This methodology enabled matched faecal and blood samples to be collected from the same bird, eliminating inter-individual variation in the comparison of results from the two sample types. Samples were collected at various times of the day (0900-2000 h), however circadian rhythms in hormone levels were unlikely to have produced any within-day variation due to the continuous 24 h daylight (Ringer, 1976 and references therein); furthermore, Vleck & Van Hook (2002) found no evidence that circadian rhythms were operating in Adelie penguins in this context.

Faecal samples were collected into cryovials and stored in a cryogenic dry shipper within 2 hours of collection. Blood samples of 0.5-1 ml were collected by venipuncture from either the brachial vein in the flipper, or from the medial metatarsal vein of the leg using a 3 ml syringe containing EDTA anticoagulant. This was done by bending the needle about 30º, to allow penetration to occur at a very slight angle. In the flipper, the vein was located by feeling the underside for a groove running laterally down the flipper, then inserting the needle at a very slight angle into the groove. On the inside of the leg, the vein was identified and the needle inserted on a slight angle and only shallowly beneath the skin. All blood samples were collected within 5 min from first approach of a bird. These are regarded as representing levels not influenced by the sampling disturbance, as Vleck et al. (2000) found no detectable plasma corticosterone increase in Adelie penguins within the first 5 min after approaching a penguin. The blood was kept from freezing until returning to camp (<2 h), at which point a small blot was made onto an FTA card (Fitzco/Whatman - FTA®) for DNA sexing, with the remainder

centrifuged and plasma extracted and transferred into a cryovial. The plasma was stored in a cryogenic dry shipper. Upon returning to New Zealand, all samples were stored at -80ºC until analysis. This study was approved by the University of Waikato Animal Ethics Committee and Antarctica New Zealand.

DNA sexing

A small (2 mm diameter) circle of blood sample was punched from the FTA card (Fitzco/Whatman - FTA®) and put into a 1.5 mL Eppendorf tube. This was then washed by adding 500 µL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and vortexing gently. Following this the TE buffer was removed and 20 µL of alkaline PEG reagent (see Chomczynski & Rymaszewski, 2006 for reagent details) was added to the sample, which was then shaken at 65ºC for 5 min to extract the DNA. Following extraction, the DNA was amplified by polymerase chain reaction (PCR) following the protocol of Griffiths et al. (1998) using P2 and P3 primers, which amplify parts of the CHD (chromo-helicase-DNA-binding) gene homologues on both the avian Z and W sex chromosomes. A Platinum® *Taq* kit (Invitrogen, Carlsbad, CA, USA) was used for the PCR, and the final reaction conditions were as follows: 1X PCR buffer (minus Mg); 0.2 mM of each dNTP; 1.5 mM $MgCl_2$; 1 µM of each primer; 1.0 unit of *Taq* polymerase; 1µL of the DNA-PEG solution. PCR was performed in a PTC-100 thermal cycler (MJ Research). An initial denaturing step at 95ºC for 4 min was followed by 30 cycles of 94ºC for 30 s, 48ºC for 30 s, and 72ºC for 45 s. On completion of the PCR, 1 unit of *Hae*III restriction enzyme (which selectively cuts only the CHD-Z gene) was added to each PCR tube and kept at 37ºC for 15 min. The cut DNA was then separated by electrophoresis on a 1% agarose gel stained with ethidium bromide, producing one band for males (ZZ, ~350bp) and two bands for females (ZW, ~350bp and ~450bp). The results of the DNA test were checked against samples in which the sexes were known (from observations of copulations), and matched in all instances.

Extraction of hormones and hormone metabolites from faeces

Faecal samples were thawed and allowed to reach room temperature. 300µg (where possible) of wet faeces was weighed and transferred to a test tube. 1.5 ml (5x sample weight) of double-distilled water was added, and the sample was vortexed. I added (making a 60% methanol solution) to the solutions 2.25 ml (7.5x sample weight) of methanol, and samples were shaken at 920-980 orbits per minute (opm) for 30 min. Samples were then centrifuged (3000g, 20ºC, 10 min). 300µl of the supernatant was then transferred to a 1.5 ml Eppendorf tube, and diluted with 150 µl of assay buffer (trishydroxyaminomethane (Merck 8382) 20mmol/l; NaCl (Merck 6404) 0.3 mol/l; 1g/l bovine serum albumin (Sigma A-4503); 1ml/l Tween 80 (Merck 822187)). Extracts were then subjected to EIAs to measure the concentrations of tetrahydrocorticosterone and testosterone.

Concentrations of estrogen, however, were too low to detect using the above extractions. Estrogen was de-conjugated using glucuronidase/arylsulfatase to test whether a significant proportion of estrogen was present in a conjugated form, and hence not being detected by the EIA. However, this made only a marginal difference suggesting that most of the estrogen was already in a de-conjugated form. Concentrated samples could not be reduced without danger of interfering molecules (e.g. lipids) affecting the EIA, so an organic extraction was developed for estrogen. The previous extracts (kept frozen after use in the previous assays) were brought to room temperature, vortexed briefly then centrifuged (3000g, 20ºC, 10 min). 1 ml of the supernatant was transferred to a new tube, to which 5 ml of diethyl-ether was added. The methanol and hormones from the previous extract is retained in the diethyl-ether phase, leaving the heavier aqueous phase sitting at the bottom of the tube. The two phases were then brought to -20ºC, and the organic phase containing the hormones decanted into a new tube, with the aqueous phase left frozen on the bottom. The samples were then dried under nitrogen, and re-suspended in 1 ml of assay buffer.

Extraction of hormones from plasma

Plasma was thawed, brought to room temperature and vortexed. 100 µl of plasma was then transferred into a new tube, and 100 µl of double-distilled water and 5

ml of diethyl-ether added. Samples were shaken for 30 min at the maximum speed possible without the liquid reaching within 1.5 cm of the top of the test tube. The samples were then frozen, and the liquid diethyl-ether decanted into a new tube, leaving the water and hydrophilic molecules frozen at the bottom. The diethyl-ether was then evaporated under nitrogen, and the samples were re-suspended in 200µl of assay buffer. I then used EIAs on these samples to measure corticosterone and testosterone concentrations. Due to the relatively low concentration of estrogen in the samples, I did not have sufficient sample volume to conduct an EIA for plasma estrogen.

Enzyme immunoassays

Double antibody biotin linked EIAs were used for the measurement of all hormones (or their metabolites). Microtitre plates were coated with antibody (anti-rabbit IGG), which binds a steroid specific or group specific rabbit antibody. After washing to remove any coating antibody not bound to the plate, the specific or group specific antibody, the sample steroid, and a biotin labelled steroid were added. The sample steroid and biotin labelled steroid compete for binding to the specific antibody. After an incubation period and washing to remove any unbound antibody, a streptavidin-peroxidase reagent was added, which binds to the biotin and creates the colour reaction upon consequent addition of the peroxidase substrate. After an incubation period the colour reaction was stopped, and the optical density was measured. The higher the concentration of steroid in the sample, the less labelled steroid was bound and the lower the optical density will be. This method of EIA achieves higher sensitivity and lower coefficients of variation than direct EIA, as two to three enzyme molecules per labelled steroid are used. All samples were assayed in duplicates. One sample with low hormone concentrations (low pool) and one sample with high hormone concentrations (high pool) were assayed on every plate, so that inter-assay variation coefficients could be calculated for both the top and the bottom of the standard curve, where variation is greatest.

For the measurement of corticosterone from faeces, an EIA for the principal metabolite tetrahydrocorticosterone (5β-pregnane-3α,11β,21-triol-20-one),

developed by Quillfeldt and Möstl (2003) and validated in Adelie penguins by Nakagawa et al. (2003), was used. Biotinylated tetrahydrocorticosterone-21-hemisuccinate (HS) was used as the label. The working dilution of the antibody was 1:40,000, and 1:8,000 for the label. The standard curve ranged from 0.82 to 200 pg/well. The intra-assay variation was 7.0%, and the inter-assay variation was 13.8% and 6.2% for the low and high pools, respectively. The sensitivity of this assay was below 1 pg/well.

For the measurement of corticosterone from blood plasma, an EIA for corticosterone-3-carboxymethyloxime(CMO):bovine-serum-albumin(BSA) was used, as described by Palme and Möstl (1997). Biotinylated cortisol-3-CMO served as the label. The working dilution of the antibody was 1:40,000, and 1:200,000 for the label. The standard curve ranged from 0.82 to 200 pg/well. The intra-assay variation was 6.5%, and the inter-assay variation was 7.1% and 5.8% for the low and high pools, respectively. The sensitivity of this assay was 0.8 pg/well.

For the measurement of testosterone from both plasma and faeces, an EIA for testosterone-3-CMO:BSA was used, as described by Palme and Möstl (1993). Biotinylated 5α-androstane-3ß,17ß-diol-3-HS served as the label. The working dilution of the antibody was 1:75,000, and 1:5,000,000 for the label. The standard curve ranged from 0.33 to 80 pg/well. The intra-assay variation was 8.0% and the inter-assay variation was 19.6% and 11.8% in the low and high pools, respectively. This assay had a sensitivity of 1 fmol/well.

To measure estrogens in faeces, an EIA described by Palme and Möstl (1993) for 17ß-estradiol-17-HS:BSA was used. Biotinylated 17ß-estradiol-17-glucuronide was used as the label. The working dilution of the antibody was 1:50,000 and 1:5,000,000 for the label. The standard curve ranged from 0.33 to 80 pg/well. The intra-assay variation was 14.5%, and the inter-assay variation was 11.7% and 12.9% in the low and high pools, respectively. The sensitivity of this assay was 0.7 fmol/well.

All assays used were group specific, and detected a range of immunoreactive metabolites, as well as the native hormones being examined. Details of cross-reactivity can be found in the reference given for each assay. Thus in this paper, 'estrogen' refers to various immunoreactive hormones of the estrogen group, and their metabolites (highest cross-reactivity with estrone, estradiol, and estriol). Similarly, 'testosterone' refers to some androgens with an immunoreactive 17ß-hydroxy group (primarily testosterone, but also dihydrotestosterone), and so on for the other assays. All antibodies used in the above assays were created by linking the hormone (Steraloids, Wilton, N.H) to bovine serum albumin and then using it as an antigen in rabbits to produce the antibody.

Apart from tetrahydrocorticosterone, the other assays used have not been biologically validated in Adelie penguins. To address this, a faecal sample with high hormone levels, following extraction using Sep-pak C_{18} cartridges, was put through a reverse phase high performance liquid chromatography (HPLC) immunogram, with a gradient elution and a starting concentration of 50 % methanol, increasing to 75 %. For testosterone, 2 main immunoreactive peaks eluted; 1 at the same position as authentic testosterone, and 1 much earlier, likely representing conjugated testosterone (see appendix 1). This provides strong evidence that what is being measured in the testosterone assays is in fact testosterone. For estrogen 2 main peaks also eluted, 1 at the position of authentic estradiol and another much earlier, suggesting a conjugated form of estrogen metabolites; however there were also many other smaller peaks in the estrogen HPLC. As estrogen occurs in lower concentrations than the other hormones being examined, there is an increased chance of some lipid interference occurring in the estrogen assay, as a more concentrated sample must be used. This must be considered when interpreting the results from the estrogen assays. Although tetrahydrocorticosterone has been previously validated in this species, an HPLC immunogram for it was also undertaken. Again immunoreactive peaks eluted very early, with only small peaks eluting at the position of authentic tetrahydrocorticosterone. To verify that this first peak was not caused by interfering substances, a second reverse phase HPLC with a gradient elution and a starting concentration of 20 % methanol increasing to 100 % was undertaken. As expected, the two peaks eluted later, showing that the early peaks seen in the first

immunogram were not due to lipid interference. This is unnecessary for plasma samples, as the extraction using diethyl-ether removes some cross-reacting steroids, especially those which are conjugated.

Statistical analysis

Following natural log (ln) transformation of all data to achieve normality or near normality, parametric tests were employed. *F*-tests were used to compare the variances between faecal and plasma measures. Within each measure, sex differences were examined using Student's *t*-tests. For each hormone (and hormone metabolite in the case of corticosterone), the relationship between faecal and plasma values was examined across the sexes using linear regression. This correlation was also examined separately at each of the breeding stages and the general trend of the correlation slopes was relatively consistent. However, reduction in sample size and strong sex ratio bias over the periods (e.g. mainly males in early incubation and females in late incubation) greatly reduced statistical power, and hindered meaningful interpretation of the data. Therefore, data were pooled across periods for statistical analyses, and only these results were presented, not the separate analyses.

Results

Plasma corticosterone and faecal tetrahydrocorticosterone

Variation in plasma corticosterone (SD=1.312) was greater ($F=4.1$, $p<0.001$, $n=121$) than in faecal tetrahydrocorticosterone (SD=0.645) (Figure 2.1)[1]. No significant difference in plasma corticosterone between sexes was detected ($p=0.51$), although males had a higher mean value than females (Figure 2.2a). Males were, however, found to have significantly higher mean faecal tetrahydrocorticosterone than females ($p<0.001$) (Figure 2.2b).

The relationship between the two measures was examined for each sex (Figure 2.3). Males showed a significant ($p=0.012$, $n=71$) positive correlation, however

[1] In original scale, variation was also greater for plasma corticosterone (C.O.V.= 1.380) than for faecal tetrahydrocorticosterone (C.O.V.= 0.582).

this correlation was weak (r=0.297). No significant correlation was found between the two measures in females (r=-0.166 p=0.248, n=50).

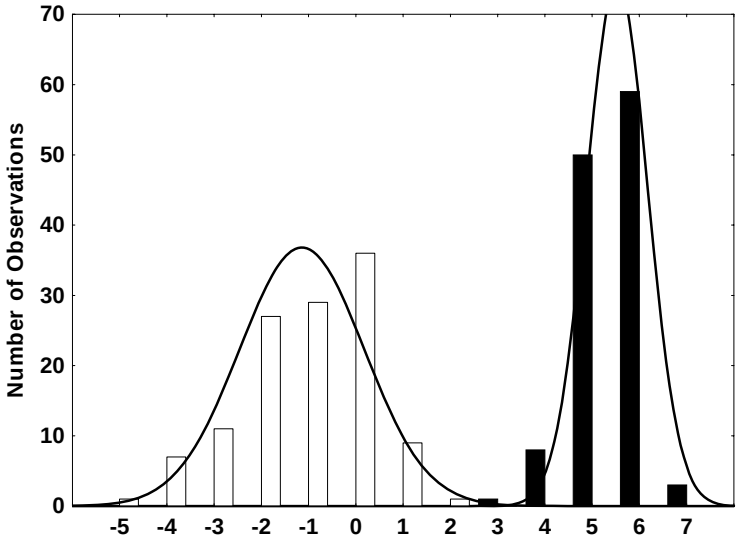


Fig 2.1: Distributions of log plasma corticosterone (ng/ml, open bars) and log faecal tetrahydrocorticosterone (ng/g, solid bars). *F*-test: F=4.1, df=240, p<0.001.

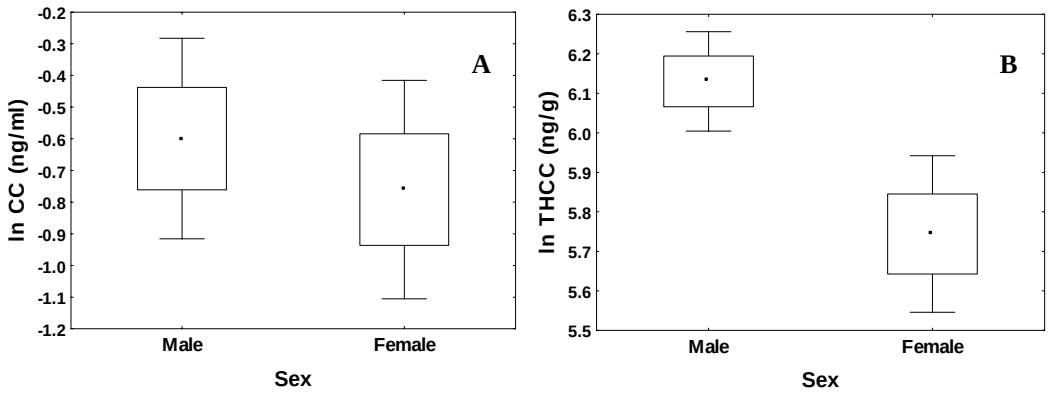


Fig 2.2: Male and female plasma corticosterone (A) levels (CC), *t*-test: t=0.664, df=119, p=0.51; and faecal tetrahydrocorticosterone (B) levels (THCC), *t*-test: t=3.383, df=119, p<0.001. Values are means ± 1 S.E. (boxes) and 95% confidence intervals (whiskers).

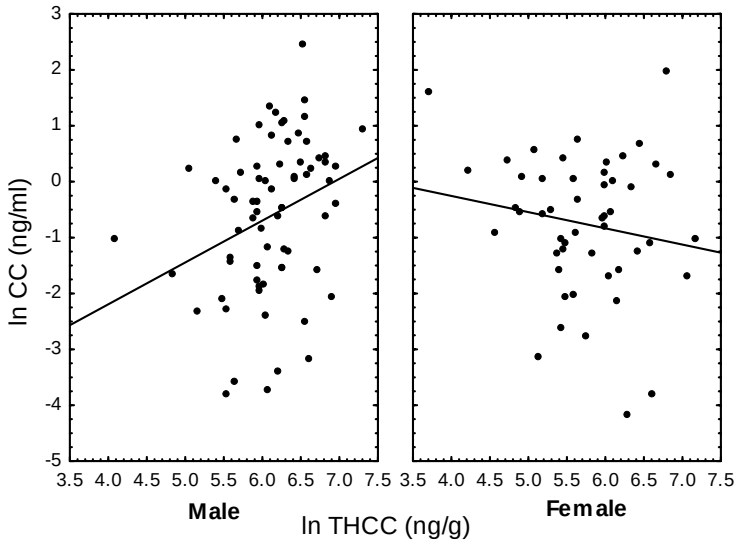


Fig 2.3: Correlation of plasma corticosterone (CC) and faecal tetrahydrocorticosterone (THCC) across the sexes. Male: r= 0.297, p= 0.012, y= -5.187 + 0.748*x; Female r= -0.166, p= 0.248, y= 0.904 - 0.290*x.

Plasma testosterone and faecal testosterone

Plasma testosterone values also showed greater variation (SD=1.087) than faecal values (SD=0.612) ($F=3.16$, $p<0.001$, $n=121$)[2] (Figure 2.4). The plasma testosterone data showed males to have significantly higher mean testosterone than females ($p<0.05$) (Figure 2.5a). Males also showed higher mean levels of faecal testosterone, however this difference was not significant ($p=0.49$) (Figure 2.5b).

Positive correlations were detected between plasma testosterone and faecal testosterone for both sexes (Figure 2.6). Males displayed a moderately strong correlation ($r=0.531$, $p<0.001$, $n=71$), whereas the correlation in females was weaker ($r=0.294$, $p=0.039$, $n=50$).

[2] In original scale, plasma testosterone also showed greater variation (C.O.V.= 1.963) than faecal testosterone (C.O.V.= 0.595).

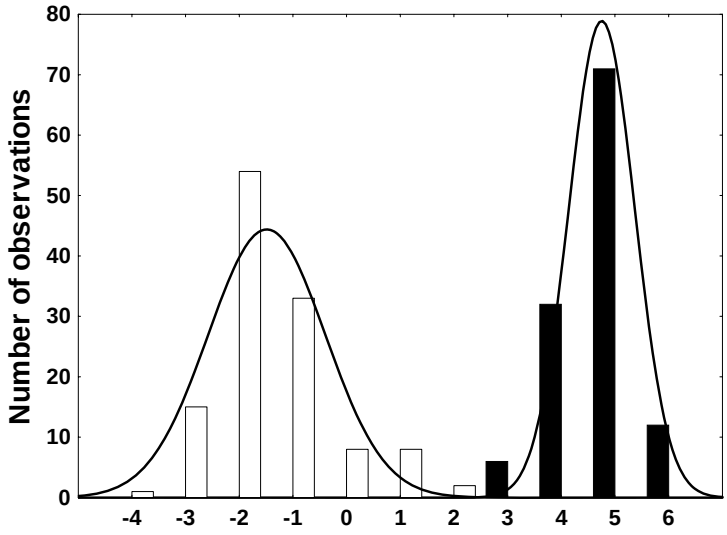


Fig 2.4: Distributions of log plasma testosterone (ng/ml, open bars) and log faecal testosterone (ng/g, solid bars). *F*-test: F=3.16 df=240 p<0.001.

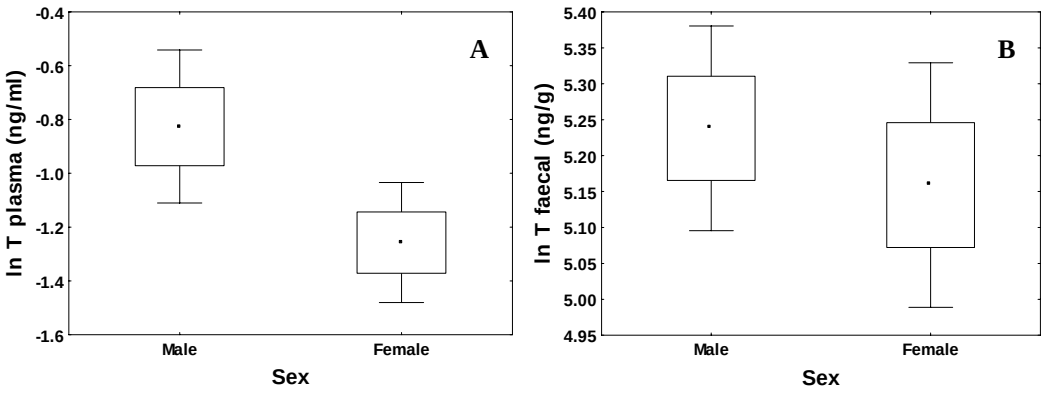


Fig 2.5: Male and female (A) plasma testosterone (T) levels, *t*-test: t=2.182 df=119 p<0.05; and (B) faecal testosterone (T) levels, *t*-test: t=0.698 df=119 p=0.49. Values are means ± 1 S.E. (box) and 95% confidence intervals (whiskers).

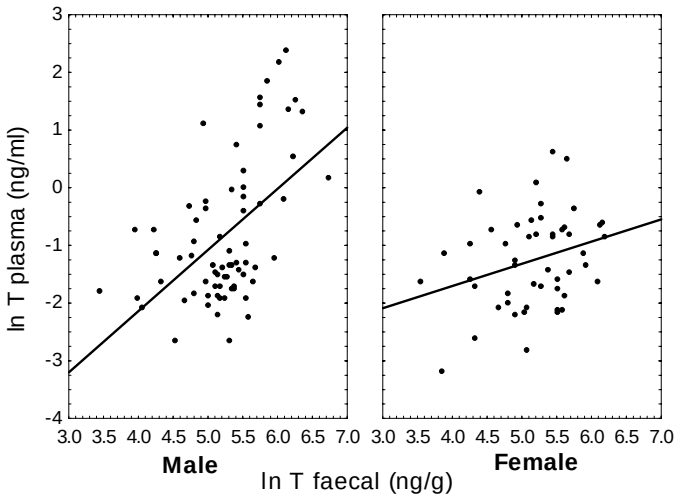


Fig 2.6: Correlations of plasma testosterone and faecal testosterone across the sexes. Male: r= 0.531, p< 0.001, y= -6.385 + 1.061*x; Female: r= 0.294, p= 0.039, y= -3.242 + 0.385*x.

Discussion

Plasma measures for both testosterone and corticosterone displayed greater variation than their faecal equivalents, and this variation was largely unaccounted for by any explanatory variables. This is not entirely surprising, given what is already known about these two measures. Circulating plasma concentrations of corticosterone in birds can change rapidly in response to a multitude of physical and psychological stressors (Siegel, 1980; Harvey et al., 1984), and this response can persist for some time afterwards (Harvey et al., 1984). Wingfield et al. (1992) collected serial blood samples over a period of 60 min of several bird species subjected to 'capture stress', and found in most instances that corticosterone was highest in the final sample, collected at 60 min. Vleck et al. (2000) blood sampled Adelie penguins subjected to handling stress over a 30 min period, and found that plasma corticosterone increased by an order of magnitude above baseline levels after 30 min. Other studies have produced similar results (e.g. Astheimer et al., 1994; Bears et al., 2003). Therefore, if a stressful event, such as an attempted predation or an agonistic encounter, occurred to an individual shortly before it was chosen for sampling, then the persistence of the corticosterone response to that stressor could confound the sample to be regarded as baseline. In this regard, the circulating corticosterone 'baseline' of an individual should be viewed, rather than a constant line, as a series of peaks and troughs; plasma corticosterone samples only gain a snapshot at an unknown point on that undulating line. Although a major stressor such as capture and restraint may still produce a detectable rise above this 'baseline' level, the variable nature of plasma corticosterone levels is particularly pertinent when it is the 'baseline' levels that are being compared between groups.

Similarly, testosterone secretion can be instigated by social stimuli in a very transitory manner, with circulating concentrations rising in response to the animal's experiences, then declining rapidly after conditions change (as testosterone has a very short half life) (Wingfield, 1994). Thus 'baseline' plasma testosterone could also be viewed as highly variable as discussed above for corticosterone plasma values, although perhaps with less chance of influencing baseline plasma samples due to the shorter persistence (shorter half-life) of elevated plasma concentrations. This point, of the inadequacy of a single plasma

sample for making sound inferences about baseline corticosteroid levels (and perhaps other hormone baseline levels), has been outlined previously (von Holst, 1998; Palme et al., 2005), yet studies still report baseline corticosteroid levels based on single plasma samples (e.g. Vleck et al., 2000; Walker et al., 2005b; Raouf et al., 2006; Ellenberg et al., 2007).

In contrast, because faecal samples represent the total production of a hormone over a species-specific time period (intestinal time passage) (Palme et al., 1996), it could be thought of as the mean value of the concentrations over that time period, thus acting to smooth out the peaks and troughs that might occur in plasma samples. The effects of these differences can be seen in the results acquired from each method. A sex difference was detected for both plasma corticosterone and faecal tetrahydrocorticosterone; however the difference for plasma was not statistically significant whilst the difference for faecal samples was highly significant, most likely due to the differences in variation within each measure. Thus, studies using plasma corticosterone to assess baseline levels, of which there are many (see above for some examples), may not appropriately detect all trends that are present, that may have been detected using measures of faecal metabolites. Indeed, Ellenberg et al. (2007) reported that male yellow-eyed penguins *(Megadyptes antipodes)* had higher basal plasma corticosterone levels than females on average, but the difference was not statistically significant. However, sex differences in basal levels of plasma corticosterone have been detected previously in Magellanic penguins *(Spheniscus magellanicus)* (Hood et al., 1998), with males having higher levels than females.

Although faecal measures appear to be better suited for measuring baseline hormone levels, an aspect of avian excretion must be noted in regards to using faecal samples to measure the acute stress response in birds. As urinary glucocorticoid excretion is faster than faecal excretion in birds (Möstl et al., 2005), but urine and faeces are excreted together, two peaks of corticosterone metabolites are observed in excreta: an earlier peak representing urinary excretion, and a later peak representing faecal excretion (Möstl et al., 2005). This point is not as important for the measurement of baseline levels, but the pattern of

excretion must be considered if using faecal samples to measure an acute stress response.

There is another difference between plasma and faecal corticosteroid measures that is not always considered, yet which is highly biologically relevant. In blood, corticosteroids occur in two portions: bound and free, the free portion being the biologically active portion. Corticosteroid-binding globulin (CBG) binds up to 95% of circulating glucocorticoids, meaning only a small fraction of circulating glucocorticoids are biologically active upon target tissues (Sapolsky, 1992). Thus plasma measurements of corticosteroids have reduced biological relevance without the assessment of bound corticosteroid proportions. In addition, CBG levels seem to be influenced by sex hormones, with estrogen inducing its synthesis causing higher levels in females (Sapolsky, 1992); further, Klukowski et al. (1997) suggested that testosterone caused increases of CBG in dark-eyed juncos (*Junco hyemalis*). Hence sex differences in corticosteroids detected from plasma measurements, in the absence of information on CBG-bound proportions, may have little biological relevance due to the likelihood of sex differences in CBG concentrations. Contrary to this, as only the free portion of circulating glucocorticoids are available for excretion, results from faecal measurements reflect only free glucocorticoid levels, thus are more biologically relevant (Palme et al., 2005).

The correlation between plasma and faecal values shown in Figure 3 lends support to the idea that results may differ depending on which method is used, with plasma corticosterone and faecal tetrahydrocorticosterone correlating significantly (but weakly) in males, while no significant correlation occurred in females. The results also suggest that the sexes should be examined separately in this species, and care should be taken to identify sex differences in studies of corticosterone in other avian species, a recommendation supported by the identification of sex differences of both basal corticosterone levels, and corticosterone acute responses in other bird species (Astheimer et al., 1994; Hood et al., 1998; Bears et al., 2003; Hayward et al., 2006; Wada et al., 2006).

A significant sex difference was detected in plasma testosterone, but not in faecal testosterone. This is difficult to interpret given that plasma testosterone showed more variation than its faecal equivalent. Potentially, due to its short half life, transient elevations in circulating testosterone could produce very high concentrations in plasma samples, whilst having only a small influence on the faecal concentration (due to the short duration of elevated levels). This could cause a significant difference to be detected from plasma that is not actually present when examined over a longer time period, which the faecal samples represent. Alternatively, as samples were collected at multiple times over the breeding season, there may be a period of elevated testosterone in males where plasma concentrations reached very high levels. Indeed, Groscolas et al. (1986) recorded elevations of plasma testosterone several fold above 'baseline' in male Adelie penguins during the courtship/copulation period. In my study, sex differences could not be examined separately at each period due to lack of power and strong bias in sex ratios within periods (e.g. males and females alternate incubation duties; the male undertakes the early incubation duties for approximately 2 weeks, then the female takes the following 2-3 weeks during the late incubation period (Ainley, 2002)). However, when the courtship period was excluded (the only period with a balanced sex ratio) there was no significant sex difference in plasma testosterone levels ($t=1.783$, $df=95$, $p=0.078$), supporting the results reported by Groscolas et al. (1986) that male testosterone levels are greatly elevated during courtship, and suggesting that they subsequently decline to levels which are not significantly different from female testosterone levels. Further studies using larger sample sizes within each period are needed to draw stronger conclusions on whether correlations change over breeding stages.

The correlation between plasma testosterone and faecal testosterone was stronger than that between plasma corticosterone and faecal tetrahydrocorticosterone, with males showing a moderate and females a weak correlation. Interestingly, although females did show a significant correlation between the two testosterone measures, it was only marginally significant compared to the highly significant male correlation. As, for both hormones, the correlation appears to breakdown in females compared to that seen in males, this reinforces that the sexes should be interpreted separately, and that different results would be reported depending on

which method is used. Angelier et al. (2006) also found that whilst a correlation between prolactin and breeding experience existed in male wandering albatrosses (*Diomedea exulans*), no statistically significant correlation was found in females.

Conclusion

This study directly compared, for the first time in a wild species, the results produced by two commonly used sampling methods for two commonly measured hormones, using individually matched samples. As faecal samples displayed less variation than plasma samples for the hormones examined, and given the potential fluctuations in circulating concentrations that these hormones can exhibit, faecal sampling appears to be a more robust method for the assessment of baseline levels in this species, and possibly other bird species given the highly conserved nature of the HPA stress response. With the additional virtues of faecal sample collection being non-invasive, and faecal samples only representing the biologically active portion of circulating corticosteroids, this sampling method could facilitate more accurate studies of baseline hormone data and of chronic stress. However plasma sampling may still be more appropriate for mapping the acute stress response. Thus researchers should recognize the distinction between plasma and faecal measures, and select the method that is most appropriate to their question (e.g. using faecal measures for studies comparing baseline hormone levels or examining chronic stress, and serial plasma samples for mapping the curve of the acute stress response). Care should also be taken to examine differences across the sexes, as there appears to be pronounced differences in circulating concentrations, and correlations between the two measures. These results contribute to the current body of literature advocating for more care and consideration in choice of sampling method, and inspection of sex differences.

Chapter 3

Colony size influences on the behavioural endocrinology and reproductive schedule of Adelie penguins in 'good' and 'bad' sea ice years

Introduction

Penguins and many other species initiate breeding synchronously because individuals respond similarly to seasonal environmental cues, like increasing photoperiod (Farner & Lewis, 1971; see also Silver, 1992) or rainfall (Sossinka, 1980; Zann et al., 1995). However, often reproduction is more synchronous than would be expected based solely on environmental seasonality (Ims, 1990); following arrival at the breeding grounds, social stimuli from conspecifics may compress the reproductive schedule further, particularly in colonial nesting species (Waas, 1995).

70 years ago Fraser Darling (1938), who was working with herring gulls (*Larus argentatus*) and lesser black-backed gulls (*L. fuscus*), proposed that social stimulation, derived from the presence or activities of conspecifics, synchronized and hastened egg-laying in colonial birds by influencing the reproductive state of females. This phenomenon became known as the 'Darling effect' (Gochfeld, 1980). One way to examine this effect was to measure the reproductive schedule in colonies of differing size. Darling suggested that, mediated via endocrine pathways, larger colonies in which individuals received more stimuli from conspecifics would have earlier and more synchronous breeding than individuals in smaller colonies.

Many studies of synchrony and colony size have provided support for the Darling effect (Burger, 1979; see Gochfeld, 1980 for review), and it has become widely accepted (Emlen, 1984). However, other studies have failed to find evidence of increased breeding synchrony with increasing colony size (e.g. Murphy &

Schauer, 1996), and some authors have conversely found breeding synchrony to decrease with colony size (Coulson & White, 1956; Vaclav & Hoi, 2002).

An ultimate explanation for why individuals in larger colonies may have earlier and more synchronous breeding is that offspring mortality could be reduced through predator swamping; predator swamping is where total predation rates do not increase past a given prey density, so reducing the temporal range that prey are available (i.e. increasing breeding synchrony) reduces overall predation (Gochfeld, 1980; Wittenberger & Hunt jr, 1985). Predator swamping has been generally accepted, and demonstrated (e.g. Hernandez-Matias et al., 2003; Kazama, 2007). Other studies, however, provide little support for this process (e.g. Weatherhead & Sommerer, 2001), and alternatives have been put forward; for example, with peripheral nests experiencing greater predation (Spurr, 1972; Oelke, 1975), differing ratios of peripheral to central nests may produce differences in survival across colony sizes (Tenaza, 1971). The benefits of individuals larger colonies having more synchronized breeding may depend on the ecology of the species and their predator(s) (e.g. whether the main predator species is a specialist or generalist), hence increasing breeding synchrony with increasing colony size might only be expected under certain conditions (e.g. when there is no predator recruitment); asynchrony may be selected for under other conditions (e.g. where non-incubating birds can mob predators) (for reviews see Wissel & Brandl, 1988; Ims, 1990).

The Darling effect and an ultimate explanation for it (i.e. predator swamping) have received much attention in the literature, however little attention has been paid to investigating the proximate (physiological) mechanisms that produce increased breeding synchrony with increasing colony size. The neuroendocrine system has been hypothesized as the mechanism underlying the hastening and synchronization of breeding in larger colonies (Darling, 1938; also see Gochfeld, 1980). Laboratory studies have demonstrated that vocal and visual cues stimulate the neuroendocrine system (Farner & Wingfield, 1980; Bluhm et al., 1984; Dufty & Wingfield, 1990; Cheng et al., 1998), and that social stimulation from conspecifics influences ovarian condition in birds (Warren & Hinde, 1961; Erickson & Lehrman, 1964; Brockway, 1965; Lehrman & Friedman, 1969;

Erickson, 1970; Bluhm, 1985; Cheng, 1988); yet studies have seldom simultaneously examined breeding synchrony, social stimuli, and endocrine parameters of free-living birds (with the notable recent exception of Setiawan et al., 2007). Information on endocrine profiles of free-living birds in different sized colonies may reveal whether any differences in breeding schedule are due to differences in social stimulation, or other factors such as differing ratios of peripheral to central nests.

The aim of this study is to test the hypothesis that increasing colony size hastens and synchronizes egg laying, in a novel species regarding this hypothesis, the Adelie penguin (*Pygoscelis adeliae*). Additionally, this study will examine levels of a metabolite of the stress hormone corticosterone, and the sex hormones testosterone and estrogen, to investigate whether these aspects of the neuroendocrine system are mediating the phenomenon of synchronization with increasing colony size, and hence elucidate a proximate mechanism. If social stimulation levels drive differences in the timing of breeding across colony sizes, corresponding differences in endocrine profiles would be expected. By also examining survival rates of chicks across colony size, this study comprises a comprehensive test of the Darling effect, examining the phenomenon and its hypothesized proximate and ultimate processes. This study will also describe both intra- and inter-annual temporal variation of baseline concentrations of the hormones stated above. Baseline hormone concentrations are important as a reference to compare experimental hormone levels against (e.g. in disturbance studies), and hence may be valuable for future hormonal studies in this species (Walker et al., 2005). Finally, major differences in sea ice conditions between years allowed me to compare the effects of 'favourable' and 'unfavourable' environmental conditions on the parameters described above.

Methods

Study site

This study was undertaken over 2 austral summer field seasons (from early November to mid January, 2004 to 2006), at two nearby (<80km) rookeries on

Ross Island, Antarctica. The first season (austral summer of 2004/2005) was conducted at the southern colonies of Cape Bird (ca 166°28'E, 77°14'S). The southern colonies are distributed over ice-free rocky substrate at altitudes from 30-70 m above mean sea level, and within 500 m of the McMurdo Sound shoreline. McMurdo Sound is icebound by extensive fast ice during the winter months, which usually breaks out to expose open water before egg laying in early November, but sometimes not till mid December, around hatching and the start of the guard period (Spurr, 1975). The second field season of this study (austral summer of 2005/2006) was undertaken at the eastern colonies of Cape Crozier (ca 169º16'E, 77º28'S). The eastern colonies of Cape Crozier comprises similar habitat to the Cape Bird southern colonies: ice-free rocky substrate at altitudes from 10-30 m above mean sea level, and within 300 m of the Ross Sea shoreline. Less fast ice is present on the Ross Sea shoreline at Cape Crozier, as it is not locked into a sound as at Cape Bird. Sea ice at Cape Crozier is often pushed offshore before egg laying in early November, but may return and not fully break out until late December during the guard stage, or even later in some years (Ainley & LeResche, 1973; Ainley et al., 1998). Differences in the annual cycle of Adelie penguins, for example time of arrival at breeding grounds, are largely driven by photoperiod, and occur on a latitudinal gradient (Ainley, 2002). Hence, as Cape Crozier and Cape Bird are very close in latitude, comparisons between these sites are thought to reflect inter-year comparisons rather than inter-site differences. Moreover, a comparison of data from a study of Adelie penguins at Cape Bird (Spurr, 1975) and at Cape Crozier (Ainley & LeResche, 1973) found the timing of the occupation period (averaged across seasons for each study) to be identical (Ainley, 2002).

For each season, 4 colonies were used and marked within the population: 1 small (S, ca 50-100 pairs), 1 medium (M, ca 200-300 pairs), 1 large (L, ca 500-600 pairs), and 1 extra-large (XL, >1000 pairs).

Timing of clutch initiation

Following arrival at the breeding grounds in early November, twenty nests within each of the four colonies (S, M, L, XL) were identified and marked, in which a

solo male was clearly occupying and maintaining a nest, prior to female arrival. Nest checks were consequently conducted every second day until egg laying had occurred, then every four days following. Nests were checked by an observer slowly approaching the nest and gently lifting the front of the bird with a pole (as described by Davis, 1982), or lifting the tail from behind, so the nest contents could be clearly identified. The onset and synchrony of breeding were measured using the date that the first egg was laid (clutch initiation).

Survival

Survival was taken as the proportion of nests that still had a chick or chicks present just prior to the onset of the crèche period. This corresponded to the 1st January ± 1 day, as not all nests in both years were able to be checked on the same day. This measurement of survival was used, as following crèching it became impractical to follow which nest any given chick originated from. Clarke et al. (2002) found that at Béchervaise Island, the majority of Adelie penguin chicks that survive to the crèche period survive through to fledging age, so it is likely that this measure of survival is reflective of survival at fledging.

Hormones

Both faecal and blood samples were collected from colonies that were not study colonies being subjected to nest checking, as to more accurately represent undisturbed baseline hormone levels. However care was taken to collect the samples from colonies that corresponded to the sizes of the study colonies. Samples were collected at 5 periods during the breeding season, corresponding to: courtship, early incubation, late incubation, guard, and just pre-crèche (refer to Chapter 1 for definitions).

Following the findings of Chapter 2, only faecal measurements of testosterone (T), estrogen (E), and the corticosterone metabolite tetrahydrocorticosterone (THCC) were considered in the analysis (refer Chapter 2 for details on sample collection and EIA measurement of hormones). All EIA's were undertaken at the

Institute for Biochemistry at the University for Veterinary Medicine, Vienna, Austria.

Due to logistical constraints, analysis of sex could not be achieved for the faecal samples, and as the majority of faecal samples and blood samples were not matched from the same bird, sex could not be inferred. Using the faecal samples that did correspond to plasma samples and hence were of known sex (n=39), it was investigated whether a coefficient based on proportions of testosterone to estrogen could be used to infer sex. However significant overlap in the range of concentrations of these hormones across sex meant this was not feasible. Students *t*-tests were carried out on these 39 samples to investigate sex differences. Differences between the sexes for testosterone and estrogen were small, and did not approach statistical significance. Males had higher THCC than females ($p<0.01$). These results correspond to those reported in Chapter 2, in which sample sizes were larger. Thus, although by using faecal measures sex was unable to be included as a factor in the full analysis, I consider the faecal sample data, based on the reasons outlined in Chapter 2, to be more biologically meaningful in the context of this study (measuring baseline levels) than using blood plasma sample data. Because Chapter 2 found no significant sex differences in faecal sex hormone levels, the importance of sex not being included in this chapter is reduced.

Statistical analysis

Clutch initiation was not normally distributed, so non-parametric tests were employed to examine the timing of clutch initiation. A Mann-Whitney *U* test was used to compare the means across years, and a Kruskal-Wallis ANOVA was used to compare the means across colony sizes. To examine differences in variability (i.e. synchrony) across sizes and between years, Levene's tests were used, as recommended by Waas et al. (2005). Due to the non-normality of these data, they were first rank transformed before applying the Levene's test. These rank transformed data of clutch initiation were also used to investigate the presence of an interaction term between colony size and year, using a factorial ANOVA.

Survival data was binomial (presence/absence of chicks at crèching), and as both continuous and categorical predictors needed to be investigated, generalized linear models (with binomial errors) were used. The full model was examined first, which contained all relevant potential explanatory variables (first egg lay date, year, size), and their interaction terms, for the response variable 'survival'. Adopting the principle of parsimony, terms were deleted sequentially until a minimum adequate model was resolved. Akaike's information criterion (AIC) (Akaike, 1974), which considers model simplicity against the fit of the model, was used to assess the best minimum adequate model. It is a useful tool in model simplification, as a model with a lower AIC is preferred to one with a higher AIC (Crawley, 2005).

Data for all three hormones were log transformed (natural log) to achieve normality or near normality. As all relevant potential predictor variables were categorical, factorial ANOVAs were used. Again, the full model was the starting point, followed by stepwise removal of terms until a minimum adequate model was reached, based on AIC.

Although p-values were calculated in the analysis of these data and presented for the reader, discussion of the results attempts to focus more on effect size, and includes discussion of some trends or differences that may not have reached the statistical significance threshold of $p<0.05$. Effect sizes and associated 95% confidence intervals are presented, to better facilitate an assessment of the biological importance of results, as recommended by Nakagawa and Cuthill (2007).

Because this study compared only 1 colony of each size in each season, sampling 20 nests from each colony, there was potential for pseudo replication. To investigate this, a colony 'id' code was created (incorporating colony size, period, and year) and included as a random effect in preliminary mixed model ANOVAs. It was found to account for a trivial amount of variation (<1%). Consequently, nests within each colony were regarded as independent and simpler factorial ANOVAs were used. This issue was not a consideration for hormone data, which were collected from different colonies from those being nest checked.

Results

Timing of clutch initiation

Colony size had no significant effect on clutch initiation date (Year 1, KW-H (3, n= 77)= 0.43, p= 0.93; Year 2, KW-H (3, n= 71)= 4.38, p= 0.22; Kruskal-Wallis ANOVA), or synchrony (Year 1, F= 0.43, p=0.74; Year 2, F= 1.43, p= 0.24; Levene's test on rank transformed first egg lay date), and no significant colony size*year interaction term was present (F= 0.88, p= 0.45; Factorial ANOVA on rank transformed first egg lay date). Clutch initiation was later in year 1 compared to year 2 (Z=5.87, p<0.001; Mann-Whitney *U* test; Figure 3.1). Year 1 also displayed greater synchrony (S.D.= 2.73 days) compared to year 2 (S.D.= 4.20 days) (F=16.40, p<0.001; Levene's test on rank transformed first egg lay date; Figure 3.1).

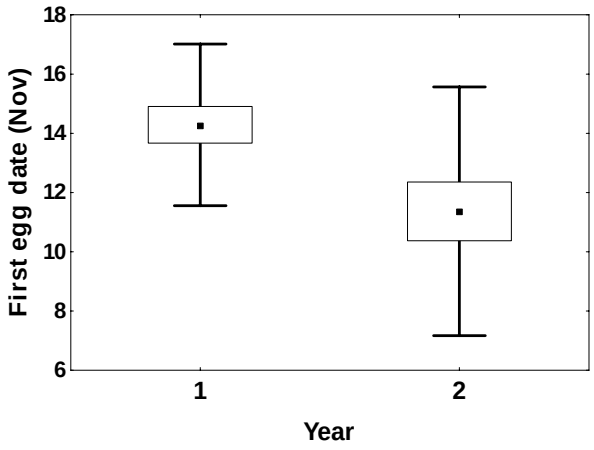


Figure 3.1: Mean first egg date (solid squares) across year's ± 95% confidence intervals (box) and ± 1 standard deviation (whiskers). Outliers and extreme values were included in the analysis, but have been excluded from the graph to more clearly display the differences.

Survival

First egg date was not a significant predictor of survival (Table 3.1, top), and was consequently omitted from the model. The minimum adequate model contained the factors year, size, and their interaction term (year*size) (Table 3.1, bottom). Year was the most significant predictor of survival (Table 3.1, bottom), with year 1 having lower survival (45% of nests were successful) than year 2 (75%) (Figure 3.2a). Size alone was not a good predictor of survival, however the year*size

interaction term was significant (Table 3.2, bottom), showing a near mirror image trend between years (Figure 3.2b).

Table 3.1: Full model (generalized linear model) for survival with binomial errors, AIC = 196 (top) and minimum adequate model (generalized linear model) for survival with binomial errors, AIC= 187 (bottom).

Effect	Degrees of freedom	Wald Stat.	P
First egg lay date	1	0.006	0.941
Year	1	11.420	0.001
Size	3	0.665	0.881
Year*Size	3	10.486	0.015

Effect	Degrees of freedom	Wald Stat.	P
Year	1	13.038	0.000
Size	3	0.667	0.881
Year*Size	3	10.557	0.014

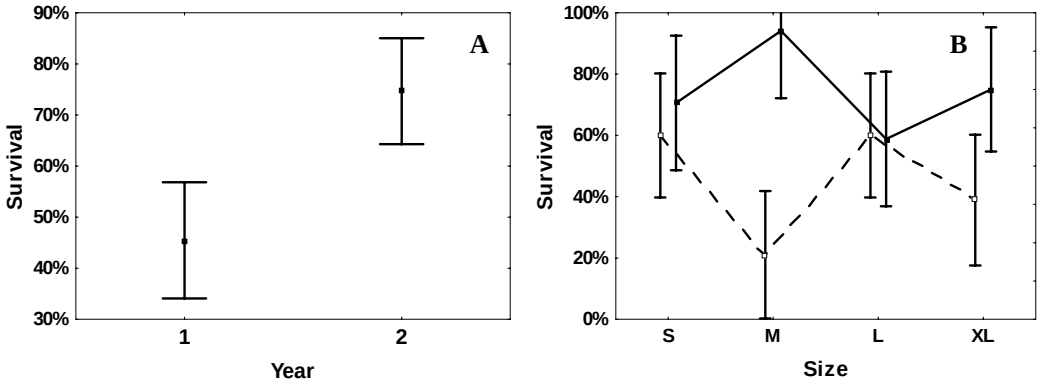


Figure 3.2: Mean proportion of nests in which chicks survived through to crèching across (A) years, and (B) size for each year (year 1= open squares & dashed line; year 2= solid squares & solid line). Vertical bars denote 95% confidence intervals.

Hormones

Colony size was a poor predictor of tetrahydrocorticosterone (THCC) levels within years (Table 3.2, top), however it was included in the minimum adequate model because of the size*year interaction term, which reached statistical significance. Post-hoc tests (Fisher LSD test) revealed that the significance of this interaction term was driven solely by disparate THCC levels of large sized colonies between years. The minimum adequate model is shown in the lower portion of Table 3.2. THCC varied significantly across years (Table 3.2, bottom), with year 1 displaying much higher levels than year 2 (Figure 3.3a). Trends of THCC across period also differed markedly between years (Figure 3.3b). Year 1 shows a conspicuous increase in THCC levels between the courtship (C) and early

incubation (E) periods, following which levels remain relatively high. Contrary to this, year 2 shows decreasing levels from the courtship to the early incubation period, with courtship representing the highest THCC levels within any period in year 2. THCC levels in year 2 were particularly low during the guard period.

Table 3.2: Full model (factorial ANOVA) for tetrahydrocorticosterone, AIC= 262 (top) and minimum adequate model (factorial ANOVA) for tetrahydrocorticosterone, AIC= 250 (bottom).

Effect	Degrees of freedom	F	P
Size	3	0.471	0.703
Year	1	10.470	0.002
Period	4	1.013	0.406
Size*Year	3	3.504	0.019
Size*Period	12	1.034	0.427
Year*Period	4	4.487	0.003
Size*Year*Period	12	1.330	0.220

Effect	Degrees of freedom	F	P
Size	3	0.335	0.800
Year	1	9.665	0.003
Period	4	1.002	0.411
Size*Year	3	3.035	0.033
Year*Period	4	4.669	0.002

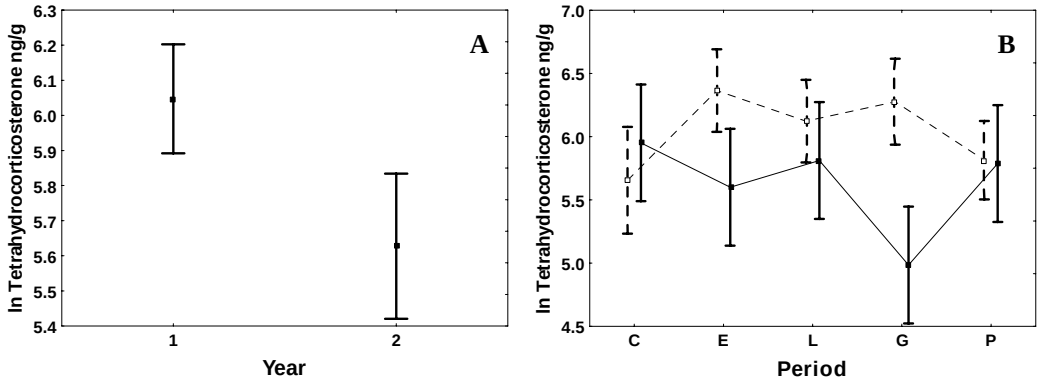


Figure 3.3: Mean tetrahydrocorticosterone across (A) years, and (B) periods for each year (year 1= open squares & dashed line; year 2= solid squares & solid line). Vertical bars denote 95% confidence intervals.

The full model for testosterone showed that colony size and all interaction terms including colony size were poor predictors of testosterone levels (Table 3.3, top). The removal of all statistically insignificant terms was found to produce the lowest AIC value following stepwise deletions, and was adopted as the minimum adequate model (Table 3.3, bottom). Year, period, and their interaction term were found to be the strongest predicting variables. Overall testosterone was higher in

year 2 than year 1 (Figure 3.4a). Year 1 displayed a marginal increase in testosterone levels from courtship to early incubation before declining, whereas conversely year 2 displayed highest levels during courtship, before consequently declining (Figure 3.4b). However, both years 1 and 2 showed small elevations at the post-crèche period (Figure 3.4b). These year*period differences approached ($p=0.069$), but did not reach a threshold of statistical significance of $p<0.05$.

Table 3.3: Full model (factorial ANOVA) for Testosterone, AIC= 223 (top) and minimum adequate model (Factorial ANOVA) for Testosterone, AIC= 200 (bottom).

Effect	Degrees of freedom	F	P
Size	3	0.390	0.760
Year	1	3.835	0.054
Period	4	13.262	0.000
Size*Year	3	0.310	0.818
Size*Period	12	0.714	0.733
Year*Period	4	1.853	0.128
Size*Year*Period	12	0.720	0.727

Effect	Degrees of freedom	F	P
Year	1	4.51	0.036
Period	4	14.63	0.000
Year*Period	4	2.24	0.069

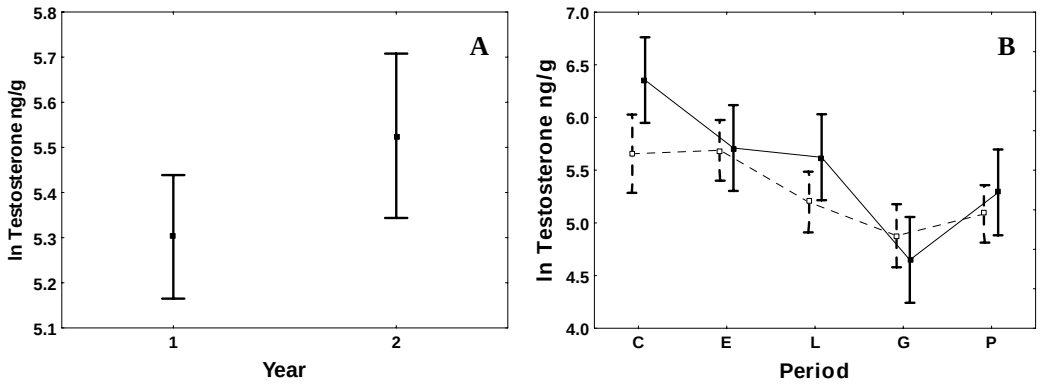


Figure 3.4: Mean testosterone across (A) years, and (B) periods for each year (year 1= open squares & dashed line; year 2= solid squares & solid line). Vertical bars denote 95% confidence intervals.

Colony Size was also a poor predictor of estrogen levels. The full model for estrogen (Table 3.4, top) showed only year and period to be statistically significant predictors, however the minimum adequate model also included their interaction term (Table 3.4, bottom). Year 2 displayed higher overall estrogens than year 1 (Figure 3.5a). The trends across the first two periods for the two years matched that of the other hormones, with year 1 showing an increase from

courtship to early incubation reaching peak values at early incubation, whereas year 2 exhibited peak levels during courtship, declining significantly to period early incubation (Figure 3.5b).

Table 3.4: Full model (factorial ANOVA) for estrogen, AIC= -29 (top); and minimum adequate model (factorial ANOVA) for estrogen, AIC= -43 (bottom).

Effect	Degrees of freedom	F	P
Size	3	0.540	0.657
Year	1	4.574	0.036
Period	4	7.380	0.000
Size*Year	3	1.378	0.256
Size*Period	12	1.054	0.410
Year*Period	4	2.109	0.088
Size*Year*Period	12	1.142	0.341

Effect	Degrees of freedom	F	P
Year	1	4.528	0.036
Period	4	7.477	0.000
Year*Period	4	1.958	0.106

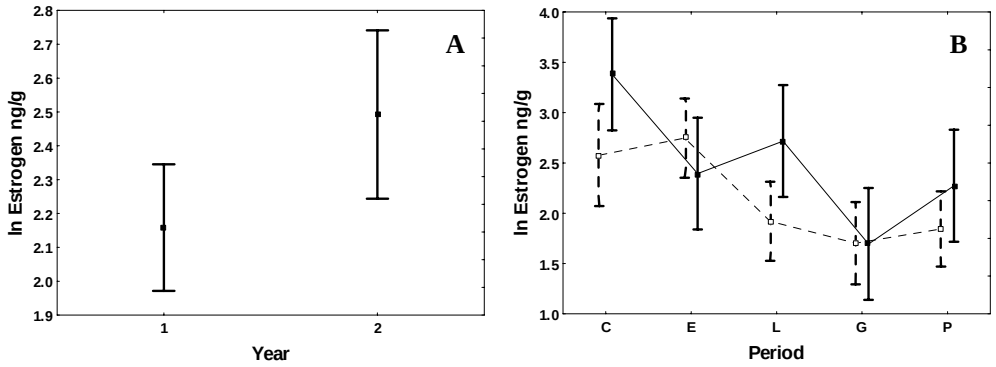


Figure 3.5: Mean estrogen across (A) years, and (B) periods for each year (year 1= open squares & dashed line; year 2= solid squares & solid line). Vertical bars denote 95% confidence intervals.

Discussion

Timing of clutch initiation

No evidence for the 'Darling effect' was detected in this study, with no differences in the mean date of first egg lay, or the synchrony of first egg lay found in colonies of differing size. This could be for several reasons. The sampling interval of nest checks (every second day) may have been too large to detect any differences that may be present, as the ability to detect differences will decrease as sampling interval increases. Waas (1995) used a sampling interval of

every 12 h to detect differences in egg laying schedule across colonies of royal penguins (*Eudyptes schlegeli*) subjected to varying intensities of colony sound playback. However, Giese (1996) used a nest check sampling interval of every second day to detect differences in hatching success and chick survival in Adelie penguin colonies of varying size and disturbance levels (although she only examined for disturbance effects within, but not across, size classes). The sampling interval in my study did detect a difference of approximately 3 days in mean first egg lay date across years, and a difference in synchrony across years. Thus it is likely that the sampling interval in this study was adequate to detect any major differences if present, and that if the differences were too small to be detected by this sampling interval they would likely be of little biological significance (although sample sizes were larger for across year comparisons than for colony size comparisons).

The lack of statistical significance of colony size in explaining survival rates or differences in hormone levels within years supports my suggestion that colony size was not important in influencing the reproductive schedule of Adelie penguins. The hypothesized ultimate and proximate mechanisms for the synchrony/colony size relationship are increased survival (through predator swamping) and neuroendocrine changes, respectively (Darling, 1938; also see Gochfeld, 1980). Hence differences in hormone levels and survival rates within years might be expected across colonies of differing size, if breeding synchrony had differed with colony size. The absence of any difference in egg laying schedule across colony sizes in this study appears biologically valid.

The absence of timing differences across colony sizes could be because my identification of a 'colony' did not match the definition that Darling, or other authors investigating breeding schedule across colony size in birds, have used. Adelie penguins nest on ice-free exposed ground, which is at a premium in extreme latitudes (Müller-Schwarze & Müller-Schwarze, 1975), and being the most numerous penguin species breeding in the high Antarctic (Giese, 1996), there is often little possibility for much dispersion of the colonies that comprise a rookery. Here I have used the 'old definitions' of 'colony' and 'rookery'(Ainley, 2002), where rookery refers to all the penguins of one species breeding at a given

location, and colony refers to the smaller scale discrete continuous groups that can be identified within that general location, as defined by Penney (1968). Ainley (2002) advocates for the use of the terms 'colony' to describe all the birds of a species breeding at a given location, and subcolony to refer to the discrete groups within that location. Though Ainley's definitions may be more appropriate, for simplicity and consistency I will continue to use the 'old definitions' throughout this thesis. Gochfeld (1980) points out the importance of defining these terms clearly, yet the difficulty to do so in some contexts. Gochfeld (1980, p. 209) states that birds in a colony are "in sufficiently close proximity to experience regular social interactions and are separated by birds in the next colony by a sufficient gap such that little or no communication occurs". The open nature of, and dense clustering of colonies into the restricted viable breeding habitat of Adelie penguins, means neighbouring Adelie penguin colonies may often not be in auditory isolation. Moreover, it is unclear whether neighbouring Adelie penguin colonies are in visual isolation. Walls (1942) suggested that penguin's eyes are notoriously myopic (nearsighted, with poor distance vision) in air, which was supported by a study on Humbolt penguins (*Spheniscus humboldti*) (Martin & Young, 1984). Other authors argue that penguin's eyes are emmetropic (have perfect vision) in both air and water (Sivak & Millodot, 1977; Howland & Sivak, 1984; Sivak et al., 1987). It is thus possible that one colony may receive social stimulation (acoustic and possibly visual) from neighbouring colonies, in which case the entire rookery would effectively be one colony in the context of Darling's hypothesis, and differences in lay date and breeding synchrony may not be expected across colonies of varying size.

Most of the study nests were on or close to the periphery of the colonies, to minimize disturbance to the colony from nest checking. This could have had a homogenizing effect on the social stimulation being experienced across the different sized colonies, as study nests in all colonies had only a few neighbours in one direction from them. Giese (1996) found no differences in survival rates of Adelie penguin nests located centrally or peripherally in colonies. Such differences might be expected to be present if differences in synchrony were also present. However, other studies on birds have detected differences in breeding success between the centre and periphery of a colony (Patterson, 1965; Coulson,

1968), including in Adelie penguins (Spurr, 1975). Thus an affect from nest orientation cannot be ruled out.

Clutch initiation was about three days later, but more synchronous, in year 1 than in year 2. This seems contrary to Darling's hypothesis. If colonies within the rookery do receive social stimulation from each other, then each rookery could be treated as a colony. As the Cape Crozier eastern rookery is about twice as large as the Cape Bird southern colonies (Lyver P., and Barton K., Landcare Research, unpubl. data), it would be expected to display earlier and more synchronous first egg laying. My sample nests from Cape Crozier did show the former, but were less synchronous than my Cape Bird sample nests. Burger (1979) found in herring gull (*Larus argentatus*) that as colony size increased, synchrony increased up to a critical number, then decreased again, suggesting that past a given number the colony broke down into discrete sub-colonies. My study found no support for this concept in Adelie penguins. The decreasing synchrony from the smaller to larger rookery could be explained by them being beyond a critical size, however no evidence was found that they break down into smaller sub-colonies.

In year 1 of this study, a large iceberg (B15a) and a smaller daughter iceberg (B15k) situated between the Drygalski Ice Tongue and Ross Island, blocked off McMurdo Sound, preventing the sea ice from breaking up and dispersing out of the sound (Figure 3.6). This likely affected the penguins, as instead of walking a short distance from their nests to open water, where they can forage or swim to foraging grounds, they had to walk much greater distances (~50 km) to reach open water. Travel over sea ice by foot is energetically costly and time consuming for Adelie penguins (Culik & Wilson, 1991; Wilson et al., 1991), which can travel rapidly over long distances if swimming (Ainley & LeResche, 1973; Clarke et al., 2002). Indeed, the importance of sea ice conditions in relation to foraging or breeding success in Adelie penguins is well reported (Yeates, 1968; Ainley & LeResche, 1973; Spurr, 1975; Rodary et al., 2000; Wilson et al., 2001; Clarke et al., 2002; Croxall et al., 2002; Kato et al., 2002; Rombola et al., 2003; Olmastroni et al., 2004; Jenouvrier et al., 2006; Yoda & Ropert-Coudert, 2007). Breeding success of Adelie penguins has shown a strong negative correlation with the extent of summer sea ice, followed by according fluctuations to the population

after a 5 year lag (the age of sexual maturity) (Clarke et al., 2002; Kato et al., 2002; Jenouvrier et al., 2006).

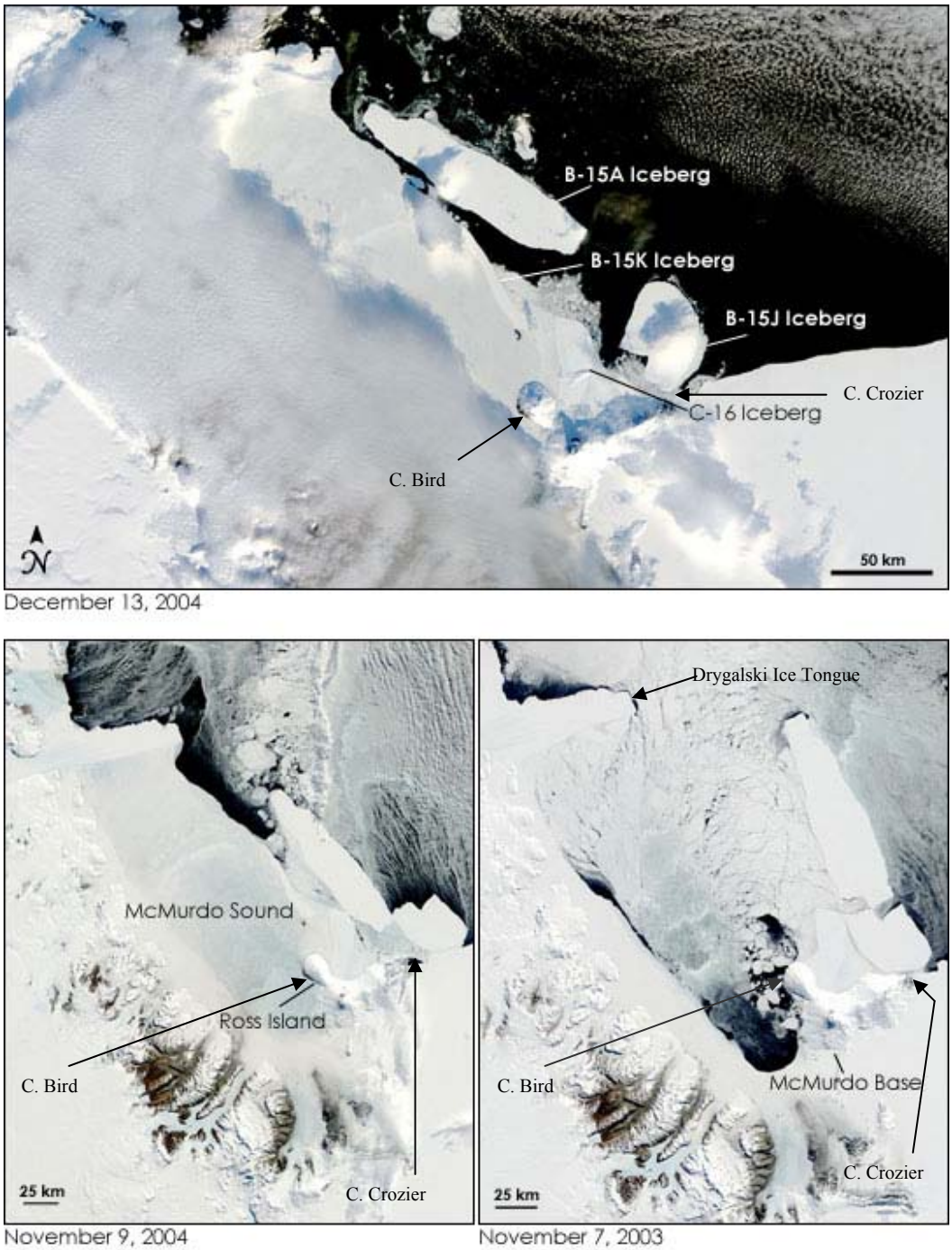


Figure 3.6: Iceberg orientation and sea ice extent in McMurdo Sound, showing break-out occurring in early November 2003, and persistence of sea ice into December in 2004. Source: NASA images courtesy Jeff Schmaltz, MODIS Land Rapid Response Team at NASA GSFC. Taken from http://earthobservatory.nasa.gov/Newsroom/NewImages/images.php3?img_id=16759 on 22/01/08.

The later laying date in year 1 could be due to the presence of the iceberg B15a. If this had increased the time taken for the penguins to reach their breeding grounds at Cape Bird, then this could have delayed their breeding schedule. Under this

scenario, those birds that may have potentially laid earlier (the early outliers in the laying distribution) may have laid later. Given the extreme seasonality at these latitudes and consequent narrow breeding window (Trivelpiece & Trivelpiece, 1995), it could be conceived that there may be a theoretical 'cut-off date' to egg laying; chicks from eggs laid after a given point may be unable to survive. If Adelie penguins use environmental cues to initiate breeding (e.g. photoperiod, see Ainley, 2002, chapter 4) and are then consequently delayed, it could squash the laying distribution against this cut-off date, synchronizing egg laying. The concept of a laying 'cut-off date' is supported by the findings that previously successful breeders at Cape Bird that failed to breed the following year had arrived late (Spurr, 1975), and that fast ice delayed the influx of penguins to the breeding grounds at Wilkes Station, but did not affect subsequent stages of the breeding cycle (Penney, 1968). Davis and McCaffrey (1986) also found that for Adelie penguins to breed successfully they must initiate breeding promptly after arriving at the breeding grounds. This could explain the year 1 birds having more synchronous, but later clutch initiation than birds in year 2.

Survival

Survival of chicks through to the crèche period was much lower in year 1 (45% of nests produced ≥1 chick) compared with year 2 (75%). This compares with 55%-66% in normal years and 43% in a bad year which Spurr (1975) recorded at Cape Bird (his estimates will be lower than mine as he accounted for 2-egg clutches in which 1 egg did not survive), and 57% and 31% in a 'good' and 'bad' year, respectively, recorded by Ainley and LeResche (1973) at Cape Crozier (their estimates will be lower than mine as they only examined birds aged 3-7 years; maximum chick raising ability is reached at 7-8 years of age (Ainley & Schlatter, 1972)). My survival results align with the finding that birds in year 1 were more stressed, as indicated by their corticosterone metabolite levels. The duration of foraging trips of Adelie penguins during the incubation and guard stages increase during seasons of low breeding success (Watanuki et al., 1993; Kerry et al., 1995; Ainley et al., 1998), and greater sea ice extent (Yeates, 1968). Years when fast-ice persists longer than normal are often associated with poor breeding seasons (Trivelpiece & Fraser, 1996; Ainley et al., 1998), most likely because Adelie

penguins spend more of their foraging trips travelling, and consequently return with smaller meals (Watanuki et al., 1997); the success and length of foraging trips and according timing of relief of incubating partners determines breeding success to a large extent (Yeates, 1968; Davis, 1982). Poor survival, elevated corticosterone metabolite levels, and later clutch initiation in year 1 is likely due to changes in foraging trip duration and success, and delayed relief of incubating parents. This is likely a result of extended sea ice conditions and the presence and orientation of the iceberg, although this cannot be confirmed in this study, as information on foraging trips was not collected. The fact that year 1 had more synchronous breeding does not align with these other factors, with the possible explanations described above.

Colony size explained very little variation within and across years for laying synchrony, and within years for survival, but was a significant predictor of survival across years. The pattern of survival across colonies of differing size showed a reverse trend between years (Figure 3.2b). This may suggest directional selection, in which medium and extra-large sized colonies had better survival rates than small and large sized colonies in year 2 (favourable sea ice conditions), yet suffered relatively greater nest failure rates in year 1 (unfavourable sea ice conditions). Colonies of differing size may be comprised of birds of differing age (as has been suggested, see Gochfeld, 1980), with younger birds being later (Coulson & White, 1956 and reference therein; De Forest & Gaston, 1996) and poorer breeders than older or more experienced birds (Yeates, 1968; De Forest & Gaston, 1996; Angelier et al., 2006), probably relating to foraging ability in Adelie penguins (Ainley & Schlatter, 1972). However, due to limited replication and sample sizes, this trend may be a random artefact. Longer-term studies with greater sample sizes are needed to verify this potential phenomenon.

Hormones

Neither tetrahydrocorticosterone (THCC), testosterone (T), nor estrogen (E) levels varied significantly across colonies of differing size, with the exception of the size*year interaction term for THCC. This interaction term is difficult to interpret, and as only THCC levels in large sized colonies differed significantly between

years, it is likely that this trend is an artefact of limited replication and sample sizes. That size was a poor predictor of hormone levels is consistent with the results for survival, and the timing and synchrony of clutch initiation. Thus, these hormones remain as a possible mechanism for mediating differences in the breeding schedule of colonies of varying size, in species in which such differences are found.

THCC trends throughout the breeding season differed between years. In year 2, highest THCC levels occurred during courtship. McQueen et al. (1999) also found highest concentrations of corticosterone during courtship in Adelie penguins, maybe due to the stress of nest establishment and defence, and the creation and maintenance of pair bonds. THCC levels in my study then declined by about 40% from courtship to early incubation (refer to appendix 2 for graphs in original scale), never again approaching courtship levels. THCC levels might decrease following courtship as the stressors mentioned above may diminish, with incubating birds settling into their incubating duties. Fluctuations observed across the following periods may occur because sampled birds spent differing lengths of time fasting, as elevated corticosterone levels during fasting occur in other bird species (Harvey et al., 1987), although the low levels of THCC during the guard phase are difficult to explain.

Conversely to year 2, THCC levels during courtship in year 1 were relatively low, approximately doubling through to early incubation. This fits with the greater extent of sea ice delaying the arrival of birds at the breeding grounds. If this reduced courtship duration, the amount of stress experienced by the birds during courtship may be reduced, explaining the lower levels of THCC at courtship in year 1. Alternatively, as testosterone and estrogen levels at courtship were much lower in year 1 compared with year 2, birds may have had fewer stressful agonistic interactions, producing lower THCC levels. Elevated THCC levels observed at the early incubation period in year 1 may be due to incubating birds being less physiologically prepared for fasting, due to the increased energy expended in reaching the breeding grounds. The consistently high THCC levels over late incubation and guard stages may also have reflected the difficulty of the birds to physiologically cope with fasting and chick provisioning, whilst likely

having to spend a greater proportion of their foraging trips travelling, and less time feeding. This may reflect lower fat reserves, as king penguins suppress secretion of corticosterone until their fat reserves become depleted (Cherel et al., 1988a; Cherel et al., 1988b). Differing physiological condition (i.e. amount of fat reserves) due to differing sea ice conditions could explain the different patterns of THCC levels between years observed in this study.

Both testosterone and estrogen varied significantly across periods. For both, the highest levels across any period in year 2 were found during courtship, with concentrations declining through to early incubation. These findings concur with those from other studies for plasma androgen and estrogen levels in Adelie penguins (Groscolas et al., 1986; Davis et al., 1995; McQueen et al., 1999), and in other penguin species (macaroni and gentoo penguins, Williams, 1992; king penguins, Cherel et al., 1994; Fiordland crested penguins, McQueen et al., 1998).

Courtship concentrations of testosterone and estrogen in year 1 were lower than those in year 2. Conversely to year 2, concentrations of both showed small increases from courtship to early incubation in year 1, where they reached their highest levels. This is consistent with the delayed clutch initiation in year 1, correlating with lower sex steroid levels early in the season, and a later occurrence of peak levels in year 1 than in year 2, probably due to sea ice conditions. The lower levels of reproductive hormones in year 1 than in year 2 are consistent with the higher THCC levels indicating the birds are more stressed, as reproductive hormones are inhibited by stress (Rabin et al., 1988; Sapolsky, 1992).

Testosterone and estrogen concentrations in both years declined from early incubation to the guard stage, before showing an increase at the pre-crèche stage. These findings differ from McQueen et al. (1999), who found plasma androgen and estrogen levels in Adelie penguins to drastically decline from courtship to clutch completion and then remain stable, compared to the gradual decline observed in this study. These differences could be due to the different sample types used between the studies (refer to chapter 2 for details on differences between plasma and faecal measurements). Estrogen concentrations increased during late incubation in year 2, likely due to gender bias across periods; usually

males take the early incubation duties, whist females take the late incubation duties (Davis, 1982; Ainley et al., 1983; Davis, 1988). This rise in estrogen concentrations during late incubation still fell well short of courtship levels. Estrogen levels in year 1 did not show an increase from early to late incubation, as was seen in year 2, lending further support to the conclusion that physiological reproductive condition was affected in year 1.

Elevations in plasma androgens during late incubation have been detected in macaroni penguins (Williams, 1992) and Fiordland crested penguins (McQueen et al., 1998); the trend is thought to be due to a rise in agonistic interactions, renewed nest defence, and pair bonding (Williams, 1992). McQueen et al. (1999) could not reconcile the absent late testosterone rise seen in Adelie penguins, as the explanations for the late rise in macaroni penguins should also apply to Adelie penguins. Additionally, the pattern of incubation duties in Fiordland crested penguins is similar to Adelie penguins (Davis, 1988), thus similar endocrine profiles may be expected between them; females take the first foraging trip, unlike all other eudyptid species (Warham, 1975). No rise in estrogen was found during late incubation by McQueen et al. (1999) in Adelie penguins, or by McQueen et al. (1998) in Fiordland crested penguins, suggesting the regression of ovaries after clutch establishment (Groscolas et al., 1986; McQueen et al., 1998). The fact that increases in both testosterone and estrogen from the guard stage to the pre-crèche stage were seen in both years in this study, suggests that Adelie penguins may still be capable of secretion of gonadal hormones late in the breeding season. The purpose of such late secretion of gonadal hormones is unclear.

Conclusion

No differences in breeding schedule or hormone levels were detected across colony sizes in either season of this study. Hence this study found no evidence that the Darling hypothesis, as tested using colony size, holds in Adelie penguins. The patterns of hormonal changes in year 1, with later peak levels of all hormones, lower peak and overall levels of testosterone and estrogen, and elevated levels of THCC, correspond to a later mean lay date and lower offspring survival than in year 2. The persistent sea ice conditions in year 1 are the most

likely cause of these inter-annual differences. This novel study thus links endocrine profiles with differences in breeding schedule, reproductive success, and environmental variability, providing new insight into the proximate mechanisms that may be mediating differences in breeding behaviour and success. This study also reveals the consequences of unusual environmental conditions on Adelie penguins, at both the physiological, behavioural, and ecological levels.

Chapter 4

Conclusions

The relationships between an animal's behaviour, ecology, survival, and life-history are complex, and are underpinned physiologically by endocrine changes. My work involved investigating the hormonal basis of the Adelie penguin breeding system. In this chapter, I will synthesize the findings I have made in the previous chapters, and discuss them in a wider context to outline what scientific contributions I have made. I will then discuss areas for future research which I have identified.

Research findings and theoretical contributions

As ecologists are usually not also biochemists and physiologists, they may often include hormonal measurements in ecological studies without spending much time considering the assumptions and limitations of the methods used (Buchanan & Goldsmith, 2004). Adkins-Regan (2005, pp. 9-11) provides a brief but stimulating discussion on this. Before measuring and interpreting endocrine levels, some critical questions should be asked, like what are you actually measuring? What do the numbers mean? Assays are not perfect, and the results produced are only estimates of true, but unknown values (Adkins-Regan, 2005). Chapter 2 focused on this point, by examining an aspect of hormone sampling methodology that had till now not been addressed.

In Chapter 2, I directly compared the hormonal results obtained from plasma and faecal samples taken from the same birds, as both of these sample types are commonly used in the literature. This study demonstrated the variability of the results that can be produced by the different methods, outlining the care required in choosing a methodology, and subsequently interpreting the results. The fact that hormone levels in faeces have been integrated over a substantial time period, compared to the snapshot of fluctuating circulating hormones represented by a

plasma sample (discussed in Chapter 2), can be either an advantage or disadvantage depending on the question being asked. Despite this, both measures have been used in similar ecological studies, for example measuring baseline hormone levels (e.g. plasma - Groscolas et al., 1986; Angelier et al., 2006; and faeces - Arlettaz et al., 2007; Kralj-Fišer et al., 2007). In Chapter 2, I found that faecal measures of hormone levels appear more suited for the measurement of baseline levels. Additionally, studies using plasma to measure hormone levels rarely quantify the proportion of measured hormone that is bound to binding globulins, which is of great importance for interpretation of biological significance; for example, hormone levels may stay the same over time, but if binding globulins reduce over time then more free hormone will be present and acting on target tissues (Breuner & Orchinik, 2002).

This study has contributed a demonstration of why careful consideration must be given to choosing a methodology to suit the question being asked, providing new evidence to support other authors which advocate the same (e.g. Möstl & Palme, 2002; Adkins-Regan, 2005; Palme et al., 2005)**.** This information was important in determining the sampling methods to be used in the second major study which comprises this thesis.

In Chapter 3, I used faecal samples to measure sex and stress hormones in an ecological study which focussed primarily on testing the Darling hypothesis, and did so under variable environmental conditions. A secondary aim of this study was to collect baseline hormonal data, using the most appropriate measures (faecal samples), of Adelie penguins at five periods throughout their breeding season, to provide a solid point of reference for future hormonal studies in this species.

Darling's hypothesis (Darling, 1938) theorizes that endocrine changes mediate increased breeding synchrony from social stimulation, which improves survival by reducing overall predation. This is a complex theory, as it addresses proximate and ultimate explanations for the behavioural phenomenon, by incorporating physiological, behavioural, and demographic variables. Gochfeld (1979) reiterated that the value of a theory is in its testability. Although testing the Darling

hypothesis in its entirety is challenging, obtaining robust tests of this theory should not be dismissed because of its complexity, as it is this very complexity that is the virtue of this theory, which has the potential to greatly aid our understanding in evolutionary ecology. Many studies have tested aspects of Darling's hypothesis, however a comprehensive test which examines all of its attributes is absent. In Chapter 3, I made progress on this, by examining the timing (including the synchrony) of breeding in Adelie penguins across colonies of four sizes, and correlated this information with measurements of hormones and survival, across years of favourable and unfavourable sea ice conditions. This study made several valuable contributions.

No evidence was found for differences in the timing or synchrony of breeding across colonies of differing sizes, indicating that the Darling hypothesis does not appear to hold in this species. Potential reasons for this could help guide future studies. Although the varying colonies appeared discrete to the human observer, they may not have been isolated enough from adjacent colonies to totally remove them from their 'social environment'. This outlines the importance of adhering to the definition of a colony that Darling intended when undertaking tests of his hypothesis, by carefully considering the perceptual system of the species being tested, and what is likely to represent 'social isolation' from other colonies.

Scale may confound tests of the Darling effect. When comparing different rookeries (a larger scale comparison than colonies), I found the smaller rookery in year 1 to display greater synchrony than the larger rookery in year 2. Larger colonies were also less synchronous than smaller colonies in herring gulls once size increased over 200 pairs (Burger, 1979). Darling's hypothesis was developed on colonies ranging from 2 to 10^2 pairs, thus it may not apply on a larger scale, such as the inter-rookery comparison I made, or the study by Orians (1961) on colonies of tricolored blackbirds (*Agelaius tricolor*) ranging from 10^2 to 10^5 pairs, which also found no evidence for the Darling hypothesis. It may thus be that the applicability of the hypothesis is limited to species or situations which fit within these criteria of size and social isolation.

The timing and synchrony of breeding, and offspring survival, differed significantly between years. This correlated with a difference in hormone levels and environmental conditions. In the first year of this study, extensive sea ice conditions persisted late into the breeding season, likely due to the presence of a large iceberg at the fringe of the sea ice. This correlated with later but more synchronous breeding, and reduced survival compared to the second season. Elevated corticosterone metabolite levels and reduced androgen and estrogen levels also occurred in the first season. The likely relationship between these correlations is that the extensive sea ice increased the time and energy spent travelling between open water and breeding grounds, producing a later initiation of breeding by delaying the initial arrival of penguins at the breeding grounds, and reducing the net energy gain from foraging trips. This was most likely responsible for the increased stress hormone levels, decreased sex hormone levels, and reduced survival in the first season. The aspect of these results that is contrary to expectations is that in the first season breeding synchrony was greater despite breeding initiation being later, sex hormone levels being lower, and the rookery being smaller compared to the second season. This indicates that late breeding may have necessitated greater synchrony in this environment, despite possible desynchronizing factors; because individuals arriving later than usual would have had to either lay sooner than they may have normally (i.e. reduced courtship time), or not lay at all, due to the intense seasonality at these extreme latitudes and the consequent narrow breeding window.

Baseline levels of hormones throughout the breeding season differed between years, also indicating a hormonal response to differing environmental conditions. Thus although hormonal mechanisms for the Darling effect could not be investigated in this study, as the Darling effect was not observed, hormonal changes were found to be the likely mechanism mediating the effect of variable environmental conditions on breeding dynamics and offspring success. Additionally, this study demonstrated that environmental constraints on the timing of breeding (e.g. short seasons at high latitudes) may confound expectations of the Darling hypothesis. Thus, robust future tests of Darling's hypothesis, and ecological studies in general, will need to examine measures of hormones,

behaviour, and reproductive success, in the context of the social, and physical environment.

This study contributes to the current body of literature (e.g. Yeates, 1968; Watanuki et al., 1997; Kato et al., 2002; Cockrem et al., 2006; Jenouvrier et al., 2006) suggesting that environmental factors, especially sea ice conditions, have a strong influence on the breeding ecology of Adelie penguins, including their foraging behaviour, breeding success, and population trends. I also make advances on such studies by describing the endocrine changes resulting from these differing conditions, and the consequent affects they have on breeding. Additionally, since the extended sea ice conditions observed in my study were attributed to the presence and orientation of a giant iceberg, this study demonstrates that icebergs can have significant and detrimental consequences on breeding Adelie penguins, in addition to the effects described for breeding emperor penguins (*Aptenodytes forsteri*) (Kooyman et al., 2007).

Future research

I found that plasma and faecal measures of sex and stress hormones correlated weakly, and produced different results. This was the first study to directly compare blood and faecal measures of these hormones in a free-living species. Vleck et al. (2000) compared blood plasma corticosterone measurements with heterophil/lymphocyte (H/L) ratios, which have been used to measure stress in other studies (e.g. Work et al., 1999), as measures of stress in Adelie penguins. They found that neither corticosterone nor H/L ratio varied with sex, consistent with my plasma data, but not with my faecal data. They also found that plasma corticosterone and H/L ratios were moderately correlated, except in comparisons between birds that were injured or had recently been fighting, probably because heterophils increase in response to injury to fight infection (Vleck et al., 2000), which would bias them as a pure indicator of stress. They did distinguish the difference from plasma, that H/L ratios show a slower reacting and longer response to stress, similar to faecal samples. Neither my study, or the study by Vleck et al. (2000) quantified corticosterone binding globulins (which may also bind androgens in birds, Breuner & Orchinik, 2002), which could potentially

account for differences between measures. More studies are needed which compare H/L ratios, faecal hormone measures, and plasma hormone measures which measure and account for binding globulins. These studies need to be conducted across multiple species, to investigate if correlations between measures and/or sex differences hold across different taxa. H/L ratios, however, are only potentially useful as a measure of stress, compared to the ability of faecal and plasma samples to be assayed for various hormones.

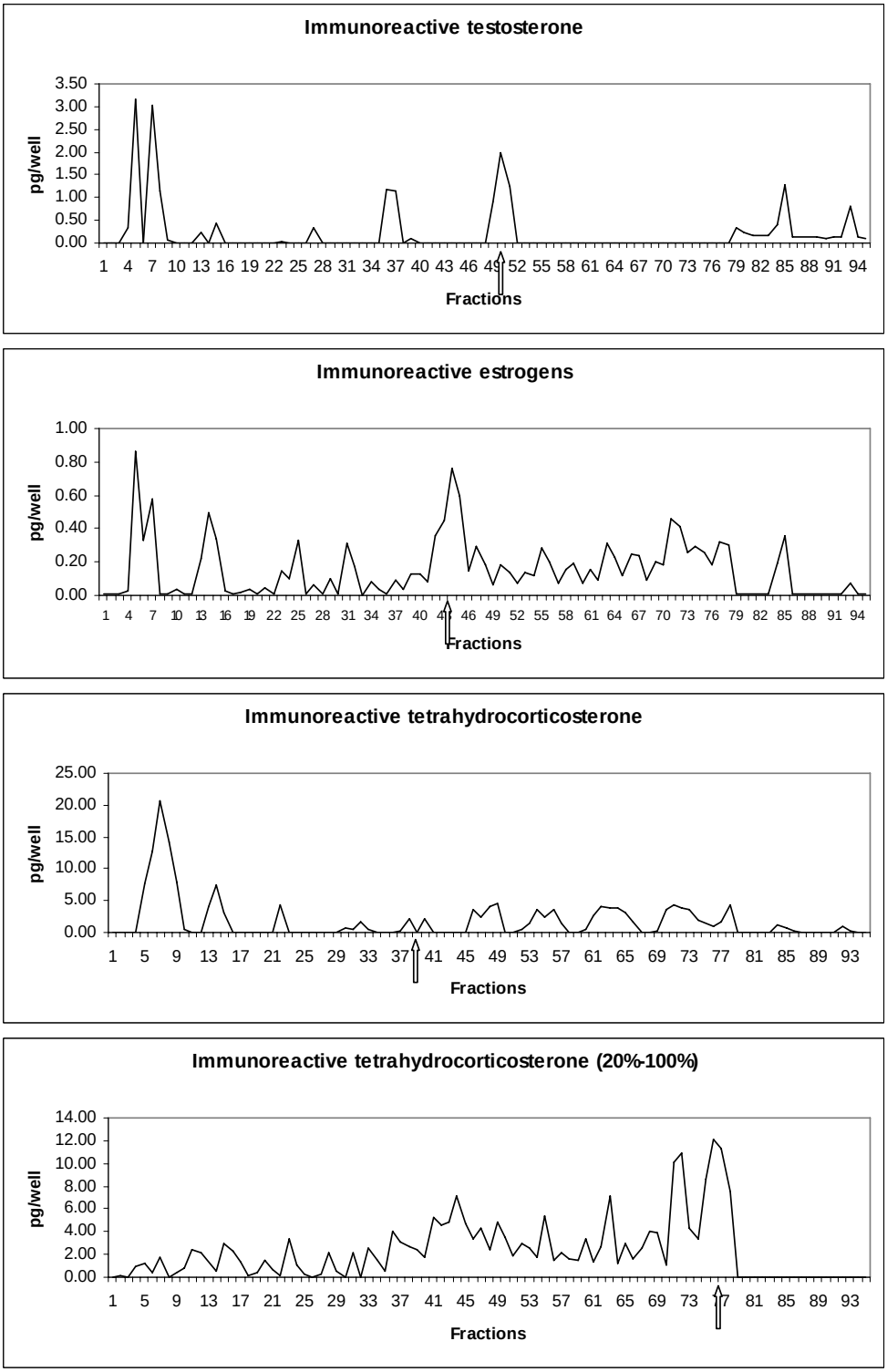
In Chapter 3, I aimed to conduct a comprehensive test of the Darling hypothesis. However, the Darling effect was not observed in this study. This could be due to the nature of the breeding ecology of this species, as has already been discussed. It may be that the Darling effect only applies to species with less stringent seasonal constraints on their breeding timing, and in which the distribution of breeding habitat allows for colonies to be clearly independent in regards to social environment; the Darling effect should be thoroughly investigated in such species before being applied to species whose breeding ecology varies from these conditions. Future studies should continue to test the Darling hypothesis, and other evolutionary theories, using the comprehensive approach (Palme et al., 2005), examining physiology, behaviour, reproductive timing and success, and environmental conditions. Although my study measured survival as representative of an ultimate mechanism (i.e. predator saturation), my study could have been further strengthened by examining predation rates.

Gochfeld (1980) reviews the concept that differences in the timing and synchrony of breeding across colony sizes could be related to age differences. Smaller colonies may represent newer aggregations of younger birds, who are poorer, less experienced breeders. This issue still remains to be clarified, and could benefit from further testing on a range of species, especially in conjunction with testing the Darling hypothesis in combination with the other variables discussed above. However, identifying the age of individuals of a colony necessitates marking birds as chicks, and hence requires long term demographic studies. Even in such studies, it would require high rates of natal philopatry.

In Chapter 3, an interesting trend emerged for survival across years. Although colony size was a poor predictor of survival within years, it was a significant predictor of survival between years. The data suggested a form of directional selection, where in the first season, with unfavourable sea ice conditions, medium and extra-large colonies suffered relatively low offspring survival rates, whereas in the second season, with favourable sea ice conditions, medium and extra-large colonies had relatively high offspring survival rates. Offspring survival rates in small and large sized colonies were relatively constant between seasons. This could possibly be due to different sized colonies comprising different age demographics, with younger birds being poorer and earlier breeders than older birds (see Gochfeld, 1980). Why age related differences would show a zigzag pattern rather than a linear pattern is unclear. This trend clearly needs further investigation with greater replication and sample sizes before it can be verified.

In this thesis I have made novel advances in assessing methodologies for endocrine measurement. I have outlined clear directions for essential future research needed to strengthen the validity of endocrine measurements, and inferences that can be made from them. I then incorporated the recommended endocrine methods into a robust test of the Darling effect on Adelie penguins, and found strong evidence that it does not occur in this species. I showed that Adelie penguin breeding is strongly confined by the extreme seasonality of their environment, and is heavily affected by sea ice conditions; poor conditions having major effects on their reproductive schedule and offspring survival, mediated through endocrine changes.

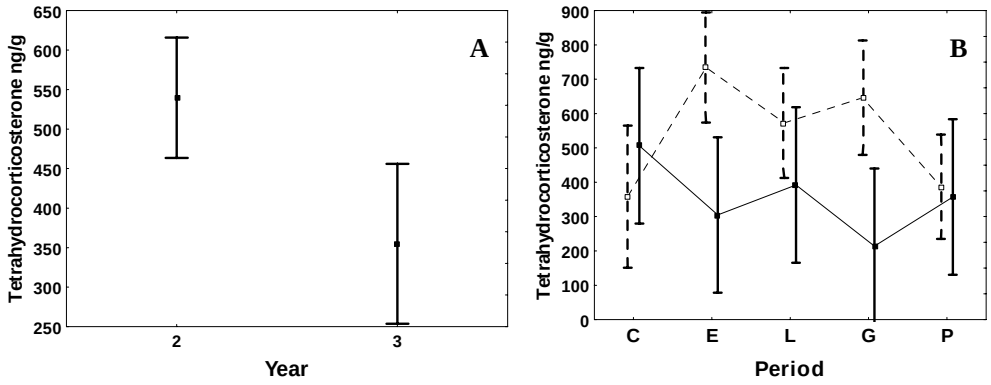
Appendix 1



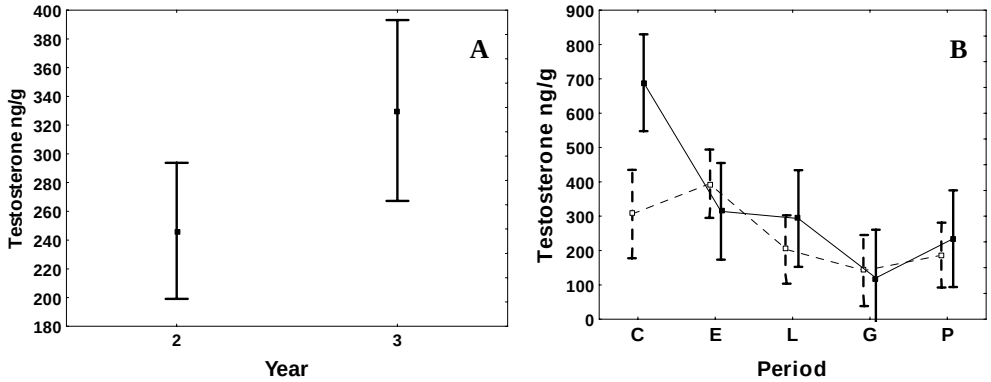
Appendix 1: Results of HPLC analysis of immunoreactive hormones and hormone metabolites in faecal samples. HPLC in the first 3 graphs used a gradient elution with a starting concentration of 50 % methanol, increasing to 75 %. HPLC in the final graph used a gradient elution with a starting concentration of 20 % methanol, increasing to 100 %. Arrows denote the elution fraction of authentic testosterone, estradiol, and tetrahydrocorticosterone in the respective graphs.

Appendix 2

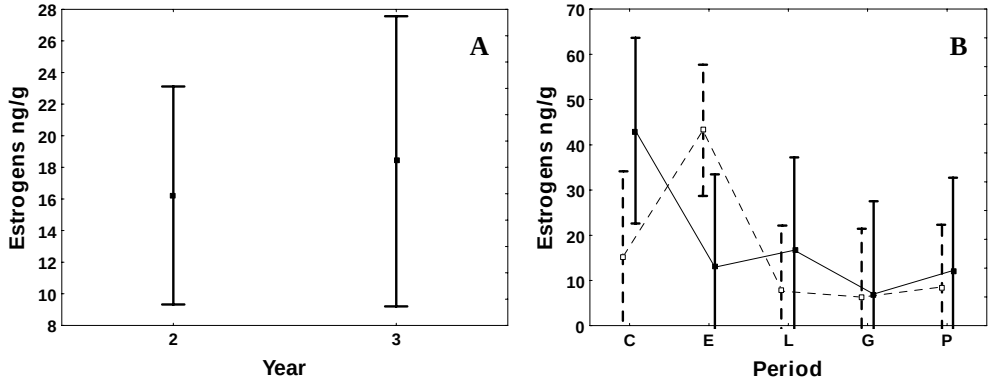
Graphs of hormone levels (from Chapter 3) in original scale



Mean tetrahydrocorticosterone across years (A), and across periods for each year (B) (year 2= open squares & dashed line; year 3= solid squares & solid line). Vertical bars denote 95% confidence intervals.



Mean testosterone across years (A), and across periods for each year (B) (year 2= open squares & dashed line; year 3= solid squares & solid line). Vertical bars denote 95% confidence intervals.



Mean estrogen across years (A), and across periods for each year (B) (year 2= open squares & dashed line; year 3= solid squares & solid line). Vertical bars denote 95% confidence intervals.

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