Indicators of and Influences on Reproductive Success in Yellow-eyed Penguins (*Megadyptes antipodes*)

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Abstract

Reproductive success is highly variable among individuals in bird populations and is often attributed to differences in individual quality. A small percentage (5.4%) of the endangered yellow-eyed penguin (*Megadyptes antipodes*) population at Boulder Beach, New Zealand, appears to contribute many more offspring to future generations compared to other individuals. To date, these high quality yellow-eyed penguins can only be identified retrospectively, using breeding data collected over their entire lifetimes. Yellow-eyed penguin numbers are dwindling as a result of numerous stressors, despite conservation management. Therefore, identifying potential indicators that would allow the superior breeders to be distinguished from average breeders would help guide yellow-eyed penguin management with respect to prioritising assistance for particularly productive breeding pairs.

Immunocompetence and feather ornamentation are well studied in birds as measures of reproductive fitness and are influential in sexual selection. Two common parameters used to infer levels of fitness are oxidative stress levels and leucocyte counts. These components of immunocompetence are obtained through haematological analyses and provide an idea of the individual's ability to resist infection (oxidative stress levels) and their stress levels (leucocyte counts) at the time of sampling. Ornament brightness, colour, and size are all widely known indicators of quality as well. I examined these parameters in relation to both short- and long-term reproductive success. In addition to this, I used 11 microsatellites to estimate internal relatedness (IR), which is a measure of inbreeding, to understand whether inbreeding levels influenced any of the putative indicators or was linked to reproductive success.

I found that superior breeders had higher levels of oxidative stress in their lifetime: this means that despite experiencing more challenging events, there were able to successfully fledge more chicks. The superior breeders were also less stressed (Heterophil/Lymphocyte ratio) in relation to the number of eggs laid for the year. Variation in the ornamentation parameters was not associated with differences in reproductive performance. Inbreeding did not have a significant effect on the indicators, which is good for the yellow-eyed penguin population since inbreeding levels are not affecting the reproductive success of these birds. This study shows that there are two promising indicators to discern breeding quality in yellow-eyed penguins (oxidative stress and H/L ratios), and that inbreeding levels are not currently a concern.

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Chapter 1: General Introduction

A glimpse of Boulder Beach, Dunedin

1.1 Reproductive Success

Reproductive success can be broadly defined as the passing of genes from one generation to the next. It is often expressed as the number of offspring a breeding pair or population produces. For studies on birds, researchers often report reproductive success as egg success (number of young leaving the nest/total number of eggs laid) or nest success (number of clutches that produce offspring/total number of clutches; Murray Jr. 2000). Reproductive success varies between years (Chastel et al. 1993; Murphy et al. 1991) and between individuals (Lescroël et al. 2009; Newton 1989) in response to fecundity-dependent factors such as female size and condition and, fecundity-independent factors such as environmental changes (Blums et al. 2002), for example, fluctuations in the oceanic climate as well as the marine food supply in the case of seabirds (Boersma 1978; Becker et al. 2007). High reproductive success rates are also linked to higher survival rates (Lescroël et al. 2009).

Identifying the causes of variations in reproductive success can be complex. Personality traits are increasingly invoked to explain among-individual variations within the population of a species, including differential rates of reproduction (Blums et al. 2005; Lescroël et al. 2009; Mutzel et al. 2013; Cram et al. 2015). For example, aggressive male blue tits (*Cyanistes caeruleus*) feed nestlings at a lower rate than passive individuals: this results in the female blue tit provisioning the nestlings more often to compensate, and is rewarded with greater reproductive success (Mutzel et al. 2013). Other potential reasons for some individuals producing fitter and larger numbers of offspring than others include genetic makeup (Bensch et al. 1994) and stronger immune systems (Sydeman et al. 1991; Moreno 1998).

It is important to monitor and record reproductive success, especially in the case of endangered species. Superior breeders are likely to possess qualities that make them appear fitter to ensure higher pairing rates and higher reproductive success rates (Saino et al. 1997; Sardell et al. 2014). Establishing how reproductive success is associated with individual quality and identifying indicators of fitness will be useful in recognising superior individuals in the population, should species numbers decline and active management interventions become necessary.

1.2 Individual Quality

Individual quality is defined as among-individual differences in fitness levels and reproductive success in a population. Within most populations some individuals have higher breeding success than others (Blums et al. 2005; Lescroël et al. 2009). Those fitter individuals, theoretically, have more opportunities to pass their genes on to future generations. Age and breeding experience, which vary between individuals, are known to affect productivity in many bird species (Wooller et al. 1990; Sydeman et al. 1991; Fowler 1995; Zhang et al. 2015); however, even controlling for these factors, some individuals are consistently more productive than others (Blums et al. 2005; Lescroël et al. 2009). Breeding success and quality differences are also associated with individuals' survival rates: successful breeders can have higher survival rates than unsuccessful breeders (Lescroël et al. 2009).

Differences in individual quality have been shown to be associated with "personality" (i.e. repeatable individual behaviours; Holtmann et al. 2015) which can influence the degree of dominance, subsequent pairing (Cram et al. 2015) and even extra-pair copulation opportunities (Thusius et al. 2001), as well as the individual's ability to respond to various stressors (Ellenberg et al. 2009). One of the most detrimental influences is inbreeding depression, whereby reproductive success is reduced, potentially driving an endangered species to extinction (Frankham 2005; Jamieson et al. 2009). Therefore, identifying high quality breeders is important, but understanding the impact that influences such as inbreeding have on individuals is also extremely important.

Individual quality has been evaluated in a number of ways (Blums et al. 2005; Lescroël et al. 2009; Wilson & Nussey 2010), including immunocompetence. The health status of birds influences their productivity. For example, late breeders are considered to be less healthy and are less productive (Sydeman et al. 1991; Moreno 1998; Moreno et al. 2002). A commonly used measure of an individual's health status is oxidative stress (Constantini 2008). Evaluating individual differences in health status, using haematological measures, can therefore be used to predict which individuals will have the highest productivity in a population (Moreno et al. 2002).

1.3 Indicators of Individual Quality

For the purpose of this thesis, I am using three proxies of individual quality. First, oxidative stress, which measures the long-term fitness levels of an individual. Second, leucocyte counts, as an indicator of stress levels at the time of sampling and provides short-term data on fitness. Third, ornamentation to determine whether quality is displayed externally by an individual. Below, I will briefly describe immunocompetence and then provide details of each component of quality involved in this thesis.

1.3.1 Immunocompetence

Immunocompetence is the body's ability to produce a normal immune response following exposure to an antigen. Immunological responses, quantified using leucocyte counts and oxidative stress levels, reflect resource competition and can therefore be linked to reproductive efforts and costs (Quillfeldt et al. 2008).

Immunocompetence is generally tested in birds by inducing an inflammatory response and estimating the ability of the individual to mount a response. Both Mougeot (2008) and Nolan et al. (2006) determined immunocompetence by assessing the T-cell-mediated immunity of red grouse (*Lagopus lagopus scoticus*) and king penguins (*Aptenodytes patagonicus*) respectively, using a standardised challenge with phytohaemagglutinin (PHA). This approach has several drawbacks: (i) it tests both acquired immune responses (T-lymphocyte production) and innate immune responses (phagocytosis) when only the former is required, (ii) a sick or recovering individual subjected to this test would skew the results (Salaberria et al. 2013), and (iii) the time of testing could coincide with an up-regulation or down-regulation phase which would influence the immunological response (Zuk & Stoehr 2002). During the breeding cycle, a species might up-regulate their immune defence from a 'normal' or baseline level to a higher level during an environmentally challenging peiod, such as the approach of winter (Zuk & Stoehr 2002). Similarly, the immune response might down-regulate from the baseline to a lower level to accommodate competing needs, such as sexual competition (Zuk & Stoehr 2002).

The more reliable and, in recent times, more widely-used approaches to establish immunocompetence are the measurement of oxidative stress, which is thought to represent long-term health, and leucocyte counts, to determine the current health state. Instead of measuring the temporary physical response, the physiological response is considered; that is

to say, the immediate effect on the body may not be as important as the permanent chemical changes which influence the normal functioning of the body. In the following sections, I will elucidate the importance of oxidative stress and leucocytes as measures of immunocompetence.

1.3.1.1 Oxidative Stress

Oxidative stress can be defined as the imbalance between the production of reactive oxygen species (ROS) and the ability of the body to produce antioxidants to detoxify harmful free radicals (Betteridge 2000). The ROS, also known as reactive oxygen metabolites (ROM), are free radicals which possess a lone electron. These unstable radicals bombard the healthy cells in an effort to stabilise. The healthy cells are damaged (i.e., lose electrons) and begin to age, and this is called oxidative stress. The antioxidants provide these harmful radicals with electrons to prevent the imminent chain-reaction by stabilising them. The ROS cause free radical chain reactions (viz. lipid peroxidation and protein damage) which are directly harmful; however, there is also secondary damage to biomolecules caused by the altered levels of ions due to oxidative stress (Betteridge 2000; Constantini 2008). Hence, the greater the difference between the number of ROS and the neutralising capacity of the antioxidant barrier, the higher the oxidative stress. The common free radicals that prove to be detrimental at a cellular level are the hydroxyl radical ($^{\cdot}OH$), superoxide anion ($O_2^{\cdot -}$), and transition metals such as iron (Betteridge 2000). The generation of these reactive oxygen radicals can result as a by-product of biochemical reactions in the body but also as an immunological response, during phagocytosis, for example (Betteridge 2000; Constantini & Møller 2009). Antioxidant capacity is species-dependent, varying with age and reproductive status of the individual (Constantini & Møller 2009).

Environmental changes force individuals of a species to counter cellular oxidation and maintain a homeostatic environment, using physiological, morphological and behavioural modifications which may, in turn, affect individual fitness (van de Crommenacker et al. 2011b). An increase in ROS can be caused by a number of factors (e.g. infections, changes in territory quality, reproduction), and various studies have linked it to a variety of possible effects. Oxidative status has been examined in the cooperatively breeding Seychelles warbler (*Acrocephalus sechellensis*) in relation to territory quality, parasitic infection, and social status (van de Crommenacker et al. 2011a; van de Crommenacker et al. 2011b; van de Crommenacker et al. 2012). The authors found that oxidative stress in warblers would

increase with short-term changes to their territory, increase with a parasitic infection, and dominant female breeders had an increase in antioxidants leading up to egg laying, which could suggest females undergo changes to optimise breeding. Oxidative stress could play a role in facilitating life-history trade-offs, as seen in individuals providing for their offspring (Guindre-Parker et al. 2013). It is a highly sensitive measure and can therefore be used to identify environmental and physiological shifts. For example, the effects of oxidative stress can be reflected in sexual traits, such as pigmented ornaments (Metcalfe & Alonso-Alvarez 2010).

Reproduction, like other physiological processes, brings about oxidative stress in birds (Constantini et al. 2014). There is likely to be a difference between the sexes, and females of some species that experience higher oxidative stress display lower fecundity and chick survival (Bize et al. 2008; van de Crommenacker et al. 2011a). Studies on populations with individuals of different reproductive quality, describe the relevance of managing antioxidant levels. For example, dominant white-browed sparrow weavers (*Plocepasser mahali*) in a population could potentially up-regulate (increase from a basal level) their antioxidant levels in order to counteract the oxidative stress brought about by their higher reproductive rates (Cram et al. 2015). This ability to regulate antioxidant levels could be prevalent in dominant or superior individuals in other bird species. It is also possible that superior breeders encounter alternative stressors to average breeders.

Compared to passerines, Sphenisciformes (the order to which penguins belong) and other large seabird families might experience oxidative stress during reproduction in very different ways. Adélie penguins (*Pygoscelis adeliae*) subject to induced breeding constraints showed an increase in antioxidants (Beaulieu et al. 2011). Both emperor penguins (*Aptenodytes forsteri*) and Adélie penguins are less susceptible to oxidative stress than other Antarctic seabirds and it was theorised that they are exposed to comparatively more oxidants which they are able to overcome (Corsolini et al. 2001). This elevated exposure to oxidation could be a result of their foraging behaviour. Unlike other Antarctic birds, penguins dive to forage and have to swim in order to eat. The oxygen reserves for penguins would be higher compared to other species. However, the authors do not consider the fact that penguins might simply be more robust. Adélie penguins also have the ability to up-regulate their antioxidant levels when stressed (Beaulieu et al. 2011), and the individual's ability to adjust in this way could separate the superior breeders from the average ones. Therefore, long-term health

assessments provided by oxidative stress measurements may explain individual adaptability and quality, making it a reliable distinguishing parameter.

To summarise, the imbalance between oxidants and antioxidants in any individual is unavoidable due to a high number of potential stressors, and hence oxidative stress will always occur. An individual that is comparatively healthier will be able to counter the oxidative stress by either being able to obtain more antioxidants (e.g. through their diet) or by resisting stressors with a strong immune system. Birds with larger broods and more chicks to feed will expend more energy and have higher oxidative stress (Deeming & Reynolds 2015). With this in mind, it is possible that they are able to have larger broods since they are not exposed to as many oxidising molecules as a less healthy bird, and tend to use the antioxidants they have to ensure that their offspring survive. In this way, oxidative stress may be detrimental to the health of an individual but fitter individuals will be able to resist and reduce the physiological damage better than weaker individuals. The fitness levels would be reflected by lower oxidative stress levels and higher antioxidant levels.

1.3.1.2 Leucocyte Counts

The effects of variations in physiological state (e.g. in reproductive performance) reflected in avian hematological parameters have been widely studied in several species. Leucocyte (white blood cell) profiles in particular are directly related to stress hormone levels and are used as indicators of physiological stress in birds (Davis et al. 2008). Birds possess erythrocytes (red blood cells) which are comparatively larger than those of mammals, and five main types of leucocytes: heterophils, eosinophils, basophils, lymphocytes, and monocytes (Clark et al. 2009).

Heterophils, eosinophils, and basophils contain unique cytoplasmic granules, and are called “granulocytes” (Clark et al. 2009). Within this group, the heterophils and eosinophils in particular, have an affinity for acidic stains and are therefore termed as “acidophils” (Clark et al. 2009). Heterophils act as the first line of phagocytic immune defense and increases in numbers are associated with inflammation, infection, and increased stress (Bickford 2007; Davis et al. 2008). Heterophils in birds are equivalent to neutrophils in mammals, in terms of immunological function (Bickford 2007; Davis et al. 2008). A high number of eosinophils often indicates an inflammatory response or defence against parasitism (Bickford 2007; Davis et al. 2008). The function of basophils in birds is still unclear; however, it is thought to

be associated with inflammation since the granules contain histamine (Bickford 2007; Davis et al. 2008).

Lymphocytes and monocytes are types of leucocytes which are referred to as "mononuclear cells" (Clark et al. 2009). These two types of cells can often be mistaken for each other, especially large lymphocytes, which tend to look like monocytes (Bickford 2007). Lymphocytes are responsible for immunoglobulin production, immune system regulation, defence against acute viral infection, and terminal viral disease (Bickford 2007; Davis et al. 2008). Monocytes are large, pleomorphic cells which carry out phagocytosis and defend against pathogens, mainly bacteria (Bickford 2007; Davis et al. 2008; Clark et al. 2009).

One of the most commonly used indicators of stress from leucocyte counts is the ratio of the number of heterophils to lymphocytes. Short-term levels of high stress are expressed as a high H/L ratio whereas individuals with lower stress levels have lower H/L ratios. Quillfeldt et al. (2008) found that the seasonal decline in adult body condition of thin-billed prions (*Pachyptila belcheri*) was reflected in an increase in H/L ratios. A similar result was described by Ochs & Dawson (2008), who noticed elevated H/L ratios in female tree swallows (*Tachycineta bicolor*) with decreased body condition due to climatic variations and increase in age. A comparative study on captive gentoo penguins (*Pygoscelis papua*), with and without bumblefoot (inflammation of the feet caused by a bacterial infection), found that the infected birds had a higher H/L ratio (Hawkey et al. 1985). The H/L ratio was reported as far more reliable measure of chronic stress in Adélie penguins (*Pygoscelis adeliae*) than a single corticosterone measurement (Vleck et al. 2000).

Maxwell (1993) reviewed the literature on the response of avian leucocytes in chickens (*Gallus domesticus*) to various stressors such as parenteral, nutritional, environmental, and psychological stressors. In this case the H/L ratio is less reliable than individual cell counts but more reliable than corticosteroid levels, and could possibly function as a two-phase reaction – a) a raised H/L ratio due to heterophilia during moderate stress and b) an increase in basophil counts (basophilia) during extreme stress. A more recent review by Davis et al. (2008) proposes that the H/L ratio is a useful indicator of the current and long-term fitness of an individual but recommends considering eosinophil counts, since these cells are more closely tied to stress than infection.

There has been some promising research done specifically on adult penguins and their chicks during the breeding seasons. At the time of egg laying, male Magellanic penguins (*Spheniscus magellanicus*) had lower leucocyte counts than females, and the females with high peripheral leucocyte counts were found to be poorer breeders i.e. they laid smaller eggs and successfully fledged fewer chicks (Moreno et al. 2002). The authors suggest that leucocyte counts can be used to determine the productivity of breeding penguins without considering long-term breeding success (Moreno et al. 2002). Multiple blood samples of breeding Adélie penguins showed a reduction in H/L ratios through the different stages of reproduction: from courtship, to incubation, to chick rearing (Vleck et al. 2000). However, it seems unusual for a bird to experience less stress during the chick rearing stage than the courtship stage, especially since adults expend more energy caring for their offspring (Deeming & Reynolds 2015).

1.3.2 Ornamentation

Most birds rely on sexual selection and have developed external ornaments to attract mates. Mate choice can be driven by the females (Thusius et al. 2001) or it can be mutualistic in monomorphic species (Kraaijeveld et al. 2007). Individual quality can be advertised to potential mates through ornamentation; e.g. feather ornaments (Siefferman & Hill 2003), colourful masks (Thusius et al. 2001), or pigmentation of the beak and feet (Dresp et al. 2005; Kraaijeveld et al. 2007). The colour of ornamental plumage determines sexual selection and reproductive success in eastern bluebirds (*Sialia sialis*; Siefferman & Hill 2003).

Avian ornaments can also reveal individual fitness in terms of how tolerant a potential mate is to oxidative stress (von Schantz et al. 1999). Ornamentation can also advertise immunocompetence or the current health status of an individual (Dobson et al. 2008). Immune defence is costly with respect to reproduction because during courtship there is a trade-off between sexual activity and immune responses, leaving individuals vulnerable to infection (Zuk & Stoehr 2002). For example, male barn swallows (*Hirundo rustica*) with longer tail feathers produced more offspring, as they were selected for by females; however, they had lower immunocompetence levels (Saino et al. 1997). An infection can have negative consequences; a parasitic infection may affect the growth of tail feathers, which thereby affects mate choice (Zuk et al. 1990; Møller 1994). Essentially, the fitness of breeding

individuals not only affects their own behaviour and immune defences but can also influence the survival of their offspring (Ilmonen et al. 2000).

A distinctive plumage pattern can be an indicator of mate quality during sexual selection where brighter plumes suggest higher quality (Zahavi 1975). There are a growing number of studies using colour reflectance and brightness of plumage or ornaments to test individual health. Measuring the brightness of feathers could potentially be a non-intrusive approach to discern the quality of breeding individuals in a population. In their study on Snares penguins (*Eudyptes robustus*), McGraw et al. (2009) found yellower crests meant better individual condition. Supposedly monomorphic birds, such as yellow-eyed penguins, generally use multiple signals during mate selection but colourful plumage could play an important role in the process.

Adult male and female yellow-eyed penguins have a characteristic yellow post-ocular stripe and bright yellow eyes. The post-ocular stripe is described as extending from the eye, backwards and encircling the crown and it can be used to distinguish the adults from the sub-adults (Massaro et al. 2003). Although this species appears monomorphic, there could be as yet undescribed variations in the width of the post-ocular stripe between the two sexes since they possess varying head lengths (Setiawan et al. 2004). It is likely that yellow-eyed penguins rely on multiple cues during mate selection (similar to other penguin species; Jouventin et al. 2008; Pincemy et al. 2009), and the size of the post-ocular stripe could play a role for this species.

Massaro et al. (2003) evaluated the hue and saturation of the yellow eye and post-ocular stripe of yellow-eyed penguins using photographs and found that they were an honest signal of parental quality, likely playing a role in sexual selection in this species. The yellowness of the plumes of the post-ocular stripe of yellow-eyed penguins has long been treated as a carotenoid-derived pigment (Massaro et al. 2003). A recent discovery suggests that in fact the yellow pigment is ‘spheniscin’, uniquely found in penguins (in the genera: *Megadyptes*, *Eudyptes* and *Aptenodytes*; Thomas et al. 2013) and is in fact not carotene at all (McGraw et al. (2007). This development could alter the implications maintained by previous research since the source, functions, and chemistry of this pigment molecule may not work like carotenoid-derived pigments. The chemistry of the molecule strongly determines how it

reacts physiologically with sex hormones and various metabolic pathways (Yu et al. 2004; Prum et al. 2012). The discovery of 'spheniscin' warrants revisiting these hypotheses using a different approach. Massaro et al. (2003) used secondary data (photographs) to evaluate pigmentation; therefore, using primary data (feather samples) and spectrometric analysis will yield more scientifically rigorous results.

In addition to colour and brightness, the size of ornaments in birds can be a reliable indicator of individual fitness. While examining plumage colouration and patterns in pigeons (*Columba livia*), Burley (1981) found that individuals selected higher quality mates based on these criteria. Studies on king penguins (*Aptenodytes patagonicus*) showed that the females are extremely selective when the ornaments of males are reduced (Pincemy et al. 2009) and the reflectance of the coloured head ornaments were indicative of sexual maturity and status (Nicolaus et al. 2007). Changes in the size of an external trait (e.g. plumage ornaments) can be used as an early indicator of both environmental and genetic stress (Leary & Allendorf 1989). These studies reaffirm the significance of analysing ornament sizes coupled with colouration. Smaller and duller plumage ornaments are less selected for by females (Jouventin et al. 2008; Pincemy et al. 2009). Computer software (e.g. ImageJ) has been used to analyse sexual ornaments from photographs taken of wild bird populations (e.g. Nolan et al. 2006; Rosen & Tarvin 2006). Measurements of the width of the eye stripe in yellow-eyed penguins could provide a non-invasive approach to identifying fitter individuals.

1.4 Consequences of Inbreeding

When closely related individuals breed, there is a genome-wide reduction in heterozygosity which in turn reduces reproductive success and survival of the individual, termed inbreeding depression (Keller & Waller 2002; White et al. 2015). This has a great impact on populations of endangered species (Hoeck et al. 2015), especially affecting early life traits. Limited numbers of individuals inevitably lead to incestuous mating, which reduces genetic diversity (Jamieson et al. 2008). The negative impacts of inbreeding in birds can be seen as increased egg infertility (Jamieson & Ryan 2000), lowered hatching success (White et al. 2015), and lowered fledging success (Jamieson et al. 2003). Some species have adapted to avoid inbreeding and its subsequent costs (Blouin & Blouin 1988; Pusey & Wolf 1996).

Genetic techniques, such as microsatellite typing, have been used to build pedigrees and estimate relatedness in species. Constructing pedigrees for a species can address questions on territorial group living, kinship, and family groups (Lillandt et al. 2003). Social pedigrees built for socially monogamous species provide an understanding of inbreeding depression and genetic variation of the population (Pemberton 2008). This approach can be used to predict the relatedness of offspring even when the parents are unknown. For example, microsatellite markers successfully reconstructed a pedigree for brown-headed cowbirds (*Molothrus ater*), an obligate brood parasite, and identified accurate parent/sibling groups (Alderson et al. 1999). They have also been used to study gene flow, genetic mapping, parentage, kinship, genetic diversity, inbreeding depression, and relatedness (Queller et al. 1993; Mudrik et al. 2014; White et al. 2015).

In penguin species, microsatellite markers have been used to estimate parentage and relatedness, as seen in a long-term study on Adélie penguins (*Pygoscelis adeliae*; Sakaoka et al. 2014). This species exhibits low levels of genetic differentiation despite substantial levels of genetic variation (Roeder et al. 2001). Genetic differentiation refers to distinctive genetic makeup between populations while genetic variation indicates distantly related individuals within a population which explains greater allelic diversity. In the endangered Galápagos penguin (*Spheniscus mendiculus*), unlike Magellanic penguins (*Spheniscus magellanicus*), populations have low heterozygosity and are highly susceptible to El Niño events (Akst et al. 2002; Nims et al. 2008) due to the resulting food shortages and low plasticity to adjust to catastrophic events. Conservation genetic analyses carried out on the Humboldt penguin (*Spheniscus humboldti*) refuted the observed distribution and suggest that this vulnerable

species should be managed as a single population (Schlosser et al. 2009). In addition, genetic analyses in Humboldt penguins showed that although extra-pair copulations are observed, none of these extra-pair matings results in fertilization (Schwartz et al. 1999).

Yellow-eyed penguins are socially monogamous, but genetic analyses are required to confirm this due to the possibility of extra-pair copulations in penguins. Long-term monitoring of the population at Boulder Beach enables an investigation into the relatedness and genetic diversity of these breeding birds. Given the declining status of the Boulder Beach population (Ellenberg & Mattern 2012), it is important to evaluate the heterozygosity of the remaining birds. Due to the close proximity of nests (Clark et al. 2015) there are opportunities for extra-pair copulation. Extra-pair mating has been observed in other penguin species (Schwartz et al. 1999) and can result in misleading pedigrees, if present (Reid et al. 2014). The incidence of extra-pair copulations in yellow-eyed penguins is unknown and could significantly bias inbreeding estimates (Reid et al. 2014). There is anecdotal evidence of extra-pair copulation in yellow-eyed penguins (Richdale 1957); however, genetic analyses would be required to confirm whether offspring were sired as a result of those extra-pair copulations. Even anecdotal evidence gives reason for a more rigorous study to identify whether extra-pair matings exist and influence reproduction of yellow-eyed penguins.

1.5 Individual Quality in Yellow-eyed Penguins

Yellow-eyed penguins (*Megadyptes antipodes*) are endemic to New Zealand. Individuals vary hugely in their lifetime reproductive success, with only 5.4% of breeders contributing offspring which bred in the next generation (Stein, 2012, unpublished thesis). Among-individual differences have also been found with respect to behavioural plasticity and stress responses (Ellenberg et al. 2009). Yellow-eyed penguins appear to have varied personalities (timid, calm or aggressive), which result in some individuals being fitter and potentially better breeders (Ellenberg et al. 2009). They also exhibit unique behavioural responses when stressed - all signs of individual quality (Ellenberg et al. 2009).

1.6 Relevance of understanding the indicators of yellow-eyed penguin reproductive success

The yellow-eyed penguin is listed as 'endangered' on the IUCN red list, with populations experiencing an overall decline around New Zealand (BirdLife International 2012), and 'nationally vulnerable' in the New Zealand threat classification (Miskelly et al. 2008). The species is susceptible to a number of threats: for example, diphtheria at a young age (Alley et al. 2004), predators on land and at sea (McKinlay 2001; Lalas et al. 2007), and anthropogenic activity such as tourism (McClung et al. 2004; Ellenberg et al. 2009). Their conservatism in foraging behaviour suggests poor adaptability to changes in the marine environment (Mattern et al. 2007).

Given the declining status of this rare species and the increasing frequency of catastrophic events (Ellenberg & Mattern 2012), early identification of superior breeders in the yellow-eyed penguin population could improve management strategies, making it possible to prioritise conservation efforts to benefit high quality individuals. This could help increase the numbers of this endangered species. In this thesis I explore potential relationships between individual quality and immunocompetence expressed as oxidative stress and leucocyte counts, and I have investigated whether feather brightness, plumage colouration, and ornament size act as indicators of individual quality. I have also examined the genetic diversity of the population in terms of internal relatedness and the potential effect of inbreeding on reproductive success.

1.7 Thesis aims and outline

The aim of this research was to investigate whether relationships exist between putative indicators of individual quality and reproductive success in yellow-eyed penguins, specifically, between the group of individuals that show consistently high reproductive success, compared to the remaining birds of average reproductive performance. Additionally, I explored the possible presence and impact inbreeding might have had on the reproductive success of yellow-eyed penguins whilst validating the relationships recorded in the DOC database by checking for genetic evidence of extra-pair mating.

With a small proportion of the yellow-eyed penguin population at Boulder Beach demonstrating high levels of reproductive success, and the species in general decline, it is important to identify the fitter individuals, since an understanding of what causes these superior individuals to be more successful might benefit management of the entire population. Is reproductive success linked to immunocompetence? And do high quality individuals display quality through low levels of oxidative stress levels and brighter ornamentation? The effects of inbreeding on fitness levels might also help improve our understanding of the differences in reproductive success. This thesis contains two data chapters as well as a general introduction (Chapter 1) and general discussion (Chapter 4).

Chapter 2 aims to determine whether reproductive success rates can be predicted using immunocompetence (oxidative stress and leucocyte counts) and ornament measurements. For immunocompetence, I analysed the relationship between levels of oxidative stress and current and long-term reproductive success. I predict that individuals with higher reproductive success would have lower levels of oxidative stress. I then determined whether there is a relationship between leucocyte counts and reproductive performance. I expected superior breeders to have lower H/L ratios, lower eosinophil counts, and lower total white blood cells (TWBC) on account of being less stressed than average individuals. And finally, I looked at relationships between ornamentation and reproductive success. I treated feather brightness, feather yellowness, post-ocular stripe size, and post-ocular ring size as potential external indicators. I predicted feather ornaments would be brighter, more colourful, and larger for the superior breeding group, allowing them to be selected for first during the courtship period.

Chapter 3 aims to determine the internal relatedness of this population, levels of inbreeding, and the influence inbreeding had on the putative indicators. I anticipated none to low levels of inbreeding since juvenile yellow-eyed penguins undertake a pelagic phase post-fledging and show less philopatry until they start breeding. However, the levels of relatedness had to be confirmed since the species is endangered and can be considered at risk for inbreeding depression.

Chapter 2: Immunocompetence as an Indicator of Reproductive Success

A yellow-eyed penguin in a weighing bag prior to sampling

2.1 Introduction

Yellow-eyed penguins have been closely monitored on Boulder Beach for the last three decades by DOC. Retrospective breeding data have shown that 5.4% of the population is significantly more successful at fledging chicks that survive and go on to breed themselves (Stein, 2012, unpublished thesis). Yellow-eyed penguins are endangered and their populations continue to decline (BirdLife International 2012), making them vulnerable to catastrophic events (Ellenberg & Mattern 2012). Hence it is important, from a conservation standpoint, to be able to identify the high quality individuals pro-actively rather than retrospectively, to guide management of the species.

Differences in individual quality within a population are reflected in immunocompetence (Quillfeldt et al. 2008) and reproductive success (Wilson & Nussey 2010). It is not uncommon for bird populations to have individuals which reproduce at different rates (Blums et al. 2005). Currently, the two prevalent approaches for estimating health status and stress levels are oxidative stress (Constantini 2008) and leucocyte counts (Davis et al. 2008). Oxidative stress is a record of the physiological challenges faced by an individual through its lifetime and there is often a trade-off between maintaining immune defence systems and caring for offspring (Lescroël et al. 2009). Leucocyte counts, in particular the Heterophil/Lymphocyte ratio, reflect the stress levels of an individual, and is variable based on illness and injury (Davis et al. 2008). It is more of a short-term indicator of fitness.

Feather ornaments and variations in plumage patterns reflect internal fitness and quality. These traits are important for sexual selection in birds where mates can be rejected for possessing smaller or duller feathers (Thusius et al. 2001; Siefferman & Hill 2003). It has been seen as an honest signal of quality, indicating a better genetic makeup (Zahavi 1975). Yellow-eyed penguins appear monomorphic; however, their yellow post-ocular colouration could advertise their quality to potential mates during courtship.

In this chapter, I investigate whether differences between highly productive and less productive yellow-eyed penguins are reflected in differences in these haematological parameters: if this was the case, they could be used as indicators of breeding quality. In addition to this, I use post-ocular yellow feather samples and photographs of the post-ocular stripes to determine whether colouration and ornament size vary between the two breeding groups. These external indicators of individual fitness could influence mate selection during

the courtship period. These two measurements could also help distinguish superior breeders from average breeders.

2.2 Methods

2.2.1 Field Data Collection

Sampling was restricted to the Boulder Beach Complex on the Otago Peninsula in Dunedin, New Zealand (45°53'S, 170°37'E). All data collection was approved by animal ethics (permit 44/2014) and obeyed the Wildlife Act Authority 39071-FAU. The Department of Conservation (DOC) conduct annual yellow-eyed penguin nest searches in September. The Beach Complex is split into four main areas: Highcliff, A1, Midsection, and Double Bay. During the nest searches, the GPS coordinates for each nest site are recorded and an orange/pink marker is attached to a plant located either directly above the nest or to the most visible point of access to the nest. This makes locating the nests much easier during the course of the breeding season. For this study, the GPS coordinates for the nest sites at Boulder Beach were uploaded on a Garmin 60CSx GPS device. Unlike other colonial penguin species, yellow-eyed penguins breed in isolation but share breeding grounds with other pairs, usually by nesting under dense vegetation (Darby & Seddon 1990). A clutch of up to two eggs are laid in September or October and incubated by both parents (Darby & Seddon 1990; van Heezik & Davis 1990). Hatching usually occurs in November (van Heezik & Davis 1990); however, during a poor breeding year, chicks could hatch as late as the end of December. Yellow-eyed penguins were sampled during the incubation stage of reproduction (November 2014) because it is easier to find and sample the birds while they are on the nest. The parental load is shared relatively equally between the males and females by trading places every day: one stays on the eggs while the other goes out to sea to feed. I attempted to sample both adults on each nest by returning to the same nests on multiple occasions.

On locating a nest, I examined the bird to ensure that it showed no signs of injury, disease or malnourishment, which was the case for all the individuals. Since the parent stays on the egg to protect it when approached by a possible threat, I had to take into account the possibility of the penguin crushing the eggs or knocking them out of the nest while being approached. Adults were lifted carefully off the eggs by their feet and put into a weighing bag. The eggs were checked to determine whether hatching had begun. If there were signs of

pipping (first stage of hatching when the chick initially cracks open the shell), that nest was left out of the sample set. Sampling was quick (<15 minutes) so as to minimise the risk of the eggs getting cold. On some occasions, the eggs were kept warm while the adults were being sampled by placing a hat or a weighing bag on them.

2.2.2 Sampling and Processing

The blood samples were collected first. The penguins were turned on their sides (while still in the bag) and a flipper was extended from the bag. The area on the ventral surface of the flipper surrounding the brachial vein was disinfected thoroughly using 70% Alcohol Prep Pads (Shanghai Yinjing Medical Supplies Co., Ltd., Shanghai, China). A 23-gauge hypodermic needle was used to draw blood out of the brachial vein. The 2ml syringes (Becton, Dickinson and Company, Singapore) used were lined with heparin by drawing ~1ml of heparin into the syringe and ejecting it after a few seconds. Between 1-4ml of blood was collected: 200µL-400µL was transferred into a BD Microtainer®™ tube lined with lithium heparin and the remaining into 5ml Cryo.s™ tubes (Greiner Bio-One, Frickenhausen, Germany). The few drops of blood remaining in the syringe were used to make blood smear slides on-site. The blood in the Microtainer®™ tube was used for hemocytometer counts and the blood stored in the Cryo.s™ tubes was used to extract plasma to measure the oxidative stress levels. The two tubes containing blood for each individual were immediately placed in an ice box. The temperature in the ice box could not be regulated and hence no sampling session lasted more than 4 hours, to ensure that the samples remained in prime condition.

A Nikon D90 camera with a Nikon 18-135mm lens was used to capture images of the yellow post-ocular stripe. Prior to photographing the yellow eye-stripe, a photograph was taken of the flipper band number (as per DOC banding records) to identify the individual in the subsequent images. Photographs were taken of both sides of the bird's head (Fig. 1). The beak was held shut by a volunteer and the head angled perpendicular to the camera lens. The distance between the lens and the head was ~30cm. There was no flash used.

Figure 2.1: An example of the photographs used for post-ocular stripe analyses (Sample No. J18865)

The final sampling step was feather collection to measure feather brightness. The yellow post-ocular stripe was divided into three regions: the right side (near the eye), the dorsal side (behind the head) and the left side (near the eye). I collected approximately 4-5 feathers from each region using a small pair of scissors. A volunteer held the bird's head still while single feathers were extracted from the eye-stripe. The feathers were not collected close together, but rather in an almost circular pattern to prevent large areas of skin being exposed. The feathers from each part of the eye-stripe were stored in separate Eppendorf tubes and labeled with 'left', 'right' or 'back' along with the band number. The Eppendorf tubes were then stored in a darkened box to preserve the colour of the feathers since light exposure leads to feather degradation.

Immediately upon returning to the lab, the Cryo.s™ tubes containing blood were placed in a MSE Mistral 3000i centrifuge at 3000rpm for 20 minutes to isolate the plasma. The red blood cells settle at the bottom of the Cryo.s™ tubes and the plasma rises to the top. The plasma was carefully pipetted into 1.5ml Eppendorf tubes, making sure no red blood cells or fibrinogen was aspirated, and frozen at -70°C. The remaining erythrocytes were refrigerated. For some individuals, <1ml of blood was collected. These blood samples were put into capillary tubes and centrifuged. The capillary tubes were then broken above the fibrinogen layer and dispensed into 1.5ml Eppendorf tubes and stored along with the other samples. The blood stored in heparin was used to make blood smears and for hemocytometer counts which were performed on the same day.

2.2.3 Reproductive Success Index

I measured reproductive success in two ways. First, reproductive output of the year, hereafter termed as, "short-term reproductive success". For this, I included number of eggs laid and number of chicks fledged per breeding individual during the 2014-2015 breeding period. Second, reproductive ouput of lifespan, hereafter termed as "long-term reproductive success" or simply "index". For this, I included the number of chicks fledged and the total number of breeding seasons (from which I have information; min =1, max=16 breeding seasons) while controlling for missing data (the number of eggs laid).

Calculating long-term productivity was complicated by the varying ages and number of breeding seasons experienced by the yellow-eyed penguins sampled. I had to first standardise the productivity data in the form of an index. I created an index using a Poisson Exposure Model (PEM) in the following manner. Since the birds were of varying age, with the majority of individuals less than 10 years old, a 'quasipoisson' distribution was used to account for the overdispersed count data (age). I divided the total number of chicks fledged by the number of breeding seasons of each sampled individual. A generalised linear model (GLM) was run to establish the index which also included an 'exposure' (by means of the 'offset' function) for the log of the total eggs produced. An exposure takes into account any missing data, here, the potential number of eggs missed during nest searches. There could be a number of nests which were not found, and hence a certain number of eggs which were missed. I obtained an output of the exponential values of the predicted coefficient estimates for each individual. These values were used as the long-term productivity index for the sampled yellow-eyed penguins. The median was calculated, rather than the mean, because of the skewed distribution of the index. The individuals with an index greater than the median were categorised as highly productive birds (broadly termed as "superior breeders") and those with an index below the median were termed as average birds (which includes average and poor breeders). This index was specifically made to distinguish superior breeders from average breeders (as suggested by Stein 2012, unpublished thesis). Unlike other studies (e.g. Massaro et al. 2003) which look at a spectrum of individuals exhibiting certain traits, I focused on analysing high quality individuals against average individuals. This long-term reproductive success index accounts for all pertinent variables needed to distinguish quality.

2.2.4 Oxidative Stress Measurements

I calculate oxidative stress measuring the imbalance between the reactive oxygen metabolites (ROMs) and the antioxidant barrier capacity in the blood as a proxy of individual condition. To achieve this, I used the d-ROMs test kit to measure ROMs, and an OXY-Adsorbant test kit to estimate the antioxidant barrier capacity. The steps taken for each of the kits and the calculation of the imbalance are as follows:

d-ROMs: The d-ROMs test is based on a colorimetric reaction in accordance with Beer-Lambert's Law. The plasma from the yellow-eyed penguins is tested to measure the level of reactive oxygen metabolites (ROMs). The reagents provided in a d-ROMs Test Kit (Diacron International, Grosseto, Italy) are: a chromogenic mixture (*N*,*N*, diethyl-*para*-phenylendiamine; R_1), an acetate buffer (pH 4.8) with preservatives and stabilisers (R_2), and a calibrator made of lyophilised serum with known hydroperoxide concentration. The calibrator is essential to calculate the oxidative damage and antioxidant capacity. These reagents were stored away from sunlight and at 2-8°C.

Before commencing, the reagents and the plasma sample were kept at room temperature for about 10 minutes. Distilled water (2ml) was added to the calibrator and 500µL of R_1 was mixed with 5µL of R_2 in 1.5ml Eppendorf tubes. The first two Eppendorf tubes were set aside for the blank and calibrator. The blank, which functions as a control, contained 10µL of distilled water. The calibrator was made up of 5µL of distilled water and 5µL of the lyophilised serum. The remaining Eppendorf tubes were filled with 10µL of plasma for each sample and gently mixed by inversion. The Eppendorf tubes were incubated for 90 minutes at 37°C. After 90 minutes, the tubes were gently inverted again to ensure mixing. They were then centrifuged for two minutes at 13,000rpm. A total of 350µL of the supernatant in each tube was transferred onto a microplate. Finally, the microplate was inserted into a FLUOstar Omega microplate reader (company) and the spectrum from 480nm to 580nm was generated with a resolution of 1nm and 100 scans per well. The spectra could then be analysed.

OXY-Adsorbant: the OXY-Adsorbent test is also a colorimetric based analysis. Here, however, the test quantifies the strength of the antioxidant barrier against oxidant damage. The reagents provided in an OXY-Adsorbent Test Kit (Diacron International, Grosseto, Italy) are: an oxidant solution (HClO-based; R_1), a chromogenic mixture (*N*,*N*, diethyl-*para*-

phenylendiamine; R_2) and a calibrator made of lyophilised serum with known antioxidant capacity. These reagents were also stored away from sunlight and at 2-8°C.

The reagents and plasma were kept at room temperature for about 10 minutes. Distilled water (2ml) was added to the calibrator. The plasma was then diluted in 1.5ml Eppendorf tubes (10µL of plasma in 1mL of distilled water). The calibrator was also diluted in the same way (10µL of calibrator in 1mL of distilled water). Each well on the microplate was filled with 200µL of R_1 (HClO). Distilled water (5µL) was filled in the first well (blank) and 5µL of the calibrator in the second well (standard). The remaining wells were filled with 5µL of the plasma sample. The microplate containing the mixture was incubated for 10 minutes at 37°C. After incubation, 5µL of R_2 (chromogen) was added to each well and mixed gently with a multichannel pipette. The microplate was placed in a FLUOstar Omega microplate reader and the spectrum from 480nm to 580nm was generated with a resolution of 1nm and 100 scans per well. The spectra could then be analyzed.

Balance of Oxidative Stress: From the spectra, the optical density (OD) at a wavelength of 552nm was used for all analyses. The d-ROM and OXY-Adsorbant results were treated separately. For the d-ROM test, I calculated the mean OD for each individual from two measures (Vassalle et al. 2008). I also calculated the mean OD for the standard. I used the formula:

$$\frac{\text{(Mean OD of the individual)}}{\text{(Mean OD of the standard)}} \times 350 = \text{ROMs (in U.CARR)}$$

This gives the concentration of oxidising metabolites in Carratelli Units (U.CARR). These results were multiplied by 0.08 to convert the unit to mM of H_2O_2.

For the antioxidant barrier capacity, I calculated the mean OD of the blank (Vassalle et al. 2008) and used the formula:

$$\frac{\text{(Mean OD of the blank – Mean OD of the Individual)}}{\text{(Mean OD of the blank – Mean OD of the Standard)}} \times 320 = \text{OXY (in mM } H_2O_2\text{)}$$

Finally, the following formula was used to estimate the ratio for oxidative stress:

$$\frac{\text{ROMs (in mM } H_2O_2\text{)}}{\text{OXY (in mM } H_2O_2\text{)}} \times 1000 = \text{Oxidative Stress Ratio}$$

2.2.5 White Blood Cell Counts

For an accurate estimation of white blood cell concentrations, two sets of values are required: those obtained from hemocytometer counts and those from differential counts. Hemocytometer counts provide the total number of heterophils and eosinophils. Differential counts give the percentage concentration of each leucocyte type. By counting a hundred cells, the percentage of heterophils and eosinophils is determined for that individual. Although the number of basophils, lymphocytes, and monocytes are not used, they are required to make up the percentage of white blood cells present. The total white blood cell count can then be calculated using the formula (Campbell 1995):

$$\text{Total WBC/µL} = \frac{\text{Total Heterophil + Eosinophil (both chambers) x 1.1 x 16 x 100}}{\text{\%Heterophils + \%Eosinophils (from differential counts)}}$$

With the aim of calculating the total white blood cell count for each individual, the total heterophil and eosinophil count was obtained using a Neubauer hemocytometer (Fig. 2.2). The Leukopet™ method (Vetlab® Supply Inc.) was adopted. These kits include prefilled tubes containing 0.1% stabilised phloxine solution. The method works on the underlying principle that the prominent granules in heterophils and eosinophils are stained orange-red by 0.1% phloxine solution.

It was carried out in the following way: a new disposable pipette tip was attached to a 25µL pipette. The cap of a prefilled phloxine tube was unscrewed and placed on a stand. I used a pipette to aspirate 25µL of anticoagulated blood. The outer surface of the pipette tip was wiped clean with a lint-free wipe so as to ensure the volume of blood was exactly 25µL. Taking care to avoid making contact with the opening of the tip, I dispensed the blood sample into the 0.1% phloxine solution. The pipette tip was rinsed thoroughly by aspirating and

dispensing the blood and phloxine mixture at least six times. The cap was placed back onto the tube and the contents were mixed well by inverting the tube several times. The tube was then placed on a stand in an upright position to incubate at room temperature for 10-15 minutes. The incubated tube was gently rotated 3-4 times to ensure complete homogenisation of the blood and phloxine dye. Using the same pipette, I aspirated the sample from the tube and charged both chambers of the hemocytometer. The hemocytometer was then allowed to stand for 10 minutes. Finally, I used the 10X objective lens on the binocular microscope to count the heterophils and eosinophils in both chambers of the hemocytometer.

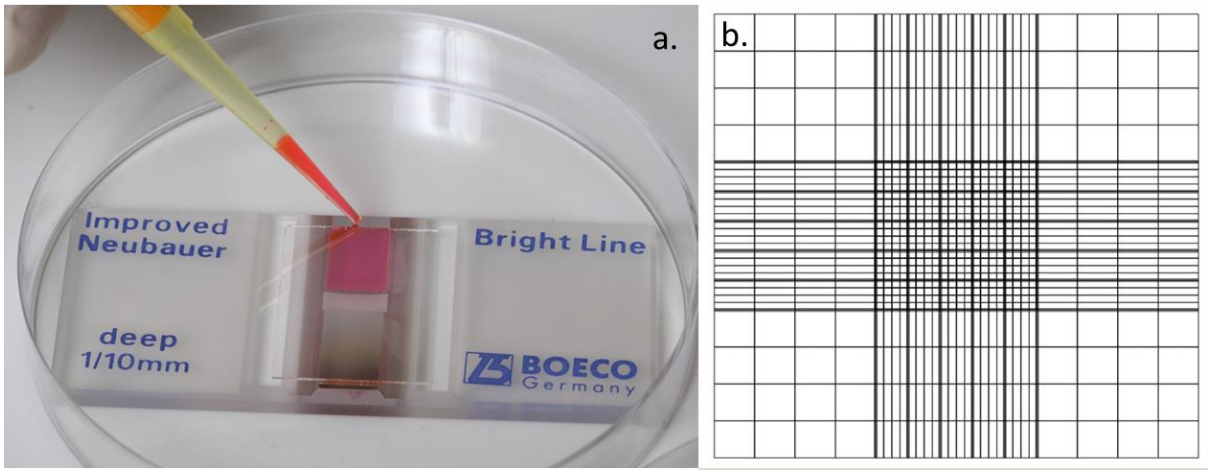

Figure 2.2: a) The Neubauer Hemocytometer being loaded with phyloxine and blood. b) An enlarged view of a single chamber on the hemocytometer showing the 9 main squares which are further subdivided.

I made blood smears from the blood samples collected. The blood smears were fixed in pure methanol using a staining dish and a swing handle slide staining rack. The slides were placed in the staining rack and immersed in 100% methanol solution for a few seconds. The slides were then allowed to dry gradually.

A Wright-Giemsa modified stain (WG16; Sigma-Aldrich Co.) was used on all the blood smears. The Dip Method was used to stain the slides (Brown 1993). Under this method, a coplin jar was filled with approximately 50ml Wright-Giemsa stain. Another coplin jar was

filled with the same volume of deionised water. The thoroughly dried smear slides were placed in the Wright-Giemsa stain for 30 seconds. They were then removed and placed in the second coplin jar of deionised water for approximately 5 minutes. The slides were then rinsed with running deionised water and air-dried thoroughly.

The slides, once dried completely, were ready for evaluation. A 9-key Economy Benchtop Counter (Universal Medical Inc., Boston, MA) was labelled with the five types of white blood cells (heterophils, eosinophils, basophils, lymphocytes and monocytes; Fig. 2.3). The slides were placed under a high-powered Olympus BX 51 binocular microscope. The leucocyte counts were started at the feathered edge of the blood smear under the oil immersion objective lens (100X). The counts were carried out for the five main types of leucocytes until one hundred cells were counted. This gave the percentage of each type of leucocyte for that individual.

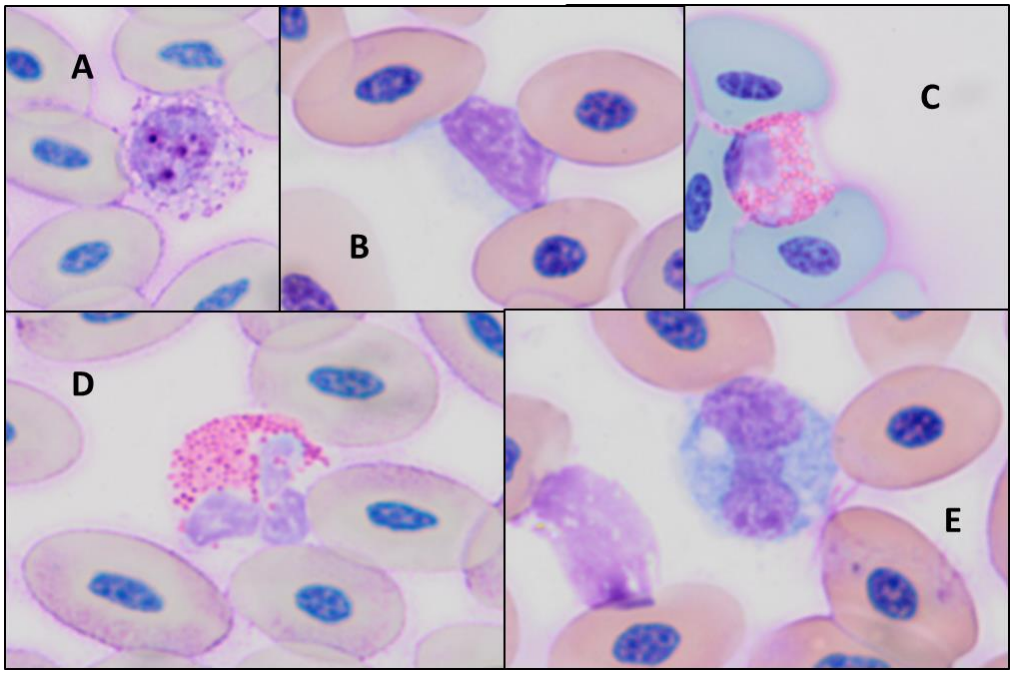

Figure 2.3: The 5 types of white blood cells used in the differential white blood cell counts – A) basophil, B) lymphocyte, C) eosinophil, D) heterophil and E) monocyte.

2.2.6 Feather Reflectance Measurements

I measured the reflectance of feathers from each zone of the post-ocular stripe separately, using an Ocean Optics USB4000 spectrometer (USB4000, Ocean Optics, Dunedin, FL, USA) with a deuterium-halogen light source (DH-2000, Ocean Optics). The reflectance spectra ranged from 300-900nm; however, for the analysis, the range was restricted to 300-700nm. I measured 41 individuals for the left and right zone and 40 individuals for the back zone (J19866 was missing).

All measurements were taken in a dark room to prevent the influence of external light. I stacked four feathers, one on top of the other, ensuring the yellow barbs would be measured rather than the white base. The feathers were positioned between a piece of black cardboard with a 6mm hole and a black cardboard surface. I measured reflectance relative to a white standard (WS02, Ocean Optics) and the black cardboard surface. Measurements were taken at a 90° angle using a Mikropack™ probe holder to prevent ambient light from affecting the readings and at approximately 6cm distance from the feather sample. I recorded reflectance at 4 second intervals by averaging 20 sequential spectra per reading. A total of five measurements were taken for each set of feathers at 1nm steps between 300nm and 700nm, using SpectreSuite software (Ocean Optics).

Spectral analyses were carried out using the 'pavo' package on R software (Maia et al. 2013). I treated the three post-ocular zones as separate segments and the summary data (mean values) for all individuals were exported. Of the 23 colorimetric variables in the output from 'pavo', I focused on the yellow spectral range (S_1.yellow) and mean brightness (B_2). I used the ICC package (*ICCest* function) on R to measure repeatability by estimating the intra-class correlation coefficient (ICC) values for the 23 colour variables (Montgomerie 2006; Wolak et al. 2012). I took the average of the mean S_1.yellow from the left and right sides to get one value per individual. The yellow range of the posterior feathers was excluded as the posterior zone is visibly duller than the sides and there is a chance that black feathers might affect the yellow range. I did the same for B_2 but included the measurements taken from the posterior zone of the stripe.

2.2.7 Post-ocular Stripe Measurements

The images were saved and analysed using ImageJ (Schneider *et al*. 2012). To standardise the images for all individuals, the image size was set at 4288x2848 pixels. To minimise error and ensure that all images were comparable irrespective of image quality, the eyeball was used as the standard. The average length of a yellow-eyed penguin eyeball is 15mm (M. Young, DOC Ranger, pers. comm.) from nasal canthus to temporal canthus. Once the image was opened on ImageJ and the image size set, a line was drawn from tear duct to tear duct (i.e. the length of the eyeball) and the scale was set at 15mm. All measurements thereafter were in relation to the eyeball for that image. Therefore, even highly pixelated images would yield measurements that could be compared with those from a high quality image. The post-ocular stripe was split up into three zones: close to the eyeball (temporal canthus), middle and away from the eyeball (curvature of the skull) as seen in Fig. 2.4a-c. A single line was drawn within each zone and measured. The eye-ring was also measured in a similar way. The eye-rings were treated as two main zones: upper and lower eye-ring. Both these zones were then separated into three zones: nasal canthus zone (Fig. 2.4d), middle zone (Fig. 2.4e), and temporal canthus zone (Fig. 2.4f). The nasal canthus zone is the area above and below the eye closest to the nasal fossa. The temporal canthus zone is the region closest to the ear. A single line was drawn using ImageJ in each zone on the photograph and the length was recorded.

This analysis resulted in nine measurements – three for the width of the post-ocular stripe, three for the width of the upper region of the eye-ring and three for the lower region of the eye-ring. I used the ICC package (*ICCest* function) on R to measure repeatability (Wolak et al. 2012). All measurements were highly repeatable (ICC >0.80) and following this the mean was calculated for each set of values i.e. mean stripe width and mean ring width.

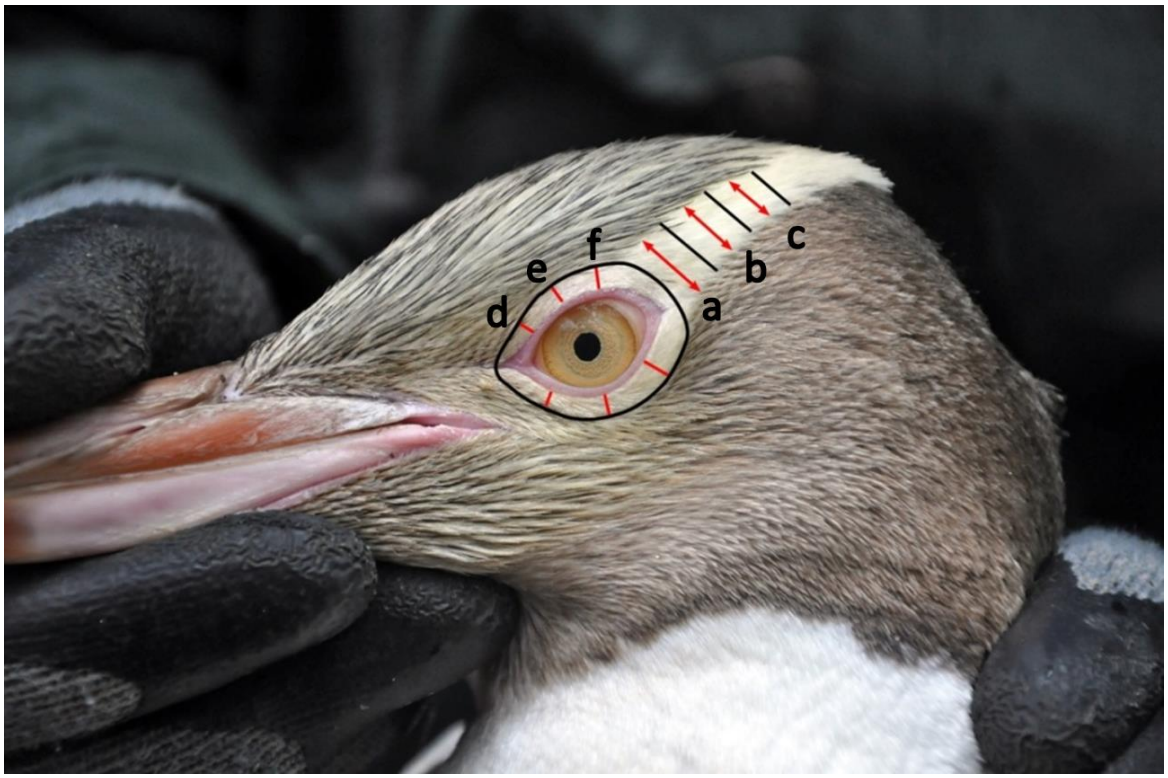

Figure 2.4: The zones of measurement for the post-ocular stripe and the upper and lower eye-ring. The black lines represent the zonal demarcations. The red lines (labelled a-f) denote the points of measurement.

2.2.8 Statistical Analyses

The variables included in the analysis of long-term reproductive success were plumage ornaments (reflectance and morphometric data) and oxidative stress data. Plumage colouration varies with age, time since last moulting, and exposure to antioxidants, since most pigment molecules consist of antioxidants. Ornament size, as well, is age-dependent and therefore all ornamentation data were analysed against long-term breeding success. Furthermore, there is a certain degree of heritability with feather characteristics. Similarly, oxidative stress levels are the result of a lifetime of stressors, making it an important factor to consider for the entire breeding life of the birds. The current health and condition, including short-term breeding success, of the birds is likely to be reflected in leucocyte counts which provide insight into the physical state of the bird at the time of sampling. Some individuals experience greater stress, and this can be estimated using leucocyte measures. Oxidative stress levels were also analysed against reproductive success of the season to examine whether the overall health of the bird influenced egg and chick production in one season.

I ran generalised linear models (GLMs) using R (R Core Team, 2015) to identify possible relationships between the reproductive performance of the samples (current or long-term) and the variables. I examined the influence of H/L ratio, eosinophil counts, total white blood cell (TWBC) counts, oxidative stress levels, and dROM levels on the number of eggs laid and the number of chicks fledged, respectively. The leucocyte variables were analysed separately from the oxidative stress variables.

The long-term productivity index was analysed against ornamentation variables (feather yellowness, feather brightness, post-ocular stripe width, post-ocular ring width). The two spectrometric variables were investigated independently from the two morphological variables. Another GLM was used to test the relationship between long-term productivity and oxidative stress variables: dROM levels and oxidative stress levels.

2.3 Results

Short-term Reproductive Success

The short-term reproductive index was considered as a) number of eggs laid, and, b) the number of chicks fledged. Of the 38 yellow-eyed penguins included, more laid two eggs than one egg. For the number of chicks fledged in the 2014-15 breeding season at Boulder Beach, the majority of birds successfully fledged one chick, a smaller number of birds fledged two chicks, and some birds fledged zero chicks. As seen in Fig. 2.5, the distribution was skewed; however, the sample set included breeding yellow-eyed penguins with different reproductive success rates.

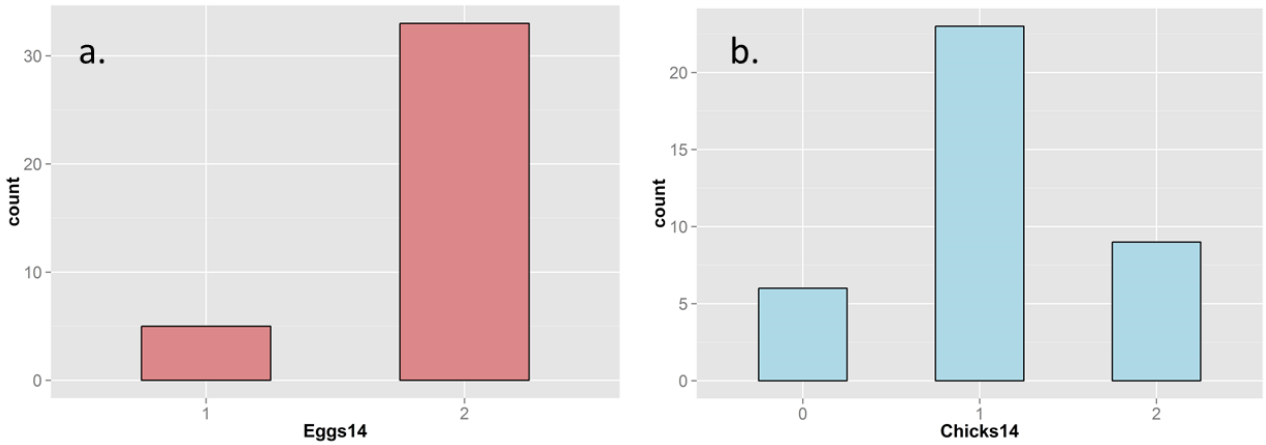

Figure 2.5: Bar graphs of the distribution of the short-term reproductive success as applied to yellow-eyed penguins at Boulder Beach. a) Red bars depict the number of yellow-eyed penguins with the number of eggs laid (1 egg, *n*=5; or 2 eggs, *n*=33). b) Blue bars depict the number of yellow-eyed penguins with the number of chicks fledged (0 chicks, *n*=6; 1 chick, *n*=23; or 2 chicks, *n*=9).

Reproductive Success Index (Long-term Productivity)

The reproductive index ranged between 0.40 and 1.30 with the median at 0.98 (n = 38) for yellow-eyed penguins at Boulder Beach. As seen in Fig. 2.6, the distribution was skewed: high quality birds (referred to as "superior", n = 19) defines as those above the median and average quality birds (referred to as "average"; n = 19) were below.

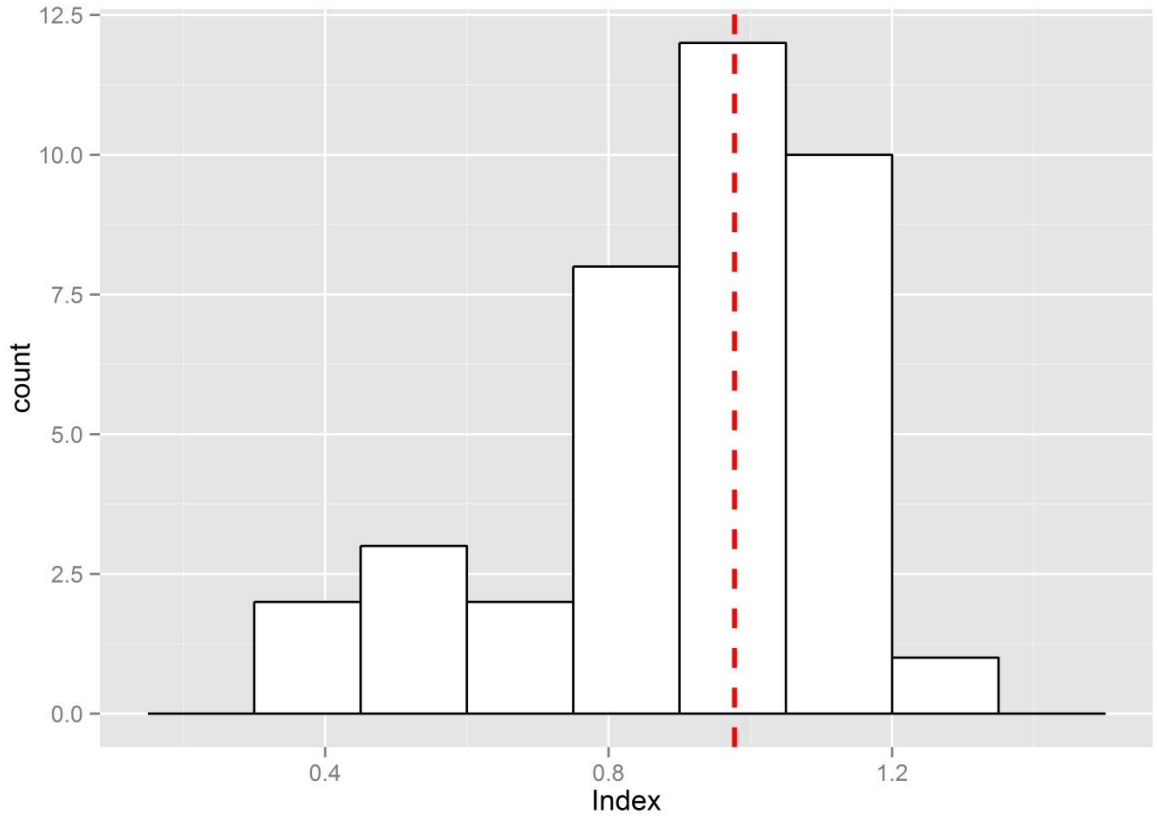

Figure 2.6: The distribution of the long-term reproductive success index as applied to yellow-eyed penguins at Boulder Beach. The median is depicted as the red dashed line.

Oxidative Stress Levels

Oxidative stress levels ranged between 6.73 and 11.42 with the median at 7.72- for yellow-eyed penguins (n = 38) at Boulder Beach. As seen in Fig. 2.7, the distribution was skewed to the right.

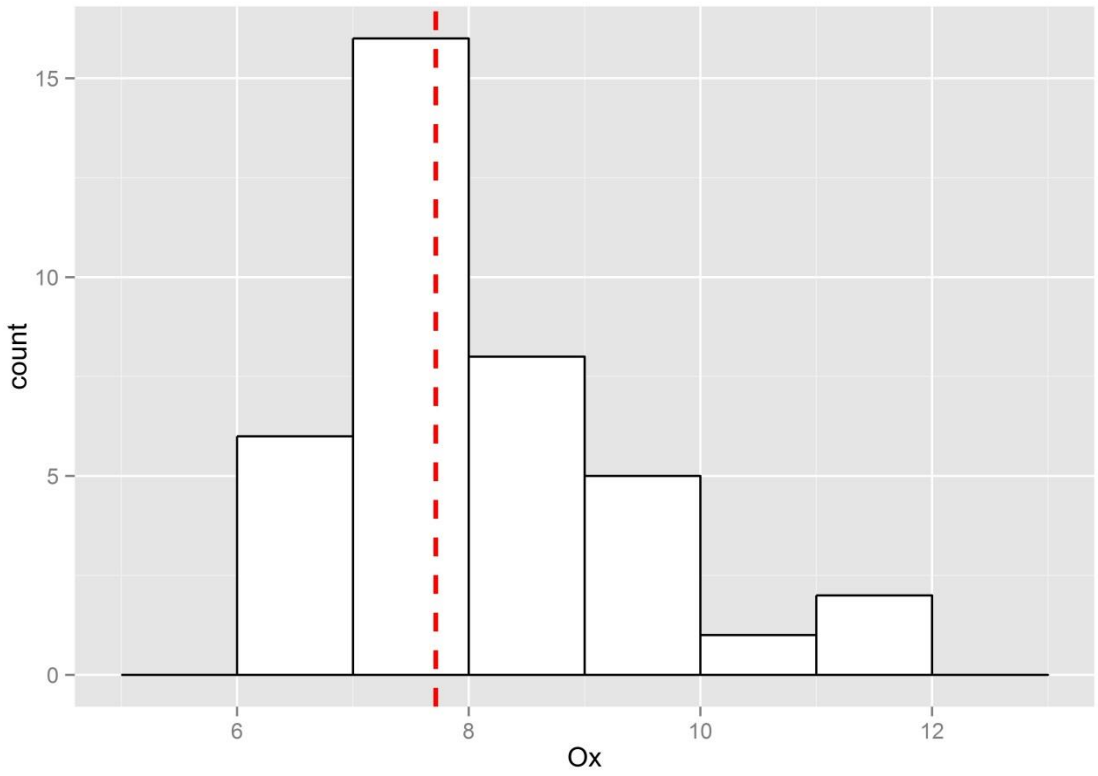

Figure 2.7: The distribution of the oxidative stress ratio as calculated in yellow-eyed penguins at Boulder Beach. The median is depicted as the red dashed line.

Short-term Reproductive Success and Oxidative Stress

While oxidative stress levels appeared higher in birds which produced more eggs (β = 0.020, t = 0.660, p = 0.514), and lower for birds which fledged more chicks (β = -0.077, t = -0.797, p = 0.431, Fig. 2.8), these differences were not significant. The opposite trend was true for levels of oxidants, in that birds that produced more eggs for that breeding season had lower oxidants (β = -0.188, t = -1.507, p = 0.141), and more chicks fledged meant a higher level of oxidants (β = 0.194, t = 0.491, p = 0.627, Fig. 2.8), but these differences were not significant.

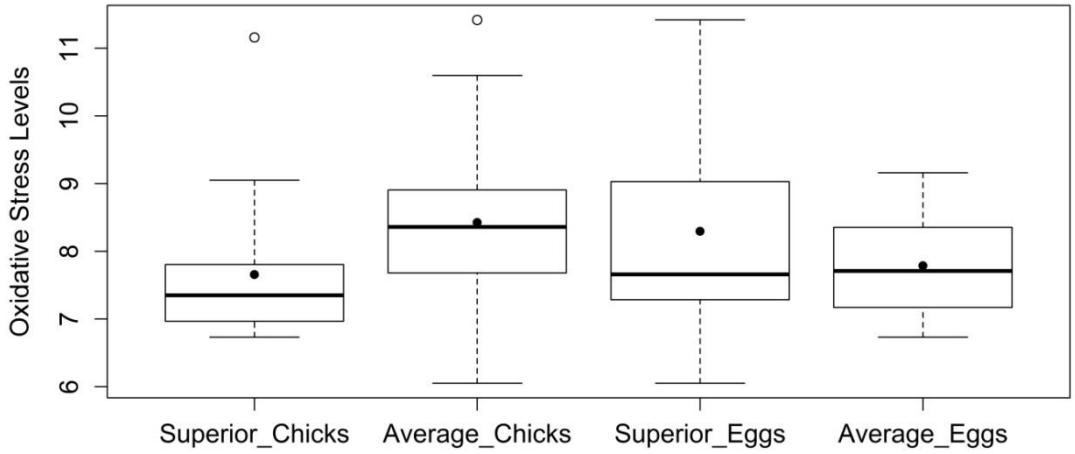

Figure 2.8: Boxplots showing the median, upper, and lower quartile values for oxidative stress levels between breeding groups based on both the number of chicks fledged (Superior_Chicks = 19; Average_Chicks = 19) and the number of eggs laid (Superior_Eggs = 19; Average_Eggs = 19) in yellow-eyed penguins. The solid black dots indicate the mean oxidative stress level for each group.

Long-term Reproductive Success and Oxidative Stress

Oxidative stress in superior and average breeders was significantly different when analysed against the long-term productivity index. Highly productive individuals exhibited higher oxidative stress levels ($\beta = 0.077$, $t = 2.109$, $p = 0.042$; Fig. 2.9) as compared to less productive birds.

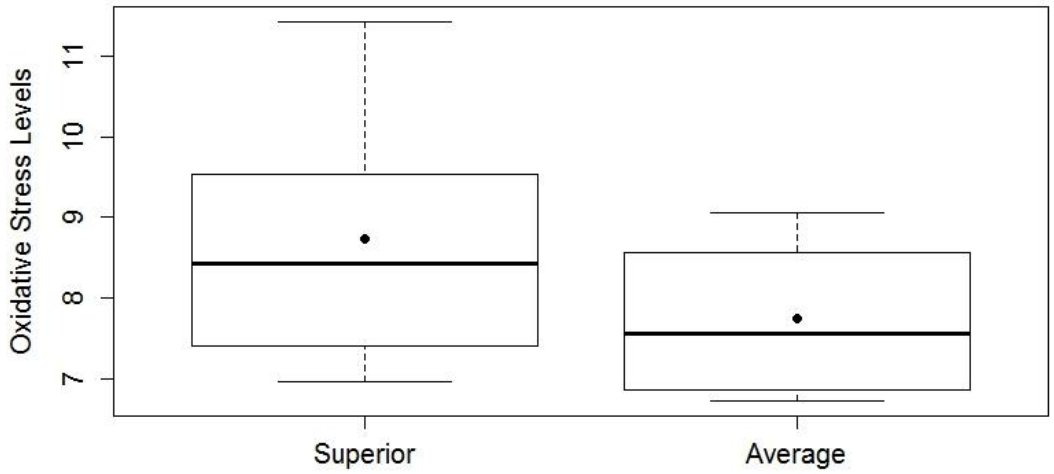

Figure 2.9: Boxplots showing the median, upper, and lower quartile values for oxidative stress levels between breeding groups (Superior and Average) based on their long-term reproductive success index. The solid black dots indicate the mean oxidative stress for each group.

Short-term Reproductive Success and Leucocyte Counts

This study has for the first time recorded leucocyte counts for yellow-eyed penguins. It essentially provides the baseline for the species during the incubation phase of reproduction. Mean leucocyte counts for the 41 birds sampled were: heterophils (43.5), eosinophils (10.0), basophils (8.9), lymphocytes (32.5), and monocytes (4.9). Based on the standard deviation (Fig. 2.10), lymphocytes were the most variable of the five WBC types. Overall, heterophils are the most plentiful, followed by lymphocytes, eosinophils, basophils, and monocytes.

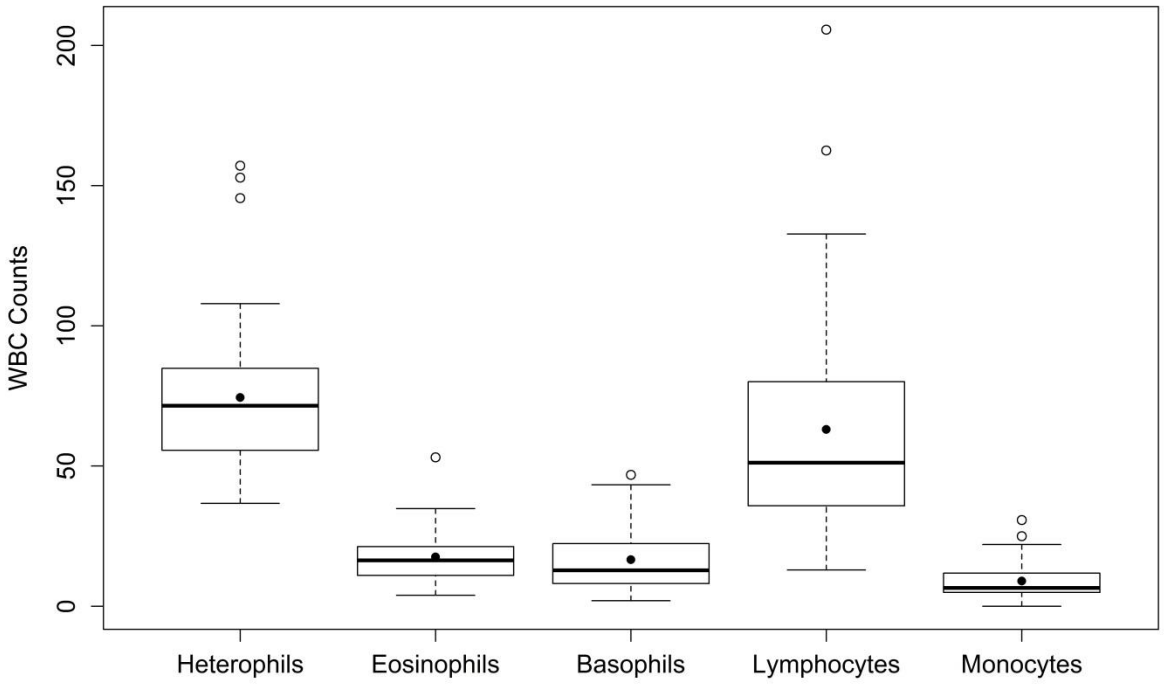

Figure 2.10: The percentage concentration for the five leucocyte cell types: heterophils, eosinophils, basophils, lymphocytes, and monocytes with SD error bars (n = 41). The solid black dots indicate the means for each blood type. The hollow circles represent outliers.

The H/L ratio was lower in adults that produced more eggs (β = -0.083, t = -2.113, p = 0.042) but not for the number of chicks fledged (β = -0.077, t = -0.602, p = 0.551). This result indicates that the H/L ratio is a reliable indicator of individual quality with respect to egg production.

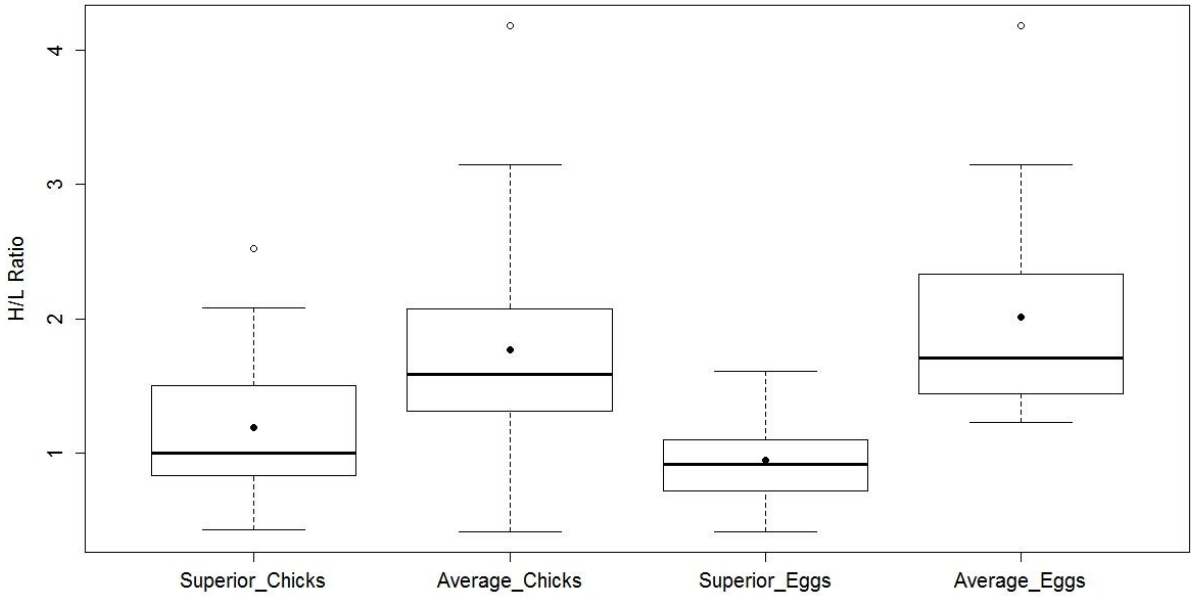

Figure 2.11: Boxplots from the two models, comparing H/L ratio against chicks fledged and eggs laid, respectively, for that breeding season. The solid black dots denote the mean H/L ratio for each group.

The two other leucocyte parameters analysed were the eosinophil count (Fig. 2.11) and total white blood cell (TWBC) count (Fig. 2.12). When analysed against egg production for the season, neither produced a significant trend (eosinophil count: $\beta = 0.006$, $t = 0.862$, $p = 0.394$; TWBC: $\beta = -8.99e\text{-}06$, $t = -0.933$, $p = 0.357$). These parameters were non-significant when analysed against chicks fledged as well (eosinophil count: $\beta = 0.031$, $t = 1.448$, $p = 0.157$; TWBC: $\beta = 2.530e\text{-}05$, $t = 0.846$, $p = 0.403$). Neither eosinophil count nor TWBC counts appear to be indicative of yellow-eyed penguin reproductive performance in the season of sampling.

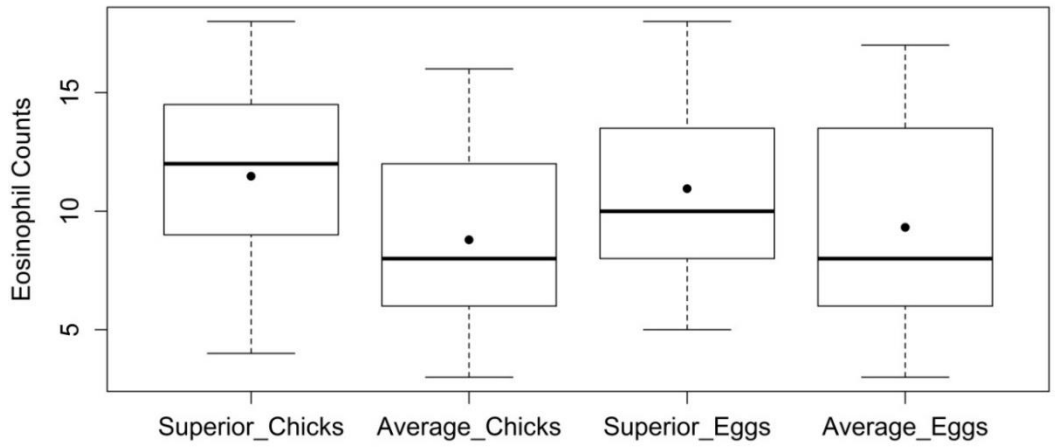

Figure 2.12: Boxplots showing the median, upper, and lower quartile values for the eosinophil counts between breeding groups of yellow-eyed penguins at Boulder Beach based on both the number of chicks fledged (Superior_Chicks = 19; Average_Chicks = 19) and the number of eggs laid (Superior_Eggs = 19; Average_Eggs = 19) in yellow-eyed penguins. The solid black dots indicate the mean eosinophil count for each group.

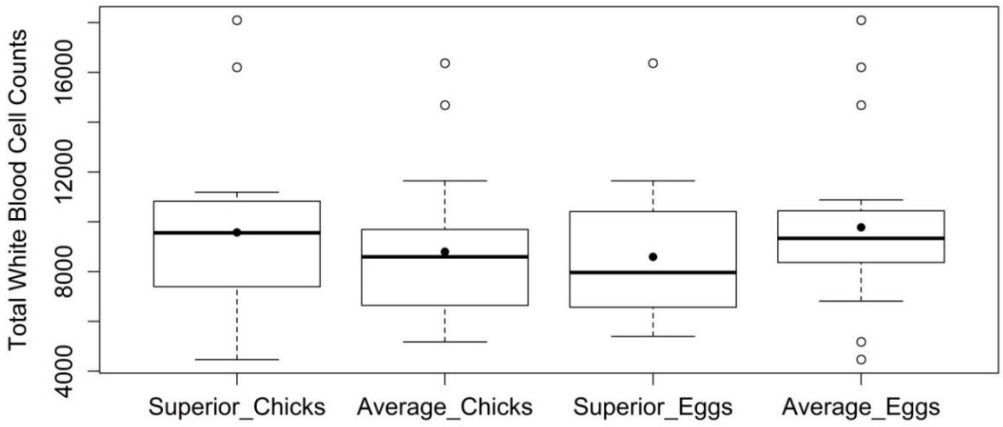

Figure 2.13: Boxplots showing the median, upper, and lower quartile values for the total white blood cell (TWBC) counts between breeding groups based on both the number of chicks fledged (Superior chicks = 19; Average chicks = 19) and the number of eggs laid (Superior eggs = 19; Average eggs = 19) in yellow-eyed penguins. The solid black dots indicate the mean TWBC for each group.

Ornamentation – Spectrometry Data

Yellowness of the post-ocular stripe ranged between 0.218 and 0.281 with the mean yellowness at 0.251 for yellow-eyed penguins (n = 38) at Boulder Beach. As seen in Fig. 2.14, yellowness was normally distributed.

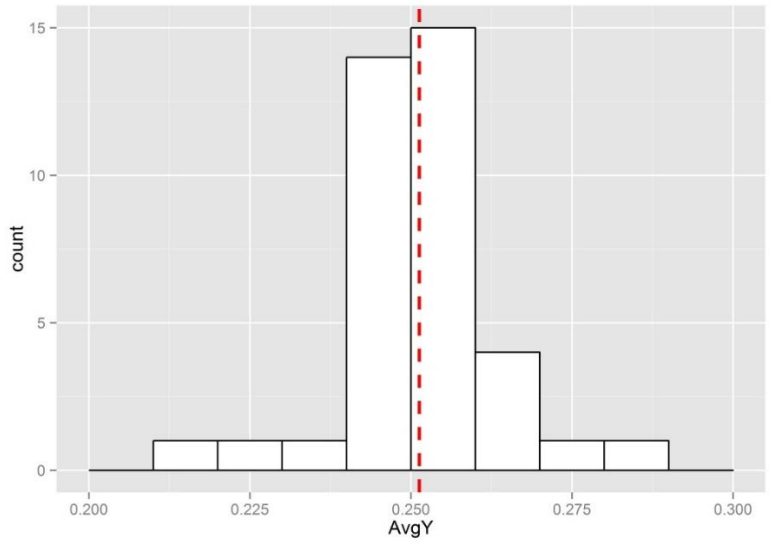

Figure 2.14: The distribution of the post-ocular yellowness as calculated in yellow-eyed penguins at Boulder Beach. The mean yellowness is depicted as the red dashed line.

Post-ocular brightness ranged between 11.77 and 60.93 with the median at 28.46 for yellow-eyed penguins (*n* = 38). As seen in Fig. 2.15, the brightness distribution was skewed to the right.

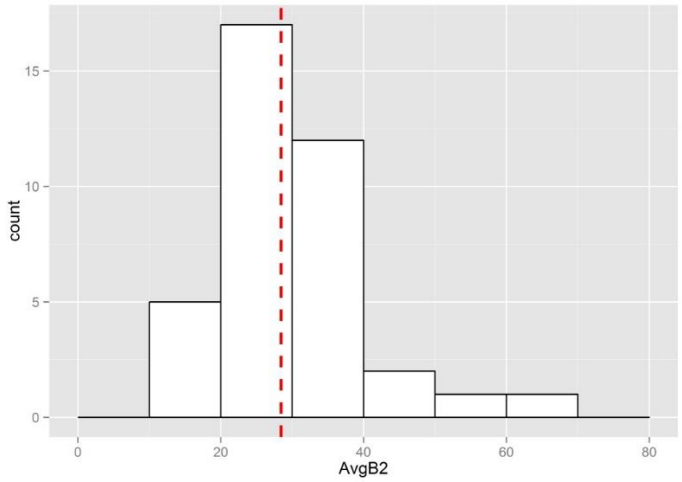

Figure 2.15: The distribution of the post-ocular brightness as calculated in yellow-eyed penguins at Boulder Beach. The median for feather brightness is depicted as the red dashed line.

Ornamentation – Morphological Data

Post-ocular stripe width ranged between 7.405mm and 13.321 mm with the median was at 9.869mm for yellow-eyed penguins (*n* = 38). As seen in Fig. 2.16, the stripe width distribution was slightly skewed to the left.

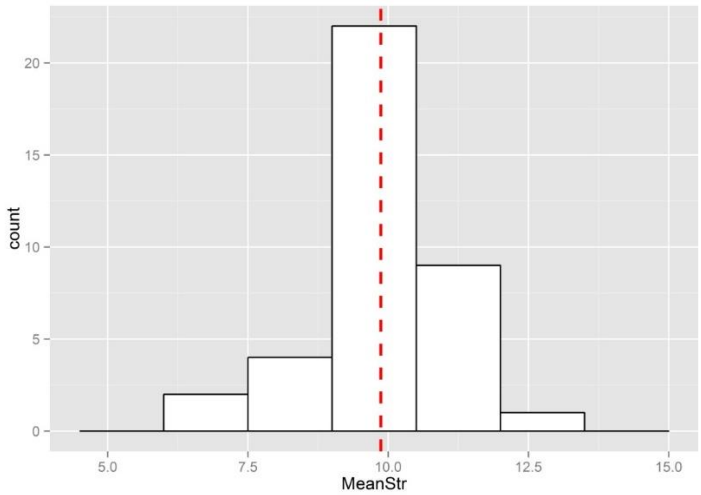

Figure 2.16: The distribution of the post-ocular stripe width as calculated in yellow-eyed penguins at Boulder Beach. The median is depicted as the red dashed line.

Post-ocular ring width ranged between 2.05mm and 4.11mm with the median was at 2.88mm for yellow-eyed penguins (n = 38). As seen in Fig. 2.17, the distribution of ring width was skewed to the right.

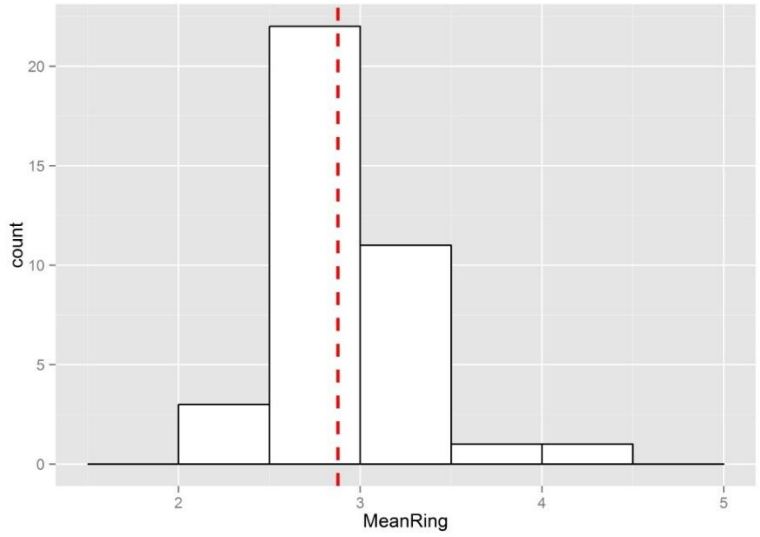

Figure 2.17: The distribution of the post-ocular ring width as calculated in yellow-eyed penguins at Boulder Beach. The median is depicted as the red dashed line.

Long-term Reproductive Success and Ornamentation

I analysed the variables related to ornamentation using the lifetime productivity index. Average brightness had a weak effect, which was non-significant ($\beta = 0.001$, $t = 0.243$, $p = 0.809$). The average yellowness of the post-ocular stripe appeared to have quite a large effect and was positively correlated with productivity ($\beta = 3.358$, $t = 0.874$, $p = 0.388$), however, this was not significant (Fig. 2.18). It is likely that a larger sample size would lead to statistical significance for feather yellowness.

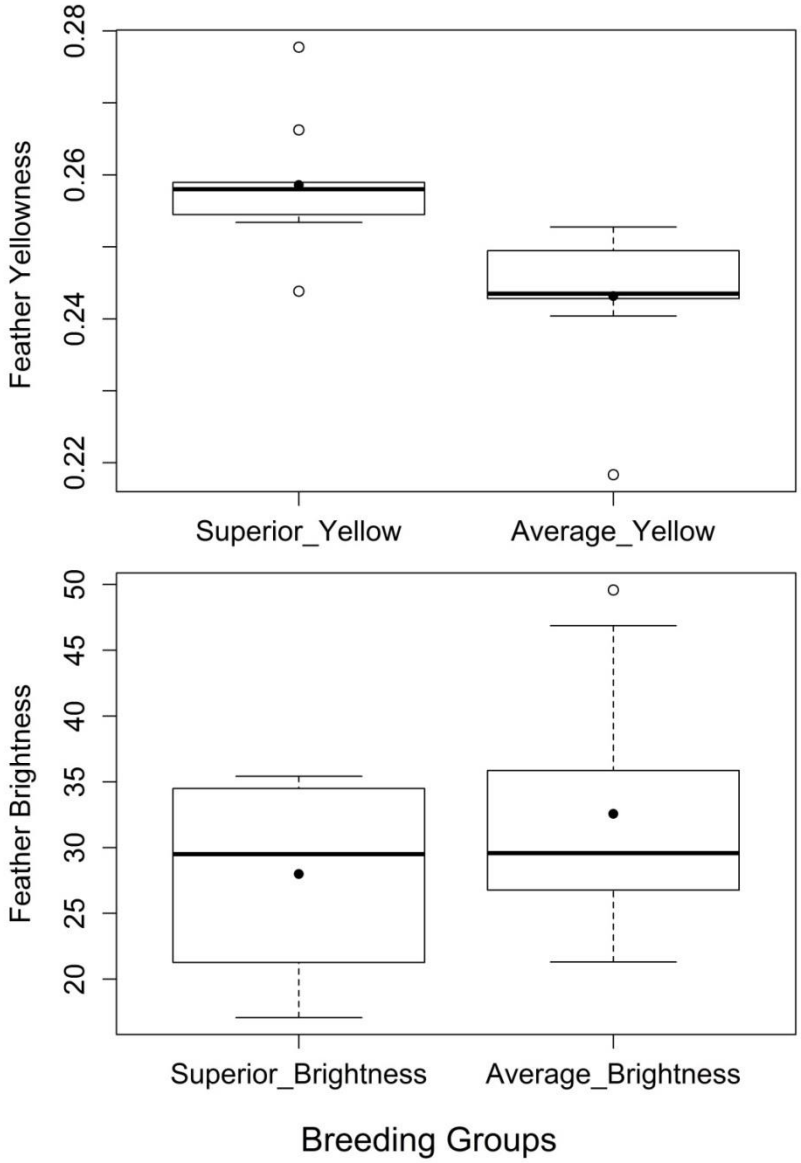

Figure 2.18: Boxplots showing the median, upper, and lower quartile values for the feather yellowness and brightness between breeding groups based on their long-term productivity index. The solid black dots indicate the mean value within each group.

Neither the mean post-ocular stripe width (β = -0.027, t = -0.555, p = 0.582) nor the mean eye ring width (β = 0.233, t = 1.506, p = 0.141) were significantly correlated with the long-term productivity index (Fig. 2.19).

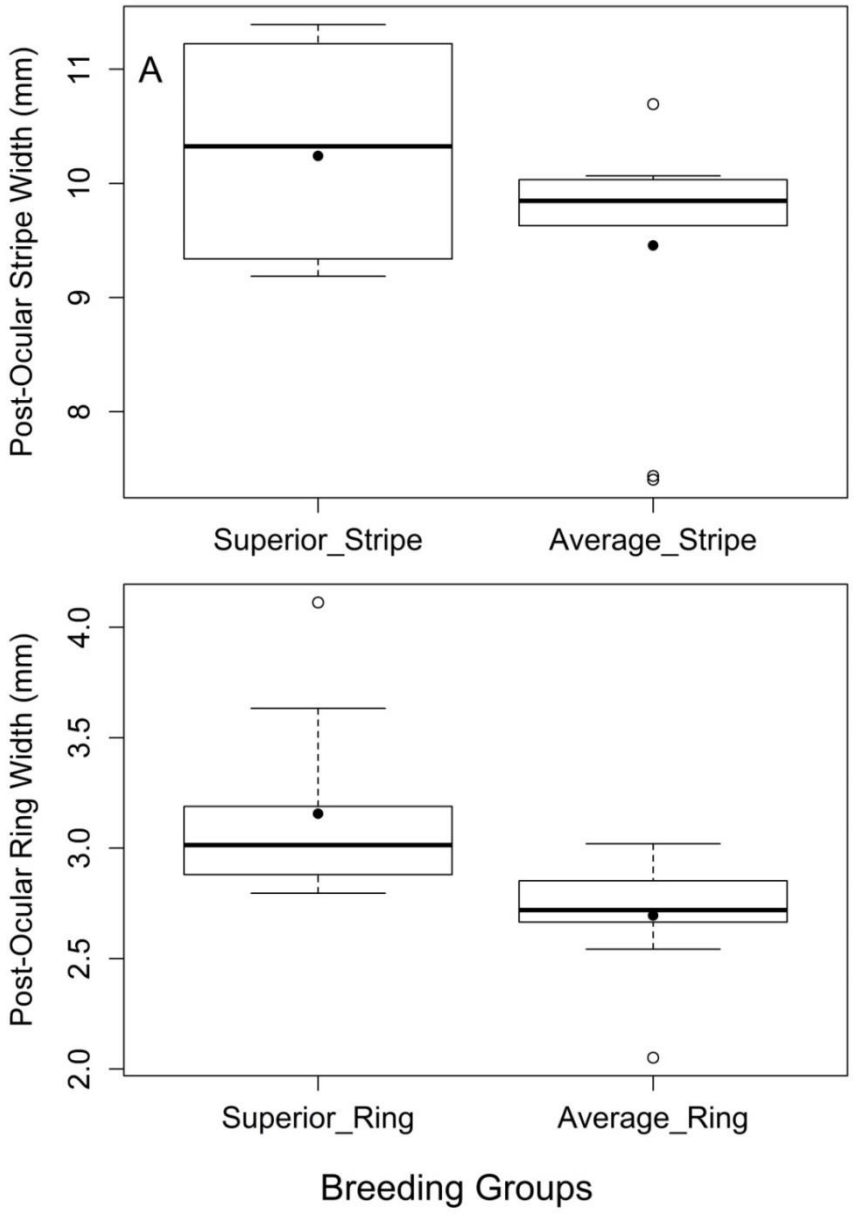

Figure 2.19: Boxplots showing the median, upper, and lower quartile values for the post-ocular stripe and ring width between breeding groups based their long-term productivity index. The solid black dots indicate the mean value within each group.

2.4 Discussion

I used breeding data accrued over the last 30 years by DOC, along with data from blood, feathers, and photographs collected during the yellow-eyed penguin incubation period to identify potentially reliable indicators which can be used to identify high quality breeders in the population. I considered physiological indicators related to immunocompetence (oxidative stress and leucocyte counts) and external traits (plumage colour and ornament size). It is important to note that yellow-eyed penguins on their first breeding attempt usually lay one egg, which is likely to be infertile, as opposed to experienced breeders, which lay two fertile eggs. First-time breeders are likely to be more stressed. The sampled individuals consisted of several young birds and their age was controlled for in the index.

Breeders identified as more productive than others experienced more oxidative stress than average breeders. These individuals appear to be producing more offspring and probably allocate their antioxidants to care for their young in the form of energy expenditure. This could be a trade-off between productivity and immunocompetence. That superior breeders consistently produce more offspring without any apparent negative impacts, suggests that these high quality individuals are able to accumulate and manage the antioxidants more efficiently. It is also likely that because they expend more energy than average breeders whilst tending to their chicks, they experience more physiological stress overall. This appears to be consistent with other birds (Guindre-Parker et al. 2013; Deeming & Reynolds 2015).

The average breeders appear to be retaining as much antioxidant as possible to counter the oxidants in their bodies, and produce fewer offspring. This can be attributed to a trade-off between saving resources for immune responses and being more productive (Guindre-Parker et al. 2013; García-Tarrasón et al. 2014). Average breeding birds cannot afford to use the antioxidants they have accumulated on incubation or chick rearing (Deeming & Reynolds 2015) because they will require antioxidants to overcome the levels of oxidants which attack them. Average breeders are potentially choosing self-preservation over reproduction, which is indicative of their lack of fitness. These individuals encounter far more stressors and therefore conserve resources for defense (García-Tarrasón et al. 2014). This finding goes against the prediction that superior yellow-eyed penguins experience less oxidative stress. The physiological processes involved in oxidative stress and reproduction are far more complex than suspected. Considering this result is based on a single collection during the incubation stage of reproduction, it is possible that superior breeders are better

equipped prior to incubation and can therefore trade-off oxidative stress for more offspring. These high quality birds probably accumulate much higher levels of antioxidants in preparation for reproduction, enabling them to produce more eggs which are also healthier than the average penguins' eggs (Beaulieu et al. 2011; Guindre-Parker et al. 2013; Cram et al. 2015). These presumptions can easily be verified by collecting sampling over the course of the breeding season to observe the trends in antioxidant levels.

The key finding with respect to leucocyte count analyses was that birds which laid more eggs were less stressed at the time of sampling. This was deduced from the lower H/L ratios of those individuals when compared to birds with fewer eggs. The other parameters (TWBC counts and eosinophil counts) proved to be no different in the superior and average breeders, both in terms of eggs laid and chicks fledged during the breeding season in which sampling took place. Interestingly, there was no difference in H/L ratios between the two breeding groups when divided according to number of chicks fledged. This could be because high quality yellow-eyed penguins have the ability to normalise (attain homeostasis) their white blood cell counts while the eggs are on the nest. However, once the chicks have hatched, there are a number of additional stressors which affect the birds, and there is no clear difference between the H/L ratios. During incubation, male and female yellow-eyed penguins take turns to incubate the eggs, and these results suggest that more productive and higher quality breeders are less stressed at this time. Either due to inexperience or the physical stress of being a young breeder, some yellow-eyed penguins produce less, possibly to alleviate the workload. Post-hatch nests can be affected by a number of factors, but there is a similar parental workload, which could be the reason why there is no clear difference in H/L ratios when analysed against fledging success.

Ornamentation parameters were the least useful as indicators of quality since neither the colouration nor the morphometric data provided indicative trends with productivity. I predicted that superior breeders would exhibit external markers of superiority, with brighter post-ocular feathers or a wider post-ocular stripe, however, there were no differences in plumage ornaments between the two groups. The mean yellowness of the post-ocular feathers seemed positively correlated with productivity, albeit not significantly. The strong effect could suggest a larger sample size might be worth studying. This approach to identifying differences might have a genetic basis and analysing phenotypic traits, such as ornaments, along with genotypes could provide more comprehensive results. Quantitative Trait Loci (QTL) analyses of the colour, brightness, and size of the yellow-eyed penguin's post-ocular

stripe linked with a genetic component would be the most reliable approach (Wright et al. 2008).

In this chapter my investigation of the use of potential indicators to identify superior breeders from average ones produced three key points: 1) oxidative stress is higher in superior breeders, 2) oxidant levels are lower in superior breeders, and 3) individuals which lay more eggs have lower H/L ratios. Given the relatively small sample size, researching stress levels from more than one yellow-eyed penguin population will be the next step. Each population is likely to be impacted by different stressors, have a different diet, and varying levels of productivity. However, the known disparities in long-term reproductive success between individuals in the Boulder Beach population and the reasonably strong sampling effort given the poor breeding season makes this study a good starting point. My results show that high quality breeders can be discriminated from average quality breeders by using stress measures. Estimating stress levels, both short and long-term, of yellow-eyed penguins can help alter current management strategies by prioritising superior breeders over average ones.

Chapter 3: Does inbreeding influence the reproductive success of yellow-eyed penguins?

Blood being drawn from the flipper of a yellow-eyed penguin

3.1 Introduction

Inbreeding depression lowers reproductive success in a number of ways which imposes a greater threat on endangered species (Keller & Waller 2002; Hoeck et al. 2015). Inbred bird populations suffer from increased egg infertility, low hatching and fledging success rates, and ultimately depletion of the genetic diversity (Jamieson & Ryan 2000; Jamieson et al. 2003; Jamieson et al. 2008; White et al. 2015). This makes identifying and quantifying inbreeding, as well as assessing the impact inbreeding has on reproductive performance, essential for avian conservation management (Hoeck et al. 2015; White et al. 2015). Inbreeding depression is a major threat to New Zealand's endemic birds (Jamieson & Ryan 2000; Jamieson et al. 2009; White et al. 2015) because their populations are small, distributions are restricted, and most species have not developed inbreeding avoidance strategies (Jamieson et al. 2009). Some of these protected species continue to experience poor reproductive success (high hatching failure), as their populations go through genetic bottlenecks (Briskie & Mackintosh 2004). With species moving quickly from 'endangered' and 'critically endangered' to extinction, understanding inbreeding depression and genetic diversity is extremely important (Frankham 2005).

More distantly related breeding birds tend to have greater reproductive success (Merilä & Sheldon 2000; Sardell et al. 2014), but inbreeding detrimentally impacts breeding productivity. For example in great reed warblers (*Acrocephalus arundinaceus*; Bensch et al. 1994) and blue tits (*Cyanistes caeruleus*; Kempenaers et al. 1996), genetically similar breeding pairs, including non-kin pairs, show greater hatching failure. This reduction in reproductive success as a result of inbreeding is attributed to increased homozygosity and the expression of recessive alleles (van Noordwijk & Scharloo 1981; Amos et al. 2001). The offspring of related parents are not only weak, but the fitness of future generations can be compromised. In the endangered red-cockaded woodpecker (*Leuconotopicus borealis*) and Hawaiian crow (*Corvus hawaiiensis*), high inbreeding levels of chicks results in severely reduced survival rates of future generations (Daniels & Walters 2000; Hoeck et al. 2015). Although some species have evolved inbreeding avoidance adaptations (e.g. splendid fairy wrens (*Malurus splendens*), where the offspring are sired by the resident male, a high percentage of breeding pairs are related and to avoid inbreeding carry out extra-pair copulation whereby inbreeding is reduced; Blouin & Blouin 1988; Pusey & Wolf 1996). These are typically uncommon. Some species have adjusted to pair with more distantly related individuals (Amos et al. 2001).

Variations in individual quality in a population are reflected by differences in the reproductive output between breeding pairs. Birds possess varying degrees of fitness which is expressed in terms of social dominance, ability to find a healthy mate, or an affinity to high quality territory (Jouventin et al. 2008; van de Crommenacker et al. 2011b; Cram et al. 2015). There is evidence that this yellow-eyed penguin population exhibits disproportionate reproductive success rates, where only 5.4% of breeding birds successfully fledge chicks which go on to breed (Stein, 2012, unpublished thesis). Given that the population appears to contain a set of superior breeders, there could be specific lineages which tend to be more productive and form this 5.4%. However, since high parental similarity between breeding individuals influences reproductive fitness (Amos et al. 2001), it is likely that these superior breeders have adapted to pair with more distantly related mates. Internal relatedness levels can be used to better explain how the genetic makeup of an individual influences its immunological fitness and reproductive fitness. Yellow-eyed penguins are endangered and their numbers are declining (BirdLife International 2012). This endemic species is subjected to terrestrial and marine threats (McKinlay 2001; Alley et al. 2004; Lalas et al. 2007). Now, facing an increasing number of stressful events (Ellenberg & Mattern 2012), an added presence of inbreeding could be devastating for this endemic species.

This variation in individual productivity can also be influenced by the levels of relatedness of breeding pairs (Amos et al. 2001). This highly productive group of yellow-eyed penguins, which make up a small proportion of the population (Stein, 2012, unpublished thesis), might be associated with relatedness. It is likely that these superior breeding pairs are the most genetically dissimilar from each other because, not only do their offspring possess higher rates of survival, they also go on to breed successfully (Stein, 2012, unpublished thesis). These findings suggest that the high quality individuals overcome the common weaknesses associated with related breeding pairs and their offspring: i.e. hatching failure, low fledging success, low offspring survival rates, and low offspring fitness (Bensch et al. 1994; Briskie & Mackintosh 2004; Hoeck et al. 2015; White et al. 2015).

Individual fitness in birds can also be estimated using oxidative stress levels (Beaulieu et al. 2011; Cram et al. 2015; Deeming & Reynolds 2015) and leucocyte counts (Moreno et al. 2002; Davis et al. 2008; Quillfeldt et al. 2008). These indicators of individual fitness could be associated with the genetic makeup of the individual. These two measures of immunocompetence using haematological samples are associated with reproductive performance (Moreno et al. 2002; Constantini et al. 2014) and can be putative indicators of

future productivity. Individual quality, related to reproduction, can be reflected by these parameters which also provide insight into variations in social status (Cram et al. 2015). The differential breeding success of yellow-eyed penguins could be a result of individual quality and fitness based on immunocompetence. The among-individual variations in fitness levels could be connected to genetic diversity (Wilson & Nussey 2010) which would subsequently influence oxidative stress levels and leucocyte counts.

Plumage colour and patterns have been suggested as honest signals of superior individual quality for avian mate choice (Zahavi 1975; Hamilton & Zuk 1982). Feathers can be an external indicator of fitness with a genetic basis. The rate of feather growth in Siberian jays (*Perisoreus infaustus*), for example, differs between individuals and is inherited (Gienapp & Merilä 2010). According to Hamilton & Zuk (1982), birds choose the healthiest partner based on the brightness of their ornamentation which is inherited, and hence genetic. Feather ornaments signal the health status (Pincemy et al. 2009), breeding quality (McGraw et al. 2009), and parental quality (Massaro et al. 2003). Although they appear monomorphic, yellow-eyed penguins possess a unique yellow post-ocular stripe which could potentially be one of the cues used during sexual selection, and which could be influenced by relatedness. Duller feathers could be indicative of an inbred individual's lower fitness levels.

The main aim of this chapter is to determine whether indicators of individual quality are associated with inbreeding levels of the individual. Here, I use microsatellite data to establish the internal relatedness of the population, compare the relatedness of sampled pairs with their status in the DOC database, and correlate relatedness with parameters describing reproductive success. Indicators such as oxidative stress levels, leucocyte counts, and plumage colouration (refer Chapter 2 for details) are presumed to be useful for distinguishing high quality breeders from low quality ones. I test the influence internal relatedness (IR) has on these variables (used in a similar capacity in White et al. 2015). Finally, using known relatives in the sampled population, I validate social relationships recorded in the DOC database. Extra-pair copulation has been observed in yellow-eyed penguins (Richdale 1957); however, extra-pair paternity is unquantified and could bias productivity estimates (Reid et al. 2014). Verifying these social relationships will provide information on the likelihood of extra-pair copulation in the yellow-eyed penguin and increase the accuracy of estimating productivity.

3.2 Methods

3.2.1 Indicators of Immunocompetence and Monitoring Data

I collected blood samples from yellow-eyed penguins (n =41) at Boulder Beach, Dunedin, New Zealand, in 2014 and accessed each individual's life history data (e.g. the number of eggs laid, the number of chicks fledged, individual age, and the number of breeding seasons) from the DOC database. Sex of each yellow-eyed penguin was determined using morphometric measurements of the head and feet with 93% accuracy (Setiawan et al. 2004). The blood samples were used to assess variations in individual quality based on immunocompetence and identify indicators of fitness levels (Constantini 2008; Davis et al. 2008). I calculated oxidative stress levels and performed leucocyte counts using the blood samples, and measured plumage patterns and ornament colouration using photographs and feather samples, respectively (refer Chapter 2 for details). The internal relatedness levels were analysed against the fitness and plumage parameters to determine whether they play a role in the manner these factors impact on reproductive success. In order to minimise ambiguity, I only used oxidative stress values and feather yellowness, since they were tested with long-term reproductive success. Although leucocyte counts are a reliable indicator of fitness, it is highly variable over time and is more accurate for short-term or immediate data analysis (Davis et al. 2008). Therefore, I finally used three proxies for reproductive success differences: oxidative stress, reactive oxygen metabolites, and post-ocular yellowness. Oxidative stress accumulates over the lifetime of a bird, feather characteristics are inherited, and therefore, both these traits can be compared to long-term breeding data where age is controlled for.

To account for the large number of young birds in the dataset, I created a standardised long-term productivity index controlling for age (refer Chapter 2 for details). First time breeders tend to be unsuccessful, mostly laying one egg which is often infertile; therefore, controlling for age and breeding experience is important. Yellow-eyed penguins, above and below the median of the long-term productivity index, were grouped as superior and average breeders, respectively. In addition to reproductive performance, I identified known relatives (n = 8) from the sampled yellow-eyed penguins. A byproduct of using microsatellite analyses to estimate genetic relatedness would be to confirm the accuracy of recorded relationships in the DOC database.

3.2.2 DNA Extraction and Sequencing

DNA was extracted and purified from blood samples adopting the 5% Chelex protocol (Walsh et al. 1991). Eleven existing polymorphic microsatellite loci (Boessenkool et al. 2008; Table 3.1) were used to genotype the 41 samples. I used a Qiagen Type-it microsatellite PCR kit in a polymerase chain reaction (PCR) to amplify the 11 microsatellite loci using a three primer methodology to label PCR fragments using the fluorescent dye labels (fluorophores) 6-FAM, VIC, NED, and PET (Schuelke 2000).

Each PCR reaction contained c. 15ng of template DNA (dried), 1 µL of Qiagen Type-it microsatellite PCR mix, 0.08 µM of each end-labelled locus-specific forward primer, 0.32 µM of each locus-specific reverse primer, and c. 0.32 µM of fluorescent-labelled M13 tags in each 2 µL PCR reaction. Thermal cycling consisted of 95°C for 15 minutes (denaturation step), 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, and finally 72°C for 40 minutes (extension step). Two multiplexes were performed post-PCR (Table 1). After PCR amplification, I added 25 µL of MilliQ H_2O to the PCR product and then used 10 µL of each PCR product (dye-labelled) in the multiplexes. Prior to genotyping on an Applied Biosystems 3730XL DNA Analyser (Applied Biosystems Inc.), GeneScan™LIZ®500 size marker and Hi-Di formamide was added to 2 µL of the pooled multiplex mix.

Table 3.1: Details of the polymorphic microsatellites loci used on adult yellow-eyed penguins from the Boulder Beach Complex on the Otago Peninsula, New Zealand (*n* = 41).

Locus	Primers (5' → 3')	Dye Label	A	H_o	H_e
Multiplex 1					
Man03	F: GCCTGAGAGACCCGTGTG	6-FAM	1	0.000	0.000
	R: CTCCCCAGTTGCCTCCTG				
Man08	F: CCTGTCTTCTATTAAACCCTC	NED	3	0.135	0.151
	R: CCACATTTGCACCAGTTG				
Man21	F: TACTGGTAGCATGGGGTG	6-FAM	2	0.306	0.361
	R: CACTGAAAGATGACAACGG				
Man27	F: GATCCTGAGAAGAGAGACAG	VIC	1	0.000	0.000
	R: GGCTGTTCATTTTGTCAC				
Man54	F: GTTTCCTATTTCAGTCTGG	NED	2	0.435	0.441
	R: TTGTGCTTTCAGTGTTGG				
Multiplex 2					
Man13	F: AACACATTTGACAGCCTG	VIC	3	0.553	0.579
	R: GTTATTCCAACACCAAGC				
Man22	F: TTTCCACTTGAGAGTGTATG	VIC	3	0.054	0.104
	R: CAAACAGAAAGGATTTGTG				
Man39	F: GATCTTTCCAGAGACCTC	6-FAM	4	0.429	0.622
	R: ACCCTGTGAGTATGAACC				
Man50	F: CCTCCACTTAGTTTTGCC	VIC	2	0.216	0.193
	R: TGGAAGCATAACCATAGC				
Man51	F: CAGAGATATTGACTCTGACATC	NED	6	0.838	0.654
	R: CCTATCACACAGAAACACTG				
Man55	F: TTGAACTAGCAAGCAGTGTAG	6-FAM	2	0.487	0.494
	R: AAGGGCATTTCCATTCTG				

A = Total number of alleles at each locus, H_o = Observed Heterozygosity, H_e = Expected Heterozygosity.

3.2.3 Data Processing and Analysis

The post-genotyping results were analysed using Geneious 6.0.6 (Kearse et al. 2012) and the alleles for each individual were scored. The raw allele scores were then manually binned. I used GenAlEx 6.5 (Peakall & Smouse 2012) to convert the data to the formats required by other software. The binned data were exported and analysed in GENEPOP 4.5 (Rousset 2008). I determined the presence of null alleles and determined whether the loci displayed Hardy-Weinberg proportions. A genotypic contingency table was generated to examine the interactions between the loci and a Bonferroni correction ($p = 0.05$/number of tests) was applied to account for the number of tests.

The Rhh package (Alho et al. 2010) in R was used to calculate the internal relatedness (IR) and homozygosity by loci (HL; Aparicio et al. 2006) for each individual. For the verification of known relationships (e.g. full siblings, parent-offspring, etc.), I used COANCESTRY (Wang 2011) to calculate the Wang relatedness estimates (Wang 2002) and Queller & Goodnight relatedness estimates (Queller & Goodnight 1989) for every pairwise combination. These two relatedness estimators were then plotted against each other to compare the values for known relatives (first and second order relatives from the DOC database) and unknown relatives. I predicted that the relatedness estimates for the known relatives would be highly correlated while unknown pairs would have varying degrees of correlation, since this approach is fairly accurate (Robinson et al. 2013).

I used generalised linear mixed models (GLMM), similar to White et al. (2015), to determine whether IR affected the proxies of reproductive success of yellow-eyed penguins. The reproductive success index was the response variable with oxidative stress, reactive oxygen metabolites (dROM), and average yellowness of the eye-stripe as covariates. I chose these three variables because they represent both long-term physiological and external fitness indicators. I expected oxidative stress and dROM to be negatively correlated with the productivity index because theoretically, more productive individuals would be less stressed. I also predicted feather yellowness to be positively correlated with index since fitter birds of other species tend to have more colourful plumage ornaments (Thusius et al. 2001; Siefferman & Hill 2003; Dobson et al. 2008). I entered the sex of the samples as a random factor. I used the 'lmer' function from the 'lme4' package (Bates et al. 2012) and the package 'arm' (Gelman & Su 2014) in order to fit the model and standardise the global models in R (R Core Team, 2015). A subset of the global models was generated using the 'MuMIn'

package (Barton 2013). This subset was ranked according to the adjusted lowest AIC_c values (Burnham & Anderson 2003). The individual IR values later replaced the variable with the lowest effect in the first model, based on the subset. This was done to ensure that IR was compared with variables exhibiting the main effects and influences on the model (Gelman 2008; Grueber et al. 2011; White et al. 2015). The mean effect size and confidence intervals of inbreeding on the variables in the models with $\Delta AIC_c < 4$ were calculated and plotted (Grueber et al. 2011; White et al. 2015). Using $\Delta AIC_c < 4$ to generate the top models, is a reliable approach (Symonds & Moussalli 2011).

3.3 Results

3.3.1 Internal Relatedness

The 41 yellow-eyed penguins were screened at the eleven microsatellite loci. Two of the loci (Man03 and Man27) were monomorphic for the population and excluded from further analysis. Of the 41 birds, samples that failed to amplify at more than four loci (n = 3) were excluded from the dataset. The mean observed heterozygosity (H_o) and expected heterozygosity (H_e) were 0.339 and 0.326, respectively. Only one locus (Man51) deviated from Hardy-Weinberg proportions (HWE), but did not deviate following a standard Bonferroni correction.

The mean internal relatedness (IR) was -0.4244 for females (n = 16), and -0.3673 for males (n = 20) suggesting the females sampled are marginally less inbred than the males (Appendix Figure A1). IR and HL were highly correlated (R^2=0.79); only IR was used for all further analyses.

3.3.2 Effect of Relatedness on Reproductive Success

I ran two GLMMs, first, comparing the long-term reproductive success index to the three putative indicators (oxidative stress, dROM, and post-ocular yellowness). The top-ranked model included oxidative stress (β=0.189, SE=0.076, p=0.017) and dROM levels (β=-0.199, SE=0.083, p=0.0196), while yellowness had no significant effect (β=0.064, SE=0.065, p=0.3423) and did not explain variations in reproductive success (Table 3.2). The overall R^2 value for the model was 0.1954 (Figure 3.1).

The second model retained oxidative stress and dROM since they had a significant effect, and yellowness was replaced by IR. The top-ranked models once again only included oxidative stress (β=0.192, SE=0.076, p=0.016) and dROM levels (β=-0.200, SE=0.083, p=0.020). The model including internal relatedness explained marginally more variance than the first model; however, the overall R^2 value (0.2125; Figure 3.2) was still quite low. Additionally, IR did not affect the other two variables (β=0.024, SE=0.065, p=0.7264), suggesting that inbreeding levels are not linked to physiological stress in yellow-eyed penguins (Table 3.3).

Table 3.2: Top-ranked models when a) IR is excluded, and b) IR is included, against the long-term productivity index. The models were selected based on a $\Delta AIC_c<4$. This table shows the corrected difference between Akaike information criterion and the best model (ΔAIC_c), the degrees of freedom (df) and weight.

Model	ΔAIC_c	df	Weight
a) Oxidative Stress + dROM	0.00	5	0.55
Oxidative Stress + dROM + Yellowness	1.93	6	0.21
dROM	3.07	4	0.12
Null	3.10	3	0.12
b) Oxidative Stress + dROM	0.00	5	0.60
Oxidative Stress + dROM + IR	2.76	6	0.15
dROM	3.07	4	0.13
Null	3.10	3	0.13

Table 3.3: Standardised coefficients (β) of predictors against productivity from a) the model excluding IR, and b) the model including IR, with the conditional standard errors (SE) and relative importance (RI). Test statistics (z) along with 95% confidence intervals (CI) and the associated p-values are included. The significant parameter estimates ($p<0.05$) are in bold.

Predictor	β	SE (β)	RI	*z*	95% CI	*P*
a) Intercept	0.913	0.032	-	27.143	0.9787 to 0.8469	<0.001
dROM	**-0.199**	**0.083**	**0.88**	**2.335**	**-0.0320 to -0.3668**	**0.0196**
Oxidative Stress	**0.189**	**0.076**	**0.76**	**2.383**	**0.3446 to 0.0336**	**0.0172**
Yellowness	0.064	0.065	0.21	0.950	0.1960 to -0.0680	0.3423
b) Intercept	0.913	0.032	-	27.043	0.9790 to 0.8466	<0.001
dROM	**-0.200**	**0.083**	**0.87**	**2.326**	**-0.0315 to -0.3694**	**0.0200**
Oxidative Stress	**0.192**	**0.076**	**0.75**	**2.410**	**0.3472 to 0.0357**	**0.0159**
IR	0.024	0.065	0.15	0.350	0.1563 to -0.1089	0.7264

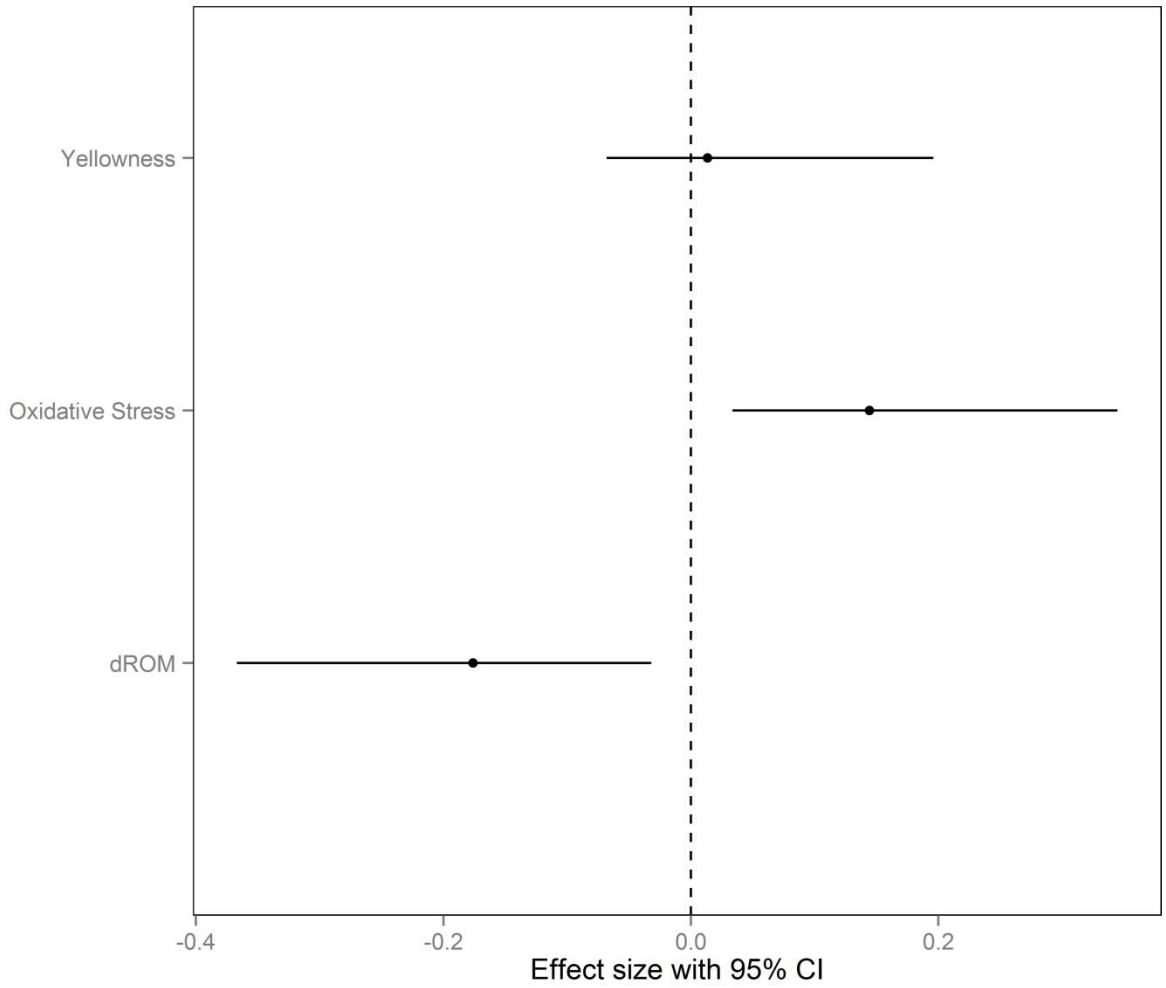

Figure 3.1: The model-averaged parameters containing feather yellowness to estimate the effect size of the model without IR. The standardised effect sizes are depicted with confidence intervals (CI) of ±95%.

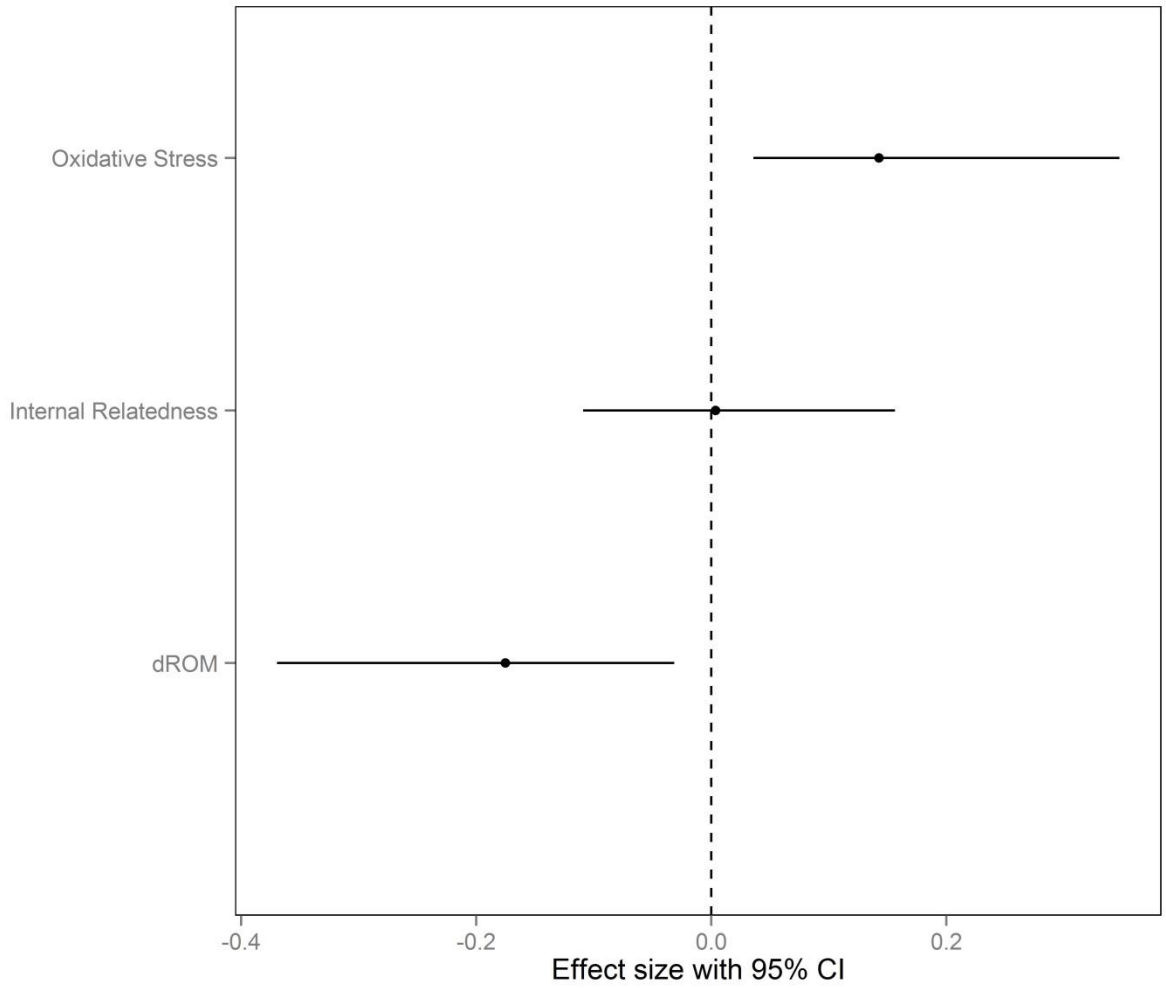

Figure 3.2: The model-averaged parameters excluding feather yellowness and including IR. The standardised effect sizes are depicted with confidence intervals (CI) of ±95%.

3.3.3 Genetic verification of the yellow-eyed penguin DOC database

From the plot of Wang and Queller & Goodnight estimates, both estimators ranged from -1.5 to 1. The putative first-order relatives in the DOC database had high relatedness coefficients, the second-order relatives were lower, and the unknown dyads had a wide range (Supplementary Material Figure S2). The level of relatedness of unknown dyads was anticipated since these dyads include both related and unrelated individuals. First-order and second-order relatives deduced from nest records in the DOC database were verified by locating the pairs on the plot (Figure 3.3). All the known related pairs (n = 8) were found to fall within the range (between 0 and 1.0 for both estimators) and the database appears to be accurate. Highly related pairs, for example full siblings, are found right at the top of the plot, which indicates a close similarity in genetic makeup. Typical second-order relatives, such as the uncle-nephew relationship, fall close to the centre of the plot. None of the known relationships from the database (i.e. parent-offspring, siblings, etc.) fell out of the range of either the first-order or the second-order relatives, into the unrelated range (close to -1.0 for both estimators).

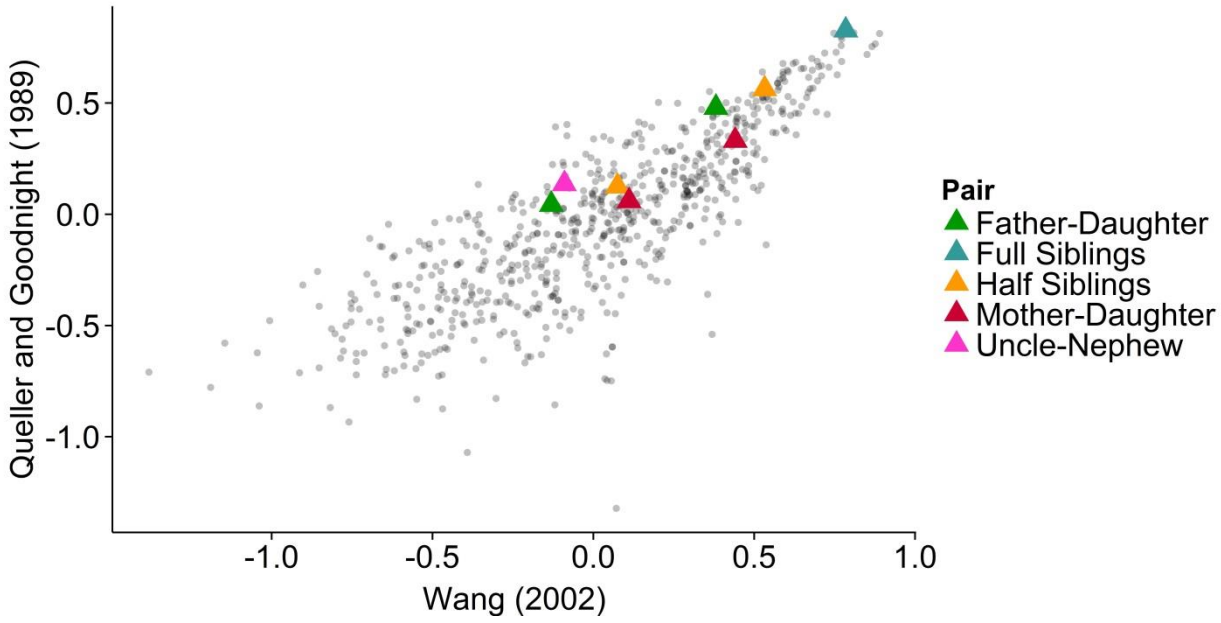

Figure 3.3: The correlates of Wang and Queller & Goodnight relatedness estimators to calculate relationships between yellow-eyed penguins. Known relationships are explained in the figure legend (n = 8) and denoted by coloured triangles. The unknown relationships (n = 695) are represented by grey circles.

3.4 Discussion

Here, I examined the role inbreeding plays in the long-term reproductive success of the endangered yellow-eyed penguin. The population at Boulder Beach includes a small percentage (5.4%) of individuals that have higher levels of reproductive success and whose offspring as just as successful – considered "superior breeders" (Stein, 2012, unpublished thesis). My aim was to determine whether inbreeding levels of yellow-eyed penguins were reflected in variation in putative indicators of reproductive success. A secondary outcome of this study was the verification of DOC's 30 year yellow-eyed penguin monitoring database. Based on the GLMM models, there is no evidence that IR has a significant effect on the proxies of reproductive success of the Boulder Beach population of yellow-eyed penguins. The indicators included in the models remained unaffected by the inclusion of IR, suggesting that inbreeding is not influencing the fitness levels of the birds. This bodes well for the endangered yellow-eyed penguins since the observed levels of inbreeding do not appear to be impacting on this population. My study also suggests that variation in physiological quality may lack a genetic basis in yellow-eyed penguins. Finally, the yellow-eyed penguin database maintained by DOC was accurate based on a small number of known relationships, thereby pointing to its accuracy.

Inbreeding depression is a very real problem for endangered birds (Jamieson & Ryan 2000; Jamieson et al. 2008). From a conservation standpoint, estimating inbreeding in avian populations is required in order to gauge genetic diversity (Frankham 2005; Hoeck et al. 2015; White et al. 2015). A population can contain birds with varying degrees of individual quality, which are expressed as social status, the ability to find the most suitable territory to nest, and an finding the best partner to mate with (Jouventin et al. 2008; van de Crommenacker et al. 2011b; Cram et al. 2015), which all translate into differential reproductive success. All these traits can also be attributed to an individual's genetic distinctiveness. In this study, the two main putative measures (oxidative stress and dROM levels) present the same effect levels, regardless of the levels of inbreeding (IR) of yellow-eyed penguins. The superior breeders consistently experience higher levels of oxidative stress and lower levels of oxidants (dROM), and vice versa for average breeders. Interestingly, this goes against the norm where higher quality individuals are those that have lower oxidative stress levels (Beaulieu et al. 2011; van de Crommenacker et al. 2011b; Cram et al. 2015). This finding may imply that environmentally-induced heterogeneity, rather than

heterozygosity, is the basis for developing high levels of immunocompetence in yellow-eyed penguins.

With endangered or highly endemic species, conservation management must consider how diverse a population is and record data from every breeding season, and preferably by maintaining a pedigree. For instance, the critically endangered kakapo (*Strigops habroptilus*) has a highly restricted range on predator-free islands and managing the species requires an accurate breeding record to prevent any chance of inbreeding (Elliott et al. 2001). Yellow-eyed penguins are not as badly affected by predation or the risk of extinction as the kakapo; however, it is pertinent that DOC continue to monitor these birds as well as they have. This study appears to confirm the accuracy of the DOC database on yellow-eyed penguins, with respect to recording relatives in the population. It is important to note that, from the 41 yellow-eyed penguins sampled here, there were only eight known relatives. Therefore, when considering the accuracy of the database, one must take into account the small sample size. Maintaining this database will improve the understanding of relatedness, dispersal behaviour, and will help prevent inbreeding depression should the species face a major catastrophic event.

It is possible that no significant effect of IR on the indicators of yellow-eyed penguin reproductive success was detected due to the small sample size. However, given the low number of breeding pairs that season, this dataset is a fair representation of the Boulder Beach population. Small sample sizes, especially for endangered species, can be analysed and interpreted just as accurately (Hernandez et al. 2006). It would appear that yellow-eyed penguins observe female-biased dispersion, since females appear marginally more outbred than the males, in order to avoid inbreeding (Greenwood 1980; Daniels & Walters 2000). Female dispersal is the predominant pattern followed by seabirds where females leave their philopatric breeding site and search for a mate at a different location (Greenwood & Harvey 1982; Hall et al. 2009). The study might have been strengthened by sampling the fledglings of the sampled adults, but this was not an option because it was a poor breeding season and further handling of chicks at the nest would have added to their stress. This study, however, presents an opportunity and can be used as the baseline for further research.

Superior yellow-eyed penguins may compromise their own health status to produce more offspring; however, the relative impact of oxidising molecules (resulting from illness and other stressors) is lower than that of an average individual. This can be interpreted as

more efficient use of antioxidants since the fitter individuals have enough to counter the effects of the oxidants and remain highly productive. Another reason for the disparity between reproductive success rates and oxidative stress levels may be that post-hatch parental care is far more costly than egg laying and incubation (Deeming & Reynolds 2015). The superior breeders with larger broods would therefore have to use far more energy to care for their offspring by depleting their antioxidant resources much quicker (Beaulieu et al. 2011). Although inbreeding levels are currently low in yellow-eyed penguins and do not appear to alter the individual fitness, it would be hard to rule out the possibility of this happening in the near future. With the yellow-eyed penguin numbers declining (Ellenberg & Mattern 2012), it would be beneficial to analyse MHC as an indicator of fitness and expand sampling across the entire range of the species. There may come a time when yellow-eyed penguins require some form of intervention by conservation managers to prevent inbreeding (e.g. increasing gene flow by translocating birds; Storfer 1999).

Chapter 4: General Discussion

A male yellow-eyed penguin mid-moult

The overall aim of this research project was to identify potential indicators of individual quality within a population of yellow-eyed penguins, with quality represented as both breeding success during the season of sampling, and longer term breeding performance estimated as an index. Ellenberg et al. (2009) have previously explained how there are individual differences in yellow-eyed penguins in the way they adapt to stress. The existence of a core group of breeders, with respect to lifetime reproductive success (Stein, 2012, unpublished thesis). These individuals may be very important for population persistence, given the steady decline of the population at Boulder Beach and other areas (Ellenberg & Mattern 2012). With the increasing frequency of catastrophic events, the endangered yellow-eyed penguin numbers could reduce drastically (Ellenberg & Mattern 2012). From a management point of view, it might be useful to be able to identify these individuals, so that proactive management can be implemented to protect them.

From this single sampling effort during the incubation stage of breeding, it was evident that the more productive birds were less stressed, based on the H/L ratio, however differences in levels of oxidative stress and oxidants were more difficult to interpret, in that more productive birds experienced higher levels of oxidative stress. This result was contrary to my prediction that the superior breeders exhibited lower levels of oxidative stress than average breeders. This prediction was made based on the idea that higher quality individuals would have better immune defenses to withstand oxidative stress, thereby having high reproductive success as well as low oxidative stress levels (Constantini 2008; Constantini et al. 2014). There was no evidence of inbreeding affecting the productivity of the population, or of oxidative stress measures being influenced by inbreeding coefficients. Therefore, the fitness of yellow-eyed penguins at the Boulder Beach complex is not affected by inbreeding. The dimensions and colouration of the ornaments also proved to be unsuitable as indicators of individual quality, and there were no correlations between ornamentation and immunocompetence. These findings suggest that oxidative stress analyses and certain haematological measures can successfully be used to distinguish high quality yellow-eyed penguins from average individuals; however, measures of reproductive success must be carefully chosen since only specific variables are reliable with respect to specific measures of productivity.

4.1 Oxidative Stress in Yellow-eyed Penguins

A number of studies have explored the impact oxidative stress might have on avian reproduction (Bize et al. 2008; Constantini 2008). Regardless of whether or not oxidative stress is directly influencing reproductive performance, it is clear that breeding birds have higher oxidative stress levels than non-breeding birds (Constantini et al. 2014). In yellow-eyed penguins, all breeding birds in the population are also likely to have a heightened level of oxidative stress compared to non-breeding birds. Studies show that breeding birds with larger broods have a lower resistance to oxidative stress, and this observation was the basis for my prediction that productive yellow-eyed penguins would demonstrate lower oxidative stress (Constantini 2008; Christe et al. 2011). This was not the case, as I observed that the more productive birds (laid more eggs) exhibited higher oxidative stress levels. It is possible that the trade-off between these individuals caring for more chicks and their survival is balanced out by their ability to handle that level of stress. That is to say, fitter individuals in the population can withstand a much higher level of oxidative stress and can therefore afford to lay more eggs. Antioxidants in the yolk and albumen benefit the growth of the egg, prevent oxidative stress, and offspring development (García-Tarrasón et al. 2014).

It is possible that the fitter individuals in the population are more productive because reactive oxidants are a result of infections, so that fewer oxidants are associated with fewer infections and hence greater fitness. Studies of pied flycatchers (*Ficedula hypoleuca*; Ilmonen et al. 2000) and Audouin's gulls (*Larus audouinii*; García-Tarrasón et al. 2014) provide interpretations that could be applied to yellow-eyed penguins. However, here, I suspect the levels of antioxidants and their uses are more relevant to the yellow-eyed penguins. Ilmonen et al. (2000) inferred that at the time of an activated immune response, the bird's reproductive success was lowered, and this might be a trade-off to avoid oxidative stress. The flycatchers compensated for the immune response by producing fewer, less healthy chicks, thereby reducing the parental workload. García-Tarrasón et al. (2014) reported two main results: 1) a marine diet is richer in antioxidants than a terrestrial (rice field prey) diet and, 2) Audouin's gulls differentially distribute antioxidants to their eggs in the order that they are laid. These gulls lay a clutch of three eggs, with levels of antioxidants decreasing with each egg, suggesting a limited availability of antioxidants to the birds (García-Tarrasón et al. 2014).

In yellow-eyed penguins, fitter individuals might have the ability to allocate antioxidants to more eggs, and choose to trade-off their health for a greater workload with

more chicks to tend. These high quality birds experience greater oxidative stress because their antioxidant levels are low, but comparatively so are the deleterious oxidising molecules. In order for individuals to show high oxidative stress, they would have to possess low levels of antioxidants as well. It is possible that yellow-eyed penguins can up-regulate antioxidants (similar to the results of Cram et al. 2015) when required; however, the superior breeders can afford to defend themselves against oxidative stress-causing factors and still produce more offspring. Average breeders appear to retain as many antioxidants as possible and choose immune defences against having high reproduction rates.

The relative decrease in antioxidants from the penguin with no offspring (least productive) to the penguin with the most offspring (most productive) implies that they are able to manage their antioxidants levels without experiencing the damaging physiological effects of oxidative stress. Yellow-eyed penguins with poor breeding success appear to retain whatever antioxidants they obtain, since they also experience the greatest impact of the oxidising agents. These individuals are therefore less fit and less productive because they cannot afford to lose any of their antioxidants. If, for example, one of these weaker birds were to have two chicks in the nest to care for, that would essentially compromise the survival of the adults as well as the chicks. This might also explain why certain breeding pairs skip a breeding season. A bad food year might translate to dangerously low levels of antioxidants, pushing the oxidative stress levels beyond a limit. The sensible adaptive response would be to wait for a better year.

The role of oxidative stress on the costs of reproductive success has been questioned in the recent past (Metcalfe & Monaghan 2013). However, there appears to be a considerable amount of evidence to support this theory (Christe et al. 2011; Constantini et al. 2014; Guindre-Parker et al. 2013), spanning a number of avian families. It would be useful to establish a baseline of oxidative stress levels of yellow-eyed penguins and how these match up against other Sphenisciformes. In order to obtain the most accurate baseline, it is necessary to calculate oxidative stress levels through the various breeding stages (possibly even during the non-breeding seasons). The deductions one can draw from this study point towards reactive oxygen metabolites having greater relevance than the overall oxidative stress levels. Yellow-eyed penguins prefer to lay more eggs despite the physiological stress they endure; however, when the oxidising agents reach a certain level, they retain the antioxidants they have absorbed. Unlike other birds, which have smaller broods with less fit offspring, yellow-eyed penguins appear to adapt and produce as many as physiologically

feasible. These findings also make it extremely important to understand the foraging behaviour and diet of the yellow-eyed penguins. Their dependence on taking in antioxidants requires us to better understand how the fluctuations in their diet affect their breeding. All these studies could lead to better management of the species.

4.2 Leucocyte Counts in Yellow-eyed Penguins

This is the first published record of leucocyte counts and proportions of the different white blood cell types in yellow-eyed penguins. Some yellow-eyed penguin blood smears have been taken for parasitology (Hill et al. 2010; Argilla et al. 2013), but my values are the first to establish a baseline for leucocyte ratios. There can be significant differences in proportions of heterophils and lymphocytes between species, even those belonging to the same family, the including penguins. My results show that yellow-eyed penguins have a similar proportion of leucocyte types to little penguins (*Eudyptula minor*) and Humboldt penguins (*Spheniscus humboldti*; Sergent et al. 2004; and the citations therein), i.e. more heterophils than lymphocytes.

Using leucocyte profiles to describe the effects of stress on birds can be quite complicated but it is reliable nonetheless (Davis et al. 2008), as there are clear links between immune responses on both short- and long-term breeding success (Hanssen 2006). An immune response in common eiders (*Somateria mollissima*) can lead to a longer incubation period (delayed hatching), lower body mass, and lower return rates, thereby having immediate and delayed consequences (Hanssen 2006). Ilmonen et al. (2000) found that pied flycatchers (*Ficedula hypoleuca*) are less productive when their immune systems are activated. The response these birds have to an infection results in changes to feeding patterns, self-maintenance, ultimately leading to fewer fledglings, which are weaker (Ilmonen et al. 2000). This chain reaction results in a trade-off whereby a balance is maintained between the effects of an immune response and a reasonable productivity level. It is possible that yellow-eyed penguins, like most birds, adopt strategies that are influenced by their current condition.

The key haematological result of this study was that yellow-eyed penguins that laid more eggs tend to have a lower H/L ratio than birds laying fewer eggs. It is possible that egg laying is more stressful than parental care for yellow-eyed penguins. Also, there are a number of factors which would influence fledging rate such as disease, emaciation, and climate change (e.g. heat stroke). Assuming these factors affect all fledging yellow-eyed penguin chicks, there would not be significant differences in stress levels of the adults. Finally,

considering that sampling was carried out during the incubation stage (while adults were on eggs); it is possible that sampling carried out while the chicks fledged may provide different results. A study on Adélie penguins found the H/L ratio was a reliable indicator of individual quality, as it differed significantly between individuals and through the various reproductive stages (Vleck et al. 2000), and injured Adélie penguins have a much higher heterophil count than normal. In Adélie penguins the H/L ratio reduces from the courtship phase, to the incubation phase, and even further in the chick rearing stage (Vleck et al. 2000). A similar trend is seen in little penguins (Evans et al. 2015). With respect to yellow-eyed penguins, the different stress levels at the time of incubation appear to be related to the quality of breeders: high quality breeders have lower H/L ratios compared to average quality breeders. This could be indicative of a lower leucocyte baseline for high quality individuals or that this group of birds can achieve homeostasis quicker, making them healthier individuals.

The H/L ratio reflects the short-term state of an individual, which can be influenced by a number of factors. Penguins are susceptible to diseases (Alley et al. 2004), parasitic infection (Hill et al. 2010), food availability (van Heezik & Davis 1990; Edge et al. 1999), climatic variations (Peacock et al. 2000), and anthropogenic factors such as excessive tourism (McClung et al. 2004; Ellenberg et al. 2009). It is difficult to prevent or control the first two; however, human disturbance and tourism can be regulated. Low stress levels could be important in order for yellow-eyed penguins to realise their potential. Ellenberg et al. (2007), where they found that high stress hormone (corticosterone) levels resulted in lower productivity in yellow-eyed penguins. Given that among-individual differences exist, management should ensure that weaker birds are not stressed as they during breeding. Fitness traits can be inherited, whereby fitter adults give rise to fitter offspring (McGlothlin et al. 2005; Ellegren & Sheldon 2008; Knafler et al. 2012; Monceau et al. 2013). If the H/L ratio is a reliable indicator of individual quality among yellow-eyed penguins, it would be interesting to sample offspring from both sets of breeding pairs to determine whether this fitness trait is inherited.

4.3 Ornamentation in Yellow-eyed Penguins

Birds possess striking feather ornaments, in the form of masks, breast feathers, or tail feathers (Møller & Höglund 1991; Thusius et al. 2001; Kraaijeveld et al. 2007). Female avian ornaments have been associated with reproductive quality in a number of bird species

(Nordeide et al. 2013). The role of ornamentation in influencing sexual selection is slightly more complex in apparently monomorphic birds (Kraaijeveld et al. 2007), such as the yellow-eyed penguin. While body size differs between the two sexes (Setiawan et al. 2004), the difference is very small, and it is also not possible for researchers to discern males from females by looking at the yellow eye stripe. However, because birds have a much larger spectral range than humans (Bennett & Cuthill 1994), it is possible that yellow-eyed penguins use the brightness or the yellowness of the post-ocular stripe to choose a mate. Massaro et al. (2003) suggest that males used colouration to determine the parental quality of the females, although they worked under the assumption that the pigmentation was carotenoid-based. Basing their study by attributing the pigmentation to be carotenoid-based would lead the authors to make assumptions using knowledge about carotene; however, pigment molecules function differently from one another and they may also be obtained from different sources (Yu et al. 2004; Prum et al. 2012; Thomas et al. 2013) which may not influence parental quality. They used hue and saturation of the eye and eye stripe, based on photographic data, to assess sexual selection and parental quality in yellow-eyed penguins, and found that the penguins potentially use eye and post-ocular stripe colour to advertise individual quality (Massaro et al. 2003). Since then, the discovery that the yellow colour in penguin ornamentation is caused by the unique 'spheniscin' pigment (Thomas et al. 2013) has warranted revisiting of this hypothesis with a different methodology because pigment molecules behave chemically different (Yu et al. 2004; Prum et al. 2012). Also, the authors used secondary data (photographs) as opposed to feather samples which may be more reliable from a biological point of view. Rather than look at hue and saturation, I looked at feather brightness and yellowness (using feather samples), and measured the width of the eye stripe (using photographs).

I did not find ornamentation to be a reliable indicator of reproductive performance in yellow-eyed penguins. Research to test this assumption requires a two pronged approach which was not possible during this preliminary study: I would use a technique to measure the yellowness and brightness of the feathers which would allow the researcher to control for the unique morphology of penguin feathers to obtain reliable and consistent data. I would also analyse feathers at every stage of reproduction and follow trends in pigmentation since mate selection takes place at the beginning of the breeding season. Yellow-eyed penguins may be accumulating antioxidants prior to the mate selection stage, to attract potential mates. It would be useful to know how antioxidant levels influence pigmentation in yellow-eyed

penguin feathers. This could potentially make ornamentation a more reliable indicator of quality.

As well as colour or brightness, birds also use the size of ornaments to ascertain certain traits in potential mates (Møller & Höglund 1991; Thusius et al. 2001). King penguins use the size of their ornaments for a number of reasons, including identifying high quality mates based on aggressiveness (Viera et al. 2008), and healthier partners based on immunocompetence (Nolan et al. 2006). In addition to variations in patch size, the length and colour of plumage ornaments of certain bird species appear to be influential in mate choice. I found that there was no correlation between ornament size and breeding productivity. In other species larger birds possessing larger morphometric traits are more attractive to mates (Møller & Höglund 1991; Thusius et al. 2001) but this may not ring true in yellow-eyed penguins. Even though the birds sampled belonged to different age groups and size ranges, the size of the post-ocular stripe did not vary according to breeding productivity. The sizes of yellow-eyed penguin ornaments are, perhaps, not as important for sexual selection as in other penguin species. It is likely that mate choice requires multiple cues, as seen in other penguin species (Pincemy et al. 2009), and ornament size is relatively less important to yellow-eyed penguins.

Further investigation could combine morphological (i.e. ornament-related) analyses with genetic analyses. Advances in genetic sequencing have led to in-depth research on links between ornamentation in birds and their genetic makeup (Nordeide et al. 2013). The use of quantitative trait locus (QTL) analysis could help determine how the phenotypic expression differs between male and female yellow-eyed penguins, and its impact on mate choice. This technique was successfully carried out on red junglefowl (*Gallus gallus*) to better explain how female combs were expressed (Wright et al. 2008).

In the case of yellow-eyed penguins, it is possible that low sample size meant I was unable to detect any significant variation in ornamentation (Nakagawa & Cuthill 2007). I sampled all the breeding adults in the Boulder Beach population, but nest numbers were much lower than in previous years. Sampling a larger portion of the population, with more sophisticated techniques, and introducing a genetic component (QTL analysis) might generate new findings and elaborate on the ornamentation of this rare bird.

Analysing feathers can be highly variable and measuring colouration of individual feathers and feathers still attached to the bird with spectrometric techniques can produce very

different results (Santos & Lumeij 2007). Similarly, my approach to measuring colouration using individual feathers was different to the approach of Massaro et al. (2003) who used photographic measurements, and might have yielded different results. These differences in results, where Massaro et al. (2003) found significant distinctions, could be a result of time of collection. It is possible that the authors photographed the birds during their nesting building or mating stage, while I only sampled incubating penguins, and this meant that antioxidants were less distinct between the breeding groups. It is likely that breeding penguins have significantly different levels of antioxidants prior to laying eggs; however, once the eggs are laid, there could be a less noticeable difference between high and average quality breeders. The authors' reproductive success calculation also differed from my index. The technique I used was more rigorous and ecologically more relevant. The use of primary data rather than secondary data also makes it much more reliable. Spectrometry can help analyse colouration parameters in the UV range which is the range some birds are known to use. Although my approach was highly repeatable, there was room for error: the size of the feathers was very small, some samples might not have overlapped as closely as others, the spectrometer might not have picked up the entire feather sample, and there was a possibility of error due to refraction (rather than reflection).

4.4 Inbreeding in Yellow-eyed Penguins

The negative effect inbreeding has on avian reproduction has been studied extensively and these effects are usually more pronounced in endangered or highly endemic birds (Jamieson & Ryan 2000; Jamieson et al. 2008; White et al. 2014). Individual quality can be highly variable within a population and is expressed in ways which influence reproductive success (Jouventin et al. 2008; van de Crommenacker et al. 2011b; Cram et al. 2015). These quality differences in birds are usually reflected through either internal indicators (oxidative stress levels, H/L ratios; Constantini 2008, Davis et al. 2012) or externally (ornamentation; Blums et al. 2005). By using these indicators as measures of individual quality based on reproductive success, the more productive individuals can be identified.

A small section of the yellow-eyed penguin population at Boulder Beach are known to produce a much larger number of offspring than the rest (Stein, 2012, unpublished thesis), which could be associated with their genetic makeup. Inbreeding lowers productivity and therefore, the superior breeders are likely to pair with more distantly related birds to avoid

inbreeding (Blouin & Blouin 1988; Pusey & Wolf 1996). By testing the effect inbreeding (using IR) had on the putative indicators – oxidative stress levels, oxidant levels, and feather yellowness – it would indicate potential links between inbreeding, fitness and reproductive success. I predicted that oxidative stress would increase when measured with IR (implying an increase in oxidants as well) and yellowness would decrease. However, I found no evidence that IR has a significant effect on the immunocompetence and ornamentation proxies of reproductive success of the Boulder Beach population of yellow-eyed penguins. Despite IR being included, superior breeders experienced higher oxidative stress but had lower levels of oxidants. This suggests that reproductive success is more dependent on how an individual manages its resources by taking into consideration the trade-off between immune defence and productivity.

The indicators in the models were not changed by the inclusion of IR, which means inbreeding is not influencing the fitness levels of these yellow-eyed penguins. This suggests that variation in physiological quality may lack a genetic basis in yellow-eyed penguins. This is a positive result with respect to this species because evidence of high inbreeding levels would have a much greater impact to the already declining populations. With the female yellow-eyed penguins being slightly more outbred than the males, it is possible that female-biased dispersion occurs in the species to prevent inbreeding (Greenwood 1980). This could imply that a female penguins move in and out of Boulder Beach rather than staying near the nest where they were fledged. Yellow-eyed penguins perhaps avoid inbreeding by successfully pairing with more distantly related mates (Greenwood 1980). Perhaps yellow-eyed penguins fall into the category of animals which have adapted to avoid inbreeding depression (Blouin & Blouin 1988; Pusey & Wolf 1996). An additional result as a consequence of this study showed the DOC's yellow-eyed penguin database was accurate based on a small number of known relationships, thereby proving its accuracy.

The year of sampling, between 2014 and 2015, had one of the lowest observed nesting rates for this species, including some nests being abandoned and egg infertility. The low nesting rates hindered sampling efforts due to the lack of active nests. It is possible that the small sample size caused the effect of IR on the indicators to not be evident. The study might have benefited from sampling both fledglings and adults, but the poor breeding season did not allow that, with the added stress to each, already stressed, bird on the nest was not an option because further handling of chicks at the nest would have added to their stress. This study, however, can be considered an exploratory study which may provide researchers to

answer more questions about this complex species. From a conservation standpoint, estimating inbreeding in avian populations is required in order to gauge genetic diversity, especially for endangered species (Frankham 2005). The continually declining yellow-eyed penguin population is at risk of inbreeding depression at some point in the future. Extending this study to other yellow-eyed penguin populations and building pedigrees might be beneficial to manage the species. This species might require captive breeding or translocation if their population continues to decline and inbreeding starts to occur (Storfer 1999).

4.5 Management Implications

Since the proportion of high quality breeders appears to be quite small (5.4%; Stein, 2012, unpublished thesis), it would be beneficial to the yellow-eyed penguin population to prioritise the care of these individuals during catastrophic events resulting in significant mortality, such as have been observed in recent years. If the current trend continues yellow-eyed penguins are at risk of being pushed from endangered to critically endangered.

By using the H/L ratio, egg productivity can be predicted, at least for older individuals. It is possible that ensuring yellow-eyed penguins are not stressed by regulating tourism and other human disturbance, productivity could improve.

It might be worth considering providing captive undergoing rehabilitation (injured or emaciated) yellow-eyed penguins with supplementary antioxidant-rich feeding. This would potentially increase productivity upon release and shouldn't have negative effects on the individual since they appear to be well-adapted to allocation of antioxidants.

It would be worth looking at relatedness levels at all yellow-eyed penguin breeding sites. By collecting this kind of genetic data, the species could either be managed as subpopulations or as a meta-population.

4.6 Recommendations for Future Research

This exploratory, cross-sectional study did face certain limitations due to the use of novel techniques for analyses, viz. spectrometry. Addressing these issues will improve the quality of future research. Here, I list suggestions for future studies and how to deal with the limitations I encountered.

1. A longitudinal study, spanning the various stages of reproduction, would provide a more comprehensive understanding of the relationship between oxidative stress levels and reproductive performance for the species. Multiple blood sampling efforts would also establish a reliable baseline for each of the white blood cell types, and allow the tracking of changes in the H/L ratio from nest building until the post-guard stage of breeding.

2. Including more breeding sites of yellow-eyed penguins would provide insights into how stress levels differ throughout their distribution. This kind of study could identify the most conducive habitat for the penguins, where they would encounter the fewest stressors.

3. Previous studies have used different approaches to measure brightness and pigmentation, either by photography or measurements of actual feather samples. I used a spectrometer directly on the feather samples to investigate how the brightness and yellowness appear to another yellow-eyed penguin. Birds can see a much wider spectral range which can be recorded using spectrometry on feather samples but not photographs. However, because of the structure of the feathers, the exact point of measurement could only be controlled to a certain degree. Post-ocular feathers are small (2-4mm long) and penguin feathers in general do not have a broad vane. Instead the vane is narrow and close to the rachis. It would be worth having another look at feather brightness and yellowness using a more sophisticated approach, such as multiple-angle spectrometry (Santos et al. 2007).

4. This thesis has verified the DOC monitoring database regarding the eight known relationships. This could potentially be taken one step further. Generating pedigrees from birds at multiple yellow-eyed penguin breeding sites would elaborate on the dispersion of individuals from one population to the next. It would indicate movement and levels of inbreeding of the species. This was outside the scope of my study. Nevertheless, the population at Boulder Beach has been the most closely monitored group of birds and it was the ideal place to confirm relatives as an exploratory study.

5. Once the baselines for leucocyte counts and oxidative stress levels are in place, it might be useful to look at the long-term impact avian diphtheria has on individual health and productivity.

6. While I recorded correlates between high quality birds and various indicators, further research on the propensity of birds to pair with superior individuals needs to be carried out. Using these indicators, the birds can be divided based on their reproductive success. Observations on how these birds pair with each other and the collection of data on whether pairs are formed between two high quality breeders or at random, would inform management efforts.

4.7 Conclusions

The two main goals of this study were to establish putative indicators of reproductive success in yellow-eyed penguins and to assess the impact relatedness had on productivity. The declining population trend of yellow-eyed penguins makes the research pertinent, since the outcome could help inform the management of this species. Three parameters appear to be reliable in identifying individuals based on reproductive quality. Superior individuals can be distinguished by higher oxidative stress levels and lower reactive oxidant levels from average individuals with respect to long-term breeding productivity. The only useful parameter associated with high seasonal productivity was the H/L ratio, taken from leucocyte counts, where superior breeders had lower H/L ratios than average breeders: this signifies lower stress levels. All these measures can be used as indicators of among-individual quality. The lack of inbreeding in the population suggests that the genetic diversity of the population is maintained by mating with more distantly related individuals. Productivity is not diminished by the observed levels of internal relatedness. The genetic analyses also indicate that the oxidative stress levels remained unchanged by the levels of internal relatedness which tells us that the fitness indicators are not related to the genetic differences between individuals.

The more productive yellow-eyed penguins displayed higher oxidative stress levels but significantly lower levels of harmful oxidants than less productive individuals. I suggest this is because fitter birds are able to manage their antioxidants levels better while less fit ones have to retain as many antioxidants as possible to overcome the high levels of deleterious oxidants. Yellow-eyed penguins which lay more eggs also appear to be exposed to fewer stressors, reflected in their low H/L ratio levels. These individuals are either able to achieve homeostasis quickly or have a basal leucocyte count more conducive for egg production. A young, inexperienced breeder will probably be more stressed due to inexperience: first-time breeders are more likely to produce only one egg and this egg is sometimes infertile (Richdale 1957). It is important to control for age when analysing these parameters and these indicators might be more reliable when penguins are at the age of five and above. Yellow-eyed penguins start breeding between the ages of two and four; therefore most individuals will be better prepared for breeding by the age of five. These three measures at the time of incubation could help distinguish superior from average breeding yellow-eyed penguins.

Confirmation of the accuracy of assumptions made concerning relatedness of birds based on observations in the DOC database is another promising result for yellow-eyed penguins. A combination of further research and adaptive management strategies will greatly benefit yellow-eyed penguins. With the existence of superior breeders (Stein, 2012, unpublished thesis), it should be fairly straightforward to prioritise certain breeding pairs by providing them greater care. A better understanding of the influence that factors such as leucocyte counts, oxidative status, and internal relatedness have on productivity could assist conservation efforts directed at this endangered bird.

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Appendix A

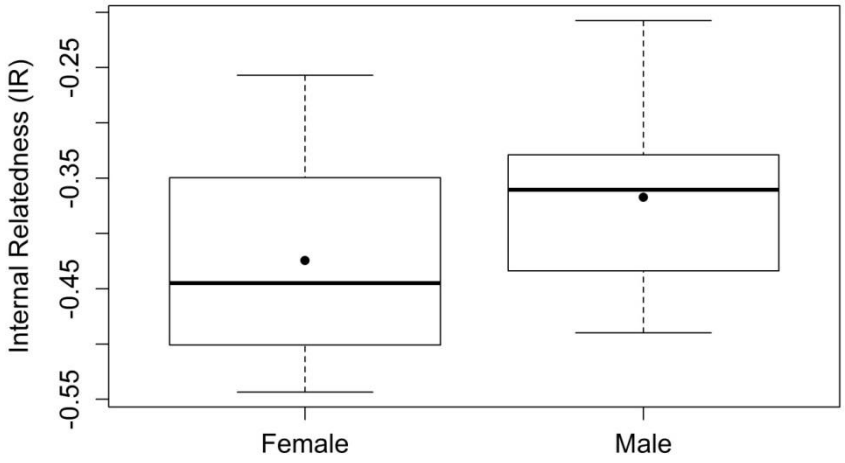

Figure A1: Box plot showing the distribution of internal relatedness (IR; Amos et al. 2001) for breeding female (*n* = 16) and male (*n* = 20) yellow-eyed penguins. The mean IR for each sex is denoted as the solid black dot.

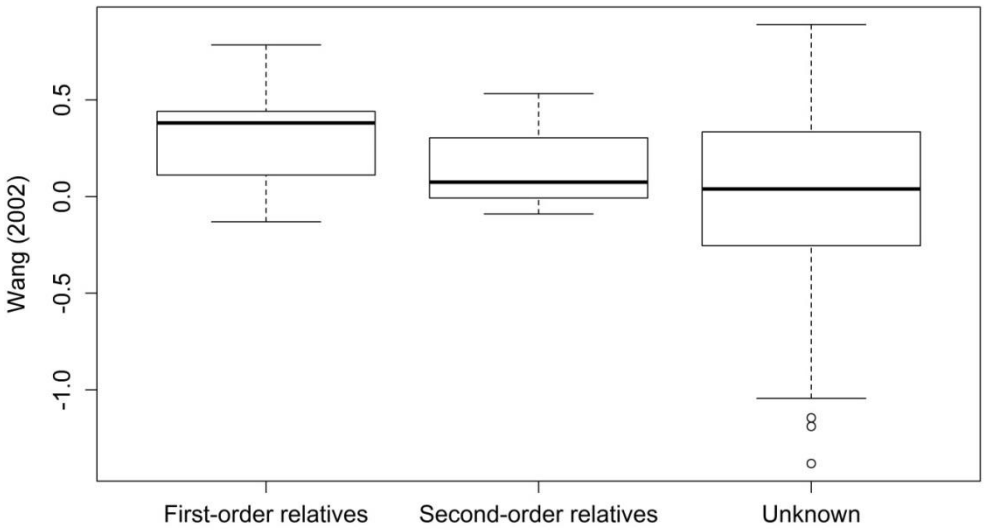

Figure A2: Box plots representing upper and lower quartiles of Wang estimator values, divided by the median, for first-order relatives (*n* = 5), second-order relatives (*n* = 3), and unknown relatives (*n* = 695). The hollow circles denote outliers.