HORMONALLY MEDIATED MATERNAL EFFECTS IN BIRDS

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CANDIDATE'S DECLARATION

I declare that the work documented in this thesis is entirely my own and is of my own composition. No part of this work has been submitted for any other degree.

Anthony Robertson

May 2009

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GENERAL ABSTRACT

The main aim of this thesis was to investigate the effects of environmental conditions, particularly unpredictable or potentially negative ones, on the maternal transmission of the primary avian stress hormone, corticosterone, to developing embryos. We currently lack information on the extent to which conditions in the maternal environment are transmitted to the offspring in birds via egg compositional changes. It is possible that maternally derived hormonal signals communicate information about the external environment to developing embryos and directly influence the fitness of their offspring in a negative or positive way. I found, using captive zebra finches, that the experimental stressor of unpredictable food availability (as these birds are used to *ad libitum* food) experienced by mothers can elevate yolk CORT concentrations, but only when combined with the additional demand of laying a replacement clutch (potentially a buffering system to prevent mild stressors impacting on CORT transmission to the embryo). I then looked at yolk CORT concentrations in two populations of gulls (herring and lesser black-backed gulls) in which the population trajectories differed depending on environmental conditions (potentially a reflection of different exposures to stressful stimuli). The results however did not support this hypothesis, as there were no differences according to habitat type or between species (where they coexist). This would suggest that the different environmental circumstances (harsher for the herring gull) experienced by these two species are not reflected in differences in their eggs (at least in terms of CORT). This could be the result of the eggs being buffered from the maternal CORT environment or it may be that the difficult environmental conditions are not occurring during the breeding season. We also identified that experimental human

disturbance during the laying period does not appear to elevate yolk CORT concentrations, although there was a trend for concentrations to be higher following the loss of the first clutch in the herring gull (as seen in the zebra finches). I also measured yolk CORT concentrations in Common Eider eggs and looked for differences according to the degree of nest shelter. I found no relationship between shelter and yolk CORT, but birds that laid in more sheltered sites had, on average, smaller eggs. This may indicate lesser quality birds are nesting in the sheltered sites and that yolk CORT is not affected by maternal condition. Finally, I looked at another mechanism through which information relating to the maternal environment could be transferred to the embryo. I investigated whether there were any links between maternally derived immunity and CORT by comparing the anti-microbial lysozyme and CORT concentrations in the albumen. I found no correlation between CORT and lysozyme, suggesting that CORT may not affect lysozyme production. It may be that other factors such as colony density and 'cleanliness' are more important in determining the concentrations of lysozyme deposited in the egg or that lysozyme production is not sufficiently costly to be influenced by the maternal stress state. The overall theme of my findings is that CORT concentrations in eggs do not appear to vary much with maternal environments. I will discuss these findings in their broader ecological and evolutionary context and discuss if stress hormones are indeed being used as adaptive signals for preparing the embryo for its postnatal environment.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE STRESS RESPONSE

Homeostasis has been termed as 'the stability of physiological systems that maintain life…and (these systems) are maintained within a range that is optimal for the current life history stage' (McEwen & Wingfield, 2003). These systems, which require constant regulation, include body temperature, sugar and pH levels in the blood, water balance and waste disposal. Allostasis is the adaptive process required to maintain stability in the face of change (Sterling & Eyer, 1988) and thus supports homeostasis. In vertebrates, the principal mediators of allostasis under stressful conditions are the hormones released by the Hypothalamic-Pituitary-Adrenal (HPA) axis. The HPA axis forms a major part of the neuro-endocrine system that reacts not only to stress, but also regulates various processes in the body through glucocorticoid (GC) secretion, including digestion and energy usage (Chester-Jones *et al*., 1972; Silverin, 1986; Fujiwara *et al*., 1996) and the immune system (Munck *et al*., 1984). Activation of the HPA axis (through a change that has the potential to disrupt the homeostatic systems) results in the release of GCs into the vertebrate bloodstream within a few minutes of exposure to a stressor ('the stress response').

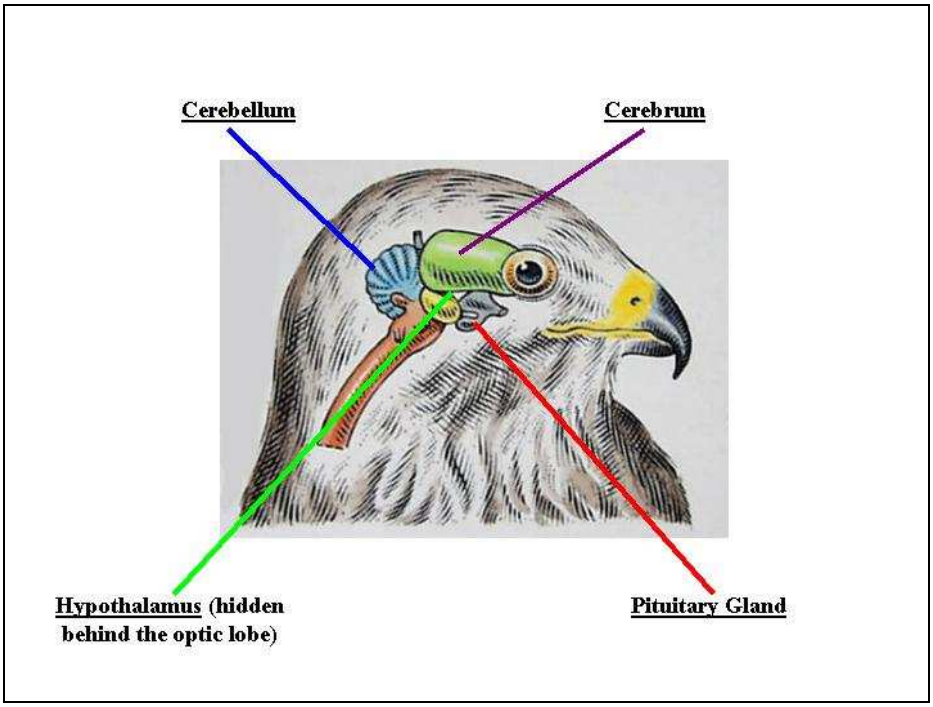


Fig.1.1. Structure and position of the avian brain. Adapted from www.dkimages.com

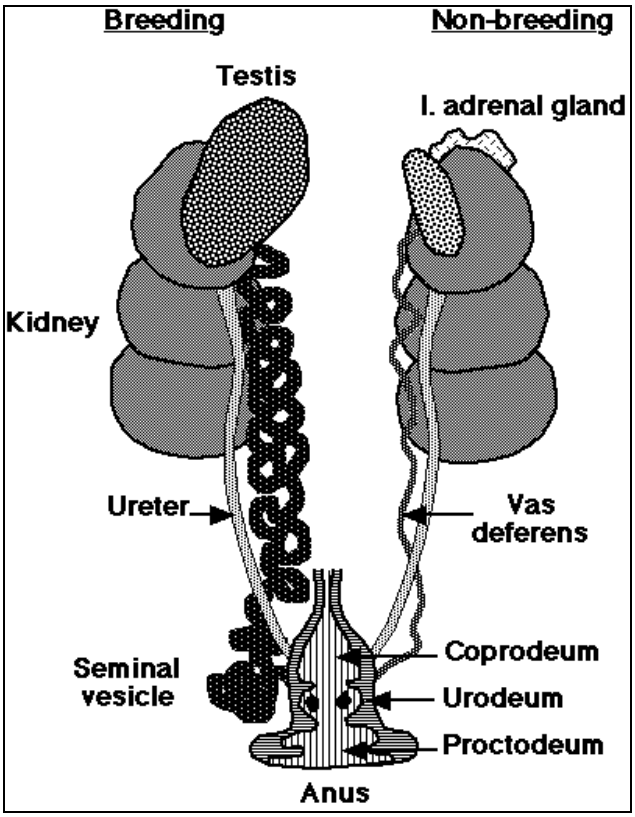


Fig.1.2. Location and structure of the avian adrenal gland (and the reproductive organs) in males during the breeding and non-breeding season. Adapted from http://people.eku.edu/ritchisong/avianreproduction.html

Corticosterone (CORT) is the primary GC in non-mammalian tetrapods and cortisol is typically the main GC in mammals (Idler, 1972). The avian HPA axis (Figures 1.1 & 1.2) has a similar morphology and many of the same feedback controls as in mammals (including humans – Fig.1.3, 1.4 & 1.5) (Carsia, 1990; Norris, 1997) and, although there may be differences compared to other vertebrates, the overall function and responses to aversive stimuli would appear to be almost identical across vertebrate taxa (Wingfield & Ramenofsky, 1999). GC release is controlled by adrenocorticotrophic hormone (ACTH), a hormone that is released by the anterior pituitary gland. ACTH is in turn controlled by the hypothalamic hormones corticotrophin-releasing factor (CRF) and arginine vasotocin (AVT) (or its mammalian homolog arginine vasopressin AVP). CRF and AVP/AVT release are ultimately under the control of higher brain centers that detect a stimulus. These brain centers must decide if this stimulus is potentially harmful and send the necessary neuronal signals to the CRF and AVP/AVT cell bodies in the hypothalamus (Weninger & Majzoub, 2001; Romero & Reed, 2005). Catecholamines (which include noradrenalin and dopamine) and cytokines are compounds also synthesized in this HPA axis and are mediators of the stress response. Although these (and other hormones and compounds) play a role in the stress response, GCs (of which CORT and cortisol are the primary ones, depending on the species) are the main focus of attention for behavioural endocrinologists. This is partly because there is already vast knowledge and laboratory protocols in place to measure GCs and because measurements of GCs have one major advantage over measurements of CRF, ACTH, catecholamines and AVT/AVP. GCs reach their maximum concentration in the bloodstream within minutes of exposure to a stressor and can remain elevated for more than one hour, allowing researchers to be confident that

these elevated concentrations are the maximum. The delay of 3 minutes before CORT concentrations begin to elevate also allows researchers to measure baseline concentrations to compare to elevated concentrations (Romero & Reed, 2005). The time taken for CRF, ACTH, catecholamines and AVT/AVP to be synthesized is earlier than GCs (as they are part of the cascade resulting in GC elevation) (Sapolsky *et al.*, 2000) and their concentrations are influenced by the negative feedback loop of GCs (Fig. 1.4), making it more difficult to assess the physiological state of the individual being sampled. In addition, taking measurements of ACTH and CRF, for example, would require the dissection of the tissues where these compounds are synthesised, meaning animals would need to be euthanised.

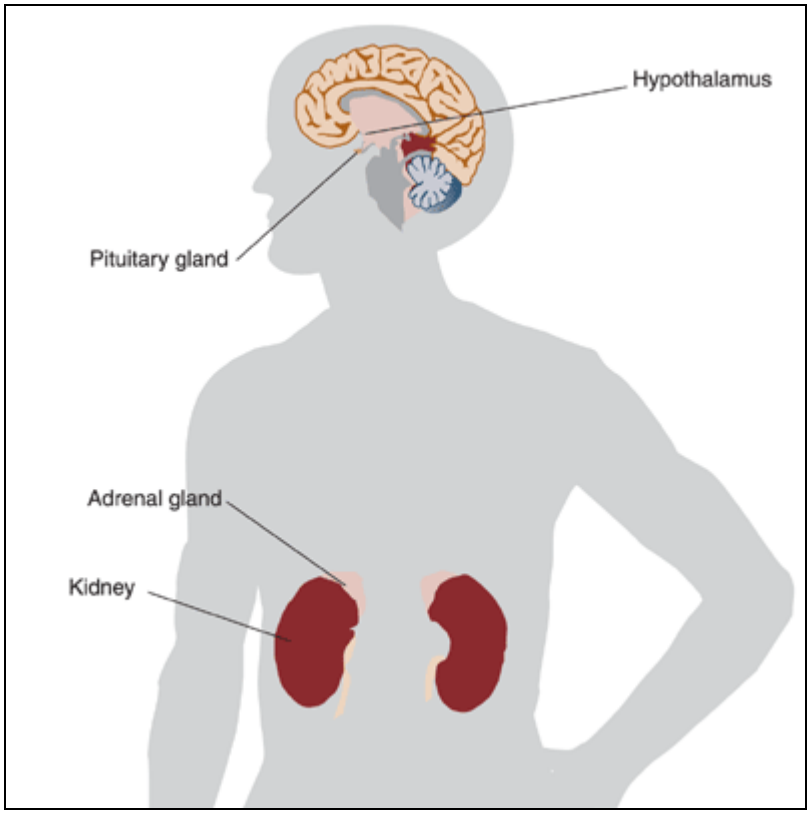


Fig.1.3. Location and structure of the HPA axis in humans.
Adapted from Adinoff *et al*. (1998).

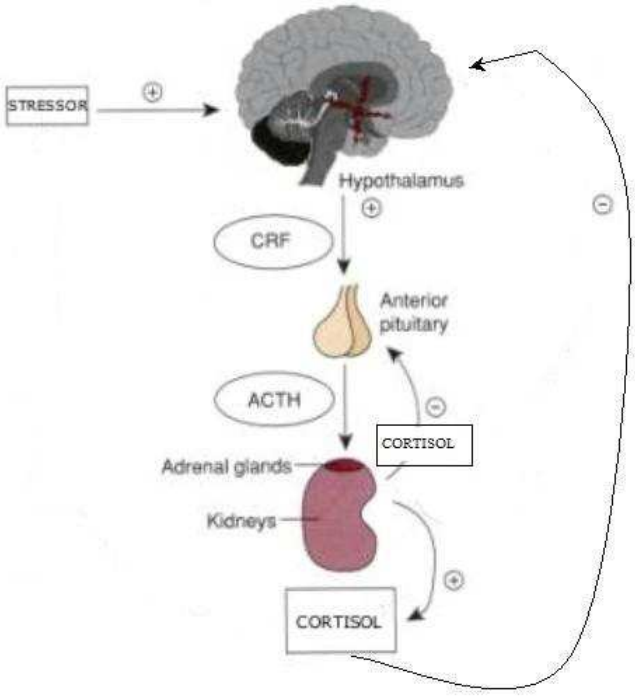


Fig.1.4. Human HPA axis.

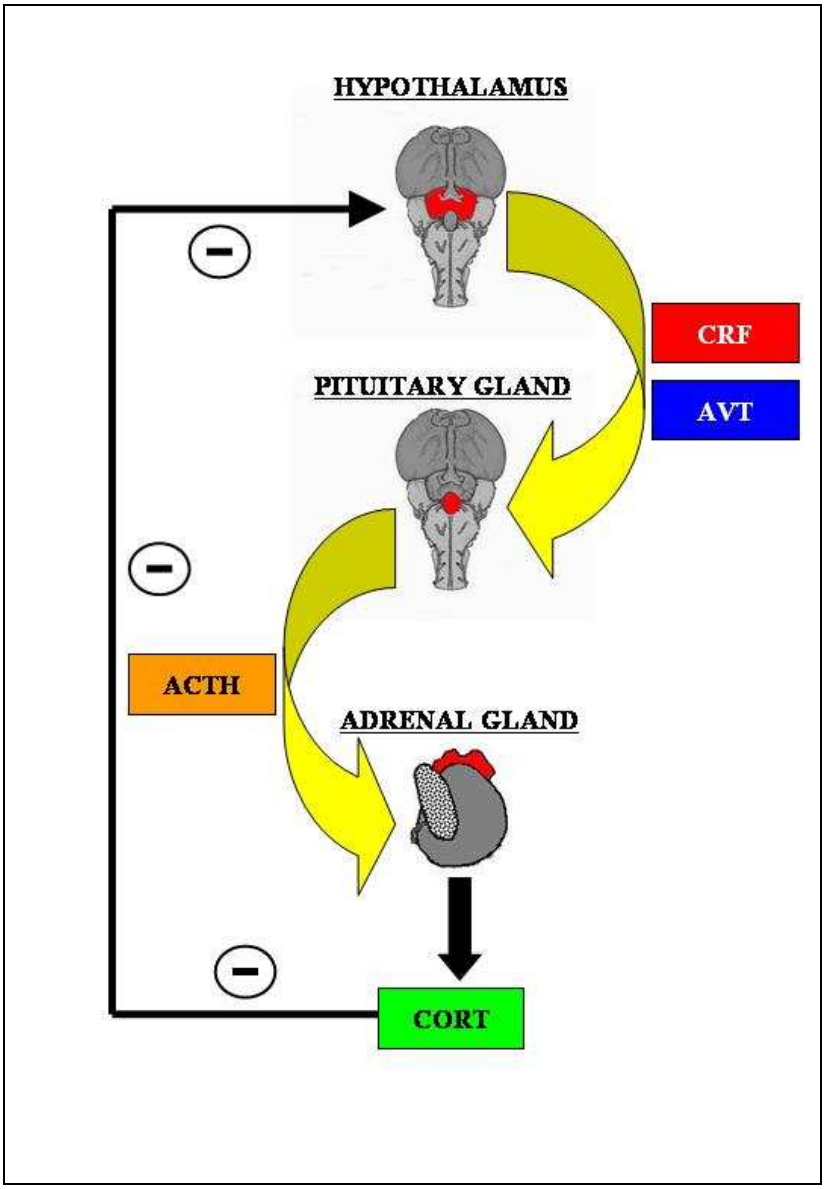


Fig.1.5. Avian HPA axis showing negative feedback loop of CORT (CRF = *corticotrophin-releasing factor*; AVT = *arginine vasotocin*; ACTH = *adrenocorticotrophic hormone*).

1.2 RESPONDING TO UNPREDICTABLE EVENTS

Characterisation of CORT concentrations in birds in recent years has lead to an increased interest in this endocrine mediator. Elevated concentrations of CORT have been shown to result in behavioural changes, such as moving away from a potential

stressor (Astheimer *et al*., 1992), altering foraging behaviour (Wingfield *et al*., 1990; Astheimer *et al*., 1992), clutch abandonment (Clutton-Brock, 1991; Silverin, 1986) and altered reproductive effort (Silverin, 1986). The nature of the behavioural change is dependent on the form and severity of the stressful conditions experienced. Short-term (minutes to hours) increases in the concentrations of stress hormones, including CORT, have been proposed to lead to adaptive changes that allow an individual to remain within its unpredictable environment (Wingfield, 1994; Wingfield *et al*., 1998). However, chronically (days to weeks) elevated concentrations of stress hormones, including CORT, have the potential to shut down reproduction and cause disease (Sapolsky, 2002). The signals or 'stressors' that promote activation of the HPA axis include physical and psychological events such as food shortages, inclement weather and perceived predation risk, but it is important for researchers to be aware that the amounts and patterns of CORT released in response to these different stressors may be different and that CORT also exhibits naturally diurnal and seasonal fluctuations (which are often species-specific) (Romero, 2002). The effects of 'unpredictable events/stressors' on the HPA axis and the CORT response in birds have been relatively well documented in several situations, and some of the most important are described below.

1.2.1 Stress during breeding

When individuals are confronted with an increase in energy expenditure and/or reduced food availability, breeding individuals will often forgo energy demanding events such as reproduction (at any of the stages from copulation through to parental care) until

conditions improve (Sapolsky, 2002). Under such conditions, concentrations of CORT within the bloodstream have been found to be elevated, and this physiological state has been shown to be incompatible with breeding and care of offspring in various vertebrates (**Birds** – Silverin, 1986;Wingfield & Silverin, 1986; Wingfield, 1994; Wingfield *et al*., 1995. **Mammals** – Sapolsky, 1987; Handa *et al*., 1994). However, it is necessary that the individual prevents abandonment of breeding behaviours when the benefits do not outweigh the costs and it is likely that this is reflected in the physiological stress responses seen. For example, comparison of pre-parental (before young are present) and parental (while feeding nestlings) plasma CORT concentrations in Savannah sparrows (*Passerculus sandwichensis*), American tree sparrows (*Spizella arborea*) and white-crowned sparrows (*Zonotrichia leucophrys gambelii*) demonstrated that CORT was elevated to a greater extent in males during the pre-parental phase (when male sparrows expend less energy on reproduction than females) (Holberton & Wingfield, 2003). However, during the parental stage, when both parents feed their young, CORT decreased in male tree and white-crowned sparrows to a concentration similar to that in the females, whose responses remained the same in both pre-parental and parental stages (due to equally high investments through egg laying, incubation and feeding).

1.2.2 Weathering the storm

Unpredictable events, such as severe changes in weather conditions, can dramatically affect the environment an animal finds itself in and the health of that animal is strongly linked to its ability to see out such an event, and respond when conditions return to

normal. Inclement weather has the potential to negatively affect foraging success (through inaccessible feeding areas), body condition (through the reduction in food availability and/or by imposing increased energy demands) and reproduction (through nest abandonment) via stimulation of the stress response and the subsequent increase in circulating CORT (Breuner & Hahn, 2003). In times such as winter, some birds living in unpredictable environments have been shown to have elevated baseline CORT concentrations relative to summering conspecifics, and it has been hypothesised that this is in an attempt to combat more frequent disruptive events (Rogers *et al*., 1993; Wingfield *et al*., 1983; Holberton & Able, 2000). Brief periods of inclement weather, such as storms, have also been shown to result in elevations in the concentrations of CORT in wintering dark-eyed juncos (*Junco hyemalis hyemalis*, Rogers *et al*., 1993); male, but not female, song sparrows (*Melospiza melodia*) during the breeding season (Wingfield 1985a & b); Lapland longspurs (*Calcarius lapponicus*) incubating their eggs (Astheimer *et al*., 1995); storm petrels (*Pelecanoides urinatrix*) when outside of their breeding season (Smith *et al*., 1994); Lapland longspurs, snow buntings (*Plectrophenax nivalis*); both adult and juvenile redpolls (*Carduelis flammea*) during moult, but not during the breeding season (Romero *et al*., 2000); and white-crowned sparrows who were feeding their offspring (Wingfield *et al*., 1983).

1.2.4 Nutrition and foraging success

Reduced food availability has been shown to elevate plasma CORT concentrations in adult Adelie penguins (*Pygoscelis adeliae*) (Vleck *et al*., 2000), black-legged kittiwakes (*Rissa tridactyla*) (Kitaysky *et al*., 1999; Buck *et al*., 2007), broilers (Knowles *et al*.;

1995; De Jong *et al*., 2003), emperor penguins (*Aptenodytes forsteri*) (Robin *et al*., 1998), Japanese quail (*Coturnix japonica*) (Scott *et al*., 1983), white-crowned sparrows (*Zonotrichia leucophrys*) (Breuner & Hahn, 2003) and White storks (*Ciconia ciconia*) (Corbel *et al*., 2008). These changes in CORT concentrations are thought to help individuals cope with environmental stressors as they induce foraging behaviours, as seen in Japanese quail (Bray, 1993) and white-crowned sparrows (Astheimer *et al*., 1992; Lynn *et al*., 2003), and increase locomotor activity, as seen in altricial White stork nestlings preparing to fledge (as parents stop feeding their young in preparation for them leaving the nest) (Corbel *et al.*, 2003).

1.2.5 Friend versus foe – the implications of social interactions

Aggression and stressors go hand in hand. Conspecific challenges and attacks are commonplace in almost all environments, particularly where individuals live within social groups. These interactions are particularly important where there is intense competition for dominant positions within the hierarchy that might lead to increased opportunities for feeding and mating. However, while the benefits of winning may be obvious, these interactions can be perceived as stressors and result in physiological change. Individuals involved in defensive behaviours against conspecific challenges, for example, guarding productive territories (male pied flycatchers - Lundberg *et al*., 1981; Alatalo *et al*., 1985; Lundberg & Alatalo, 1992; Silverin, 1998), or access to a mate (greylag geese, *Anser anser*, - Hirschenhauser *et al*., 2000 Kotrschal *et al*., 1998) exhibit elevated concentrations of plasma CORT (and testosterone).

1.2.6 Predation

Predation threat is another important stimulus in activating the stress response. It has been shown that stonechats (*Saxicola torquata*) faced with either common fiscals (*Lanius collaris* - Scheuerlein *et al*., 2001) or tawny owls (*Strix aluco* - Canoine *et al*., 2002) (both predators of stonechats) showed elevated plasma CORT concentrations, although females threatened with common fiscals showed no such elevation. Free-living great tits (*Parus major*) have failed to exhibit elevated plasma CORT concentrations in the presence of a stuffed, slow moving Tengham's owl (*Aegolius funereus*), but captive great tits did (Cockrem & Silverin, 2002). It is most likely that this effect is a result of the direct threat assessed by an individual, with captive great tits housed within a few feet of the predator and with no means of escape. Finally, it has been shown in song sparrows (*Melospiza melodica*) that reductions in food in a high predator location elevated plasma CORT concentrations to a greater extent than when individuals were faced with one of the stressors in the absence of the other (Clinchy *et al*., 2004).

1.2.7 Human interactions

Interaction with humans and human products is not part of the natural life cycle for most birds and mammals and, as such, is often perceived as a threat and thus a stressor. In a recent review of human disturbance, 64 studies were cited in which the investigators themsleves, visitors or aircraft / watercraft had effects on colonial nesting waterbirds (Carney & Sydeman, 1999). The main distress to birds following human

contact is thought to be due to the perceived predation risk (Walther, 1969, Frid & Dill, 2002). The response of an animal to human exposure, however, may be complex and related to the proximity, the number of interactions and patterns of visitations of humans (Beale & Monaghan, 2004*a*). In addition, it is likely that human contact during development can change the responsiveness of the stress axis. For example, in a study on the differences between undisturbed and tourist-visited colonies of Magellanic penguin chicks (*Spheniscus magellanicus*), Walker *et al*. (2005) found that newly hatched chicks in the visited areas had elevated stress-induced CORT responses, but similar baseline concentrations compared to unvisited individuals. By fledgling age (40-50 days), however, chicks from both locations showed almost identical CORT stress responses when captured, but 'undisturbed' chicks fled from the nest when humans were a greater distance away than 'visited' chicks. Adult Magellanic penguins are known to have less-sensitive stress responses to capture and restraint than found in chicks (Walker *et al*., 2006), which is likely to be a consequence of some form of continued habituation (which has been shown above to start as early as the fledgling stage of life), in conjunction with other factors such as time spent fasting.

1.3 MATERNAL EFFECTS

In addition to the effects of hormones produced by adults and chicks on their own physiology and behaviour, it has been shown that mothers have the ability to pass hormonally mediated 'information' onto their offspring during development. Changes in hormone concentrations can shape an embryo's development in preparation for the environment in which they may be born (Bateson *et al*., 2004). This is often referred to

as a ‘maternal effect’. Thus, we can define maternal effects as ‘effects on the phenotypic development of the offspring that arise from the behaviours and physiological state of the mother’. Such maternal effects are not limited to single-generation inheritance, with the potential for changes to occur for several generations even if the initial environmental factor has changed or is no longer present (Mousseau & Fox, 1998). Examples of known maternal alteration in egg content, both passive and active, that result from environmental changes (in birds, but in various taxa also) include changes in **nutrients / carotenoids** (Wilson, 1997; Berthouly *et al*., 2008; O’Brien & Dawson, 2008), **hormones** (Verboven *et al*., 2003; Love *et al*., 2005; Sockman *et al*., 2008) and **immunoglobulins / antibodies** (Hargitai *et al*., 2006; Pihlaja *et al*., 2006; Gasparini *et al*., 2007; Kilpimaa *et al*., 2007).

Although other animals can be used, birds and their eggs provide an ideal system to study such maternal effects. We already know a considerable amount about the life histories, physiology, behaviour and development of many avian species and egg sampling allows the hormones of maternal origin to be more easily isolated from any produced or used by the developing embryo (important in studying maternal effects) when collected within a suitable period after laying (dependant on the species). Hormones are laid down in the yolk over the course of approximately one week (depending on the species) and in the last 24 hours into the albumen (Conrad & Scott, 1935; Warren & Scott, 1935). Therefore, yolk measurements can give us an idea of the physiological state of the mother over a relatively long timeframe, where as albumen is limited to a much smaller series in time. Birds also have the advantage that effects arising from the pre-laying/laying environment can be more easily isolated from those

of the rearing environment via cross-fostering experiments. Eggs allow for a non-invasive sampling method (important when dealing with the stress response) and allow for sampling of maternal blood (invasive) and faeces (non-invasive) if needed.

Since Schwabl (1993) identified that testosterone was deposited in egg yolk, work to date relating to maternal effects has primarily focused on the effects of sex steroids, such as testosterone and oestradiol. Work on black-headed gulls (*Larus ridibundus*) has also shown that the increased concentrations of these androgens within the eggs result in phenotypic changes that include earlier hatching (Eising *et al*., 2001); enhanced begging behaviour and alertness (Eising & Groothuis, 2003); enhanced growth (Eising *et al*., 2001; Groothuis et al., 2005*a*); suppressed immune function (Groothuis *et al*., 2005*a*), and lower mortality (Eising & Groothuis, 2003; Groothuis *et al*., 2005*b*). Further, it has been shown that alterations to the maternal environment, for example by increasing nest densities (Groothuis & Schwabl, 2002; Groothuis *et al*., 2004) and food availability (Verboven *et al*., 2003) can result in effects on yolk steroid concentrations and subsequent chick performance (Lipar & Ketterson, 2000; Schwabl & Lipar, 2002; Strasser & Schwabl, 2006; Eising et al., 2006; Groothuis & Schwabl, 2008).

Sex steroids are mainly produced in the cell layers of the females' ovaries (Porter *et al*., 1989; Kato *et al*., 1995; Gomez *et al*., 1998) with small concentrations produced in the adrenal glands – Schlinger *et al*., 1999), hence there is the potential for direct transfer of steroids into the egg during its formation. CORT, however, is produced outside the ovaries by the adrenal glands and therefore can only enter the yolk

and albumen from the maternal bloodstream. Evidence from Hayward & Wingfield (2004), Hayward *et al*. (2005) and Love *et al*. (2005) has shown that elevated maternal plasma concentrations of CORT in birds correlate with elevated deposition of CORT in egg yolk. However, it is not possible to say from these studies if the transfer occurs via diffusion or active transport. It is therefore important to assess the effects of different environmental conditions on maternal CORT concentrations and when/whether maternal CORT is transferred to the egg to try and shape chick growth and development, and the adult phenotype (Bateson *et al*., 2004).

CORT exposure during development has been shown to correlate with slowed growth and increased HPA activity under acute (capture) stress during later life, in captive Japanese quail (*Coturnix japonica*) (Hayward & Wingfield, 2004). Although major changes to the well-evolved stages of embryonic development are typically unwanted, maternal information received by an embryo during this critical (but phenotypically plastic) phase may be adaptive and increase fitness after hatching (Monaghan, 2008). During unpredictable conditions (e.g. food shortages), reduced growth can theoretically reduce the burden on parental care and especially during food shortages, reduce the metabolic requirements of developing chicks (Frigerio *et al*., 2001; Hayward *et al*., 2005). Reduced growth rates in turn have also been linked with altered personalities (Jones, 1996; Jones *et al*., 1997), whereby chicks that grow slower also show increased 'fearfulness'/anxiety, a potentially adaptive strategy when faced with an unpredictable environment outside the relative safety of the nest. It has also been reported that there are additional benefits and potential adaptive consequences of elevated CORT in nestlings. For example, elevated CORT concentrations just after

hatching have been found in greylag geese (Frigerio *et al*., 2001) and it has been shown that this initial high steroid dose may help social imprinting. Maternal CORT during the first few hours after hatching may also aid young birds upon exposure to unique environmental conditions before their stress response system is fully developed and active (Sims & Holberton, 2000) and remain elevated for the first few days when precocial offspring need to imprint on their parents, feed themselves and face sibling competition to establish their rank order (Kalas, 1977; Nakamura *et al*., 1978; Lorenz, 1988). In addition, early experiences in life (in conjunction with genetic make-up) may determine the effectiveness of the stress response in adulthood (de Kloet *et al*., 1998; Meaney, 2001; Workel *et al*., 2001; de Kloet *et al*., 2002), with poor 'performance' in the nest affecting later fitness levels. Rubolini *et al*. (2005) found that the eggs of yellow-legged gulls (*Larus michahellis*) with experimentally elevated concentrations of CORT show a range of effects on the offspring including delayed hatching (without affecting hatching success), increased mass loss during incubation (except the last-laid eggs), reduced rate and volume of late embryonic vocalisations, reduced begging displays and reduced T-cell mediated immunity (an immune response which does not involve antibodies and is most effective in removing virus-infected cells).

In summary, we already know a great deal about the physiology, behaviour and development of birds and much of this knowledge has the potential to be used in comparisons with other vertebrates. CORT is an important hormone with many effects, one of those being as a response to changing environmental conditions and possibly communicating information to developing offspring about these environments.

1.4 THESIS CONTENT

The main aim of this study was to develop techniques and sampling methods to be used to identify whether there might be differences in the hormonal environment in which avian chicks develop that could be related to environmental factors experienced by the mothers. Subsequently, this work has focused on using avian eggs as a non-invasive means of investigating these maternal effects.

The development and validation of assays to measure CORT in yolk and albumen in avian eggs are addressed in **Chapter 2**. **Chapter 3** investigates the effects of unpredictable food availability on yolk CORT concentrations (in the zebra finch) and **Chapter 4** aims to investigate if yolk CORT concentrations vary in relation to population trends in two species of closely related gulls. We investigate if human disturbance can alter the deposition of CORT into yolk in the same gulls (**Chapter 5**), as well as presenting work on the effects of shelter and predation risk on yolk CORT concentrations in the common eider (*Somateria mollissima*) (**Chapter 6**). The final experimental chapter (**Chapter 7**) investigates if albumen CORT concentrations are related to lysozyme concentrations (a measure of immunity) in the gulls used previously. Finally, the findings of the thesis are discussed in a general context (**Chapter 8**).

CHAPTER 2

ASSAY VALIDATION AND DEVELOPMENT

2.1 ABSTRACT

The increasing popularity of endocrine measurements to help explain ecological and behavioural observations in captive and free-living animals has provided some fascinating results in recent years. However, for these measurements to provide meaningful results it is of critical importance that the techniques are validated for each species and sample type. This thesis will analyse the corticosterone concentrations in blood samples and eggs from zebra finches (*Taeniopygia guttata*) and eggs from two species of closely related gulls, the lesser black-backed (*Larus fuscus*) and herring (*Larus argentatus*) gull, as well as the common eider duck (*Somateria mollissima*). While the corticosterone assay has already been validated for use in zebra finch blood and egg yolk, validation studies have not been conducted for the other species and media, thus we present details of our extraction methods and radioimmunoassay for measuring corticosterone in the yolk and albumen.

2.2 INTRODUCTION

The increasing popularity of endocrine measurements to help explain ecological and behavioural observations in captive and free-living animals has provided some fascinating results in recent years. However, for these measurements to provide meaningful results it is critically important that the techniques undergo the proper validation studies for the species and samples being tested (Buchanan & Goldsmith, 2004). Failure to do so will lead to misinformation, both with regard to scientific findings and for other investigators who wish to repeat the published methodologies. As noted in the commentary by Buchanan & Goldsmith (2004), publication of validations is no longer a requirement in most journals, particularly as the marriage of endocrinology, ecology and animal behaviour has expanded the range of journals in which such results might be published. This has created problems for researchers keen to crossover into this field, but who lack a full understanding of these issues. One solution that exists for ecologists (and other researchers) is to form collaborations with expert endocrinologists.

This PhD has involved collaboration between two research groups. The group led by Prof. Monaghan has interests in the long term consequences of early life conditions, working on wild and captive populations of birds (but also other taxonomic groups), while Prof. Evans' group has interests in the ability of mothers to influence offspring development by manipulation of endogenous steroid conditions during development. Subsequently, my research has focused on maternally derived hormones

in avian eggs and their impact on offspring development, as well as using the measurement of these egg hormones as a non-invasive means to assess the physiological state of these mothers. Key to measurement of any hormones is the proper validation of extraction techniques and radioimmunoassay (RIA). Here, I present details of these validations for measurement of the primary avian stress hormone, CORT, in the yolks of the common eider duck (*Somateria mollissima*) and in the yolks and albumen of two closely related gulls, the lesser black-backed (*Larus fuscus*) and herring (*Larus argentatus*) gull. In addition, I address some issues found in the development and validation of the assay, as well as including the full RIA protocols.

2.3 METHODOLOGY

The basic theory of an RIA is that the hormone (CORT in this case) in the samples/standards will compete with a known amount of tritiated (radioactive) CORT to bind to a known amount of a specific CORT antibody. Thus the percentage tritiated CORT bound to the antibody will decrease as the concentration of unlabelled (non-radioactive) CORT within the sample/standard increases. Thus, the amount of tritiated CORT measured in samples can be quantified by comparison to known standards.

2.3.1 Yolk Extraction

The first stage of the radioimmunoassay involved the extraction and purification of the desired hormone from the sample substrate i.e. removal of any substances (such as

lipids and proteins) that can interfere with the RIA (Rash *et al.*, 1980). CORT was extracted from yolk samples following modification of the method described by Verboven *et al.* (2005). In detail, eggs were thawed and the shells, albumen and yolk separated. 1g of yolk was mixed with an equal amount of deionised water in an Eppendorf tube and homogenised with glass beads that had been cleaned with 70% ethanol prior to being added to the tubes. For each sample, approximately 500mg of the homogenised yolk/water mix (1:1) was weighed and transferred to a 12x75mm glass borosilicate tube. To each tube, 100µl of tritiated CORT ([1,2,6,7-^{3}H] Corticosterone, TRK406, Amersham Biosciences, UK) at approximately 4000-6000 counts per minute (cpm) was added, to allow assessment of recovery efficiency. Triplicates of the tritiated CORT (100µl) solution added to the samples were aliquoted into plastic assay tubes (Sarstedt, Leicester, UK), 1ml of scintillant added (Ecoscint A, National Diagnostics, Hull, UK) and the tubes counted on a Packard Tri-Carb Liquid Scintillation Counter (PerkinElmer Life And Analytical Sciences, Connecticut, USA) for a measure of the total number of counts added (a measure of 100% recovery efficiency). Comparison of the number of counts remaining in the samples after the two main stages of extraction (the methanol stage and the column stage) to these 'total counts' allowed assessment of extraction efficiency for each sample. The sample tubes were vortexed briefly following tritiated CORT addition and placed at 4˚C. After 24 hours incubation, samples were mixed with 2.5ml of 100% methanol (MeOH) (HPLC Grade Methanol, Rathburn Chemicals, Walkerburn, UK) and the tubes vortexed for one hour, before being centrifuged for 10minutes at 4000rpm. The resultant supernatant was poured off into new 12x75mm glass tubes and the used tubes decontaminated in diluted Decon 90 (Decon Laboratories, UK). From each sample, 500µl of supernatant was transferred to a

plastic assay tube, scintillant added (as above) and the tubes counted on the scintillation counter to obtain the MeOH recovery estimate by comparison with the total counts tubes. A further 1500µl of each sample was mixed with 13500µl of water, to give a 10% methanol solution before the samples were further cleaned by being passed through a C18 column (200mg, 3ml C18-220-0020-B, Isolute, International Sorbent Technology, UK). Prior to application of the sample, the columns were washed with 3ml of 100% MeOH (to clean), followed by 3ml of 10% MeOH (to condition). Following application of the samples, the columns were washed with 3ml of water and the CORT eluted from the column with 3ml of 80% MeOH. 500µl of this eluent was transferred to a plastic assay tube, scintillant added and the tubes counted on the scintillation counter, as before, to allow calculation of the column recovery estimate for each sample. A further 1500µl of each sample was added to a new 12x75mm glass tube and dried down on a heat block (60°C) under a stream of air using a sample concentrator (Teche, Cambridge, UK). Dried samples were resuspended in 330µl of assay buffer (Phosphate Buffered Saline with 0.25% BSA), and triplicates of 100µl transferred to plastic assay tubes ready for assay.

2.3.2 Albumen Extraction

For each egg to be analysed, 5 grams of albumen was placed in a 16x100mm borosilicate glass tube, which was then spiked with 300µl of tritiated CORT containing approximately 4000-6000 cpm, to allow later assessment of recovery efficiency. Triplicates of the tritiated CORT (300µl) were added to plastic assay tubes, scintillant

added and the tubes counted on the scintillation counter for a measure of the 'total counts'. Sample tubes were vortexed briefly following tritiated CORT addition and placed at 4˚C. After 24 hours incubation, samples were mixed with 5ml of 100% MeOH and the tubes vortexed for one hour before being centrifuged for 10minutes at 4000rpm. The resultant supernatant was poured off into 12x75mm glass tubes and the used tubes decontaminated in diluted Decon 90. Samples were then dried down on a heat block (60°C) under a stream of air using a sample concentrator. Dried down samples were resuspended with 3ml of 80% MeOH and vortexed for one hour. Following mixing, samples were centrifuged at 4000rpm for 10minutes and the supernatant poured off into new 12x75mm glass tubes. 500µl of this eluent was transferred to a scintillation vial, scintillant added and tubes counted on the scintillation counter, as before, to allow calculation of the extraction efficiency. A further 2100µl of each sample was added to new 12x75mm glass tubes and dried down on a heat block (60°C) under a stream of air using a sample concentrator. Dried down samples were resuspended with 330µl of assay buffer (Phosphate Buffered Saline with 0.25% BSA), and triplicates of 100µl transferred to plastic assay tubes ready for assay.

2.3.3 Radioimmunoassay

To measure CORT concentrations, samples were run in a double antibody RIA along with a standard curve of known amounts of the hormone (20ng/ml – 0.038ng/ml), using a modification of the methods described by Wingfield *et al.* (1991).

For the CORT assay used in this thesis, three plastic assay tubes were set up which contained 100µl of tritiated CORT (4000-6000 cpm) in PBS buffer (**Totals**); three tubes contained 200µl of normal rabbit serum (NRS) diluted (1:400) in PBS buffer and 100µl of tritiated CORT (**Non-specific Binding or NSB**); and three tubes contained 100µl of buffer, 100µl of anti-corticosterone antibody (B3-163, Esoterix Inc., Calabasas Hills, California, USA) diluted 1:175 with PBS buffer with NRS (1:400) and 100µl of tritiated CORT (**Maximum Binding or B_0**). Tubes containing either samples (100µl) or standards (100µl) also received 100µl of anti-corticosterone antibody (at 1:175 with NRS-mixed buffer as before), followed by 100µl of tritiated CORT. The tubes were then vortexed and incubated at 4ºC for 24hours. Following this, 100µl of a second antibody (Goat anti-rabbit IgG; Sigma-Aldrich, Dorset, UK), diluted 1:50 in PBS buffer, was added to all tubes except the Totals and the tubes vortexed before being incubated at 4ºC for a further 24 hours. Following this, 400µl of microcellulose (Sigmacell Cellulose, Type 20, Sigma-Aldrich, Dorset, UK) diluted in assay buffer (0.1g/100ml) was added to all tubes except the Totals. The tubes were spun for 50minutes at 2000rpm and the supernatant aspirated. The remaining pellets were reconstituted with 50µl of 0.1M sodium hydroxide, vortexed for 1minute and 1ml of scintillation fluid added before counting on a scintillation counter as before.

Counts obtained for the standards and unknown samples were analysed and converted to concentrations in the unknown samples using the universal assay calculator Assay Zap (vers.2.69, Biosoft, Cambridge, UK).

2.4 ASSAY VALIDATION

In order to validate and assess the accuracy of our RIA I assessed the vital components highlighted by von Engelhardt & Groothuis (2005), addressing each in turn.

2.4.1 Accuracy (variation from the true value)

In order to assess the accuracy of the assay, a known amount of hormone contained within the sample medium (a pool of several samples – 'pools') must be run through the complete assay procedure (including extraction). Pools can be tested by one of three methods. Firstly, pools can either be stripped of endogenous hormones using a charcoal solution before the addition of a known amount of unlabelled (non-radioactive) steroid (Schwabl, 1993). Charcoal stripping involves adding 1ml of charcoal solution (10mg/ml in PBS) to the sample, vortexing for 30 minutes and centrifugation at 4000rpm for 10 minutes at 4°C. The remaining eluent is separated from the charcoal pellet simply by pouring off and is then stored for use in the assay. We have not used this technique with the yolk samples as it has been found to alter the consistency of the yolk and therefore may not act in a similar fashion to an unaltered sample. As an alternative to charcoal stripping, pooled samples can be spiked with the varying concentrations of unlabelled hormone (Williams *et al.*, 2005). This technique assumes that the hormone concentration in the pools which were not spiked with hormone (the 'zeros') will be similar (if not identical) across replicates and this value must be subtracted from the

pools which were spiked with CORT to achieve the true value measured using the assay. We used this method for validating our assay procedure for CORT in the yolk of eider duck and herring and lesser black backed gulls. Specifically, we added 100µl of unlabelled CORT (50, 25, 12.5 or 6.25ng/ml) to triplicates of 500mg eider or gull yolk samples, as well as 100µl of buffer to the zero (0ng/ml) (again in triplicate). We calculated that the expected final concentration of hormone to be approximately 4 (50), 2 (25), 1 (12.5) and 0.5 (6.25) ng/ml (minus the zero) after taking into account the volumes used through the extraction procedure and into the assay. This calculation is achieved as the concentrations measured in the assay will be 12.5 times lower than the initial cold CORT added to the samples, as each sample is diluted through the extraction procedure and full volumes of extracts are not run through the assay. The CORT results obtained from the assay of these samples using yolk samples from lesser black-backed and herring gulls and the common eider are shown in Figures 2.1, 2.2 and 2.3 respectively. These figures show that there does not appear to be any other substances interfering with the assay and we can be confident we are measuring our compound of choice. The difference between observed and expected concentrations for all three species are summarised in Table 2.1. In addition to the above, to ensure there is no affect of sample media in the validation of an assay, increasing amounts of a sample should be run through extraction and assay. This was conducted for the albumen of eggs from the two gull species. Specifically, we extracted 2, 4 and 6g of sample from a single pool. The results of the assayed samples are shown in Figure 2.4.

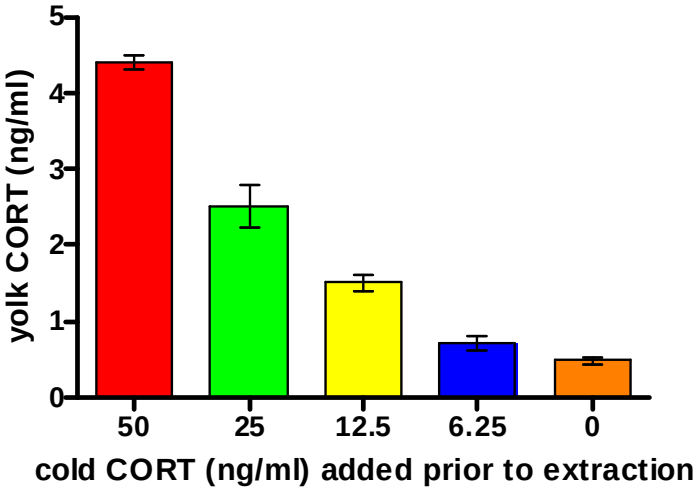


Fig.2.1. Mean yolk CORT concentrations (ng/ml) ± s.error for **lesser black-backed gull** samples spiked with cold CORT (50-0 ng/ml).

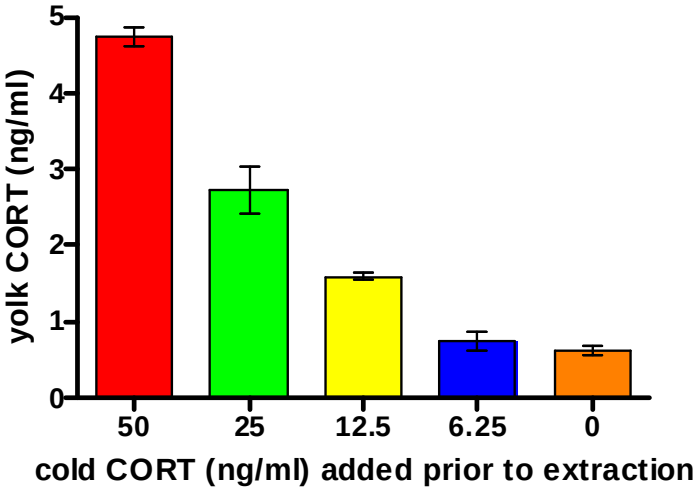


Fig.2.2. Mean yolk CORT concentrations (ng/ml) ± s.error for **herring gull** samples spiked with cold CORT (50-0 ng/ml).

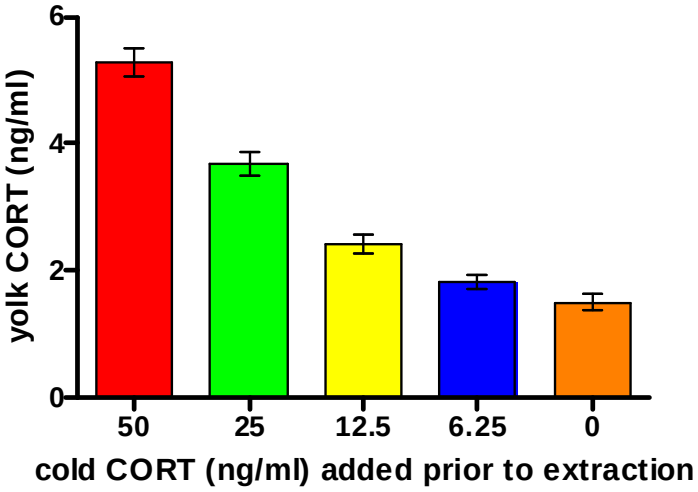


Fig.2.3. Mean yolk CORT concentrations (ng/ml) ± s.error for **eider duck** samples spiked with cold CORT (50-0 ng/ml).

Table 2.1. Observed and expected mean yolk CORT concentrations (ng/ml) for lesser black-backed and herring gull and eider duck samples spiked with cold CORT (50-0 ng/ml).

Cold CORT (ng/ml)	Lesser b-b gull		Herring gull		Eider duck	
	Observed	Expected	Observed	Expected	Observed	Expected
50	4.40	4.49	4.94	4.62	5.28	5.50
25	2.50	2.49	2.72	2.62	3.69	3.50
12.5	1.51	1.49	1.59	1.62	2.42	2.50
6.25	0.71	0.99	0.75	1.12	1.83	2.00
0	0.49		0.62		1.50	

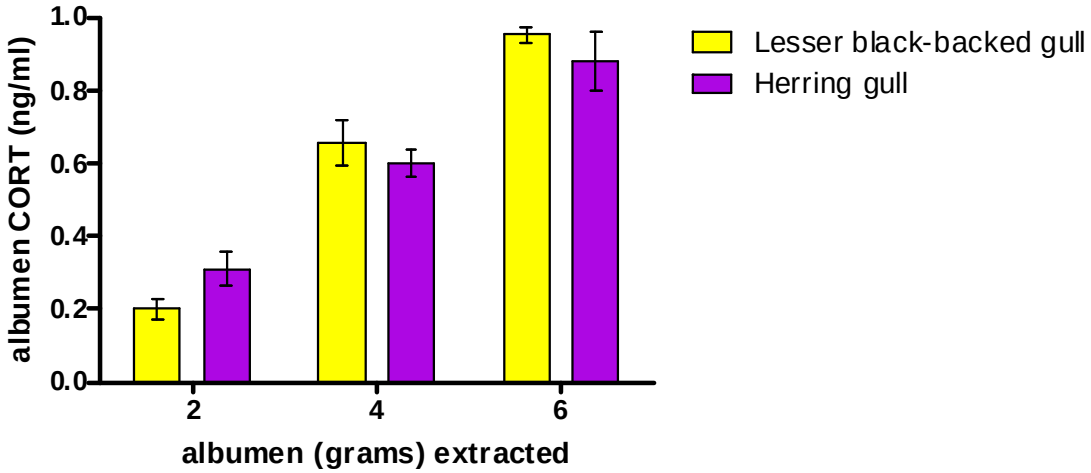


Fig.2.4. Mean albumen CORT concentrations (ng/ml) ± s.error for increasing amounts of albumen samples extracted (2g, 4g and 6g) for the lesser black-backed and herring gulls.

2.4.2 Extraction efficiency

Recovery efficiency of each sample is the measure of the accuracy of the extraction procedure, with known amounts of radioactive-labelled hormone added to samples before extraction and then recounted after extraction (see **sections 2.3.1 & 2.3.2** for details). We have evaluated our extraction efficiency to average roughly 80% for yolk and 90% for albumen, but this varied between samples and it is important to note that some samples had very poor extraction efficiency and needed to be re-extracted. It is becoming more common for this measure of extraction efficiency to be included in published work, but some studies use average extraction efficiencies (for example, from their Quality Controls / QCs, see Section 2.4.4). Average extraction efficiencies do not take into account individual variation within samples and extraction runs (see Love *et al,* 2005), which will add to errors in the data.

2.4.3 Specificity (cross-reactivity with other substances)

The antibody selected for an assay must be reviewed prior to running the RIA using the data sheet provided by the manufacturer. It is important to know if the antibody will detect the compounds of interest and if it cross-reacts with other compounds (giving false results). If such a sheet is not available, researchers will need to test their antibody for its cross-reactivity with other steroids. For our antibody (B3-163, Esoterix Inc., Calabasas Hills, California, USA), this data sheet (summarised in Table 2.2) is available on request from the manufacturer. Table 2.2 shows that our antibody has low cross-

reactivity with other hormones and hormone-metabolites, meaning we can be confident any binding in the assay is due to CORT being present rather than any other substances.

Table 2.2. Steroid cross-reaction with Esoterix corticosterone antibody, code B3-163.

COMPOUND	% CROSS-REACTION
Aldosterone	0.02
Cortisol	0.4
Cortisone	0.2
Desoxycorticosterone	4
Desoxycortisol	0.2
Dexamethasone	0.05
DHEA	0.03
Estradiol	0.01
Estriol	0.01
Estrone	0.03
17-hyrdoxy Pregenolone	0.01
17a-Hyrdoxy Progesterone	0.2
20a-Hydroxy Progesterone	0.2
20b-Hydroxy Progesterone	0.2
Prednisone	0.04
Prednisolone	0.01
5a-Pregnanedione	1
5b-Pregnanedione	1
Pregnanetriol	0.02
Pregnenolone	0.04
Progesterone	0.6
Testosterone	0.1
Tetra Hydro Cortisol	0.01
Tetra Hydro Cortisone	0.02

2.4.4 Precision (intra- and inter-assay variation)

The variation within triplicates (intra-assay variation) is an important aspect of the assay that should be reported when publishing results and is usually given as the intra-assay coefficient of variation (COV) (von Engelhardt & Groothuis, 2005). COV is calculated as the percentage variation across triplicates, summed and averaged for the total number of samples. When measuring yolk and albumen samples, intra-assay variation was seen

to be higher when compared to plasma assays. This can be due to the effects of extraction and as a mathematical artefact due to the lower concentrations of hormone seen in these media relative to plasma samples. Depending on the assay, this variation has been recorded at 10-21% in this thesis. In addition, inter-assay variation must also be included if more than one assay is required to measure your samples. Quality controls (QCs) run through these assays is the standard measure used to calculate this. QCs are samples with known amounts of unlabelled CORT that are run through all assays. Each triplicate of these samples contains either 100µl of 10ng/ml (low concentration QC) or 50ng/ml (high concentration QC) of unlabelled CORT, as the dilution factor of the extraction and assay means samples should appear on the reliable area of the bound/unbound standard curve. The inter-assay variation is defined as the percentage difference between the concentrations achieved for the low and high QCs between assays (i.e. low QCs from each assay are compared with each other and the same is done for the high QCs). This is calculated as the standard deviation of the means of the QC triplicates divided by the grand mean of the QC triplicates, multiplied by 100. Depending on the assay, this variation has been recorded at 6-8%. There is no universal scale for acceptable intra-assay variation, but we have set an arbitrary value of a 10% maximum.

2.4.5 Sensitivity (minimum hormone concentrations measurable)

Considerable time has been spent during the development of this assay on the development of an efficient and reliable method for extraction and assay. In all cases for a usable assay, the concentration of hormone in the sample has to be matched to the

concentration of hormone present in the standard curve and must fall on the linear part of the curve (Fig. 2.5) as to prevent incorrect measurements of the hormone concentrations present in a sample. The sensitivity of a radioimmunoassay can be increased by decreasing the concentration of primary antibody used (in our case from 1:100 for plasma to 1:175 for yolk and albumen), decreasing the maximum binding (from 20-40% down to 12-15%) and therefore shifting the curve to measure accurately at lower concentrations. If this is not possible or feasible, especially as CORT is assayed following extraction and resuspension, it may be possible to modify the 'amount' of hormone being assayed in each sample by either sample dilution or concentration. Greater quantities of yolk and albumen can be used, but this can make the process more time consuming and many species studied will not provide yolk or albumen samples large enough to justify redevelopment of the assay procedure. Calculating the sensitivity is an important step and should be recorded in any published works. Sensitivity is commonly defined as "two or three standard deviations (sd) above the blank value (B_0)" (von Engelhardt & Groothuis 2005) and provides a value of the minimum hormone that can be reliably measured. For our assay, we have calculated this figure as two standard deviations below the maximum binding (B_0) raw counts (i.e. the radioactive counts per minute / cpm), as this is the maximum antibody binding in the absence of any sample (which should give 0ng/ml). For our yolk and albumen assays, this has ranged from 0.11 to 0.2ng/ml respectively.

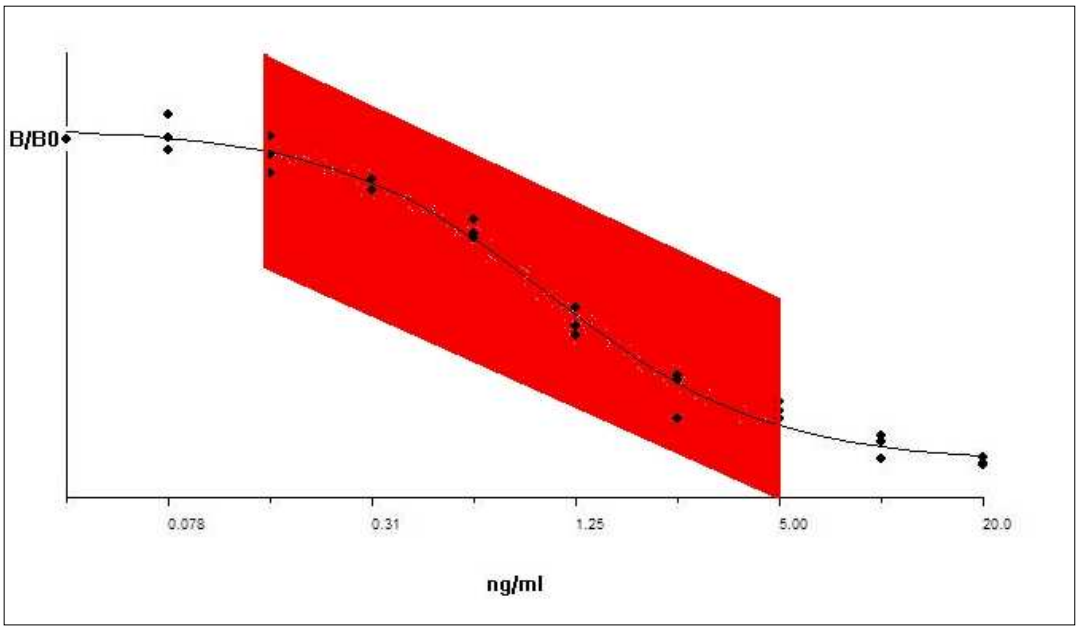


Fig.2.5. An example of a standard curve, including the linear area of the curve (highlighted) used as the reliable area of the curve for measuring CORT concentrations. Y axis (B/Bo) represents the bound to unbound ratio of antibody and the X axis (ng/ml) represents the concentration of hormone present according to the known standards.

2.5 COMPARISONS WITH PREVIOUS STUDIES

As mentioned already, published work using CORT measurements in yolk and albumen have varied in their reporting of recovery efficiencies, assay sensitivities and inter- and intra-assay variation. Table 2.3 summarises these values, where available, and shows that our recovery efficiencies and inter-assay variations are similar to those already reported, while intra-assay variation is similar to Love *et al.* (2005) & Rubolini *et al.* (2005), but typically higher than the other papers. While it is unknown why our intra-assay variation is high, the results for these studies show that varation exists across studies using the same methodology and species (Love & Williams, 2008, Love *et al.*, 2005, 2008). It is interesting that the only seabird study (on yellow-legged gulls –

Rubolini *et al*., 2005) shows similar intra-assay variation to our gull and eider assays. All these species have typically high fat, high protein diets (from eating fish and, at least in the gulls, human discards). It may be that the eggs of wild birds, especially seabirds, contain compounds that are not completely removed in the extraction procedure and possibly interfere with the assay to a greater degree than seen in the other species.

We have attempted to present evidence and advice on the development and validation of a CORT assay for gulls and eiders, but also hope that this will provide a guide for researchers trying to develop their own assays for measuring yolk and albumen CORT in these species (as well as being useful for other species, sample types and hormones). It is important for all researchers measuring hormones that although time consuming, proper validation and assay development is simply another part of good scientific practice.

Table 2.3. Summary of assay details and results from previous studies investigating avian CORT concentrations in yolk and albumen.

Study	Species		Sample medium	Assay type	Recovery efficiency	Assay sensitivity	Intra-assay variation	Inter-assay varia
Schwabl, 1993	Zebra Finch	(*Taeniopygia guttata*)	Yolk	Esoterix Antibody	44%	8pg / tube	n/a	
Hayward et al., 2005	Japanese Quail	(*Coturnix japonica*)	Yolk	Esoterix Antibody	60-61%	n/a	6-9%	
Love et al., 2005	European Starling	(*Sturnus vulgaris*)	Yolk	Assay Designs EIA Kit	83-85%	32pg / well	14.30%	
Rubolini et al., 2005	Yellow-legged Gull	(*Larus michahellis*)	Yolk	ICN Biomedicals I125 Kit	n/a	n/a	14%	
Saino et al., 2005	Barn Swallow	(*Hirundo rustica*)	Albumen	ICN Biomedicals I125 Kit	89.4-91%	n/a	4%	
Hayward et al., 2006	Japanese Quail	(*Coturnix japonica*)	Yolk	Esoterix Antibody	92%	n/a	6%	
Navara et al., 2006	Eastern Bluebirds	(*Sialia sialis*)	Yolk	Esoterix Antibody	66%	n/a	7.40%	
Love et al. 2008	European Starling	(*Sturnus vulgaris*)	Yolk	Assay Designs EIA Kit	94.60%	32pg / well	7.60%	
This study, Chapters 4 & 5	LBB & Herring Gull	(*Larus fuscus & L.argentatus*)	Yolk	Esoterix Antibody	81.3 - 87.6%	0.13 - 0.18ng/ml	14.4 - 21.1%	5.95 -
This study, Chapter 7	LBB & Herring Gull	(*Larus fuscus & L.argentatus*)	Albumen	Esoterix Antibody	90.40%	0.19 - 0.23ng/ml	16.5 - 17.5%	
This study, Chapter 6	Common Eider	(*Somateria mollissima*)	Yolk	Esoterix Antibody	85%	0.27 ng/ml	18%	

CHAPTER 3

UNPREDICTABLE FEEDING CONDITIONS AND THE MATERNAL TRANSMISSION OF CORTICOSTERONE

3.1. ABSTRACT

Experimentally restricted food availability in the laboratory, or changes in prey dynamics in the wild, can activate the stress response and elevate plasma corticosterone (CORT) concentrations in many species. In breeding female birds, these environmentally induced changes in CORT concentrations have been shown to correlate with concentrations of CORT in egg yolk. In addition, poor environmental conditions have the potential to negatively impact on reproductive output. In this study, we investigated whether unpredictable food availability increased yolk CORT concentrations in first and replacement clutches and maternal plasma CORT concentrations. We also examined the effect of unpredictable food availability on clutch sizes and how this related to changes in CORT concentrations in the zebra finch (*Taeniopygia guttata*). We found that unpredictable feeding conditions elevated yolk CORT concentrations in the replacement clutch, but not in the first clutch. We propose that there is a cumulative effect of laying a second clutch and unpredictable feeding conditions, resulting in high CORT concentrations in the yolk. In addition, females reduced their clutch size under unpredictable feeding conditions, possibly as a means of maximising their reproductive success under the prevailing conditions.

3.2 INTRODUCTION

As already described in **Chapter 1**, the unpredictable occurrence of poor environmental conditions can disadvantage an individual and result in physiological change from a normal hormonal state to what is often referred to as a stressed state. One result of this change, which has been reported in various species, is to elevate concentrations above baseline levels of a class of steroid hormones referred to as glucocorticoids (CORT in birds and cortisol in mammals). Experimentally restricted food availability in the laboratory or changes in prey dynamics in the wild can activate this change. As described in **Section 1.2.4**, previous work has shown that reduced food availability elevates plasma CORT concentrations in several species of bird. These changes in CORT concentrations are thought to help individuals cope with environmental stressors as they induce foraging behaviours (Astheimer *et al*., 1992; Bray, 1993; Lynn *et al*., 2003) and increase locomotor activity, as seen in altricial White stork nestlings preparing to fledge (as parents stop feeding their young in preparation for them leaving the nest) (Corbel *et al.*, 2003).

Food availability outside a laboratory environment is extremely difficult to control, and can be confounded by other environmental variables. This makes it difficult to assess the extent to which unpredictably of food and poor body condition are involved. The less complex captive environments (Chamove & Anderson, 1989; Buchanan-Smith, 1997) make it possible to tease these apart. Food availability is an

easy feature to manipulate in captive birds, as these individuals are normally exposed to *ad libitum* food. Therefore, any restrictions in food availability could be perceived as stressful and elevate plasma CORT concentrations in the absence of any change in body condition. Indeed, this protocol has been shown to elevate CORT concentrations in captive red knots (*Calidris canutus*) (Reneerkens *et al*., 2002). Here we investigate whether unpredictable food availability can activate the stress response in breeding, captive zebra finch (*Taeniopygia guttata*) females.

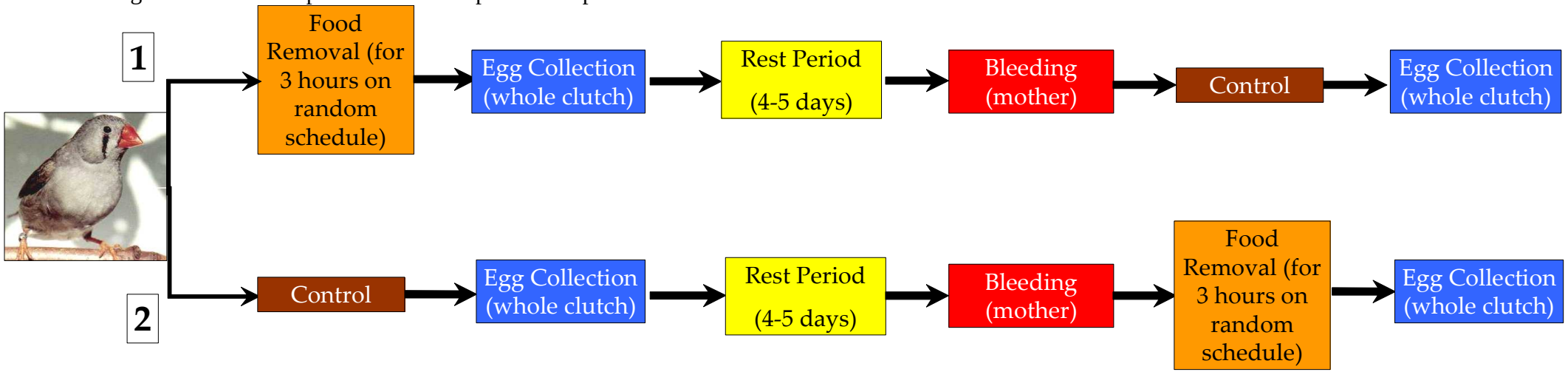
As described in **Chapter 1**, Hayward & Wingfield (2004) have shown that Japanese quail mothers, in which circulating CORT concentrations were experimentally elevated (using slow-releasing CORT pellets placed under the skin), deposited greater amounts of CORT in their egg yolks. We, therefore, also wished to investigate whether any increase in maternal plasma CORT concentrations in response to unpredictable food availability also result in increased deposition of CORT into eggs. Bernardo (1996) postulated that there is a trade-off between allocation of resources to offspring and the need to meet your own energy demands. Therefore, under poor environmental conditions (for example, low food availability, low temperatures or inclement weather), individuals might be physiologically stressed and may reduce the number of offspring produced (Perrins, 1970). Results obtained by Salvante *et al.* (2007) support this hypothesis, as zebra finches breeding under lower ambient temperatures lay smaller clutches. However, the physiological mechanisms leading to such clutch size reduction are not known. It could be hypothesised that under decreased temperatures, CORT concentrations could be elevated and that this increase in CORT could lead to reduced reproductive output. The same group, however, have previously shown that CORT

implants alone have no effect on clutch size (Salvante & Williams, 2003). This suggests that poor environmental conditions promote changes in resource allocation and egg production, but that the physiological mechanism behind this change may be independent of a direct effect of CORT or a prolonged steady increment in CORT as would be produced by the CORT implants. Therefore, in addition to determining if CORT concentrations are elevated in the yolk of eggs laid by mothers experiencing unpredictable food availability, we wished to see if this environmental stressor was accompanied by a change in clutch size.

The experiment was conducted using captive-bred zebra finches that were normally provided with *ad libitum* food. We exposed breeding pairs to unpredictable, short-term food removal and blood samples were collected from mothers (basal plasma concentrations). Clutch size and CORT concentrations in egg yolk were also measured. This protocol enabled us to address the following hypotheses:

1. Unpredictable food availability increases CORT concentrations in both maternal plasma and in egg yolk.
2. Maternal plasma CORT concentrations are correlated with the concentration of CORT deposited into the yolk of their eggs.
3. Females will reduce their clutch size when faced with unpredictable food availability.

Fig.3.1 Schematic representation of experimental protocol



3.3 MATERIALS AND METHODS

3.3.1 Experimental protocol

The experimental design is summarised in Figure 3.1. Ten pairs of captive adult zebra finches were used in this experiment (October 2006), housed and tested under Home Office licence. During the experiment, one male died leaving a sample size of nine pairs. Each pair was allowed to breed twice – once under unpredictable food availability, the other with access to *ad libitum* food (control). Unpredictable food availability was created by removal of food from the cage for three hours a day, on a random schedule, between the hours of 7am and 7pm, seven days a week (i.e. for 25% of the daylight hours). Food availability treatments began two days into nest building for the first clutch (allowing 2-4 days of experimental unpredictable food availability before laying began) and continued until the clutch was complete (between 2-6 eggs). Eggs were collected on the day of lay and replaced with dummy eggs (made from 'Fimo'; Staedtler, Pontyclun, UK), which warm upon incubation to promote normal incubation behaviour. The (dummy) clutches were removed within 24 hours of clutch completion and birds were allowed a 4-5 day rest period with *ad libitum* food, but without being allowed to enter the nest box to attempt relaying. Midway through this rest period, females were captured, mass was recorded and a blood sample obtained by brachial venipuncture (within sixty seconds of capture) for determination of basal plasma CORT concentrations. Measurement of maternal CORT was not possible at the time of egg laying under the two feeding conditions as the handling involved would

have added an additional stress to the birds. We also did not measure stress-induced plasma CORT concentrations in order to minimise any effects of prolonged handling stress on the second breeding attempt. Once the rest period was complete, pairs were transferred to their next treatment, with the order they experienced control or unpredictable food being randomly selected.

3.3.2. Study species

The zebra finch is a small (12-15g), sexually dimorphic, colonial passerine, native to Australia and the Lesser Sunda Islands (Birkhead & Fletcher, 1995; Zann, 1996). Zebra finches are known as 'opportunistic breeders' in the wild as they will breed following sufficient rainfall and grass seed growth (which are used for feeding young). This willingness to breed at any time of the year given suitable conditions has promoted their widespread use in laboratory studies, including investigations of avian physiology and behavioural ecology (Williamson *et al*., 2008). Eggs are typically laid at a rate of one per day and incubation starts after the last egg is laid. A typical clutch is between two and five eggs, although they can lay up to 8. The female does the majority of the incubation (although the male does contribute a significant amount) and the eggs will begin to hatch (asynchronously) after approximately thirteen days. Eggs mass has been found to increase with egg order in captive-bred zebra finches (Williams 2001; Rutkowska & Cichon, 2002; Royle *et al*., 2003), possibly counteracting the disparities between early- and late-laid eggs (Rutkowska & Cichon, 2005).

3.3.3 Radioimmunoassay

CORT was extracted from yolk and plasma samples and measured by radioimmunoassay using the protocols described in **Sections 2.3.1 & 2.3.3**. CORT was measured in all samples in one assay. Extraction efficiency averaged 85.3 ± 8% (sd); the intra-assay coefficient of variation was 10.9% and assay sensitivity averaged 0.11ng/ml.

3.3.4 Statistical analysis

A repeated measures linear mixed model (SPSS ver.15, SPSS Inc., Illinois, USA) was used for the analysis of yolk CORT concentrations as females were exposed to both control and unpredictable food availability, with female identify included in the model as a random factor and egg order and clutch number (1^{st} or 2^{nd}) as repeated variables. Egg order, treatment (food availability), clutch size and clutch number were also included in the model, and female plasma CORT concentration was used as a covariate. A repeated measures GLM was also used to analyse the effect of unpredictable food availability on clutch size in each female, with treatment order (control followed by unpredictable food and vice versa) included as a between subjects comparison and female plasma CORT concentration included as a covariate. Analysis of effects of unpredictable food availability on plasma maternal CORT concentrations was done by GLM, with female mass and order the birds were bled included (to account for any handling differences as blood samples were taken) as covariates.

3.4 RESULTS

Eggs collected from clutches laid by birds maintained under unpredictable feeding conditions were found to have significantly higher concentrations of CORT in their yolks compared to eggs taken from clutches laid to pairs maintained on *ad libitum* food (Fig.3.2, $F_{(1,29)} = 16.49$, $p < 0.001$). There was a significant overall effect of clutch number on yolk CORT concentrations (independent of food availability), with eggs from 2nd laid clutches showing higher CORT concentrations ($F_{(1,40)} = 7.921$, $p = 0.008$). There was also a significant interaction between clutch number and food availability on yolk CORT concentrations (Fig.3.3, $F_{(1,30)} = 9.569$, $p = 0.004$), with the effects of unpredictable food availability being much greater when this occurred as birds were laying the second clutch. Statistical analysis indicated that yolk CORT concentrations were significantly correlated with basal maternal CORT concentrations (used as a covariate) taken during the rest period ($F_{(1,23)} = 23.942$, $p < 0.001$). This result was confirmed by Linear Regression to be a positive relationship between maternal plasma and yolk CORT concentrations (Fig.3.4., $F_{(1,67)} = 9.138$, $p = 0.004$, $r^2 = 0.122$). There was no effect of laying order (egg number) on yolk CORT concentrations ($F_{(1,35)} = 1.808$, $p = 0.187$) and this term was removed from the final model.

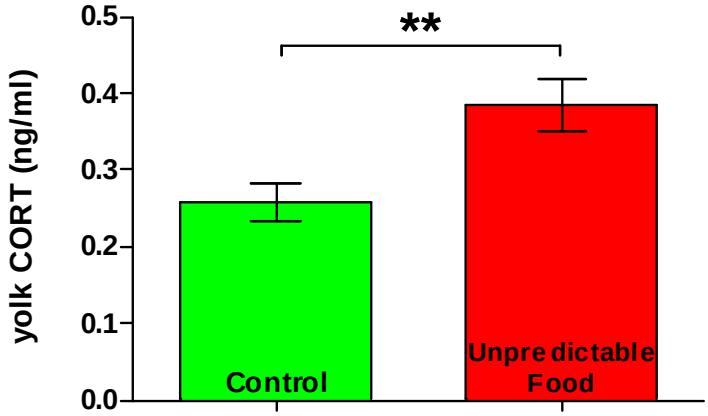


Fig.3.2. Mean yolk CORT concentrations (ng/ml) ± s.error for control (*n* = 32) and unpredictable food availability (*n* = 36) conditions (** = p<0.01)

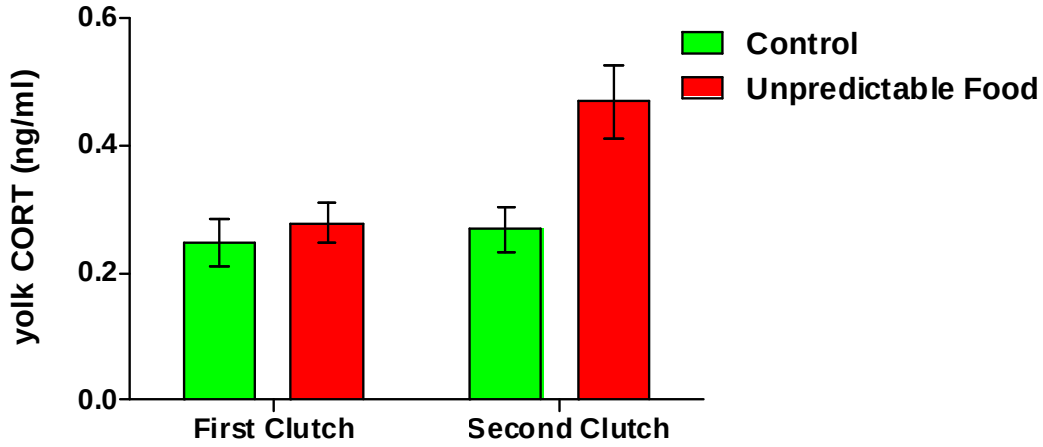


Fig.3.3. Mean yolk CORT concentrations (ng/ml) ± s.error for control and unpredictable food availability conditions split according to clutch number. Control First Clutch *n* = 12, Unpredictable Food First Clutch *n* = 19, Control Second Clutch *n* = 20, Unpredictable Food Second Clutch *n* = 17. (clutch*treatment, p=0.004).

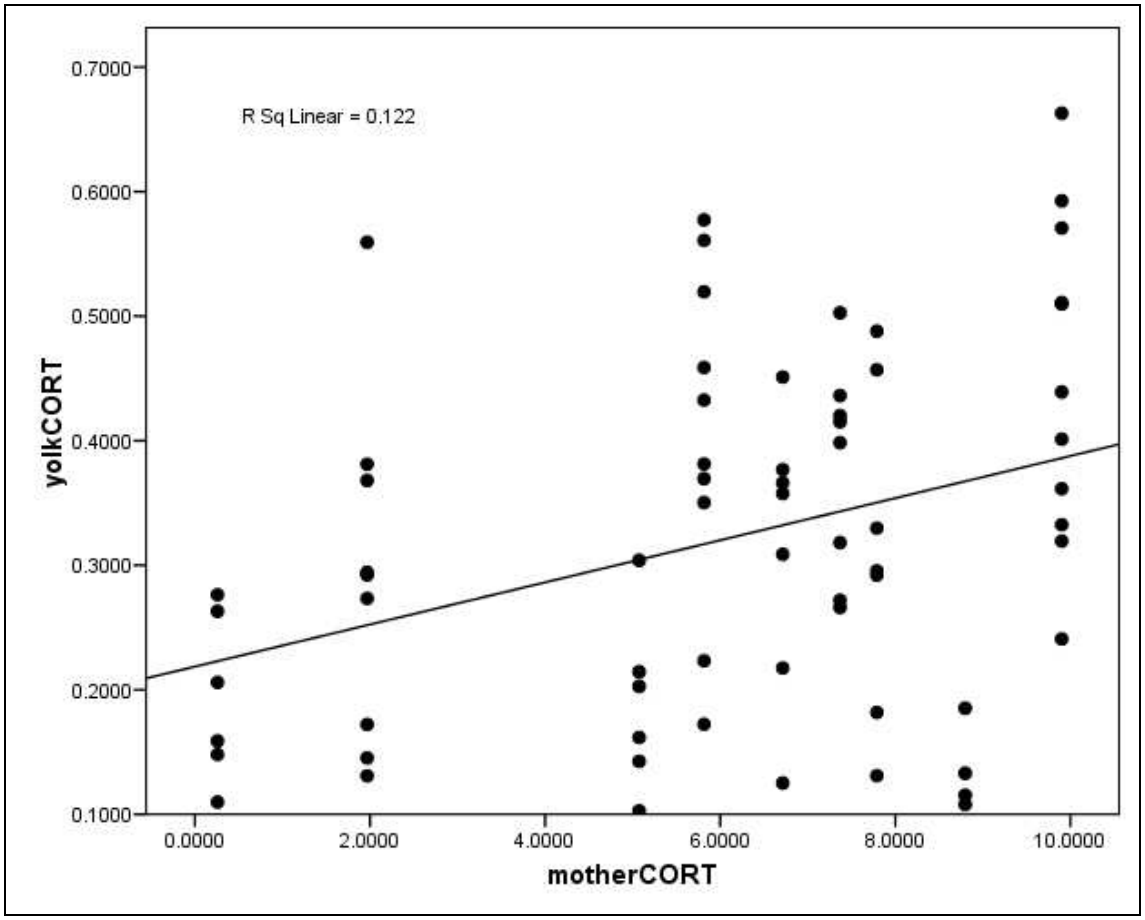


Fig.3.4: Linear relationship between maternal plasma CORT (motherCORT) and yolk CORT measured using Linear regression. $p = 0.004$, $r^2 = 0.122$.

When examining the effect of food availability on clutch size it was found that unpredictable food availability significantly decreased the size of clutch produced by a female (Fig.3.5, $F_{(1,7)} = 7.658$, $p = 0.028$), and this was relationship did not vary according to treatment order ($F_{(1,7)} = 0.479$, $p = 0.511$). There was no significant relationship between basal maternal plasma CORT concentrations and clutch size ($F_{(1,7)} = 0.237$, $p = 0.644$), so this term was removed from the model.

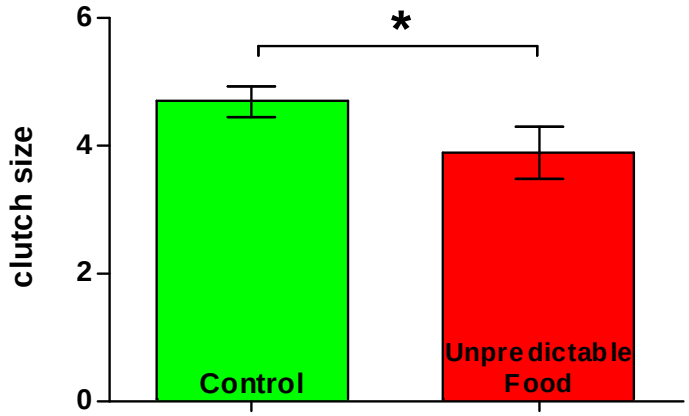


Fig.3.5. Mean clutch sizes ± s.error laid under control (*n* = 9) and unpredictable food availability (*n* = 9), where * : p = 0.028)

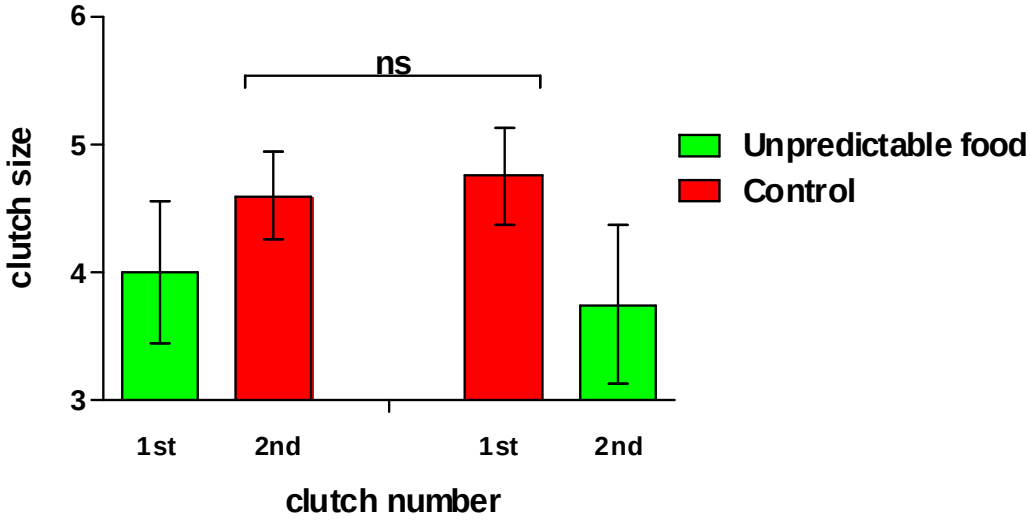


Fig.3.6. Mean clutch sizes ± s.error during control and unpredictable food availability according to treatment order, where *ns*: p = 0.511. Unpredictable food followed by control (*n* = 5) and control followed by unpredictable food (*n* = 4).

Basal plasma maternal CORT concentrations, as measured between the two breeding attempts, did not vary with food availability during the preceding breeding attempt (although there is a weak trend towards higher CORT concentrations with unpredictable food availability) (Fig.3.7, $F_{(1,9)} = 3.477$, p = 0.099). Body mass was also

not affected by food availability and there was no correlation between body mass and CORT concentrations ($F_{(1,9)} = 1.355$, $p = 0.283$). Body mass was subsequently removed from the model. The order the birds were bled showed no significant relationship with maternal plasma CORT concentrations and this variable was also removed from the model ($F_{(1,9)} = 0.707$, $p = 0.433$).

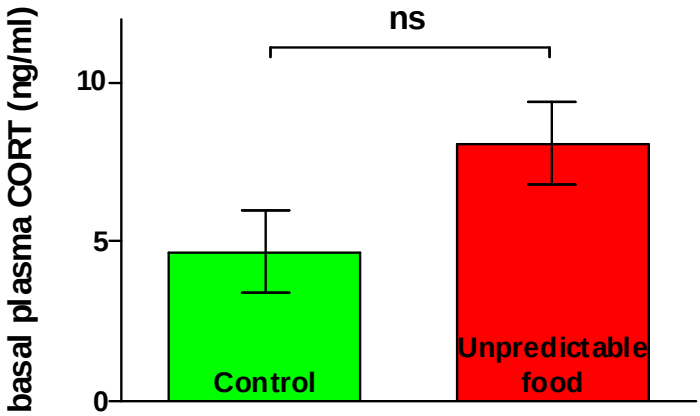


Fig.3.7 Mean maternal basal plasma CORT concentrations (ng/ml) ± s.error for control (*n* = 4) and unpredictable food availability (*n* = 5) conditions. *ns*: p = 0.099.

3.5 DISCUSSION

These results demonstrate that unpredictable feeding conditions can raise plasma CORT concentrations in female zebra finches and that unpredictable feeding conditions are an important factor in the deposition of CORT into yolk, but this relationship is not a simple one. Although we have shown yolk CORT concentrations increase under unpredictable feeding conditions, this effect was only significant in the 2nd clutch. The increases in yolk CORT concentrations in the 2nd clutch may be the result of the additive pressure of laying a 2nd clutch and being faced with the potential of lower

energy resources (through unpredictable food), thereby putting more strain on parents and possibly activating the stress response to a greater extent (and passing this information on into the yolk). Alternatively, females may expect that conditions will remain stable during the production and laying of the 2nd clutch. When this is not so, it may activate the stress response and in turn increase the deposition of CORT into yolk.

We were only able to measure maternal plasma CORT approximately 4 days after the completion of clutch one. As such, we are aware that it only provides an index of the activity of the HPA-axis during the first breeding attempt and assumed that this correlated with concentrations during laying. We found a weak trend for female plasma CORT to be elevated after the 1st clutch was laid under unpredictable feeding conditions. Interestingly, this elevation in plasma CORT was not reflected in the egg yolk concentrations (where there was no difference between treatment groups). Previous work on the relationship between maternal plasma CORT and egg yolk CORT is not straightforward as although Hayward & Wingfield (2004) have shown that elevated concentrations of CORT in maternal plasma (reflecting stress-induced levels) in Japanese quail are reflected by elevated yolk CORT concentrations in their eggs, the same group also found plasma baseline concentrations do not correlate with maternal deposition of CORT into the egg (Hayward *et al*., 2005). Rather, they are related to the high or low stress response sensitivity in the mothers (i.e. the elevated plasma CORT concentrations, where high = large elevation above baseline levels). High response females had more CORT in their eggs than low stress response females, both without any stress-induced experimentation, and this difference was multiplied following stress exposure. They suggested that this leaves the door open for a potential control

mechanism in yolk deposition, rather than simply environmental influences alone. The trend of elevated plasma CORT in the current study was protracted, since it was evident in basal CORT after a rest period of 4 days on *ad libitum* food. It is therefore proposed that the unpredictable feeding condition used in this experiment was a strong enough stressor to activate the stress response, in such a way that CORT concentrations remained elevated for four days with food freely available. This response by females could confer a possible adaptive advantage if unpredictable conditions were to return, as an individual may be 'primed' to react quickly, but requires either prolonged exposure to these negative conditions and/or other negative/costly factors (e.g. having to lay a second clutch) for females to transmit information about the environment (in the form of CORT) to the offspring (Jenni-Eiermann *et al*., 2008).

Egg production and incubation in birds are known to be energetically costly (Perrins, 1970; Monaghan & Nager, 1997; Vezina & Williams, 2002, 2005). Therefore, we propose that the high costs associated with laying two clutches in rapid succession, as occurred in this experiment, induces females to reduce reproductive effort (Drent & Daan, 1980; Stearns, 1992). We have confirmed that unpredictable food availability can influence reproductive investment by females via reductions in clutch size.Under conditions of unpredictable food availability, we have shown that females lay smaller clutches compared to those in control conditions (as predicted). This effect can be seen in both the 1st and 2nd clutches, despite the fact yolk CORT concentrations are only elevated in those laying a 2nd clutch. This finding would support the results found by Salvante & Williams (2003) and Salvante *et al.* (2007), where elevated CORT does not result in decreased clutch sizes, but a poor environment (low temperature) does,

suggesting that CORT is not directly involved in the mechanisms of reducing egg production. However, clutch size reductions may be caused by females reducing their clutch under poorer conditions as an initial means of minimising the effect of unpredictable feeding on the offspring and thereby maximising reproductive success (as, for example, 3 chicks are easier to feed to their required level for growth than 4) during early breeding attempts. Then, as reproductive investment increases with the 2nd clutch and individuals are faced with poor feeding conditions as well, females may limit their clutch size rather than risk decreasing their body mass (as seen with the clutch size reduction in the 1st clutch), but also deposit greater amounts of CORT into the yolk. This deposition of CORT may be unavoidable (as suggested above) or an attempt to prepare the chicks for an unpredictable post-hatch environment. A study by Frigerio *et al*. (2001) found that greylag geese chicks had elevated plasma CORT concentrations just after hatching and during this time growth was limited, which would appear contradictory to the selective pressure to grow quickly and decrease the time taken to fledge. However, this may be an evolutionary compromise between the need to compete and survive during the first few hours and days, and the need to grow and fledge as quickly as developmentally possible (Arendt, 1997). This would support the findings of Hayward & Wingfield (2004), who found that chicks that hatched from eggs containing elevated yolk CORT concentrations had increased HPA activity and reduced growth. If food becomes unpredictable, reducing growth (through elevated CORT) would reduce the demands for high food levels to satisfy the high metabolic costs of rapid growth (Gotthard, 2001), thereby giving these chicks an adaptive advantage over nestlings not prepared for an unpredictable environment.

Although unpredictable feeding conditions have the potential to increase CORT deposition in eggs (and possibly in adult plasma concentrations too), other environmental conditions, genetic make-up, previous breeding attempts and body condition (in both partners) all may interact to determine CORT transmission. It may be important in future studies to measure both basal and stress-induced CORT concentrations in not only females but also males after the final egg has been laid to determine any potential paternal effects (e.g. breeding with a poor quality male may promote changes in CORT deposition by females to compensate for paternally-inherited negative traits). Perhaps a longer rest period between treatments would allow plasma CORT concentrations in females to return to baseline.

In summary, we have shown that unpredictable feeding conditions can alter the concentrations of CORT found in egg yolks of captive-bred zebra finches and that these conditions may have the potential to elevate circulating CORT concentrations in breeding females. However, there are significant effects of reproductive effort in a season (laying two clutches) which can alter the deposition of CORT into yolk. What is unknown is the mechanism of control a female has in transmitting these signals of environmental change directly to their developing offspring and if the transmission of CORT is linked to the genetically-based sensitivity to stress. Finally, the role (if any) that stress and CORT play in reducing clutch sizes is still not clear, but it would appear that poor environmental conditions signal to females that a reduction in clutch size may be an early and low cost way of maximising reproductive success.

CHAPTER 4

ENVIRONMENTAL CONDITIONS AND THE MATERNAL TRANSMISSION OF CORTICOSTERONE

4.1 ABSTRACT

Herring gulls (*Larus argentatus*) and lesser black-backed gulls (*Larus fuscus*) are closely related species that breed in overlapping colonies. In non-urban environments, herring gull numbers have declined in Britain and Ireland over the last three decades, while the lesser black-backed gull numbers have increased. This suggests that the two species are experiencing differences in environmental harshness, which might be reflected in differences in corticosterone (CORT) concentrations. Such differences, if transferred to the offspring via eggs, could have phenotypic consequences. Yolk CORT concentrations can be used as an indicator of the maternal stress state and elevated yolk CORT can have detrimental affects on embryonic development and offspring fitness. We present results from several locations in Scotland comparing yolk CORT concentrations within species between urban and non-urban breeding sites, as well as comparing between the two species at sites where they coexist. We have found no significant differences in yolk CORT concentrations between the environments or the species. This could suggest that environmental stressors have little impact on the transfer of CORT into yolk, possibly an evolutionary adaptation to minimise elevations in CORT being detrimental to embryonic development, or that environmental conditions are not sufficiently stressful to activate the stress response during the breeding season.

4.2 INTRODUCTION

Herring gulls (*Larus argentatus*) and lesser black-backed gulls (*Larus fuscus*) are closely related species that breed in overlapping colonies, not only in coastal zones, but also in urban areas. In non-urban environments, herring gull numbers have declined in Britain and Ireland over the last three decades (and possibly longer) while the lesser black-backed gull numbers have increased (Fig.4.1; Table 4.1) (Monaghan, 1979; Creme *et al*., 1997; Raven & Coulson, 1997; Mitchell *et al*., 2004). During this time, both species have increasingly inhabited the urban environment where breeding success can be higher than in the non-urban habitats, possibly due to reduced predator load, improved thermal environment and abundant food sources (Kadlec & Drury, 1968, Spaans, 1971; Verbeek, 1977; Burger, 1981; Patton, 1988; Belant & Dolbeer, 1993; Wanless et al., 1996; Belant, 1997; Creme et al., 1997; Raven & Coulson, 1997). In urban habitats in the UK, herring gull colonies have increased in size 6-fold in the last 30 years, while lesser black-backed gull numbers have increased more than 30-fold, according to survey data (Fig.4.1; Table 4.1). As the rise in herring gull numbers in urban habitats does not equal the decline in non-urban areas, and as gulls are also known to show high site fidelity during their lives (Ludwig, 1962; Drury & Kadlec, 1974; McNicholl, 1975), it suggests that the population dynamics in the herring gulls are not solely due to relocation but in part to the death of individuals, fewer adults breeding each season and/or reduced breeding success (Forrester *et al*., 2007). The further contrast in lesser black-backed gull numbers which have increased in both urban and non-urban environments raises the questions as to what is the cause of the

difference in population trends seen between these two species in the non-urban environments and why is this difference not also seen in the urban populations?

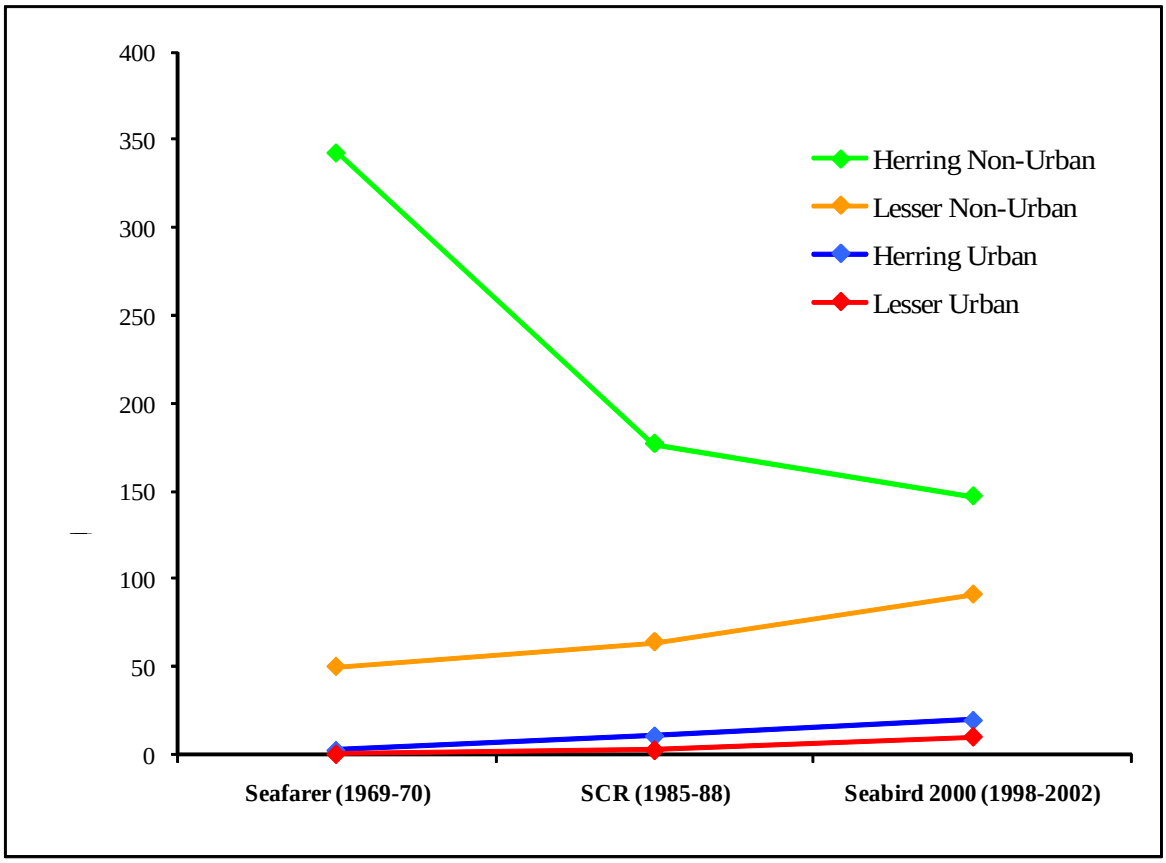


Fig.4.1. The number of adult breeding pairs for non-urban and urban populations of herring and lesser black-backed gulls, taken from the Seafarer survey (1969-70), the Seabird Colony Research census (1985-88) and the Seabird 2000 census (1998-2000) (Mitchell *et al.*, 2004)

Table 4.1. The number of breeding adult pairs for non-urban and urban populations of herring and lesser black-backed gulls, taken from the Seafarer survey (1969-70), the Seabird Colony Research census (1985-88) and the Seabird 2000 census (1998-2000) (Mitchell et al., 2004)

	Lesser Non-Urban	**Herring Non-Urban**	**Lesser Urban**	**Herring Urban**
Seafarer (1969-1970)	50,000	343,000	300	2900
SCR (1985-1988)	64,000	177,000	2500	11,000
Seabird 2000 (1998-2002)	91,000	147,000	10,000	20,000

When considering this question a number of issues become apparent. As both species often nest in overlapping colonies with direct competition appearing to be limited in terms of available nesting sites (particularly due to the declines in herring gull numbers) and food (Garthe *et al*., 1999, Kim & Monaghan, 2006), negative factors in the non-urban habitats that impact upon the herring gulls should also affect the lesser black-backed gulls. For example, it has been shown that the incidence of botulism does not differ between the species (Mitchell *et al*., 2004), and hatching success and chick survival are also comparable between the two species (Kim & Monaghan, 2006; Forrester *et al*., 2007). Alternatively, if environmental conditions (food, predation, warmth) are, on a whole, equal for both species, the difference in population trends might arise if one species, in this case the lesser-black-backed gulls, are better able to cope when faced with the negative aspects within the environment. One mechanism used by animals to initially cope with negative and/or unpredictable conditions is the elevation of glucocorticoids in the blood (as described in **Chapter 1**), with elevated CORT concentrations usually reflecting more stressful, negative and/or unpredictable conditions. In turn, the difference between baseline and stress-induced CORT concentrations reflect an individual's ability to physiologically respond to and ideally cope under these conditions. So, a relevant question to ask if we wish to investigate the differential population dynamics of the two gull species in the non urban environments might be 'are the herring gulls showing elevated CORT concentrations compared to lesser black-backed gulls in this environment?' If this were found to be true, we would then predict that CORT concentrations should be more similar in the urban environment where both populations are increasing in number and specifically that CORT should be lower in the urban compared to the non-urban herring gulls.

Table 4.2 Conservation priority criteria for UK birds (adapted from 'The population status of birds in the UK – Birds of conservation concern: 2002-2007'; RSPB)

PRIORITY	DEFINITION	SPECIES EXAMPLES
RED	– Globally threatened – Historical population decline in UK (1800-1995) – Rapid (= or > 50%) decline in UK breeding population over last 25 years – Rapid (= or > 50%) contraction of UK breeding range over last 25 years	Aquatic warbler *(Acrocephalus paludicola)* Bullfinch *(Pyrrhula pyrrhula)* White-tailed eagle *(Haliaeetus albicilla)* Quail *(Coturnix coturnix)*
AMBER	– Historical population decline (1800–1995), but recovering; population size has more than doubled over last 25 years – Moderate (25-49%) decline in UK breeding population over last 25 years – Moderate (25-49%) contraction of UK breeding range over last 25 years – Moderate (25-49%) decline in UK non-breeding population over last 25 years – Species with unfavourable conservation status in Europe (SPEC = Species of European Conservation Concern) – Five-year mean of 1–300 breeding pairs in UK – = or > 50% of UK breeding population in 10 or fewer sites, but not rare breeders – = or > 50% of UK non-breeding population in 10 or fewer sites – = or > 20% of European breeding population in UK – = or > 20% of NW European (wildfowl), East Atlantic Flyway (waders) or European (others) non-breeding populations in UK	Dunnock *(Prunella modularis)* Peregrine *(Falco peregrinus)* Puffin *(Fratercula arctica)* Kingfisher *(Alcedo atthis)*
GREEN	– No identified threat to the population's status	Tawny Owl *(Strix aluco)* Sparrow hawk *(Accipiter nisus)* Robin *(Erithacus rubecula)* Little auk *(Alle alle)*

The easiest way in which to test these predictions in CORT concentrations would be to obtain plasma samples, but blood sampling can in itself be stressful and negatively affect breeding behaviours (e.g. can induce clutch abandonment). It is also very difficult to obtain baseline blood samples as the birds need to be trapped on the nest, distressing them (plus all other birds in proximity) on approach of the experimenter. Blood sampling is also difficult to perform on such large, colonially breeding birds and these birds are currently listed as amber conservation importance by the Royal Society for the Protection of Birds (RSPB) (see Table 4.2 for details of criteria) due to the decline in numbers, making it necessary to find alternate ways to measure the physiological stress response of the birds while minimising any 'researcher effects'. One method that has become of great interest recently due to its non-invasive nature is the sampling of eggs and the measurement of yolk CORT concentrations. Hayward & Wingfield (2004) have found that experimentally elevated maternal CORT concentrations correlate with elevated yolk concentrations in captive bred Japanese quail and therefore it is thought that they provide a true reflection of the maternal physiological condition at the time of laying. If our hypothesis is correct, and non-urban herring gulls have elevated plasma CORT concentrations, we would predict that this would be mirrored in the CORT concentrations in their eggs. These elevated yolk CORT concentrations have the potential to alter embryonic development and could have long lasting, intergenerational consequences in terms of embryonic development, growth and the sensitivity of the HPA axis. These alterations can affect long-term fitness and survival (Hayward & Wingfield, 2004; Rubolini *et al*., 2005), and therefore population numbers. Although the studies by Hayward & Wingfield and Rubolini have helped promote the measurement of yolk CORT concentrations as a reflection of

maternal stress-induced condition, most studies focus on manipulating a single, potential stressor (and therefore introducing a negative environmental condition) in a laboratory-based environment. For this study we are interested in investigating how CORT may be affected by non-manipulated environmental conditions, taking population-level effects into account as habitats are made up of complex, interacting environmental factors that may have positive or negative impacts under controlled conditions, but which may not reflect the true environmental conditions faced by an animal.

There are some considerations when sampling eggs, as well as some important drawbacks (see **Section 1.3** for details). Briefly, birds generally do not lay all the eggs in their clutch at the same time. Depending on the onset of incubation, relative to the time of laying, eggs may or may not hatch synchronously. A greater literature exists with regards yolk androgens (e.g. testosterone), which have been found to increase with laying order in a number of species (Schwabl, 1993; Sockman & Schwabl, 2000; Royle et *al*., 2001; Eising *et al.*, 2001), but a recent study (Love *et al*., 2008) is the first to study within-clutch yolk CORT variation and has found that there is a significant difference between first- and last-laid eggs in yolk CORT concentrations in the European starling (*Sturnus vulgaris*) (with the last laid egg having significantly higher CORT concentrations). Therefore, in considering CORT concentrations in the eggs of herring and lesser black-backed gulls, we need to assess whether egg CORT concentrations vary with laying order.

The overall aim of this study was to examine the degree of physiological stress exhibited by herring and lesser black-backed gulls in urban and non-urban populations. We had the following specific objectives, which can be split into those required to validate the approach taken and those required to address the overall aim.

We aimed to determine if:

1. Yolk CORT concentrations vary with laying order.
2. Yolk CORT concentrations vary within species according to habitat type (urban or non-urban).
3. There is a species difference in yolk CORT concentrations at sites where the two species coexist

Our predictions were that:

1. There would be no difference in yolk CORT concentrations according to laying order.
2. That yolk CORT concentration would vary with habitat type and follow the patterns seen by population trajectories, with the non-urban herring gulls having elevated yolk CORT levels compared to their urban counterparts. For the lesser black back gulls, we expected to see no significant difference between urban and non-urban colonies.
3. In non-urban conditions, the herring gulls would show elevated yolk CORT concentrations compared to the lesser black backs.

4.3 MATERIALS AND METHODS

4.3.1 Study sites

Eggs were collected at seven field sites throughout Scotland (Figure 4.2).

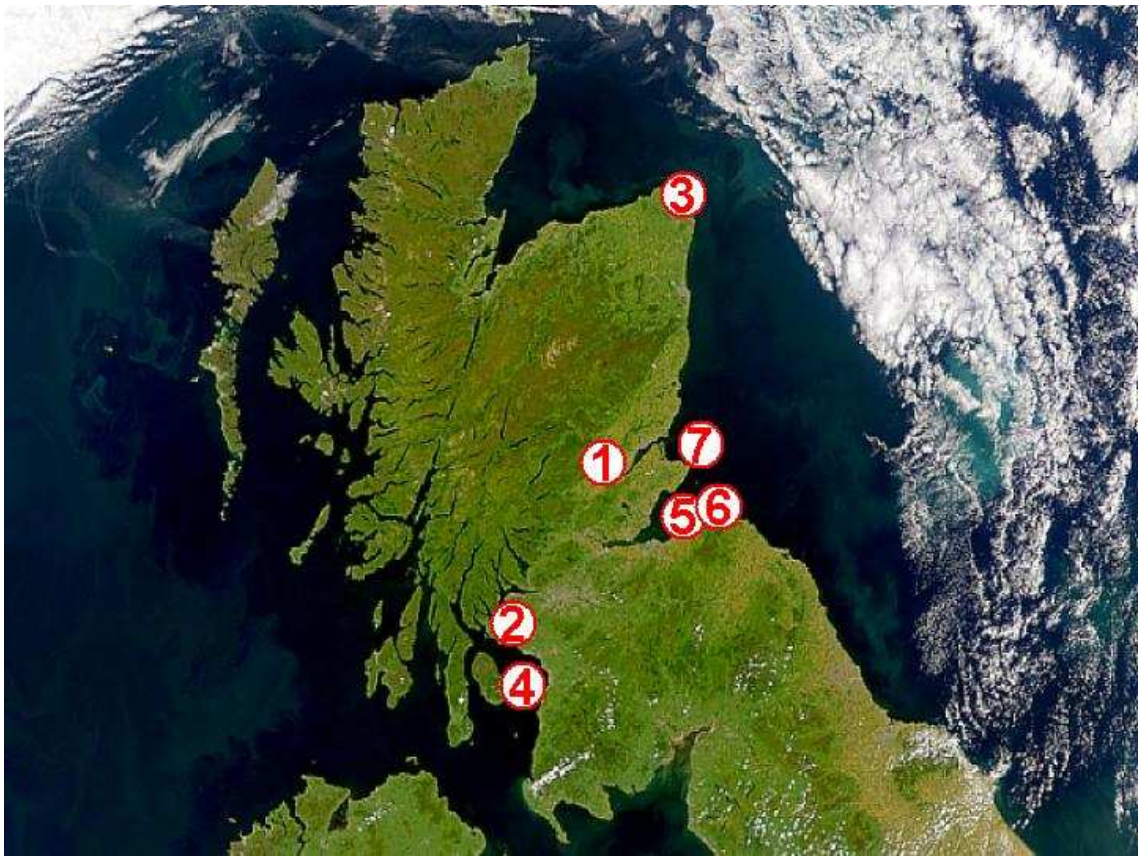


Fig.4.2. Field locations:

1. St. Serf's Island, Loch Leven, Perth, Kinross (Scottish Natural Heritage National Nature Reserve)
2. Inchmarnock Island, Bute, Firth of Clyde (private island and farm)
3. Peterhead, Aberdeenshire (town)
4. Ailsa Craig, Firth of Clyde (RSPB reserve)
5. Dunbar, East Lothian (town)
6. Musselburgh, East Lothian (town)
7. Isle of May, Firth of Forth (Scottish Natural Heritage National Nature Reserve)

St. Serf's, the largest of the seven uninhabited islands on Kinross' Loch Leven, is approximately 46 hectares and is situated three quarters of a mile from the south east side of the loch. It holds a population of lesser black-backed gull nesting pairs (613 - SNH) and a small population of herring gull pairs (estimated at 30 - SNH). It is important to note that the nest counts on the island have not distinguished between herring and lesser black-backed gulls, but observations of adults have allowed estimates that approximately 5-10% of the population is comprised of herring gulls. Data gathered by Scottish Natural Heritage (SNH) (Brooks, 1998 and from more recent surveys dating to 2007, unpublished) estimate that both populations increased from 1997 (after culling had ended, with an undetermined mixture of approximately 600 herring and lesser black-backed gull pairs) until 2002, with both species showing stable numbers each year since. The habitat is predominantly grass, which is grazed by sheep housed on the island during spring and summer, and the gulls breed in overlapping colonies on the south side of the island. Visitors are only allowed on the island with formal permission from SNH due to its status as a National Nature Reserve (NNR). Disturbance levels are estimated to be low.

Inchmarnock (NS 020, 600) is a privately owned island just over 2km off the west coast of the Isle of Bute in the Firth of Clyde. It measures 267 hectares, with the current owners having converted the central and west parts of the island into grazing land for the introduction of a herd of 180 highland cattle. Populations of herring and lesser black back gulls breed on the westerly and southerly coasts of the island, where the cattle and farmers rarely venture. These populations previously spread to the north coast of the island, but for unknown reasons have almost completely disappeared from

this part of the island over the last 20 years. The herring gulls predominately nest on the rocky cliffs, with the lesser black-backed gulls nesting on the grass adjacent. According to the Seabird 2000 Census, there were 200 lesser black-backed gull pairs on the island, 76% down from the previous SCR Census count (’85-’89), but it is not known if these declines were solely from the northern side of the island. The Seabird 2000 Census also estimated that there were 1550 pairs of herring gulls present on the island, 63% up from the SCR count. Current human disturbance levels are estimated to be low, but previous disturbances from visitors and poachers before the land was purchased by the current owners have to be taken into account.

Peterhead (NK 129, 466) is a small, coastal town roughly 50km north of Aberdeen, on the easternmost point of mainland Scotland, with a population of approximately 18,000 people. Across the four main towns in Aberdeenshire (Peterhead, Fraserburgh, Banff and Macduff) there are approximately 11,500 nesting pairs of herring gulls (Aberdeenshire Council), but no urban lesser black-backed populations are known. In Peterhead, the gulls nest on various buildings within the town and are subject to egg removal and nest destruction by pest controllers hired by the local council during the breeding season. Roughly 240 nests and 500 eggs have been removed between May and July, 2005-2007. Human disturbance is estimated to be high.

Ailsa Craig (NX 019, 997) is an uninhabited island in the outer part of the Firth of Clyde, approximately 16km west of Girvan and measures 104 hectares. The island was mainly used as a quarry for its high quality granite until the 1970s and has been an

RSPB reserve since 2004. Its steep cliffs make it an important site for nesting seabirds, including kittiwakes, guillemots, razorbills, and gannets. According to the Seabird 2000 Census there were 1,450 herring gull pairs and 400 lesser black-backed gull pairs on the island, down 38% and 78% respectively on the SCR Census (’85-’89). In recent years, the University of Glasgow and SNH have exterminated the rat population that had proved a particularly harmful predator to many of the breeding seabirds. Numbers are now believed to be stable and are possibly on the increase (RSPB). Visitors are only allowed on the island with permission from the RSPB. Disturbances levels are estimated to be low.

Dunbar (NT 675, 785) is a small coastal town in East Lothian, approximately 58km east of Edinburgh, with a population of ca. 6,500 people. According to the Seabird 2000 Census and the SCR Census (’85-’89), there were 3,500 herring gulls nesting in the entire East Lothian area between 1985 and 2002. While only 23 were nesting on rooftops in 1995 (Raven & Coulson, 1997), council estimates state that there are over 50 pairs of herring gulls nesting throughout the town of Dunbar alone, mainly on the rooftops and chimneys of the buildings on the main street. The council currently undertake an annual egg oiling and chick removal programme in the area between May and July. Human disturbance is high.

Musselburgh (NT 325, 724) is a town on the River Esk in East Lothian, approximately 9km east of Edinburgh, with an estimated population of 22,000 people. According to the Seabird 2000 Census, lesser black-backed gulls in the East Lothian area numbered 1,470, 30% up on SCR Census (’85-’89) and over 300% up from the

Seafarer survey (’69-’70). While the last published roof-top nesting counts recorded only one pair of lesser black-backed gulls throughout East Lothian in 1995 (Raven & Coulson, 1997), there is now known to be an extensive breeding colony throughout the town of Musselburgh (typically on residential rooftops and chimneys close to the town centre). The main colony is situated on the rooftop of the town’s bus station and is estimated by the local council to exceed several hundred nesting pairs. The council currently undertake an annual egg oiling and chick removal programme in the area between May and July. Human disturbance is high.

The **Isle of May** (NT 655, 995) is situated in the north of the outer Firth of Forth, approximately 8km south east of the fishing village of Anstruther and measures roughly 75 hectares. The island has been designated as a National Nature Reserve since 1956 and has been managed by SNH since 1989. According to the Scottish Ornithologists Club (SOC), over 200,000 seabirds from 12 species nest on the island during the breeding season. Herring gull numbers were estimated at 2,845 pairs in the Seabird 2000 Census (1998-2002), 35% up on the previous count from the SCR census (‘85-‘88). Lesser black-backed gull numbers were estimated at 1,203 pairs in the Seabird 2000 Census, 131% up on the previous estimates from the SCR census. Disturbance is estimated to be low.

4.3.2 Study species

Fig.4.3. Herring gull

Fig.4.4. Lesser black-backed gull

Herring gulls (Fig. 4.3) are large seabirds (55-67cm with a wing span of **130-158 cm**) with light grey backs and wings (with black and white wing-tips), white under parts, yellow bill and pink legs. The congeneric lesser back-backed gull (Fig. 4.4) is slightly

smaller than the herring gull (52-67cm with a wing span of 128-148cm), with dark grey to black backs and wings, yellow bill and yellow legs. Both species are sexual size dimorphic, with males being larger than females (Cramp & Simmons, 1983). They have very similar ecologies and life histories, although there are some differences. Herring gulls are resident all year round, with a general southerly migration over small/medium distances depending on sex and age, whereas the lesser black backs are typically a summer visitor (although this is changing, especially with the urban colonies) (Forrester *et al*., 2007). Both are colonial breeders, often breeding in the same sites, particularly in coastal areas, although the herring gulls' range inland is much smaller than that of the lesser black-backed gulls (accounting for less that 1% of the total herring gull population (Forrester *et al*., 2007). Females typically lay three eggs, with the third-laid (C) egg often smaller than the earlier laid (A and B) eggs (Parsons, 1970) and laying peaks in mid-May (herring gull) and the 3rd week of May (lesser black backs). For both species, incubation lasts roughly 4 weeks and chicks fledge after 30-40 days. Chicks hatch asynchronously, meaning the last chick can be disadvantaged compared to its older siblings (Parsons, 1975). The feeding ecology of both species is still not well understood, but both are scavengers on marine fish and on agricultural land / refuse tips. However, the lesser black-backed gulls are believed to travel further and are generally more marine feeders than the herring gull (Forrester *et al*., 2007).

4.3.3 Egg collections

Each of the seven locations described above were visited between 26th April and 24th May 2006. At the non-urban locations, nests with either one or two eggs present (A and B eggs of the maximum 3 egg clutch) were marked and observed to identify the species present. By collecting either the A or B egg at this stage allows females to replace the egg they have lost and improves the likelihood that any hormone concentrations measured are of maternal origin and have not been used up by the developing offspring and/or the offspring has begun producing its own hormones. A maximum of 10 eggs from each species were collected, with one egg removed per nest. Only nests containing one or two eggs were selected. During collections, the number of eggs present in the nest and the distance to the nearest neighbour were also recorded, as was the habitat surrounding the nest (e.g. on grass or rocks). For urban populations, nests were located according to previous records held by the pest controllers and either a hoist (in Dunbar & Musselburgh) or ladders (in Peterhead) were used by the pest controllers to gain access to the roofs where the nests were located. Any other eggs removed by the pest controllers were destroyed. All eggs were returned to the laboratory within a maximum of 12hours after collection, where they were weighed, the length and breadth measured (allowing for an estimate of volume to be made) and then frozen at -20°C before further analysis.

The Isle of May site was used to collect complete clutches from 10 nests of both herring and lesser black backed gulls. Nests were monitored from 25th April 2006, with the species present recorded. The distance to the nearest neighbour and details of the

habitat at the nest site were recorded for each nest from which eggs were collected. Eggs were only removed once the complete clutch had been laid (with the final clutch being completed on 11th May). The study site was searched for nests early in the season and fresh nests (with no eggs) were marked with numbered canes. Nests were visited daily, with fresh eggs individually labelled (using a non-toxic marker pen) in the order they were laid (A-C). Eggs were returned to the on-site field station, where they were weighed, and the length and breadth measured (to allow for an estimation of volume), before being frozen at -20°C until further analysis.

All collections were carried out under Scottish National Heritage licence and with the permission of the landowners and reserve managers as appropriate.

4.3.4 Radioimmunoassay

CORT was extracted from the gull egg yolks using a methanol extraction and measured by radioimmunoassay using the protocol described in **Sections 2.3.1 and 2.3.3**. Extraction efficiency averaged 87.6 ± 10% (sd). Inter-assay variation averaged 5.95% (2 assays), with an intra-assay coefficient of variation of 14.4%. Assay sensitivity averaged 0.13ng/ml.

4.3.5 Statistical Analysis

Linear mixed models (SPSS ver.15, SPSS Inc., Illinois, USA) (one for each species) were used to investigate the differences in yolk CORT concentrations according to laying order with female/nest number included as a random factor and egg order as a repeated variable. General linear models (GLM) (one for each species) were used to investigate within species differences according to habitat type (urban / non-urban) and a separate GLM was used to compare the two species at the non-urban sites where they coexisted. In all the models, collection date or laying date (if known), egg weight and nearest neighbour data were included as covariates and removed where non-significant (see section 4.4 below for details).

4.4 RESULTS

We found no effect of egg order on yolk CORT concentrations for either the lesser black-backed ($F_{(2,20)} = 0.351$; $p = 0.708$) or herring gulls ($F_{(2,16)} = 0.771$; $p = 0.479$) from the Isle of May (Fig. 4.5.). In addition, we found no effect on yolk CORT concentrations of laying date, egg weight or nearest neighbour for either the lesser black-backed (summarised in Table 4.3) or herring gulls (summarised in Table 4.4). These terms were subsequently excluded from the model.

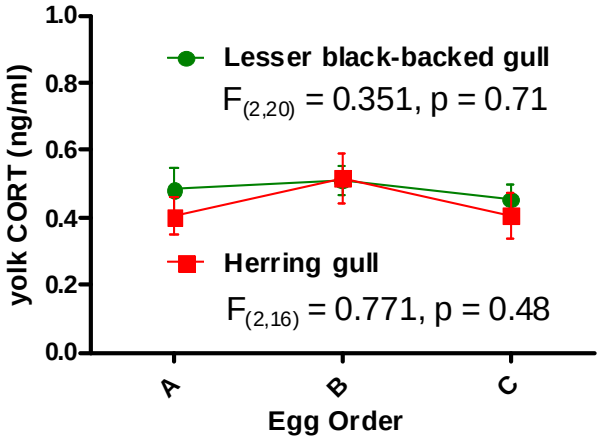


Fig.4.5. Mean yolk CORT concentrations (ng/ml) ± s.error for lesser black-backed (*n* nests = 10) and herring (*n* nests = 10) gulls according to egg laying order

Table 4.3. Summary of covariate effects on yolk CORT in the lesser black-backed gull egg order analysis

Source	Numerator df	Denominator df	F	Sig.
Laying date	1	22	0.003	0.9654
Egg weight	1	22	0.167	0.687
Nearest neighbour	1	22	0.399	0.244534

Table 4.4. Summary of covariate effects on yolk CORT in the herring gull egg order analysis

Source	Numerator df	Denominator df	F	Sig.
Laying date	1	23	0.758	0.395
Egg weight	1	23	0.956	0.338
Nearest neighbour	1	23	0.53	0.474

We also examined the effect of habitat type (urban or non-urban) on yolk CORT concentrations, but found no significant effect of habitat type in either the lesser black-backed ($F_{(1,42)}$ = 1.00; p = 0.323) or herring ($F_{(1,53)}$ = 0.33; p = 0.568) gull in terms of yolk CORT concentrations (Fig. 4.6). The effects of collection date, egg weight and nearest neighbour distance on yolk CORT concentrations are summarised in Tables 4.5 & 4.6, and where these covariates were non-significant (all except egg weight in the lesser black-backed gulls) they were removed from the model.

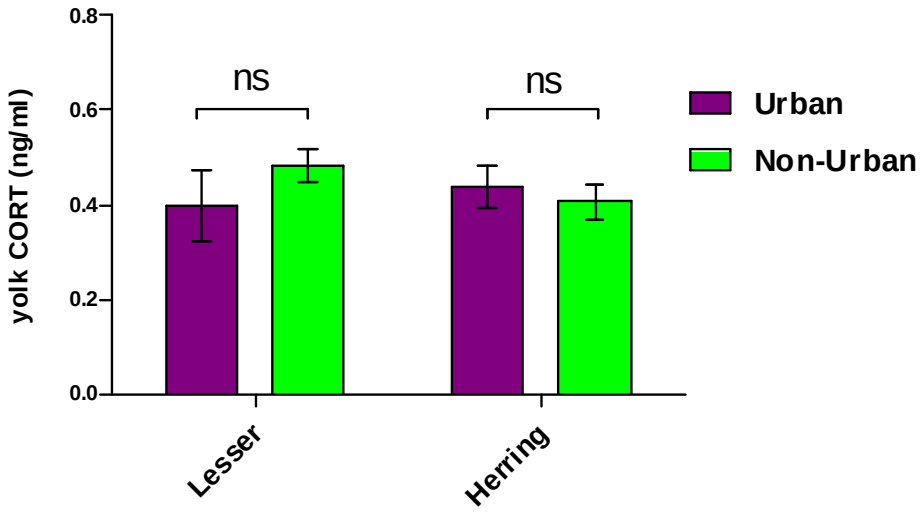


Fig.4.6. Mean yolk CORT concentrations (ng/ml) ± s.error compared between urban and non-urban habitats for lesser-black backed (urban *n* = 8; non-urban *n* = 35; where *ns*: p = 0.323) and herring (urban *n* = 21; non-urban *n* = 33; where *ns*: p = 0.568) gulls.

Table 4.5. Summary of covariate effects on yolk CORT from the urban / non-urban comparison in the lesser black-backed gull

Source	Numerator df	Denominator df	F	Sig.
Collection date	1	42	0.039	0.844
Egg weight	1	42	4.017	0.052
Nearest neighbour	1	42	0.06	0.808

Table 4.6. Summary of covariate effects on yolk CORT from the urban / non-urban comparison in the herring gull

Source	Numerator df	Denominator df	F	Sig.
Collection date	1	42	0.284	0.597
Egg weight	1	42	0.701	0.406
Nearest neighbour	1	42	3.33	0.074

Finally, we investigated whether there was a species difference between these gulls in their yolk CORT concentrations by comparing yolk samples taken from sites where both species co-exist. These four sites were all non-urban locations – St. Serf's, Inchmarnock, Ailsa Craig and Isle of May. We found no effect of collection date, egg weight or nearest neighbour distance on yolk CORT concentrations (summarised in Table 4.7) and these terms were subsequently removed from the model. We found no effect of species ($F_{(1,67)} = 2.70$; $p = 0.106$) or site ($F_{(1,67)} = 0.198$; $p = 0.897$), nor any interaction between species and site, in terms of yolk CORT concentrations ($F_{(3,67)} = 0.32$; $p = 0.811$) (Fig. 4.7). It is noteworthy from visual inspection of the data (Fig. 4.7) that there was a similar trend at three of the sites for the levels in lesser black backs to be higher than in the herring gulls. Applying a Fisher's combined probability test (Fisher, 1932; Sokal & Rohlf, 1995) to these three sites however showed that this effect was not significant ($\chi^2 = 6.9$, $df = 6$, $p > 0.4$).

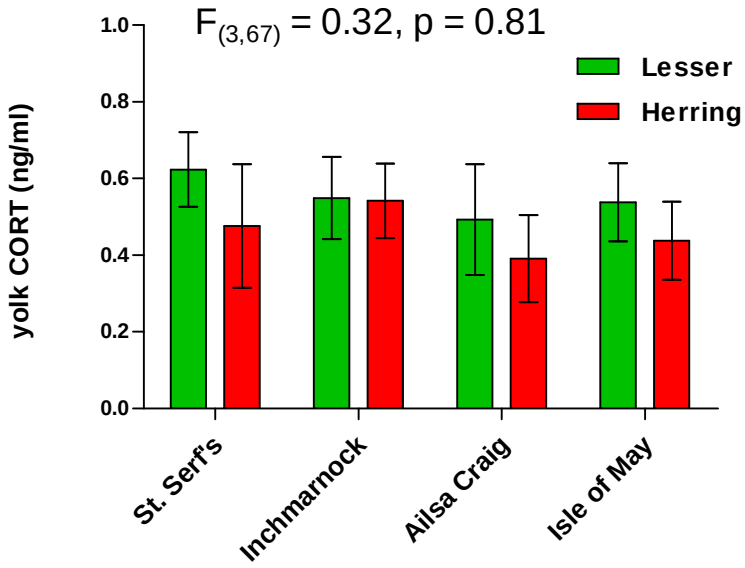


Fig.4.7. Mean yolk CORT concentrations (ng/ml) ± s.error for herring (*n* = 33) and lesser black-backed (*n* = 34) gulls from the four sites where they coexist.

Table 4.7. Summary of covariate effects on yolk CORT from sites where the two species coexist

Source	Numerator df	Denominator df	F	Sig.
Collection date	1	67	0.752	0.39
Egg weight	1	67	0.055	0.815
Nearest neighbour	1	67	2.379	0.129

4.5 DISCUSSION

This study found that yolk CORT concentrations in both herring and lesser black-backed gull eggs do not vary according to laying order. This result contrasts with that seen for other yolk steroids in other species (Schwabl, 1993; Sockman & Schwabl,

2000; Royle et *al*., 2001; Eising *et al.*, 2001) and CORT in the European starling (Love *et al*., 2008), which have all been shown to increase with laying order. It is proposed by Love *et al*. (2008) that this increase is a mechanism to assist in brood reduction if environments become poor after laying, with the later-laid chicks showing slower growth and more likely to be outcompeted by their older siblings. The lack of an effect of laying order on yolk CORT here possibly indicates that females are not trying to compensate for the negative effects on the offspring of being the last laid egg or attempting to promote brood reduction under poor post-hatch conditions. Alternatively, it may be that the laying order differences in other hormones such as testosterone, which we have found to elevate with egg order (see **Section 4.6**, Fig. 4.8) in these same eggs, have a strong enough effect in compensating for being the last laid egg that elevating CORT as well would counteract any positive effects of elevated testosterone.

As we do not have maternal plasma CORT samples we do not know if female CORT concentrations correlate with the concentrations seen in the yolks. Although the evidence provided by Hayward & Wingfield (2004) is strong and suggests a direct correlation between plasma CORT and yolk CORT, we have shown in **Chapter 3** that this may instead be a correlation between maternal condition and yolk CORT. If, however, the yolk CORT concentrations were to match maternal plasma CORT concentrations it may be that females have a suppressed stress response during the breeding season, resulting in low concentrations in the plasma, which are subsequently transferred to the yolk. Based upon published data from other birds, as well as amphibians, mammals and reptiles however, this explanation appears unlikely as stress hormone concentrations are normally highest (both baseline and stress-induced) during

the breeding season (Romero, 2002). An alternative explanation is that plasma and yolk CORT concentrations may not be correlated, where females might be able to regulate the amounts of CORT deposited in their eggs independent of laying order. This provides an advantage as it could be used to programme the embryonic development of the HPA axis (Hayward & Wingfield, 2004) and, if the adults are under severe stress, could prevent major alterations in the development of the embryo/chick (Liggins, 1994; McLusky & Naftolin, 1981; McEwen, 2001; Eriksen et al., 2003). What we can be certain of from our results is that these two species show no difference in the patterns of yolk CORT deposition over the clutch and in their mean yolk CORT concentrations. These samples were taken from nests on the same island (where both species have been increasing in numbers over the last 10 years) therefore, we did not expect to see any significant difference in yolk CORT between species as a result of different interactions with the environment. This result suggests that there may not be any inherent species difference present in terms of yolk CORT, however this was investigated further using the remaining clutches and is discussed later. Importantly as there was no effect of laying order on yolk CORT, these results validate the measurement of CORT in either 1st or 2nd laid eggs (where complete clutch collections were not feasible), as both represent the CORT concentrations that we would expect in the whole clutch.

As the population dynamics of the two gull species differed within the two environments (urban and non-urban), we aimed to use this difference to allow us to ascertain if the changes in population numbers were related to the 'environmental stress' the birds were subject to. We predicted that in the herring gulls we would see elevated CORT concentrations in those eggs taken from non-urban relative to the urban locations

(non-urban numbers are decreasing, urban colonies are increasing). However, we found that there was no significant difference in yolk CORT concentrations in the herring gull eggs collected from the two habitat types. This may be because adult birds are not experiencing any stress or adult herring gulls may find the non-urban habitat a challenging and stressful place to live, but do not pass this information onto their developing offspring (to minimise the chances of any negative effects of elevated CORT concentrations on embryonic development as discussed above). Another possible explanation is that only certain individuals in the population are sufficiently 'stressed' by living in the non-urban habitat, and these females choose not to (or cannot) breed under such negative conditions. Therefore, by definition, we will only be sampling eggs from individuals who are better able to cope with the environmental stress at the time of breeding and hence we do not see a complete picture of the coping ability of these birds from their eggs alone. Finally, it could be that the stressors occur mainly outwith the breeding season (and therefore we do not identify the effects come breeding – see below) or it is juvenile birds that are most affected by stressful conditions. Perhaps these birds, with 'inexperienced' stress responses are failing to cope under the current conditions, subsequently reducing the numbers of higher-quality breeders each year and in turn the decreasing numbers of herring gulls.

Finally we compared yolk CORT concentrations between our two gull species, but only in sites where they coexist. We found that there was no difference between species in yolk CORT concentrations in the four sites where the birds both breed, suggesting once again that yolk CORT is not influencing the species differences seen in their population trajectories. Unfortunately, the only sites we were able to perform a

direct comparison between species were from non-urban sites. It is the case that many of the urban sites in Scotland are restricted to one of the two species, but this may be an indicator of why these two species are coping differently. Perhaps there is some environmental factor not yet studied that is resulting in direct interspecific competition in sites where the two species nest (not present in urban habitats or only of importance where the species coexist) or there may be environmental factors impacting on these birds differently during non-breeding times. Over-wintering behaviour does vary between the birds, with herring gulls largely resident, while the lesser black-backs tend to migrate south (although these behaviours may be changing in the urban breeders, with lesser black-backed gulls increasingly over-wintering close to their breeding sites) (Forrester *et al*., 2007). It could be that food or disease exposure varies during these times and subsequently results in decreased body condition in the herring gulls, reducing breeding attempts. This would be expected to elevate plasma CORT concentrations, but we cannot be certain of this without blood sampling. What is certain is that more detailed studies are needed to investigate the differences in movements, disease occurrence, interactions/conflicts and foraging behaviours of these two species outwith the breeding season. Although maternally derived CORT in the egg yolks may not have indicated any differences between the species, direct comparisons of adult plasma CORT concentrations (baseline and stress-induced) could still reveal inherent species differences to explain why the lesser black backs are coping better in the non-urban environments.

In conclusion, our results suggest that herring and lesser black-backed gulls show no significant differences in their yolk CORT concentrations and the deposition of

yolk CORT by mothers of both species does not vary according to egg order or breeding circumstances.

4.6 APPENDIX

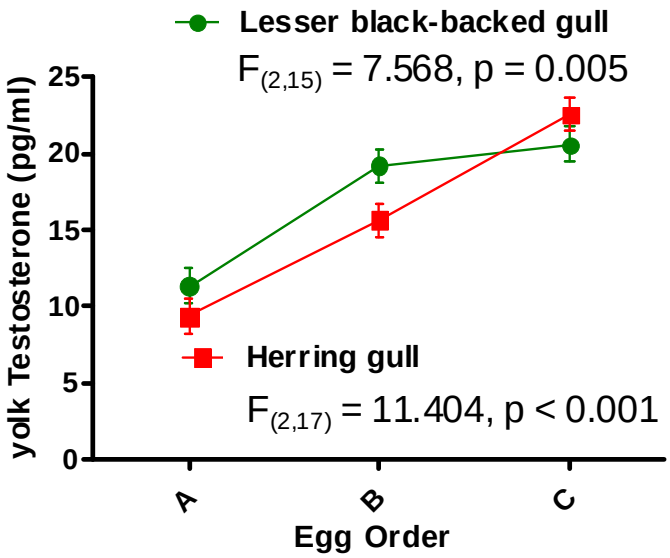


Fig.4.8. Mean yolk testosterone concentrations (pg/ml) (1pg = 0.001ng) ± s.error for lesser black-backed and herring gulls according to egg order.

CHAPTER 5

HUMAN DISTURBANCE AND THE MATERNAL TRANSMISSION OF CORTICOSTERONE

5.1 ABSTRACT

Although there has been considerable research on how environmental conditions (natural and anthropogenic) can activate the stress response in free-living animals, only a handful of studies have investigated how these environmental perturbations can influence the maternal transmission of glucocorticoids. Here we present results on the yolk CORT concentrations of two species of closely related gulls breeding on an island experiencing disturbance-based management in order to relocate them to allow terns to return and breed. We have shown that human presence and disturbance does not elevate yolk CORT in these birds. Smaller herring gull clutches contained significantly higher yolk CORT concentrations. The removal of the first clutch reduced the average replacement clutch size and herring gulls showed a trend for elevated yolk CORT concentrations in their replacement clutches. We discuss the relevance of these findings in relation to the recent declines in the UK herring gull population.

5.2 INTRODUCTION

As described in **Chapter 1**, environmental perturbations such as food shortages or increasing predator density (but also changes in season and reproductive status) can elevate circulating glucocorticoid concentrations in many free-living species. These changes, however, are not restricted to just fluctuations in the animal's immediate environment and natural host/prey interactions, as human-animal interactions are becoming more common through increased urbanisation, habitat loss, habitat fragmentation and real or perceived elevation in predation risk. Human activity has been found to be positively associated with increased glucocorticoid concentrations in several bird species [Northern spotted owls (*Strix occidentalis caurina*) (Wasser *et al*., 1997), Carolina chickadees (*Poecile carolinesis*) (Lucas *et al*., 2006), capercaillie (*Tetrao urugallus*) (Thiel *et al*., 2008), 7-day old Magellanic penguins (*Spheniscus magellanicus*) (Walker *et al*., 2005) and 40-day old hoatzin chicks (*Opisthocomus hoazin*) (Müllner *et al*., 2004)]. The effects of human exposure have also been shown to be dampened or even removed if animals become habituated to human contact (Walker *et al*., 2005; Partecke *et al*., 2006). It is thought that animals unfamiliar with human contact perceive humans as a predation threat and mount similar behavioural and physiological changes to those seen in the presence of animal predators, such as increased glucocorticoid secretion and movement away from breeding, feeding or nest sites (both temporary and permanent) (Frid & Dill, 2002; Beale & Monaghan, 2004*b*). Although there has been considerable research on how environmental conditions

(natural and anthropogenic) can activate the stress response in free-living animals, only a few studies have investigated how maternal transmission of glucocorticoids is affected by such environmental perturbations.

In birds, it is possible that the mother transmits information to the embryo/chick via deposition of hormones or alteration of the composition of eggs (Schwabl, 1993; Arnold, 2002; Hayward & Wingfield, 2004). Recent work that has investigated yolk CORT concentrations in captive birds by Oliver Love (Love *et al*., 2005 and Love & Williams, 2008) and Lisa Hayward (Hayward & Wingfield, 2004; Hayward *et al*., 2005) has demonstrated that maternal glucocorticoids (CORT in this case) can be transferred to avian yolk and can potentially influence the post-natal stress response, growth, fitness, and survival. As described in **Chapter 1**, birds are particularly appropriate for studies of the maternal transmission of hormones as sampling newly laid eggs allows the hormones of maternal origin to be more easily isolated from any produced or used by the developing embryo. We hypothesise that, like naturally occurring environmental changes, human disturbance can activate the stress response in free-living birds and fluctuations in the maternal blood system may be expressed in the concentrations of CORT found in the yolk of their eggs. In order to study this, we designed an experiment in conjunction with the Royal Society for the Protection of Birds (RSPB) where we collected full clutches of eggs laid by two species of closely related gulls (herring and lesser black-backs) with or without an acute human disturbance, using colonies that breed on the uninhabited island of Inchmickery, on the east coast of Scotland. This island was chosen as the birds are not routinely exposed to human contact and the RSPB are currently undertaking a program to promote tern

breeding by displacement of gulls from one area of the island to another area unsuitable for breeding terns. The island was artificially split in two and following collection of first-laid clutches, birds on one side of the island were disturbed through regular human contact, physical barriers and egg removal (detailed in **Section 5.3.3**). Experimental (human disturbed) birds were allowed to relay in the disturbed area (but all other birds in this area were prevented from doing so) so that a second clutch of eggs could be collected from these birds. In addition, 1st laid clutches were collected from gulls breeding in the low disturbance area, so as to force them to lay a second clutch. This design allowed us to identify if changes in yolk CORT concentrations were due to human disturbance or simply related to laying a second clutch.

From previous work on the maternal transmission of CORT to yolk, in lesser black-backed and herring gulls (see **Chapter 4**), we have shown that supposedly poor environmental conditions (non-urban habitats for the herring gulls) did not elevate yolk CORT deposition by mothers. However, results from a separate study also indicated that laying a second clutch (as these birds will do), when combined with an unpredictable (poor) environment, can result in elevated deposition of CORT in egg yolk in the zebra finch (**Chapter 3**). When combined with information from previous studies that have demonstrated that human disturbance elevates maternal CORT concentrations, we predicted that our experimental human disturbance would be enough to significantly elevate plasma CORT concentrations in female gulls and that this would be reflected in the CORT concentrations in their egg yolks in the second clutches. Our experiment also gave us the chance to compare the yolk CORT concentrations deposited into eggs in nests from the two sites prior to the disturbance. We also investigated if

human disturbance had any impact on replacement clutch sizes. As described in **Chapter 3**, one of the ways of reducing the costs of breeding is to reduce the number of offspring produced. We found in captive zebra finches that laying two clutches in one 'season' results in females reducing their clutch size in both predictable and unpredictable (food availability) conditions. We therefore predicted that upon relaying, lesser black-backed and herring gulls would reduce the size of their clutch in both low and high disturbed areas, with no difference between the areas expected.

5.3 MATERIALS AND METHODS

5.3.1 Study site

Inchmickery (NT 207,805) is a small island, roughly 1.6km north of Edinburgh in the Firth of Forth (Figs. 5.1 & 5.2). The site was originally designated as a Site of Special Scientific Interest (SSSI) in 1985 and is also an RSPB reserve. The island is home to large populations of herring and lesser black-backed gulls, as well as a small number of breeding eider ducks, fulmars (*Fulmarus glacialis*) and shags (*Phalacrocorax aristotelis*). Currently, the RSPB are implementing a 5 year strategy to create an area suitable for terns breeding on the east side of the island by removing all breeding gulls from this site. The gulls have been cited as the main factor preventing the return of the roseate tern (*Sterna dougallii*) to the island, as they occupying all suitable tern nesting sites.

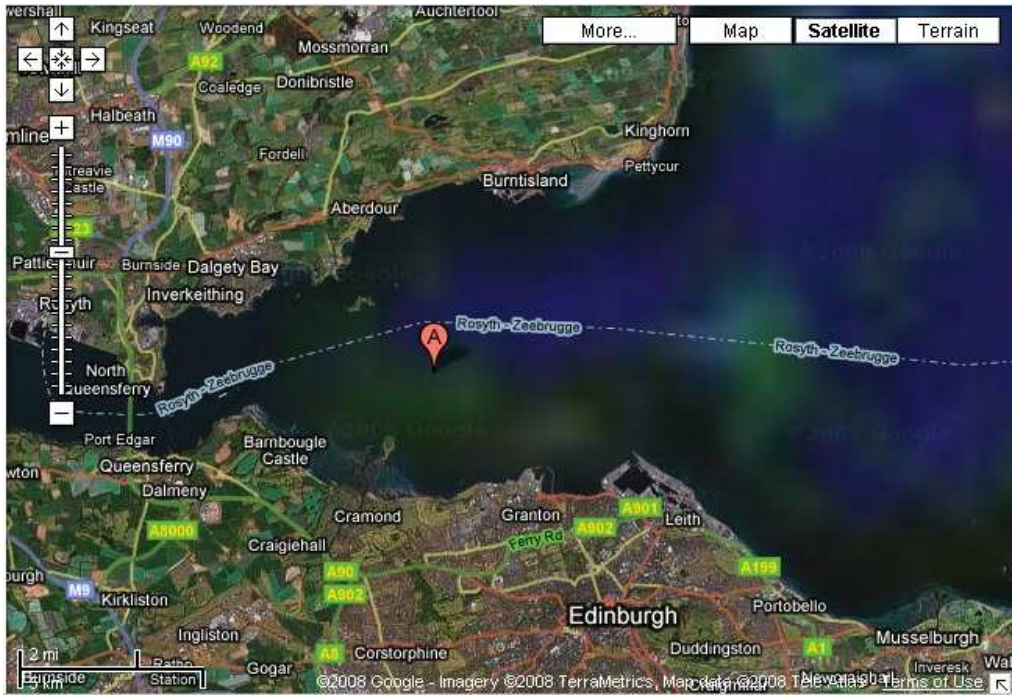


Fig.5.1. Satellite image of the location of Inchmickery (A). Copyright Google maps, 2008.

Fig.5.2. Inchmickery Island, May 2007

5.3.2 Study species

See **Section 4.3.2** for details of the study species.

5.3.3 Egg collections

The island was visited at various times throughout May and June 2007, with the first visits used to identify, mark and map all appropriate nest sites. During these initial visits, the area identified as the high disturbance site was marked using spray paint to separate it from the low disturbance site (Figure 5.3). Nests chosen for this study were marked using numbered bamboo canes to allow easy identification upon returning to the island. Nests chosen for the study were those that had no eggs present at the first visit. Dates of lay, the surrounding habitat (for example, shingle, nettle, grass or rocks) and the species using the nest were recorded for each studied nest. During the study period, the pest control team employed by the RSPB destroyed all non-experimental nests and eggs in the high disturbance area. Attempts were also made to disturb the birds laying in this area by prolonged exposure to the disturbance team, as well as the erection of physical barriers in the form of hazard tape around the study area. First laid clutches were collected after three eggs had been laid (a complete clutch) or four days had elapsed after laying of a second egg. Nest sites continued to be monitored during laying of second clutches, while the disturbance work continued. Second laid clutches were collected as before. We attempted to minimise the disturbance to the birds in the low disturbance area of the island by limiting our visits in terms of duration and frequency,

with only one individual being allowed to enter this area at a time. In addition, a maximum of 10 nests per species were selected for clutch collections. All eggs were returned to the laboratory within 10 hours of collection, where they were weighed, the length and breadth measured (allowing for an estimation of volume to be made) and then frozen at -20°C before further analysis.

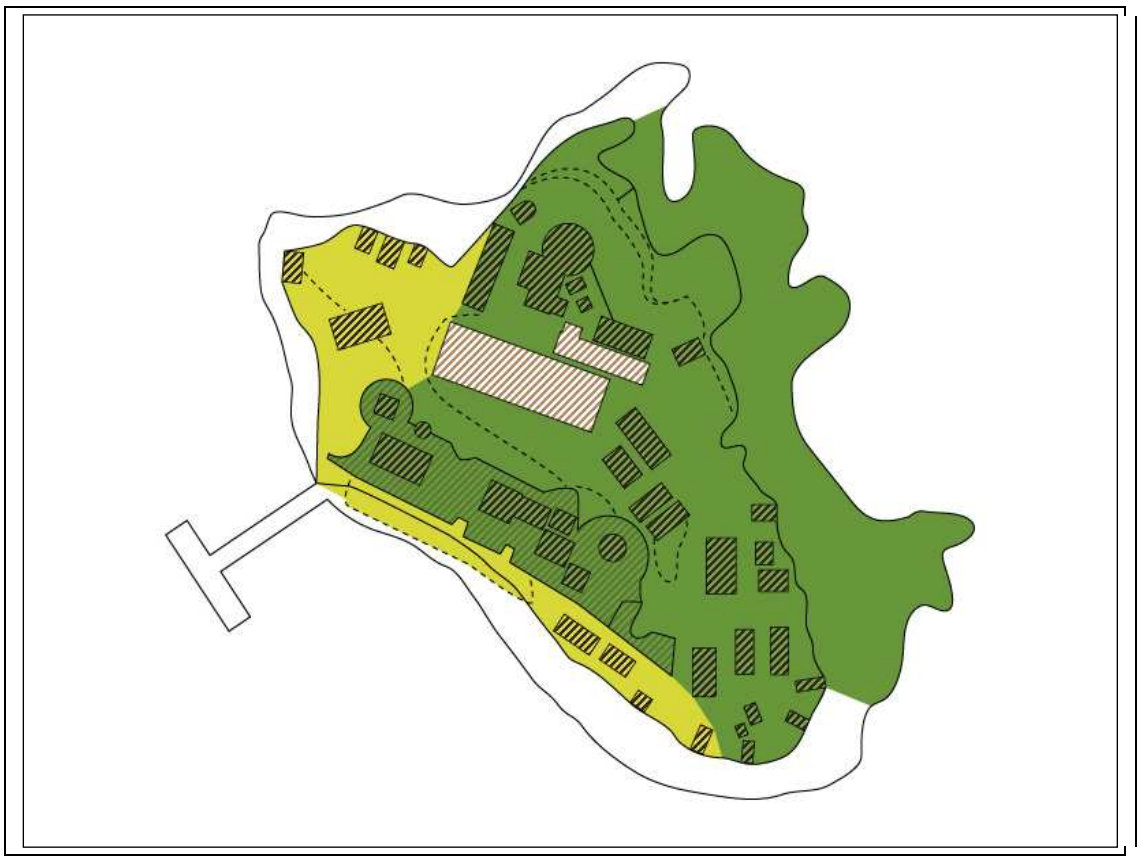


Fig.5.3. Drawing of Inchmickery Island. Hatched areas represent disused buildings and hatched areas without colours represent unsafe, disused buildings where disturbance work was not carried out. Yellow area represents the low disturbance area. Green area represents the high disturbance area. White areas within island outline represent areas not accessible.

All collections were carried out under Scottish National Heritage licence and with the permission of the RSPB who manage the site. In addition, all disturbance work (including nest and egg destructions) was carried out by a pest controller hired by the RSPB.

5.3.3 Radioimmunoassay

CORT was extracted from the gull egg yolk samples using a methanol extraction and measured by radioimmunoassay using the protocols described in **Sections 2.3.1 and 2.3.3**. Extraction efficiency averaged 81.25 ± 7.8% (sd). Inter- and intra-assay coefficients of variation were 7.89% (2 assays) and 21.1 ± 1.2% (s.error) respectively. Assay sensitivity averaged 0.18ng/ml.

5.3.4 Statistical Analysis

A linear mixed model (using SPSS ver.15, SPSS Inc., Illinois, USA) was used for the analysis of yolk CORT concentrations (log transformed to make normally distributed). Treatment (high/low disturbance), clutch number (1^{st} or 2^{nd}) and clutch size were included as fixed factors (along with interactions between the three), with clutch number and egg order as repeated variables and female identity as the subject/random factor. Egg order and egg weight were included as covariates and where these factors were not significant they were removed from the model. In order to identify whether there were any differences between high and low disturbance areas in terms of yolk CORT concentrations prior to the disturbance, we split the data according to species and clutch number (although we were only interested in the effects on the 1^{st} clutch). We then used a linear mixed model with log transformed yolk CORT concentrations included as the dependent variable, female identity as a random factor, egg order as a repeated variable and treatment included as the only fixed factor. As before, egg order

and egg weight were all included in the model and only removed if not significant. We also wanted to identify if there was any effect on clutch size when birds nested in the high disturbance area of Inchmickery. As the results for clutch size were not parametric, we used separate Wilcoxon Signed Ranks Tests for each species under each treatment (disturbed/undisturbed).

5.4 RESULTS

5.4.1 CORT concentrations in 1st vs. 2nd clutch eggs

Eggs collected from lesser black-backed gull nests on Inchmickery did not differ significantly in their yolk CORT concentrations between 1st and 2nd laid clutches ($F_{(1,35)} = 0.343$, $p = 0.562$), treatment (high versus low disturbance) ($F_{(1,58)} = 2.475$, $p = 0.121$) or with clutch size ($F_{(1,38)} = 0.015$, $p = 0.905$). No interaction between clutch number and treatment was present (Fig. 5.4; $F_{(1,60)} = 0.939$, $p = 0.336$), nor was there any significant interaction between clutch number and clutch size ($F_{(1,33)} = 0.02$, $p = 0.888$) or treatment and clutch size ($F_{(1,76)} = 0.111$, $p = 0.74$). Egg order and egg weight proved non-significant (summarised in Table 5.1) and were removed from the model.

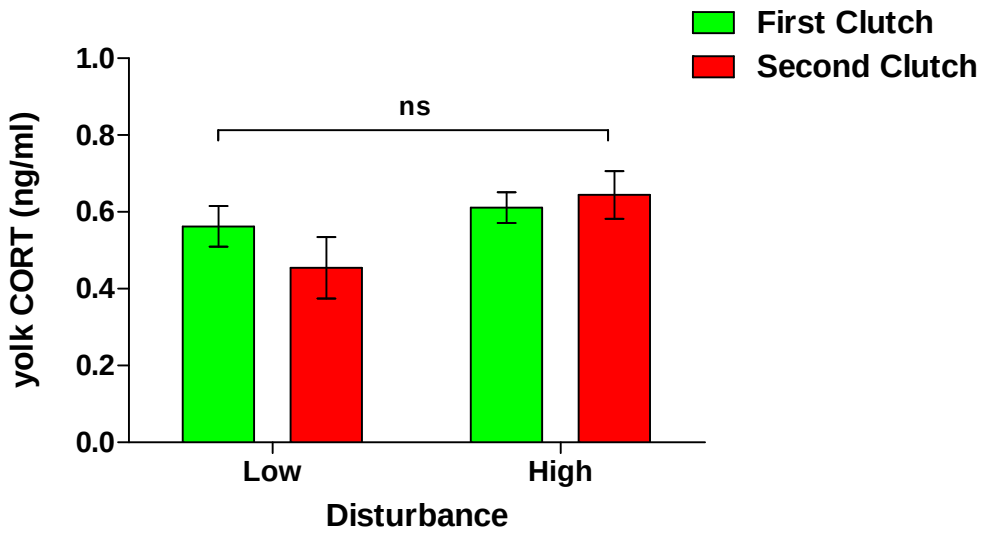


Fig.5.4. Mean yolk CORT concentrations (ng/ml) ± s.error for lesser black-backed gulls from the high and low disturbance areas according to clutch number, where ns: clutch number * treatment, p = 0.336.

Table 5.1. Summary of covariate effects on yolk CORT in the lesser black-backed gull comparison between 1st & 2nd clutches.

Source	Numerator df	Denominator df	F	Sig.
Egg order	1	61	0.199	0.657
Egg weight	1	68	0.708	0.403

Eggs collected from herring gull nests on Inchmickery were found not to significantly differ in their yolk CORT concentrations when comparing 1st and 2nd clutches ($F_{(1,34)}$ = 3.432, p = 0.073) (although there is a trend for yolk CORT to be elevated in the 2nd clutch) or treatment (high versus low disturbance) ($F_{(1,53)}$ = 1.111, p = 0.297). No interaction between clutch number and treatment was present (Fig. 5.6; $F_{(1,53)}$ = 0.07, p = 0.793), nor was there any significant interaction between clutch number and clutch size ($F_{(1,30)}$ = 0.241, p = 0.627). However, eggs from smaller herring

gull clutches were found to have significantly higher yolk CORT concentrations (Fig. 5.6; $F_{(1,30)} = 5.663$, $p = 0.024$). Egg order and egg weight all proved non-significant (summarised in Table 5.2) and were removed from the model.

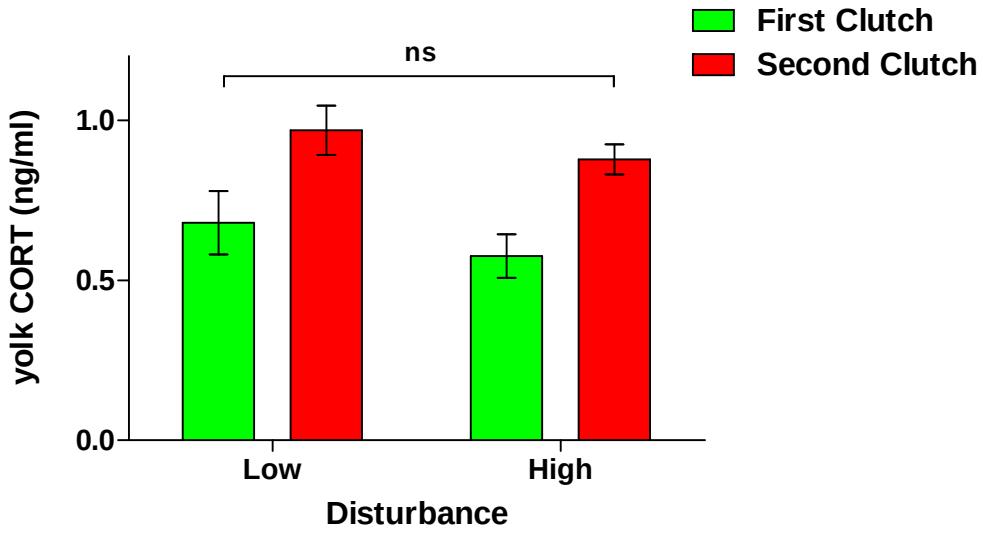


Fig.5.5. Mean yolk CORT concentrations (ng/ml) ± s.error from herring gulls from the high and low disturbance areas according to clutch order, where ns: clutch number * treatment, p = 0.73.

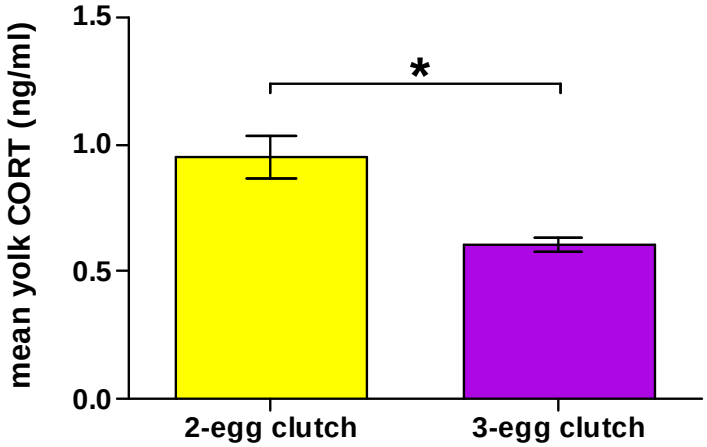


Fig.5.6. Mean yolk CORT concentrations (ng/ml) ± s.error from herring gulls with 2-egg versus 3-egg clutches, where *: p = 0.024.

Table 5.2. Summary of covariate effects on yolk CORT in the herring gull comparison between 1st and 2nd clutches.

Source	Numerator df	Denominator df	F	Sig.
Egg order	1	33.914	1.045	0.314
Egg weight	1	41.413	2.124	0.153

5.4.2 CORT concentrations prior to disturbance

We also investigated if the high and low disturbance areas differed in terms of the yolk CORT concentrations seen in the eggs of the two species prior to disturbance. We found that there was no significant difference in yolk CORT concentrations between the high and low disturbance sites for the lesser black-backed gulls (Fig. 5.7, $F_{(1,55)} = 0.304$, $p = 0.584$), but yolk CORT concentrations were higher in herring gulls nesting in the low disturbance site (Fig. 5.8, $F_{(1,27)} = 4.204$, $p = 0.05$). Egg order and egg weight were removed from both the lesser black-backed and herring gull models as all proved non-significant (summarised in Tables 5.3 and 5.4).

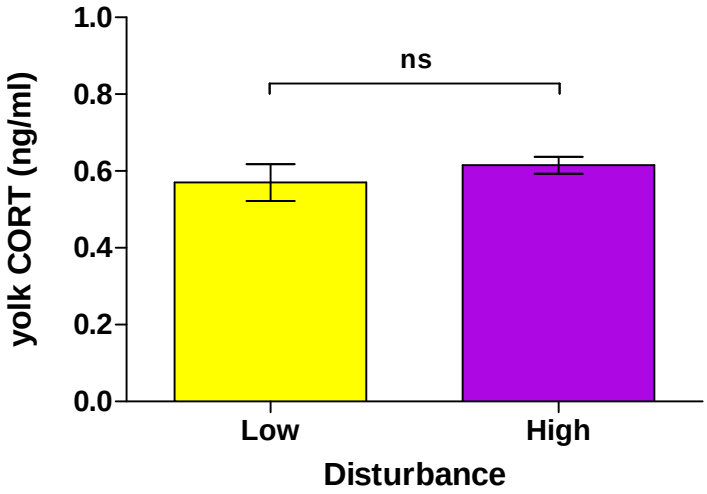


Fig.5.7. Mean yolk CORT concentrations (ng/ml) ± s.error of 1st clutch eggs of lesser black-backed gulls from the low (*n* = 11) and high (*n* = 53) disturbance areas, where ns: p = 0.584.

Table 5.3. Summary of covariate effects on yolk CORT in the lesser black-backed gull 1st clutch analysis

Source	Numerator df	Denominator df	F	Sig.
Egg order	1	34.33	1.174	0.286
Egg weight	1	39.253	0.749	0.392

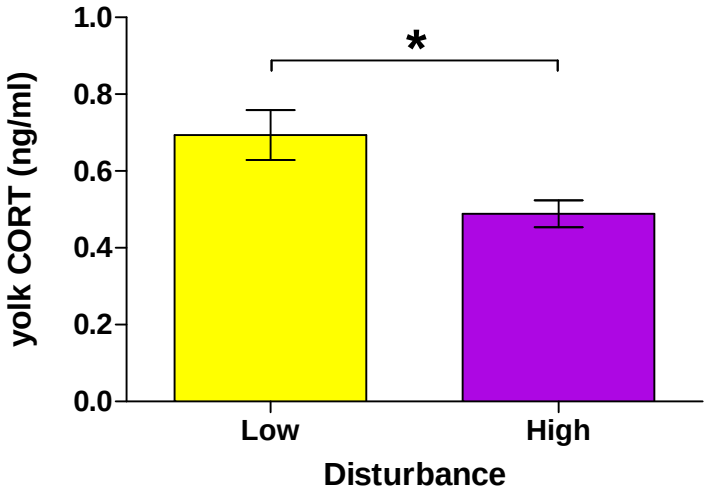


Fig.5.8. Mean yolk CORT concentrations (ng/ml) ± s.error of 1st clutch eggs of herring gulls from the low (*n* = 6) and high (*n* = 28) disturbance areas, where *: p = 0.05.

Table 5.4. Summary of covariate effects on yolk CORT in the herring gull 1st clutch analysis

Source	Numerator df	Denominator df	F	Sig.
Egg order	1	20.123	0.035	0.853
Egg weight	1	31.849	1.037	0.312

5.4.3. Effect of disturbance on clutch size

We found that in lesser black-backed gulls, the average size of the replacement clutch was significantly reduced when laying in the high disturbance area (z = -0.4796, p < 0.001; where 23 birds reduced their clutch size and 4 laid the same number), but not in the low disturbance area (z = -1.414, p = 0.157; 4 reduced their clutch size, 1 increased it and 1 laid the same size clutch) (Fig. 5.9). In the herring gull, high disturbance females reduced the size of their 2nd clutch (z = -0.2558, p = 0.011; with 8 females

reducing their clutch and 9 laying the same number), while there was no significant reduction in the low disturbance area (z = -0.272, p = 785; where 2 females reduced their clutch, 2 increased it and 2 laid the same number) (Fig. 5.10). It is important to note that in the low disturbance clutches, average clutch size was lower, which may explain why clutch size was not significantly reduced in the replacement clutch. The reasons for this will be addressed in the discussion.

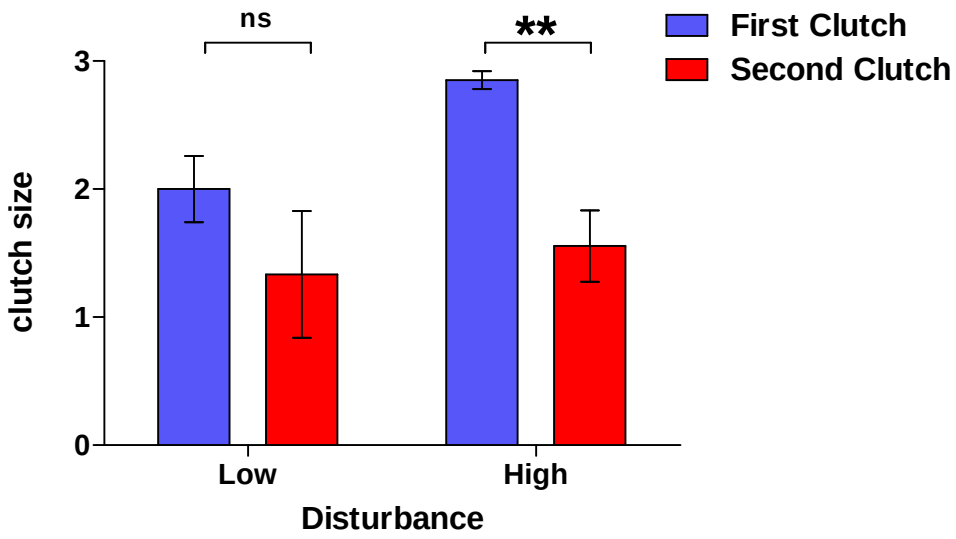


Fig.5.9. Mean clutch sizes ± s.error for 1st and 2nd clutches from lesser black-backed gulls in the low disturbance area (*n* = 6 nests), where ns: p = 0.157, and high disturbance area (*n* = 27 nests), where *: p < 0.001.

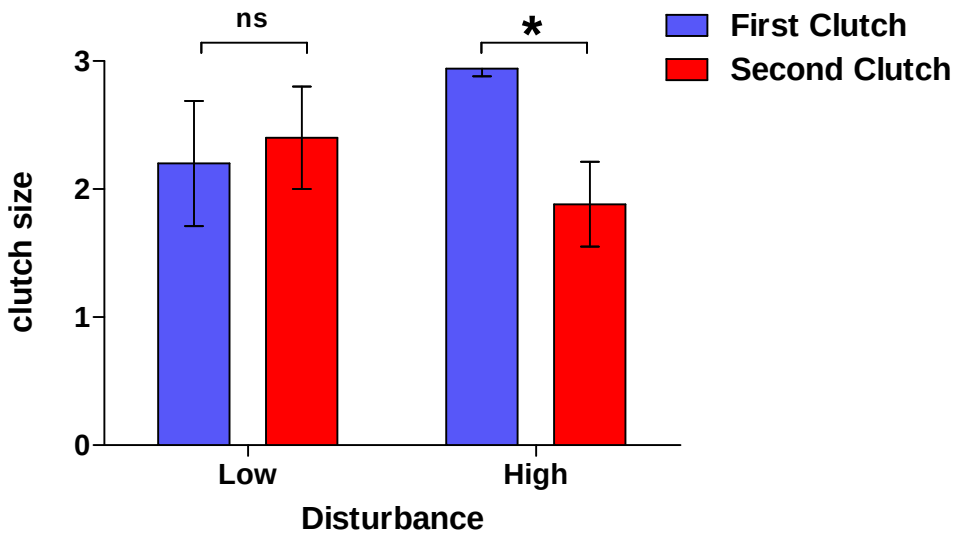


Fig.5.10. Mean clutch sizes ± s.error for 1st and 2nd clutches from herring gulls in the low disturbance area (*n* = 5 nests), where ns: p = 785, and the high disturbance area (*n* = 17 nests), where *: p = 0.011.

5.5 DISCUSSION

This study has found evidence of a difference between the lesser black-backed and herring gulls in their patterns of yolk CORT deposition. Herring gulls displayed a (non-significant, p = 0.073) trend for depositing greater CORT concentrations into the yolk of their eggs following the loss of their first clutch and when they laid smaller clutches, their eggs typically had higher concentrations of CORT. This could suggest possible differences in the ability to cope with negative environmental conditions (in this case the loss of a clutch) between the species. Herring gulls have been declining massively over the last three decades in non-urban sites in the UK, while lesser black-backed gull populations have been stable. It has been proposed that these declines could be linked with different coping abilities under stressful conditions, but no evidence was found when measuring yolk CORT concentrations in first laid clutches previously (**Chapter**

4). Ground-nesting gulls commonly lose their first clutch to causes such as floods and predators, and have evolved the ability to replace this lost clutch within the same breeding season (Brown & Morris, 1996; Zador *et al*., 2006). However, replacement clutches require the diversion of energy resources, often resulting in reduced investment in these replacement eggs (Gasparini *et al*., 2006). If the increase in yolk CORT in the herring gulls was an adaptive strategy to help prepare their chicks for an unpredictable environment, we would expect to see the same method used by the lesser black backs. It is more likely that the loss of the first clutch is activating the stress response in the adult herring gulls (at least in the females) and this is being transmitted to the egg. Increased CORT can affect embryonic development and fledgling fitness as eggs/chicks subjected to elevated CORT show slower growth, high HPA activity under stress (Hayward & Wingfield, 2004), delayed hatching, increased mass loss during incubation and reduced begging (Rubolini *et al*., 2005). In addition, birds that breed later have typically been found to have reduced reproductive success, suggested to be caused by reduced chick provisioning and high predation when colonial birds do not lay synchronously (Hatchwell, 1991); low food availability and poor weather conditions (Brinkhof *et al*., 1993; Wiggins *et al*., 1994); slow growth and high conspecific predation (Hunt & Hunt, 1976); or poor territory and/or parental quality (Wiggins *et al*., 1994; Verhulst *et al*., 1995). Therefore, herring gull chicks may be finding themselves at a disadvantage compared to their cousins and this could be playing a part in the population declines.

Interestingly, high human disturbance had no effect on CORT transmission to the yolk in either the herring or lesser black-backed gulls. It may be that both of these species have become used to the presence of humans in relatively close proximity to

their feeding and nesting grounds (as seen with the ease in which they have taken to breeding in the urban environment). As clutch loss can be common (Brown & Morris, 1996; Zador *et al*., 2006), perhaps the source of this loss being human rather than animal predators, poor weather or conspecifics, has no greater impact. The original experimental design involved greater disturbances, with more physical obstacles to nesting and increased noise levels (through radios playing continually and gas bangers) planned. However, due to constraints in the licensing, manpower and funding, RSPB decided to scale down the operation in the first year to assess the effectiveness of the disturbance. Determining the difference between human contact and human disturbance is essential if we are to understand the affects we as humans may be having on the wildlife around us. Disturbance can only be confirmed through experiments and it is possible we underestimate the resilience of animals to our presence in their natural environment.

We were able to identify that the human disturbance/contact resulted in both the lesser black-backed and herring gulls reducing their clutch sizes following direct human removal of their first clutches. As explained in **Chapter 3**, this may be an early and relatively easy means of maximising reproductive success when conditions become poor (Gustafsson & Pärt, 1990). We did not see any reduction between 1st and 2nd clutches for either species in the low disturbance area, which could provide evidence that the presence of humans was having a negative effect on these birds. However, birds breeding in the low disturbance area had smaller 1st laid clutches on average compared to those from the high disturbance area, which may have prevented a significant decline being observed. It may be that this area of the island (chosen to relocate the gulls to)

provides poorer conditions for chick survival and females may lay smaller clutches to minimise the impact of this (i.e. laying one less egg is less energetically costly than losing one chick from the egg not hatching or dying post-hatch). The birds nesting on the low disturbance side could also be younger or poorer condition birds, which are known to often occupy lower quality territories in terms of location, size and shelter (Pugesek & Diem, 1983; Reid 1988; Pärt, 2001) and, in general, lay smaller clutches (Ainley *et al*., 1983; Harvey *et al*., 1985; Hébert & Barclay, 1988; Forslund & Larsson, 1992; Pyle *et al*., 1991; Pärt, 1995). The low clutch numbers and low number of birds breeding on the low disturbance side suggest that this side of the island may not be the most suitable for relocating these birds as it may result in a decline in the size of the breeding colony. In addition, herring gull yolk CORT concentrations were typically higher in the first laid clutches in the low disturbance area prior to the disturbance work being carried out. This could be further evidence for this side of the island containing inferior nesting sites and/or poorer quality birds. In addition the herring gulls' response in terms of elevated yolk CORT concentrations could suggest a different response to stressful stimuli / poor conditions compared to the lesser black backs. This evidence could be important in shaping the management strategy implemented by RSPB on this, and possibly future, projects.

In summary, we have shown in this study that human 'disturbance' does not appear to elevate CORT concentrations in the yolk of lesser black-backed or herring gulls on the island of Inchmickery. 2-egg herring gull clutches were shown to have elevated yolk CORT concentrations compared to 3-egg clutches and experimental egg removal also resulted in a trend for elevated CORT concentrations in herring gull yolk

in the replacement clutch. These results suggest that there may be a difference in the coping strategies of these two birds and this may negatively impact on their reproductive success and their offspring's fitness. Clutch sizes were typically smaller in the low disturbance area prior to disturbance, possibly suggesting that either poorer or younger birds are using this area to breed or that this area is detrimental to reproductive success. This finding could be important in determining future management strategies for this, and other, RSPB sites.

CHAPTER 6

THE EFFECT OF NEST SHELTER & MATERNAL QUALITY ON YOLK CORTICOSTERONE IN BREEDING EIDER DUCKS

6.1 ABSTRACT

Nest site selection can provide microclimates that reduce thermoregulatory demands in parents and offspring; conceal the adults, eggs and chicks from predators; and/or limit nest/brood parasites. Negative conditions brought about by poor nest site quality could result in elevated plasma CORT concentrations in mothers, reflected in the yolk of their eggs. However, the effects of nest site on breeding performance can be confounded by variation in parental quality if better quality birds obtain better nest sites. We investigated whether nest shelter was related to the concentrations of CORT in egg yolk in the Common Eider, *Somateria mollissima,* and if there was a relationship between nest shelter and egg production (egg weight and clutch size), since females nesting in well-sheltered sites may be themselves better quality individuals. We found that nest shelter had no impact on yolk CORT concentrations or clutch sizes, but heavily sheltered nests contained lighter eggs. These results contrast with previous work suggesting better quality birds (those that lay larger clutches) prefer sheltered nest sites. The lack of any pattern between nest shelter and yolk CORT / clutch size suggests that in this study site, female Eiders may not be limited in their nest choice and that environmental conditions were not negative enough to limit food or decrease temperatures (resulting in reduced investment in offspring) or result in increases in the transfer of CORT to the yolk.

6.2 INTRODUCTION

As already discussed in this thesis, there are several environmental perturbations and cues that can activate the stress response in birds (and other species), with the elevated concentrations of plasma CORT that result potentially being passed onto a mother's developing offspring. During the breeding season, females may try to minimise the number and degree of stressful stimuli experienced, as increased concentrations of CORT transmitted to an embryo can have major developmental effects, unless these changes in maternal CORT can be used to confer information to the offspring about an unpredictable environment (see **Chapter 1**; Monaghan, 2008). However, as the breeding season progresses, elevated plasma CORT concentrations have been shown to promote nest abandonment (Silverin, 1986) or decrease nest attentiveness (Criscuolo *et al*., 2005). In addition, exposure to difficult environmental conditions, for example poor weather, can increase the energetic costs experienced by females (trying to stay warm and incubate eggs), reducing body condition (and elevating CORT concentrations) and potentially reducing breeding success (Meathrel *et al.*, 1987; Chastel *et al.*, 1995*a* & *b*; Wingfield & Ramenofsky, 1999). Therefore, females (and males) should select the best nest sites available to minimise the exposure to stress and maximise reproductive success.

Nest site selection can provide microclimates that reduce thermoregulatory demands in parents and offspring (Gloutney & Clark, 1997; Kilpi & Lindström, 1997; Fast *et al*., 2007; Hepp *et al*., 2006); conceal the adults, eggs and chicks from predators

(Ricklefs, 1969; Martin, 1992); and/or limit nest/brood parasites (Loye & Zuk, 1991). For ground nesting birds, a particularly important aspect of nest site selection is vegetation cover, as this simple feature can help conceal against predators (Martin, 1993), improve microclimatic conditions (lower wind speeds and provide milder air temperatures (Kim & Monaghan, 2005*a*); and provide less variable air temperatures (D'Alba *et al*., in press)), is associated with positive chick growth (Kim & Monaghan, 2005*b*) and is positively correlated with egg weight (Kim & Monaghan, 2005*a*) (heavier eggs suggesting better parental condition – Bolton, 1991; Bolton *et al*., 1992; Risch & Rohwer, 2000). However, the effects of nest site on breeding performance can be confounded by variation in parental quality if better quality birds obtain better nest sites (Kim & Monaghan, 2005*b*).

We wished to investigate whether the concentrations of CORT in yolk in eggs of the Common Eider, *Somateria mollissima* (Fig.6.1) varied in relation to nest shelter. We also examined the relationship between nest shelter and egg weight and nest shelter and clutch size, since females nesting in well sheltered sites may be themselves better quality individuals (as seen in the herring gull – Kim & Monaghan, 2005*a*) and/or the shelter may increase the resources that can be invested in egg production.

The Common Eider is a species of sea duck in which females nest on the ground and females incubate their eggs without assistance from their mates. As female Eiders rarely feed during incubation, they can lose up to 40% of their body weight during incubation (Gabrielsen *et al*., 1991), with this loss of body weight found to be greater in exposed nest sites (Kilpi & Lindström, 1997; Fast *et al*., 2007). However, it is not clear

if such effects arise because of poorer quality birds nesting in exposed areas or because the exposure to inclement weather at the nest site directly influences mass loss (or both) (but see D'Alba, 2007). In addition, nest site selection can also be influenced by other factors (for example, predation threat). We predicted that females using nests with intermediate and tall vegetation / shelter would be under less 'stress' than females nesting in exposed sites due to reduced predation threat (Ricklefs, 1969). However, it has already been shown that maternal baseline plasma CORT concentrations measured during incubation do not vary according to nest shelter type in the Eider duck (D'Alba, 2007). Therefore, we predicted that yolk CORT concentrations would not vary according to shelter type in this study. We also predicted that females laying in sheltered sites would have larger clutches and the heaviest eggs, as these nest sites are more likely to be used by the highest quality females (D'Alba, 2007; D'Alba *et al*., in press). It has been shown previously that female mass loss is negatively correlated with baseline plasma CORT concentrations in female eiders (D'Alba, 2007), potentially caused by better quality females being able to invest more in incubation (increasing mass loss), but importantly being able to cope with this larger mass loss without becoming 'stressed'. We therefore predicted that yolk CORT concentrations would be negatively correlated with egg weight i.e. better quality females would both produce larger clutches, larger eggs and have the lowest CORT concentrations.

Fig.6.1. Typical Common Eider nest

6.3 MATERIALS AND METHODS

6.3.1 Study site

Fieldwork was carried out by Liliana D'Alba between May and July 2007 in a breeding colony of the common Eider in Sandgerdi, South West Iceland (Fig.6.2), where approximately 2,000 pairs nest in a fenced area where local people harvest the Eider down lining of the nests at the end of the incubation period for commercial purposes. Nesting birds in the area are accustomed to close and regular presence of the farmers. Hence, any extra disturbance caused by our visits was likely to have been minimal. The area is covered by a mixture of salt marsh grasses dominated by Creeping Bent (*Agrostis stolonifera*) and Common Saltmarsh Grass (*Puccinellia maritime* / *Poa maritima*), with patches also lined exclusively with the brown algae, Channelled Wrack (*Pelvetia canaliculata*).

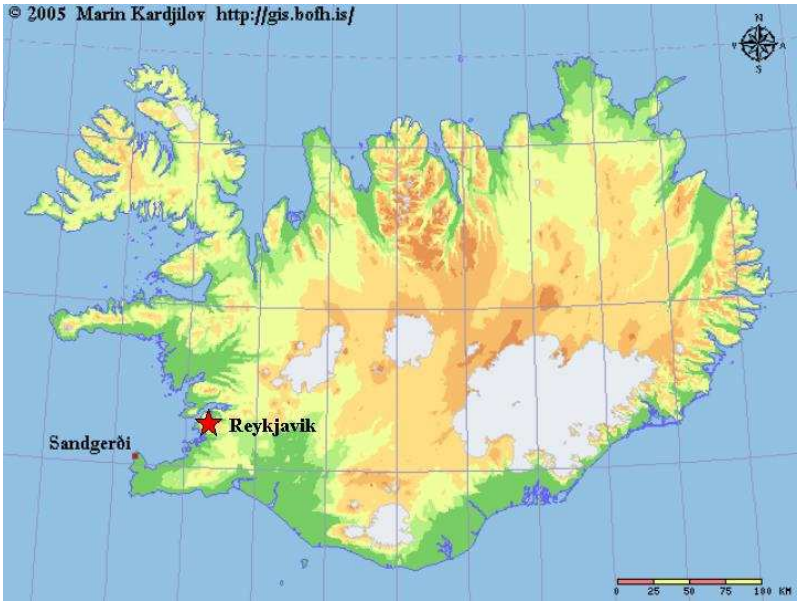


Fig.6.2. Map of Iceland showing the study site of Sandgerdi in relation to the capital Reykjavik.

6.3.2 Study species

The Common Eider, *Somateria mollissima*, is a relatively large (50-71cm body length) sea duck and is distributed over the northern coasts of North America, Europe and eastern Siberia. Eiders nest on coastal islands in colonies and their nests are lined with the down plucked from the female's breast. Females incubate their eggs for 24-26 days (Cramp & Simmons, 1983) (and fast while doing so) and rear their young without any assistance from their mate. Males and females are sexually dimorphic, making both easily identifiable in the field (Fig. 6.3).

Fig.6.3. Male (l) and female (r) Common Eider ducks.

6.3.3 Egg collections

The colony was searched for Eider nests early in the season (May 1st 2007) and fresh nests (with no eggs) were marked with numbered canes. Nests were visited daily, with fresh eggs individually labelled (using a non-toxic marker pen) in the order they were laid (A-E) and weighed. Eggs were collected when the clutch was completed (approximately 4 days after the 1st egg was laid, with clutch size ranging from 2 to 5) and returned to the field station to be placed on ice within 10 hours of collection. For each nest, the vegetation surrounding the nest was measured as the percentage of the nest circumference covered by surrounding vegetation and/or rocks within a 0.5metre radius of the nest cup and the average height of the surrounding vegetation and/or rock. A Principal Component Analysis including average height of plants and/or rocks and percentage of the nest circumference surrounded by them produced a first principal component (PC1) that explained 86.6% of the variance. PC1 was used as a composite measure of nest shelter and showed a tri-modal distribution (D'Alba, 2007). Therefore, nest shelter was classed as a categorical factor with three levels:

1. **Exposed**: no vegetation or rocks surrounding the nest
2. **Intermediate**: 40% covered with vegetation (height <17cm)
3. **Sheltered**: at least 80% of nest circumference surrounded by vegetation or rocks (average height 20cm).

6.3.4 Radioimmunoassay

CORT was extracted from the gull egg yolks using a methanol extraction and measured by radioimmunoassay using the protocols described in **Sections 2.3.1 and 2.3.3**. CORT was measured in all samples in one assay. Extraction efficiency averaged 85 ± 9.8% (sd), the intra-assay coefficient of variation was 18% and assay sensitivity averaged 0.27ng/ml.

6.3.5 Statistical Analysis

A linear mixed model (SPSS ver.15, SPSS Inc., Illinois, USA) was used for the analysis of yolk CORT concentrations according to shelter type, with female identity included as a random factor, shelter type as a fixed factor and yolk CORT (log transformed for normality) as the dependent variable. Egg order and egg weight were included as covariates and where non-significant, were removed from the model. We ran a Linear Regression to determine the relationship between egg weights and yolk CORT, controlling for egg order effects (D and E eggs are lighter – see **Section 6.6,** Fig. 6.8) by including only the A, B and C eggs and assessing the mean CORT concentrations and

mean egg weights from each female. We used another linear mixed model to investigate whether egg weight differed according to shelter type. For this, we included egg weight as the dependent variable, female identity as a random factor and shelter type as the fixed factor. Egg order was included as a covariate ($F_{(1,44)} = 4.855$, $p = 0.033$). Clutch size was assessed using a Kruskal-Wallis test, comparing clutch sizes between shelter types.

6.4 RESULTS

We found no effect of shelter type (exposed, intermediate or sheltered) on yolk CORT concentrations ($F_{(2,45)} = 1.686$, $p = 0.197$) (Fig. 6.4). Egg order and egg weight were non significant and removed from the model (Table 6.1). Figure 6.4 suggested that eggs from intermediate nests might have higher CORT concentrations than those from the other nest types. Hence, we ran separate models comparing intermediate eggs with exposed ($F_{(1,35)} = 2.958$, $p = 0.094$) and sheltered ($F_{(1,22)} = 2.861$, $p = 0.105$) eggs, but both were non-significant.

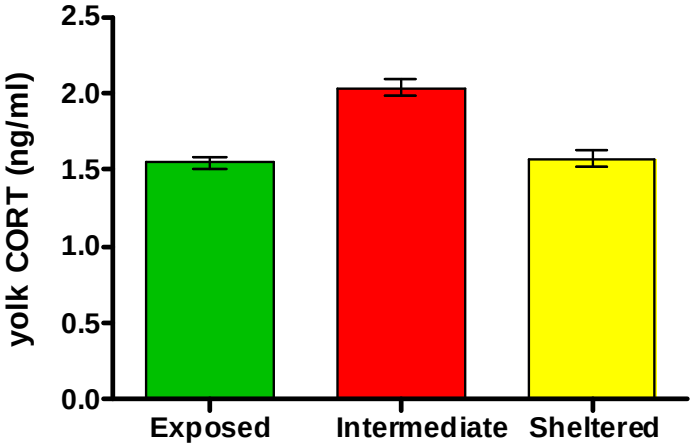


Fig.6.4. Mean yolk CORT concentrations (ng/ml) ± s.error for exposed (*n* = 24), intermediate (*n* = 13) and sheltered (*n* = 11) nest types.

Table 6.1. Summary of covariates tested in the Linear Mixed Model assessing the effect of shelter on yolk CORT concentrations

Source	Numerator df	Denominator df	F	Sig.
Egg order	1	44	0.005	0.943
Egg weight	1	44	0.768	0.386

Egg weights were found to significantly differ according to shelter type ($F_{(2,44)}$ = 27.529, $p < 0.001$) (Fig. 6.5), with eggs in sheltered nests significantly lighter than those from exposed ($F_{(1,33)}$ = 34.222, $p < 0.001$) or intermediate ($F_{(1,21)}$ = 51.919, $p < 0.001$) nests. Egg weights did not differ between exposed and intermediate nest types ($F_{(1,35)}$ = 1.849, p = 0.183). Clutch size was not found to differ significantly between shelter types (Fig. 6.6; H = 3.172, *df* = 2, *n* = 13, p = 0.205).

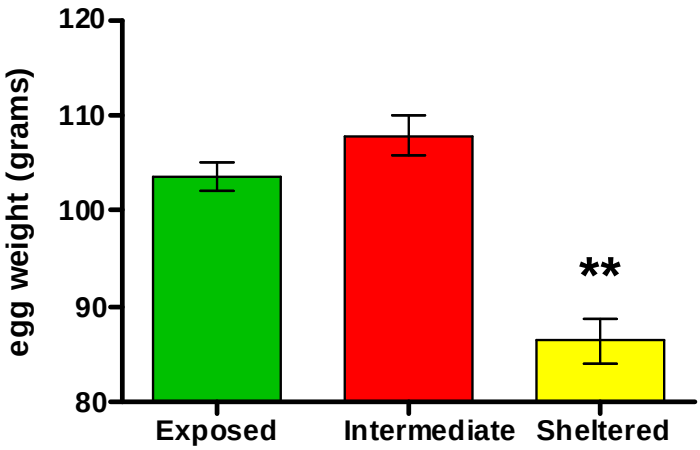


Fig.6.5. Mean egg weights (grams) ± s.error for exposed (*n* = 24), intermediate (*n* = 13) and sheltered (*n* = 11) nest types. **: p <0.001.

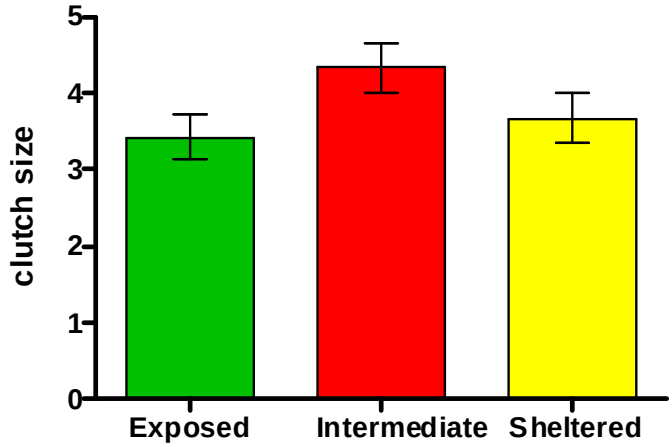


Fig.6.6. Mean clutch sizes ± s.error for exposed (*n* = 7), intermediate (*n* = 3) and sheltered (*n* = 3) nest types, where p = 0.205.

A Linear Regression analysis of mean yolk CORT concentrations and mean egg weights for each female showed no significant correlation between the terms ($F_{(1,11)}$ = 0.798, p = 0.393, r^2 = 0.074) (Fig. 6.7).

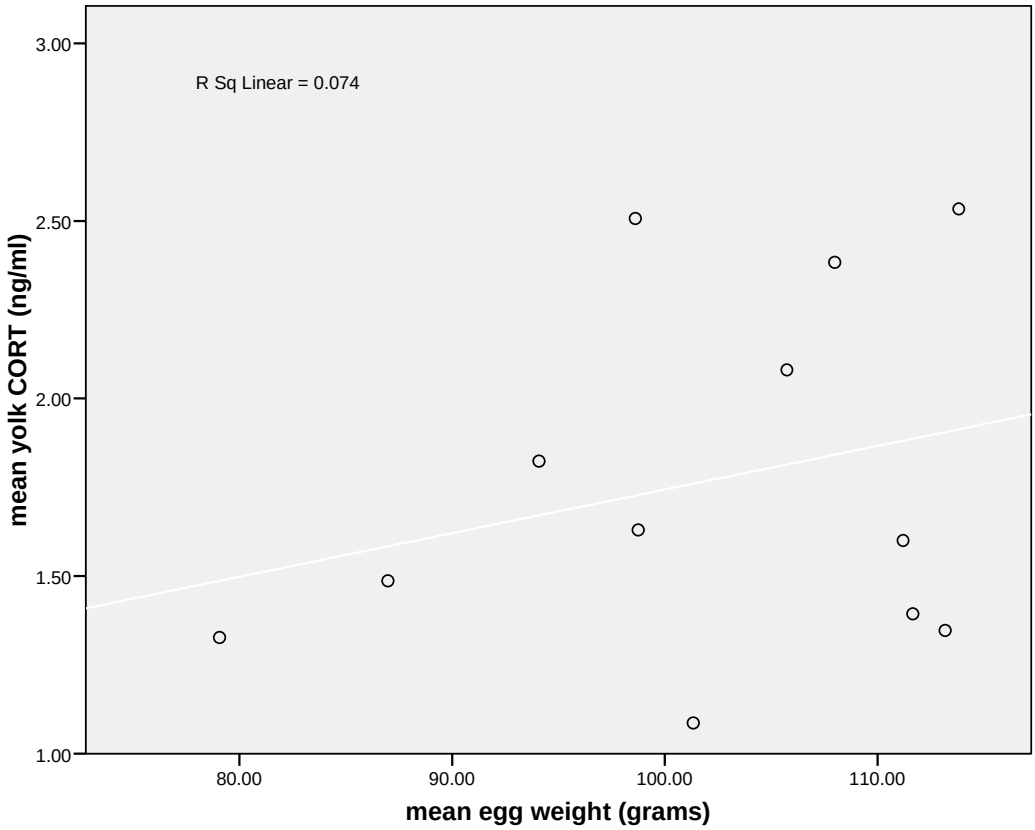


Fig.6.7. Mean yolk CORT concentrations (ng/ml) plotted against mean egg weight (grams) (means of the A, B and C eggs of each female), where $r^2 = 0.074$.

6.5 DISCUSSION

We have shown here that no difference exists in yolk CORT concentrations according to shelter type in eiders, suggesting that there were either no particularly stressful stimuli present during this study or, as seen previously (D'Alba, 2007), (maternal plasma) CORT concentrations do not vary according to nest type, but rather (negatively) correlate with mass loss during incubation. An alternative explanation is that females can suppress their stress response during the breeding season to minimise

negative effects of elevated CORT on embryonic development (but see Romero, 2002). In addition, there would appear to be no relationship between egg weight and yolk CORT concentrations. Once again this could be due a lack of stressful stimuli that may otherwise expose the differences between better and poorer quality females.

We found clutch sizes did not differ according to shelter type, but eggs were lighter in the sheltered habitats. It has previously been shown that egg weight is positively correlated with nest quality (Kim & Monaghan, 2005*a*), and better breeders (that typically choose the best nest sites) lay larger eggs (Bolton, 1991; Bolton *et al*., 1992; Risch & Rohwer, 2000). In addition, it has been shown that female gulls in better body condition lay both larger clutches and more nutrient rich eggs (Houston *et al*., 1983). Eiders that lay larger clutches have also been shown to increase reproductive effort (Hanssen *et al*. 2003), have higher breeding success (Erikstad & Tveraa 1995), lower rate of duckling abandonment (Erikstad *et al*. 1993) and higher breeding site return rates and survival (Yoccoz *et al*. 2002), therefore suggesting these are higher quality females. It remains unclear if the females nesting in the most sheltered nests are indeed lower quality breeders, as clutch size does not decrease with shelter, but egg weight does. It could be that these heavier eggs have simply more albumen (water) content, but not greater nutritional value (Meathrel et al., 1987). Therefore, the lighter eggs may not necessarily be lower quality. If however, these eggs are from poorer quality birds, why would the better breeders prefer the less sheltered nest sites?

The different trends for preferential nest type may be attributed to changes in predator abundance or weather conditions according to year (D'Alba *et al*.- collections

took place in 2005, compared to 2007 in this study) and the costs and benefits associated with particular nest attributes. For example, tall vegetation provides protection from inclement weather, but also makes it difficult for adults and chicks to see predators approaching (Götmark *et al*., 1995). Intermediate nests had the greater mean egg weights, although not significantly higher than those in the exposed sites. It may be possible that nests with intermediate vegetation cover can provide a suitable compromise between complete exposure and complete concealment under certain environmental conditions. For example, improved weather conditions in a breeding year (less wind and milder temperatures) could allow females to utilise the more exposed nest sites without the potential costs associated with complete concealment in the most sheltered sites. D'Alba *et al.* (in press) found that artificial shelters did not improve hatching success in exposed sites, suggesting that the higher reproductive performance observed in 'natural' sheltered nests is mainly determined by quality of females nesting at those sites rather than the quality of nests. Hence, better quality individuals may occupy the more favourable nest sites, resulting in a positive relationship between nest site quality and reproductive performance. Therefore, it is possible that the best quality nest sites vary each year with the better quality females adapting their choice to suit the conditions and maximise their reproductive success.

6.6. APPENDIX

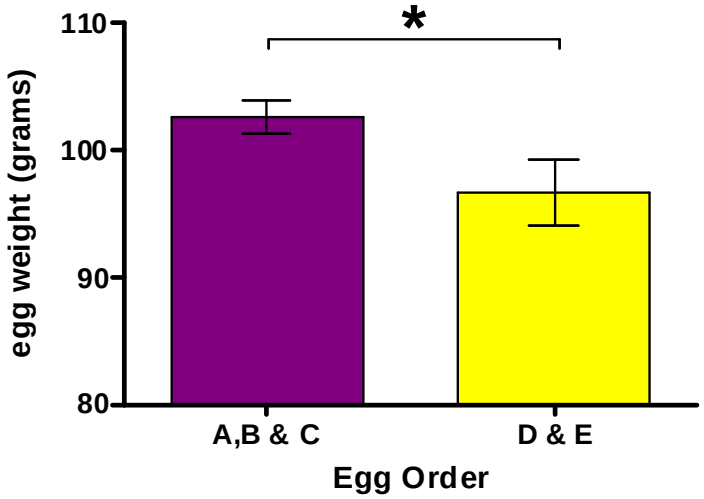


Fig.6.8 Egg weights (grams) according to grouped egg order (see Section 6.3.5) – A, B & C (*n* = 46) vs. D & E (*n* = 12). *: p = 0.046. Results obtained using a Linear Mixed Model with female identity included as a random factor.

CHAPTER 7

CORTICOSTERONE & INNATE IMMUNITY

7.1 ABSTRACT

Lysozyme forms part of an animal's innate immune response and is one of six major anti-microbial proteins deposited in avian albumen to help protect the embryo from bacterial infection, as embryos must develop in germ-free conditions. Lysozyme concentrations transmitted to a developing embryo may be influenced by female state (and possibly stress condition), especially if there are costs involved in the production and transmission of lysozyme to offspring, where females may reduce lysozyme concentration when they are in poor condition or stressed. On the other hand, certain environmental conditions, for example high pathogen presence, may favour increased albumen lysozyme concentration. We examined the relationship between concentrations of the stress hormone (corticosterone/CORT) and lysozyme concentrations in the eggs two closely-related species of *Larid*, herring and lesser black-backed gulls, which breed in overlapping colonies. We found albumen CORT did not correlate with lysozyme concentrations, suggesting that either the two are unrelated, or that CORT needs to be elevated (perhaps even chronically) in the maternal bloodstream to negatively impact on lysozyme production. In addition, we investigated whether albumen CORT and lysozyme concentrations varied between the species in the two different breeding colonies from which we obtained samples. At one of the colonies, we found albumen CORT to be significantly higher in herring than in lesser black-backed gull egg albumen, while lysozyme concentrations were significantly higher in lesser black-backed gull eggs. We discuss these findings in relation to the different potential pressures present at the two sites.

7.2 INTRODUCTION

Innate immunity is a non-specific immunity that is conferred by all those elements with which an individual is born and which are always present and thus available to protect the individual from challenges by foreign invaders (Benjamini *et al*., 2000). These elements include biological (e.g. inflammation and phagocytosis), chemical (e.g. pH, enzymes and interferons), general (e.g. fever) and physical (e.g. skin and mucous membranes) 'barriers' against infection (Miller & Harley, 1994). Lysozyme forms part of an animal's innate immune response and is one of six major anti-microbial proteins deposited in avian albumen to help protect the embryo from bacterial infection (Sibley, 1960, 1970; Board & Fuller, 1974, 1994). Laschtschenko (1909) was the first to identify the lytic properties of chicken albumen on bacteria, but it was Alexander Fleming who identified the substance involved and gave it the name 'lysozyme' (Fleming, 1922). Although lysozymes from different animals vary in their structure and composition, they exhibit the same biological function (Jollès, 1964). The role of lysozyme is to catalyse the lysis of Gram-positive bacteria (and to a lesser extent some Gram-negative bacteria – Burley & Vadehra, 1989; Bera *et al*., 2005) by hydrolysing the peptidoglycan (a polymer) of their cell walls (Rogers & Perkins, 1968). This lyses the cells as peptidoglycan is used to provide structural strength to the cell wall and counteracts the osmotic pressure of the cytoplasm (Board & Fuller, 1974). Therefore, concentrations of albumen lysozyme could be important in defending against bacteria and helping embryos to successfully reach the hatching stage, as embryos must develop in germ-free conditions (Board & Fuller, 1974). Furthermore, levels of innate agents, such as

lysozymes, transmitted to a developing embryo may be related to maternal state, especially if there are costs involved in the production and transmission of lysozyme to offspring (Saino *et al.*, 2002, 2007). Females in poor condition or experiencing stressful circumstances may have difficulty in producing lysozymes due to reduced resource availability, or preferential deployment of resources to other activities.

In addition to anti-bacterial proteins such as lysozyme, mothers transfer other substances into their eggs including nutrients, antioxidants and hormones, all important to the development of their offspring (see **Chapter 1** for details). As mentioned in previous chapters, one such substance is the stress hormone, CORT, which female birds transfer not only into the yolk of their eggs, but also the albumen (Eriksen *et al*., 2003; Rubolini *et al.*, 2005; Saino *et al.*, 2005; Downing & Bryden, 2008). Albumen is laid down in the 24 hours prior to laying (Warren & Scott, 1935). Therefore, concentrations of CORT in the albumen are likely to be a result of the stress-condition of the female within the 24 hours prior to laying (Downing & Bryden, 2008). On the other hand, yolk is laid down over several days (Conrad & Scott, 1935) and may reflect maternal hormonal condition over this longer timeframe. It is known that chronically elevated levels of glucocorticoids in the blood (such as CORT) can weaken the immune system, thereby increasing the risk of infection and reducing resistance to parasites and autoimmune diseases (Apanius, 1998). However, how increases in CORT concentrations in the maternal blood system affect the transfer of immune agents, and CORT itself, into the eggs for the developing embryos are unclear. Although egg and blood lysozyme differ in their production sites, with blood lysozyme produced by macrophages (Bandlow & Kühne, 1980) and those for egg deposition produced by

tubular gland cells in the oviduct (Palmiter & Gutman, 1972), tubular glands still have a blood supply and could be affected by CORT concentrations in the maternal blood system. It is possible that, if the final 24 hours before laying have been stressful to a mother, the elevated CORT concentrations in her bloodstream may negatively impact on the production of lysozyme to be transferred to the albumen. If albumen CORT is a true reflection of (and therefore correlated with) maternal plasma CORT (Downing & Bryden, 2008), we might predict that albumen CORT concentrations would then be negatively correlated with lysozyme concentrations. It is of course also possible that the alternative may occur, that is that the occurrence of poor environmental circumstances cause mothers to strategically elevate lysozyme concentrations in order to provide additional protection to the developing embryos, especially if the poor environmental circumstances might delay the onset of full incubation (as earlier-laid eggs are at greater risk of infection because of the rapid growth of microbes on unincubated eggs (Cook *et al*., 2005).

The main aim of this study was to examine the relationship between concentrations of the stress hormone CORT and concentrations of lysozyme in the albumen of eggs collected from two wild species of *Larid*, the lesser black-backed gull and the herring gull. We were also interested whether there were any species differences in CORT or lysozyme concentrations in the albumen. Herring gulls in Scotland have been declining for at least three decades in wild colonies, where as lesser black-back numbers have been stable or even increasing (Monaghan, 1979; Creme *et al*., 1997; Raven & Coulson, 1997; Mitchell *et al*., 2004). We have already found no difference in yolk CORT concentrations between the two species (**Chapter 4**), and would therefore

expect this pattern to continue for albumen CORT. Elevated lysozyme concentrations have been shown to correlate with increased hatching success and continue into early life (Saino *et al*., 2002). Given that the two species feed on similar prey prior to breeding, and breed in the same colonies, it is unlikely that exposure to pathogens would differ between them. We therefore predicted that these two species would not differ in their lysozyme concentrations in the albumen of their eggs. We also chose to sample two populations to help identify if any differences were brought about because of differences between colonies rather than direct species differences and included egg order to identify if this had any effect on the transfer of CORT and lysozyme to albumen.

7.3 MATERIALS & METHODS

7.3.1 Study sites, species & egg collections

Eggs were collected from two sites in the Firth of Forth (Isle of May, 2006 and Inchmickery, 2007). The Isle of May is situated in the north of the outer Firth of Forth. It is a designated National Nature Reserve and is managed by SNH (see **Section 4.3.1** for full details). Inchmickery is a small island situated north of Edinburgh. The site is designated as a Site of Special Scientific Interest (SSSI) and is managed by the RSPB (see **Section 5.3.1** for full details). Details of the study species can also be found in **Section 4.3.2**. See **Sections 4.3.3** (Isle of May) and **5.3.2** (Inchmickery) for details of the egg collections. For this study, only first laid clutches were used from both sites.

7.3.2 Lysozyme assay

Unlike the RIA used to measure CORT in albumen, there is no extraction procedure required when using this lysozyme assay as the other proteins in albumen do not interfere with the assay. Lysozyme concentrations were measured directly from samples of albumen by comparison with known amounts of lysozyme standard (L-6876, Sigma-Aldrich, Dorset, UK). 10µl of standard lysozyme solution (10µg diluted in 1ml PBS buffer – see **appendix 7.6.1** for buffer recipe) was added in triplicate to the first three wells of a 96-well plate (655180, 96W, Flat Bottom with Lid, Sterile Cellstar® Tissue Culture Plate, Greiner Bio-One, Stonehouse, Gloucestershire, UK). 10µl of serial dilutions of the 10µg/ml lysozyme standard (5, 2.5, 1.25, 0.6125, 0.3125, 0.15625 & 0µg/ml) were then added, in triplicate, to the next wells. 10µl of each sample were then placed in remaining wells, in triplicate and the plates gently shaken for one minute. 150µl of a bacteria/agarose solution was then added to all 96 wells in each plate. In order to make the bacteria/agarose solution, 0.5g agarose powder (Lonza SeaKem LE Agarose, Fisher Scientific, Loughborough, UK) was added to 50ml PBS in a glass bottle and autoclaved. In order to keep the agarose in solution it must be kept at 50-60ºC in a water bath prior to bacteria addition. In a bacteriology-grade fume hood, 25mg dried *Micrococcus lysodeikticus* (M3770, Sigma-Aldrich, Dorset, UK) was dissolved in 1.2ml PBS in a glass beaker. The agarose solution was then cooled in the fume hood (approx. 4 minutes) and added to the *M.lysodeikticus*. 50ml bacteria/agarose solution prepared as above can be used for a maximum of 2 plates before it begins to solidify. Following addition of the bacteria/agarose solution plates were kept in a CO_2 incubator

(Heraeus HERAcell® 240 CO_2 Incubator, Thermo Scientific, Waltham, Massachusetts, USA) at 37ºC, humidity 95% and with 5% CO_2 for 24 hours. Bacterial density was then read using a photometric microplate reader (Multiskan Ascent Photometric microplate Reader, Thermo Labsystems / Thermo Scientific, Waltham, Massachusetts, USA) at an absorbance of 850nm and results recorded to 3 decimal places (using Labsystems' Ascent software vers.2.4.1). Lysozyme concentrations in the unknown samples were calculated using the universal assay calculator Assay Zap (vers.2.69, Biosoft, Cambridge, UK). Inter-assay variation averaged 7.2% (18 assay plates), with an intra-assay coefficient of variation of 7.4 ± 1.1% and the assay sensitivity averaged 1.51µg/ml.

7.3.3 CORT Radioimmunoassay

CORT was extracted from the gull egg albumen using a methanol extraction and measured by radioimmunoassay using the protocols described in **Sections 2.5.2 and 2.5.3**. Extraction efficiency averaged 91 ± 8% (SD). Inter-assay variation averaged 6.3% (2 assays), with an intra-assay coefficient of variation of 17 ± 1.1% and the assay sensitivity averaged 0.21ng/ml.

7.3.4 Statistical analysis

We ran two Linear Regressions to assess the extent of the relationship between albumen CORT (log-transformed for normality) and lysozyme concentrations, one for each

species (**Section 7.4.1**). A linear mixed model was used to assess the effect of species on albumen CORT concentrations (**Section 7.4.2**), with female identity as a random factor and species and site as fixed factors (including an interaction terms between the two). We also included egg order as a fixed factor and an interaction between egg order and species. This model was repeated substituting lysozyme concentrations for albumen CORT concentrations (**Section 7.4.3**). Egg volume were included as a covariate and removed from the model where non-significant.

7.4 RESULTS

7.4.1 Albumen CORT concentrations

We found a trend for albumen CORT concentrations to be higher in the herring gulls ($F_{(1,109)} = 3.382$, $p = 0.069$) and there was a significant effect of site, with concentrations being lower in eggs from Inchmickery ($F_{(1,110)} = 6.374$, $p = 0.013$). However, there was no interaction between species and site ($F_{(1,110)} = 0.435$, $p = 0.511$; Fig. 7.1). There was a trend for albumen CORT concentrations to decrease with egg order ($F_{(2,77)} = 2.596$, $p = 0.081$), but no interaction between egg order and species ($F_{(2,76)} = 0.012$, $p = 0.989$; Fig. 7.2). Egg volume was significantly correlated with albumen CORT concentrations ($F_{(1,100)} = 12.138$, $p = 0.001$; Fig. 7.3), with larger eggs having lower CORT concentrations.

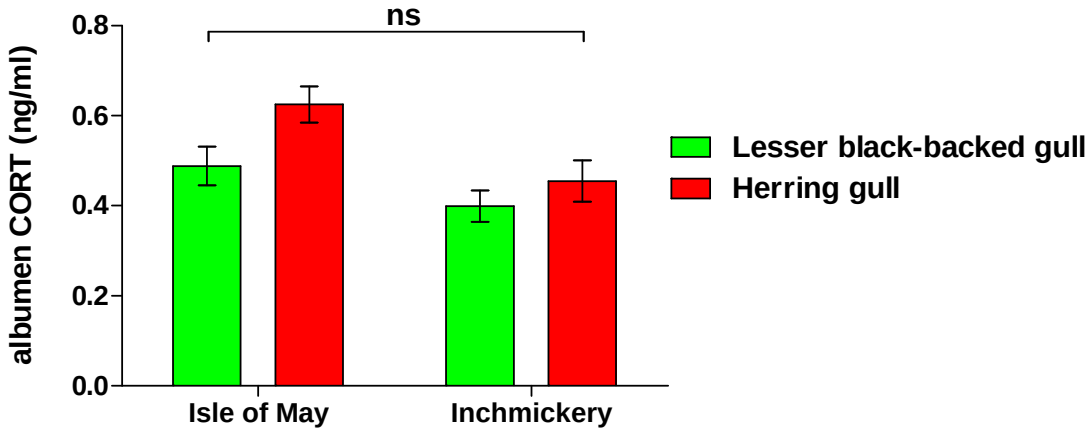


Fig.7.1. Mean albumen CORT concentrations (ng/ml) ± s.error for lesser black-backed and herring gulls according to site, where ns: site*species; p = 0.511. Lesser black-backed gull: Isle of May *n* = 29; Inchmickery *n* = 34. Herring gull: Isle of May *n* = 26; Inchmickery *n* = 31.

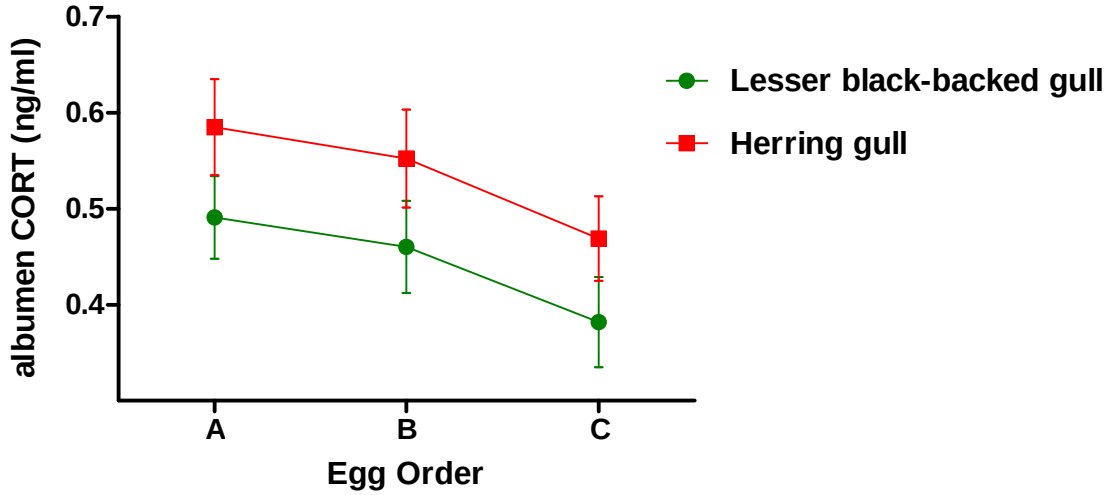


Fig.7.2. Mean albumen CORT concentrations (ng/ml) ± s.error according to egg (laying) order for the lesser black-backed (*n* = 24 nests) and herring (*n* = 20 nests) gulls.

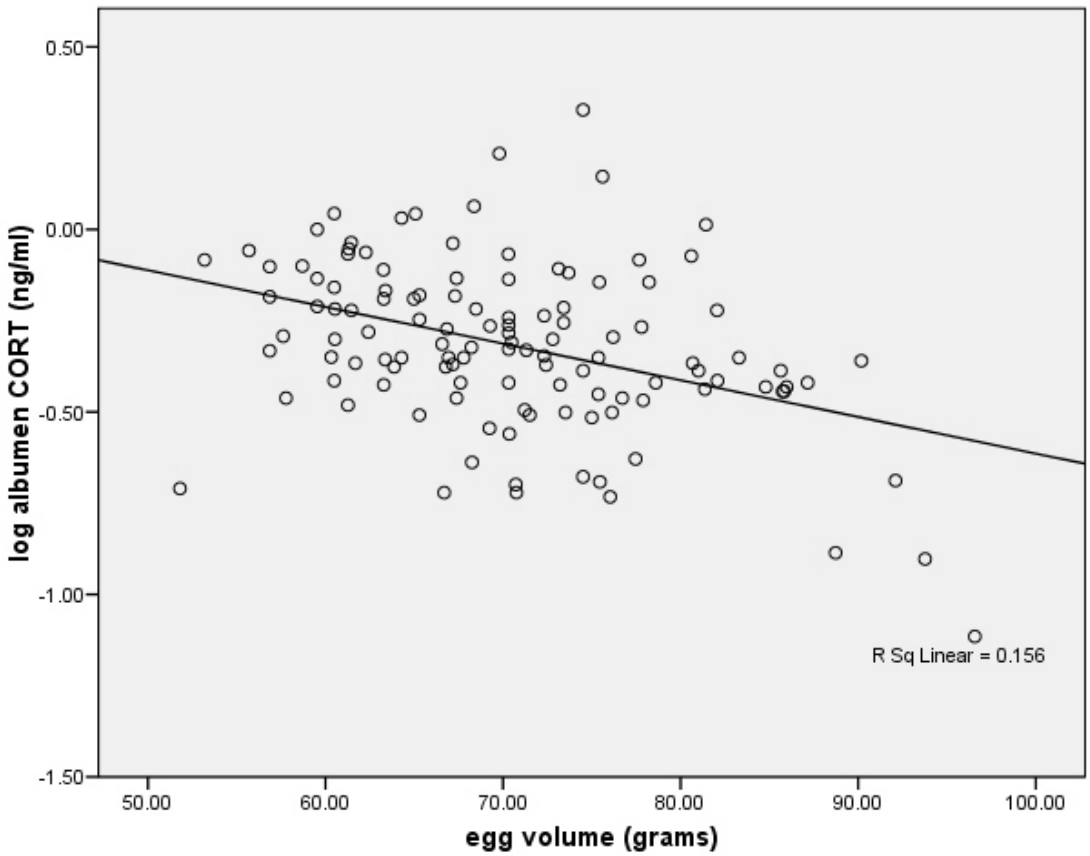


Fig.7.3. Relationship between log albumen CORT concentrations (ng/ml) and egg volume (grams), where $r^2 = 0.156$.

7.4.2 Albumen lysozyme concentrations

For lysozyme concentrations, there was no effect of site ($F_{(1,131)} = 0.083$, $p = 0.774$), but concentrations were significantly higher in the lesser black-backed gulls ($F_{(1,126)} = 4.358$, $p = 0.039$). There was also an interaction between site and species ($F_{(1,131)} = 11.075$, $p = 0.001$; Fig. 7.4), with concentrations in lesser black backed gulls being higher than herring gulls only on the Isle of May. Lysozyme concentrations did not vary according to egg order ($F_{(2,105)} = 0.051$, $p = 0.95$), nor was there any interaction between egg order and species ($F_{(2,105)} = 0.169$, $p = 0.845$; Fig. 7.5). Egg volume was not

significantly correlated with lysozyme concentrations ($F_{(1,122)}$ = 0.879, p = 0.35) and was removed from the model.

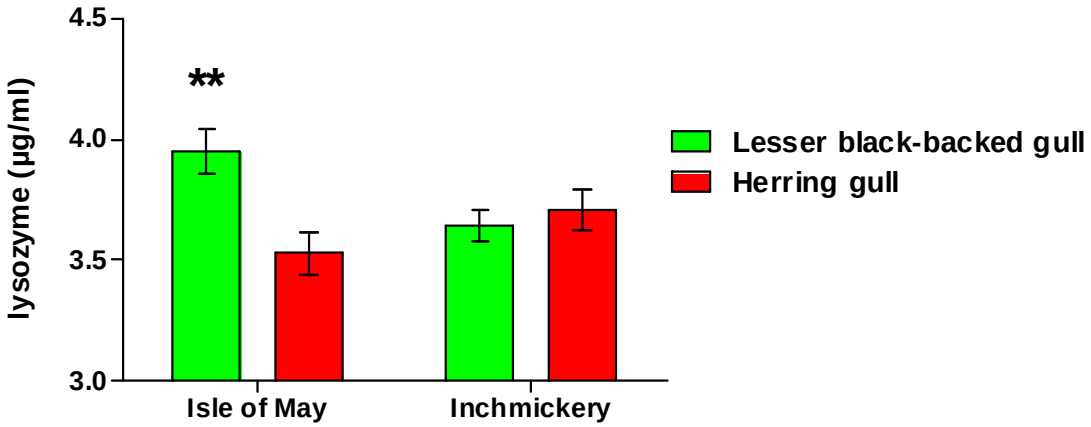


Fig.7.4. Mean lysozyme concentrations (µg/ml) ± s.error for lesser black-backed and herring gulls according to site, where **: site*species; p = 0.001. Lesser black-backed gull: Isle of May *n* = 30; Inchmickery *n* = 57. Herring gull: Isle of May *n* = 29; Inchmickery *n* = 33.

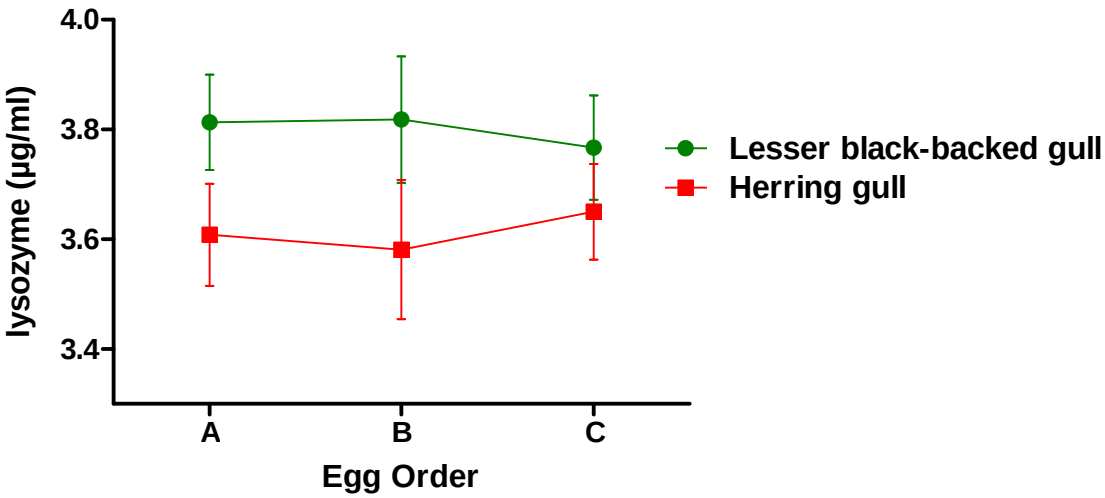


Fig.7.5. Mean albumen lysozyme concentrations (µg/ml) ± s.error according to egg (laying) order for the lesser black-backed (*n* = 31 nests) and herring (*n* = 24 nests) gulls.

7.4.3 Relationship between albumen CORT and lysozyme concentrations

Linear regressions showed no significant relationship between albumen CORT and lysozyme concentrations for either the lesser black-backed ($F_{(1,87)} = 0.141$, $p = 0.708$, $r^2 = 0.002$; Fig. 7.6) or herring ($F_{(1,93)} = 0.0.37$, $p = 0.848$, $r^2 = 0.0004$; Fig. 7.7) gulls. The coefficient of variation (CV) in CORT concentrations for the lesser black-backed and herring gulls were 61.7% and 47.8% respectively, while lysozyme CV for the lesser black-backed and herring gulls were both 13%.

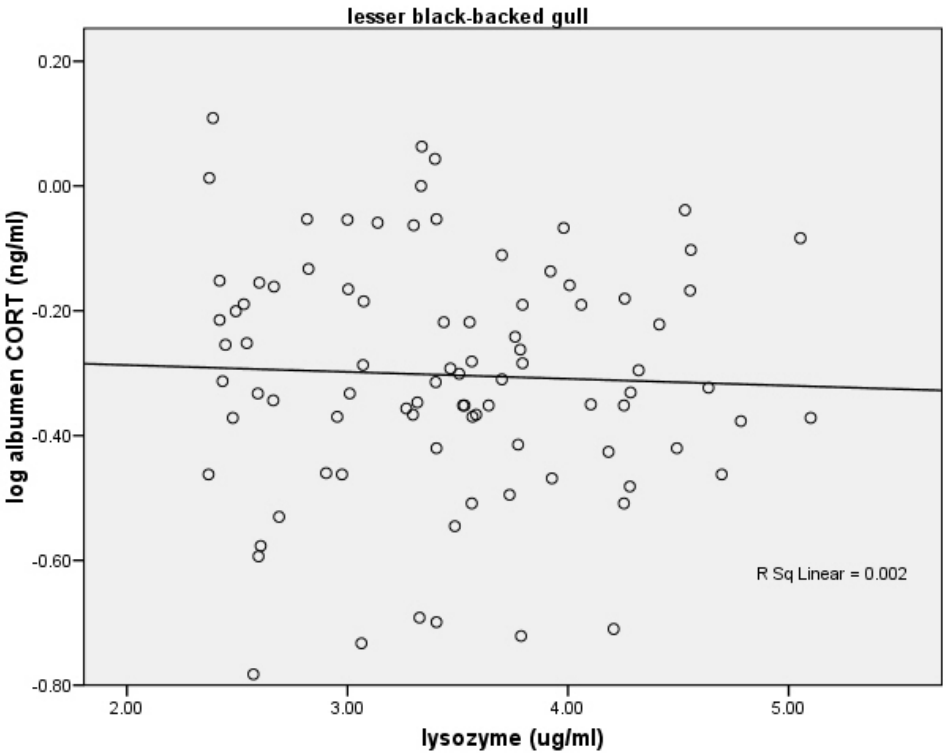


Fig.7.6. Relationship between log albumen CORT concentrations (ng/ml) and lysozyme concentrations (µg/ml) in the lesser black-backed gull, where $r^2 = 0.002$.

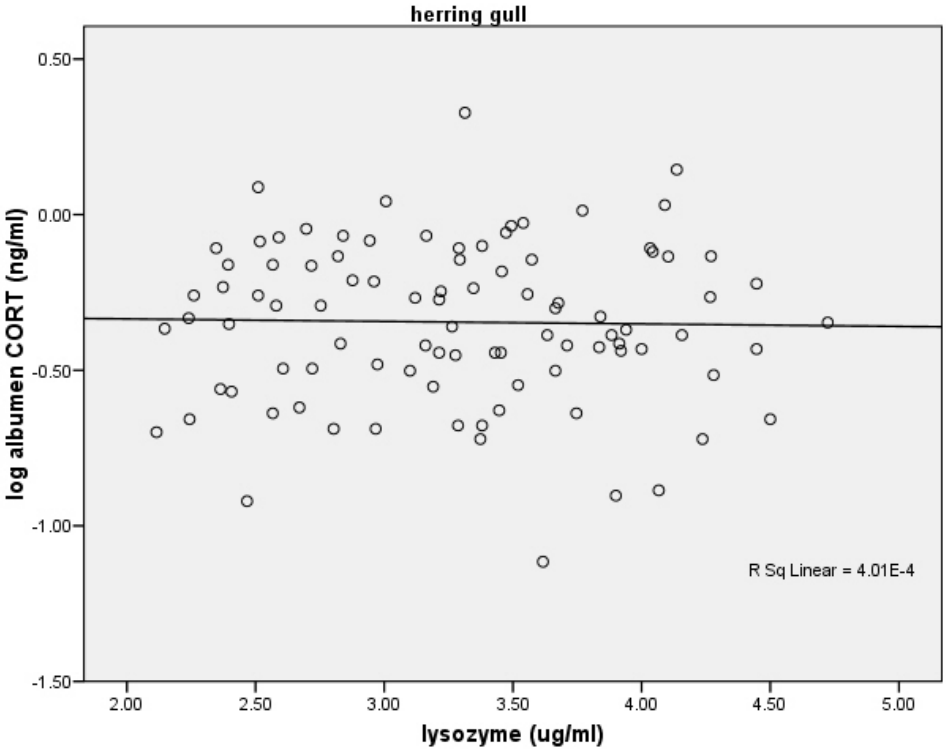


Fig.7.7. Relationship between log albumen CORT concentrations (ng/ml) and lysozyme concentrations (µg/ml) in the herring gull, where $r^2 = 0.0004$.

7.5 DISCUSSION

The results of this study have shown that there does not appear to be any relationship between albumen CORT and lysozyme concentrations. This may because the CORT concentrations in the bloodstreams of the birds sampled are not sufficiently elevated to result in any major effects on their immune systems (and subsequently the deposition of lysozymes into the albumen). It is likely that acute or chronic stress is needed to sufficiently impact on the immune system to result in changes in the concentrations of lysozyme that females transfer into their eggs (and under such acute / chronic stress females may not even breed). It may be that lysozyme is not costly to produce (Shawkey *et al.*, 2008) or is at least no more costly compared to other albumen

components (Tristram, 1953). Therefore, lysozyme deposition would not be significantly altered with female condition, or there are mechanisms in place for females to control the levels they deposit in their eggs. It is also possible that other factors such as the parasites present in the nest or nest densities have more influence on the deposition of lysozymes into the albumen than CORT.

Analysis of CORT concentrations in the two sites and between the two species revealed that albumen CORT concentrations were lower in Inchmickery eggs compared to Isle of May eggs. Preferably, this study would have included data from more than just two sites, but due to logistic reasons full clutches were only available from these two sites. The Isle of May varies from Inchmickery in that it is visited during the breeding season more frequently by researchers and tourists. Therefore, it could be the effect of increased human exposure which is increasing CORT concentrations in the albumen. Although there may be a statistically significant difference between the sites, the biological consequences, if any at all, are unclear. Elevated CORT in the albumen has previously been shown to have consequences for development, with eggs injected with CORT into their albumen shown to have reduced hatching success and produced fledglings with smaller body size and slower plumage development than controls (Saino *et al*., 2005). However, the concentrations we are dealing with are unlikely to be sufficiently elevated to result in major phenotypic changes as seen in the Saino study. In the model analysing the effects of species/site/egg order on albumen CORT, we noted that CORT concentrations decreased with egg volume. Egg size can be an important index of female quality as it reflects the amount of yolk reserves available for embryonic development and is associated with chick survival (Bolton, 1991). This

could provide evidence for a female quality effect on concentrations of CORT deposited into the egg. However, yolk CORT and albumen CORT from these samples do not correlate (see **Appendix 7.6.2**, Fig. 7.8), suggesting that other factors beside female condition are playing a role in CORT transfer (although differences in the activation of the stress response over the course of yolk and albumen production may play a role – see below). Finally, we found a trend for CORT concentrations to decrease with egg order, possibly a means of counteracting the negative effects of being the last-laid egg (brought about by hatching asynchrony and being typically smaller - Mock & Parker, 1997; Hillström *et al*., 2000). This could be the result of specific stressors on different days activating the stress response in females and altering CORT deposition or could be a signal of reducing female condition with the breeding season and energy expenditure dedicated to egg laying. The same pattern was not seen in yolk CORT concentrations from the same eggs (See **Appendix 7.6.2**, Figs.7.9 & 7.10), but this might be due to the longer production time for yolk compared to albumen, meaning that any changes in maternal condition over the laying period could be masked by earlier, more stable conditions.

As well as comparing albumen CORT concentrations between species and sites, we also compared lysoszyme concentrations. Lysozyme concentrations were found to be higher in the lesser-blacked gulls when compared to the herring gulls. However, this was driven by the lesser black-backs having higher concentrations only on the Isle of May. It is unclear why this might be the case and if there is any biological significance. It could be that females in better condition are able to deposit more lysozyme into their eggs with no effect on the other albumen components (although our only female

condition index, egg volume, did not correlate with lysozyme concentrations) and/or it is an inherited trait that has developed to allow these lesser black-backs on the Isle of May to be able to deposit higher concentrations of lysozyme, again without affecting the other albumen components. If lysozyme is costly, it could indicate that the other birds are in poorer condition, although this does not appear to affect breeding success. Our results for egg order effects also agree with those published by Shawkey *et al.* (2008), who showed that lysozyme concentrations do not vary according to egg order in several species (mainly passerines). However, our and Shawkey's results contrast with those of Saino *et al.* (2002), who reported that lysozyme concentrations decreased in last-laid eggs in barn swallows, which they suggested was an effect of decreasing female condition by the later stages of egg laying. When considering the reasons for these differing results, however, it must be remembered that gulls and barn swallows differ in their breeding behaviour as, while incubation in both species does not start until the penultimate egg is laid, gulls only lay 3 eggs (compared to 2-7 in barn swallows) and barn swallows typically lay more than one clutch per season (this only happens when gulls lose their first clutch) (Cramp & Simmons, 1983; Saino *et al*., 2002). This results in hatching asynchrony in the gulls (which is much smaller in the barn swallows), meaning the last-laid chick hatches somewhat smaller than its siblings (as is typically a smaller egg in the gulls -) and can suffer reduced competitive ability and poorer survival compared to their older, larger siblings (Mock & Parker, 1997; Hillström *et al*., 2000). The gulls sampled in this experiment had laid a maximum of 3 eggs and this was their first clutch of the breeding season. As we did not measure if there was any change in female body condition during our experiment, we cannot be sure if female condition was stable across the laying of the three eggs. If lysozyme

production is costly, we may see decreases in lysozyme concentrations when these birds lay a second clutch. If it is not costly, why would females not deposit greater concentrations of lysozyme in earlier laid eggs to compensate for time spent in the nest unincubated (Cook *et al*., 2005)? As mentioned before, it may be that lysozyme concentrations are limited because increasing them could decrease the levels of proteins and/or nutrients deposited in albumen that are essential for optimal embryonic development (Klasing, 1998; Shawkey *et al*., 2008), possibly supported by the relatively small variation and species similarities in lysozyme concentrations (CV = 13% for both species). Alternatively, differential deposition of anti-microbials within clutches may be unnecessary, as birds can increase anti-microbial activity through incubation (Board & Fuller 1974).

7.6 APPENDIX

7.6.1 Buffer recipe

4. Dissolve 9.5g Disodium Phosphate (Na_2HPO_4) (S5136, Sigma-Aldrich, Dorset, UK) in 1 litre of deionised water (A).
5. Dissolve 9.1g Potassium Dihyrdogen Phosphate (KH_2PO_4) (P0662, Sigma-Aldrich, Dorset, UK) in 1 litre of deionised water (B).
6. Mix 1 part A to 2 parts B (pH must not be lower than 6.3).

7.6.2 Yolk CORT

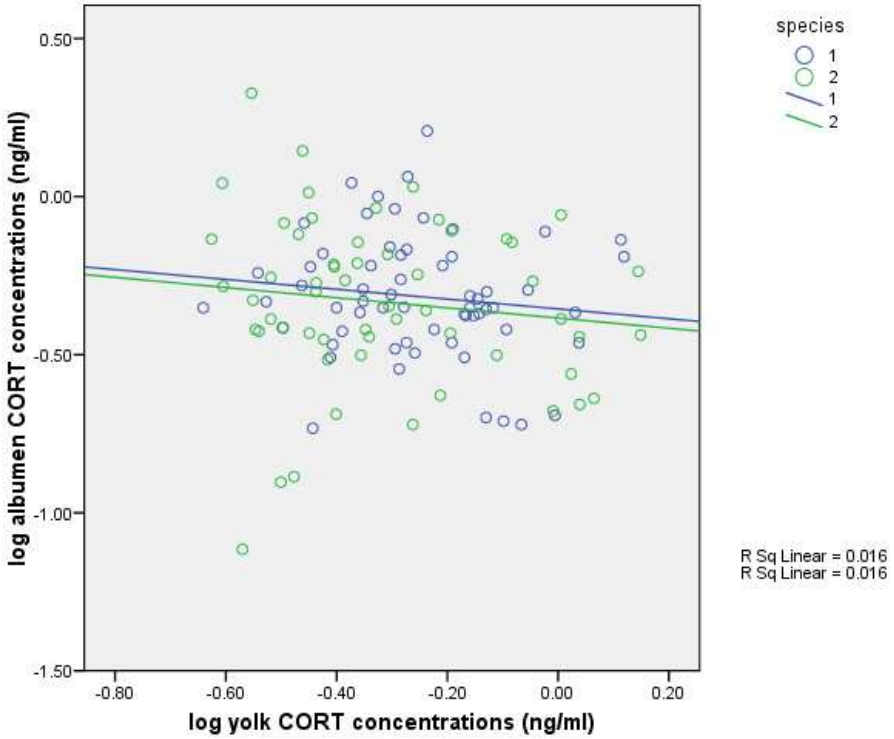


Fig.7.8. Relationship between log albumen CORT concentrations (ng/ml) and yolk CORT concentrations (grams), where 1 (blue) = lesser black-backed gulls ($F_{(1,61)}$ = 0.994, p = 0.323, r^2 = 0.016) and 2 (green) = herring gulls ($F_{(1,56)}$ = 0.903, p = 0.346, r^2 = 0.016).

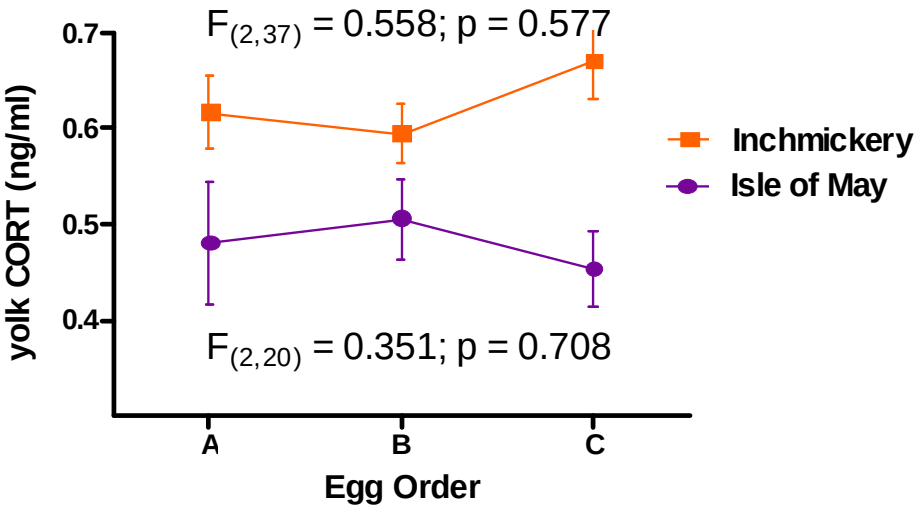


Fig.7.9. Mean yolk CORT concentrations (ng/ml) ± s.error for lesser black-backed gulls from Inchmickery and the Isle of May according to egg order.

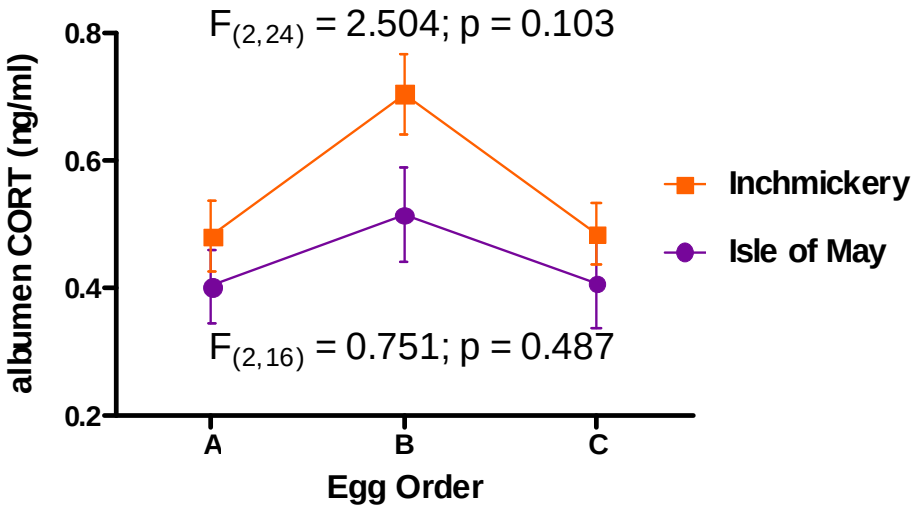


Fig.7.10. Mean yolk CORT concentrations (ng/ml) ± s.error for herring gulls from Inchmickery and the Isle of May according to egg order.

CHAPTER 8

GENERAL DISCUSSION

This chapter will briefly summarise the results of this thesis and I will discuss them in the context of their broader ecological and evolutionary significance. I will address some of the limitations of our findings and some potential studies that could follow on and enhance this work.

8.1 REVIEW OF FINDINGS

The main aim of this thesis was to investigate the effects of environmental conditions, particularly unpredictable or potentially negative ones, and their effects on the maternal transmission of the primary avian stress hormone, corticosterone, to an offspring. Birds are an excellent model to test hypotheses about maternal effects, as viviparous species (which birds are exclusively) have embryos that develop outwith a mother's body. This allows researchers to more easily quantify and manipulate environmental conditions (for example, stressful stimuli) or parental quality (for example, incubation behaviour) on embryonic development and in this study, hormone concentrations.

We found, using zebra finches in the lab, that unpredictable food availability (a potential stressor) experienced by mothers can elevate yolk CORT concentrations, but only when combined with the additional demand of laying a replacement clutch (likely to be a cumulative effect of unpredictable energy resources and the additional energy expenditure with producing two clutches) (**Chapter 3**). To further investigate whether environmental conditions influenced the maternal transmission of information relating to maternal habitat to the offspring, we looked at yolk CORT concentrations in two

populations of gulls in which the population trajectories differed depending on environmental conditions (proposed to reflect different levels of stress) (**Chapter 4**). The results however did not support this hypothesis, as there were no between species or between breeding site differences regardless of population dynamics. This would suggest that the different environmental circumstances (harsher for the herring gull) experienced by these two species are not reflected in differences in their eggs (at least in terms of CORT). This could be the result of the eggs being buffered from the maternal GC environment or it may be that the difficult environmental conditions are not occurring during the breeding season. We also identified that experimental human disturbance does not appear to elevate yolk CORT concentrations, although there was a trend for concentrations to be higher following the loss of the first clutch in the herring gull (as seen in the zebra finches in **Chapter 3**) (**Chapter 5**). This could suggest possible differences between the species in the ability to cope with negative environmental conditions (in this case the loss of a clutch). It also raises questions as to how we define human 'disturbance' and the potential habituation of these animals to human contact. In **Chapter 6**, we measured yolk CORT concentrations in Common Eider eggs and looked for differences according to the degree of nest shelter. We found no relationship between shelter and yolk CORT, but birds that laid in more sheltered sites had, on average, smaller eggs. This may indicate lesser quality birds are nesting in the sheltered sites and that yolk CORT is not affected by maternal condition. Finally, we looked at another mechanism through which information relating to the maternal environmental condition could be transferred to the embryo. We investigated whether there were any links between maternally derived immunity and CORT by comparing the anti-microbial lysozyme and albumen CORT concentrations (**Chapter 7**). We found

no correlation between CORT and lysozyme, suggesting that CORT (at least under 'basal' stress conditions) may not affect lysozyme production. Other factors such as colony density and 'cleanliness' might be more important in determining the concentrations of lysozyme deposited in the egg.

The general theme of our findings is that CORT concentrations in eggs do not appear to vary much with maternal environments. However, the extra investment needed in producing replacement embryos within a short timeframe would appear to have more influence on the maternal transmission of the primary avian stress hormone. So what does this tell us about the influence of changes in the maternal environment in influencing the transmission of signals to the developing offspring?

8.2. ADAPTATION OR BY-PRODUCT?

There is already considerable knowledge of the positive and negative effects that stress hormones can have on the development/fitness of offspring, making it possible to propose theories as to the adaptive and evolutionary significance of these maternally derived signals (see **Chapter 1**). However, we lack evidence that proves whether mothers can use these hormonal signals to communicate information about the external environment to their developing embryos and directly influence the fitness of their offspring. It has been shown in (female) guinea pigs (Lingas & Matthews, 2006), rats (reviewed in Kapoor *et al*., 2006), monkeys (Clarke *et al*., 1994; Coe *et al*., 2003), pigs (Haussmann *et al*., 2000) & quail (Hayward & Wingfield, 2004), that prenatal stress increases various aspects of glucocorticoid (GC) reactivity in offspring, a potential

adaptive benefit (Hayward & Wingfield, 2004; Kapoor *et al*., 2006). The theory of hormesis predicts that 'exposure to a low dose of a chemical agent or environmental factor induces an adaptive, beneficial effect on the cell or organism, but can be damaging at higher doses' (Mattson, 2008). Therefore, 'forewarning' your offspring about an unpredictable environment could help prepare them through changes in their development, including for example, reduced growth (if food is in low supply) or decreased stress sensitivity or risk-taking (if stressors such as predators, or perceived predators, are prevalent) (Bateson *et al*., 2004). This strategy has its risks though, as high concentrations of GCs transferred to a developing embryo could severely impair its development, including negatively impacting on learning, memory and reproduction (Sapolsky *et al*., 2000). Furthermore, if environmental conditions improve later in life, offspring 'prepared' for a poor environment may find themselves at a disadvantage. This has been shown in humans born with low birth weights, who develop increased risk of impaired glucose tolerance (Hales *et al*., 1991) and metabolic syndrome (a combination of medical disorders that increase the risk of developing cardiovascular disease and diabetes) later in life (Barker *et al*., 1993). The 'thrifty phenotype' concept (Hales *et al*., 1992; Hales & Barker, 2001) hypothesises that poor early life conditions (in this case nutrition) induce changes in the structure and function of organs and endocrine systems that will ensure that the glucose supply to the brain is maintained. This 'rationed investment' can have positive effects on survival through to reproductive age, but result in a sub-optimal phenotype not suited for survival later in life. Therefore, it would be predicted that maternal GC concentrations should correlate with those transferred to the offspring if a mother is trying to communicate information about the external environment to her offspring (if GCs are indeed the coding signal).

We found that negative/unpredictable conditions do not simply correlate with changes in GC concentrations transferred to the developing embryo. In the study on feeding conditions and stress in the zebra finch (**Chapter 3**), we found that yolk CORT concentrations were similar across control and 'experimentally-stressed' eggs, while their mothers' plasma CORT concentrations showed a trend for being elevated under the stress condition. The fact these measurements of plasma CORT were taken 4 days after the stress treatment had been removed suggested plasma concentrations would have been higher during the laying period. Then when the birds laid a second clutch and were faced with the feeding-stress, elevated yolk CORT concentrations were found. For GCs to be used in an adaptive sense, we would perhaps expect maternal and yolk concentrations to correlate with those in eggs. So, why block these signals when producing your first group of offspring, but allow them to be passed onto your offspring in the second group? This suggests that GCs could still be acting as adaptive signals when conditions become sufficiently negative, so as to either necessitate that this information is passed on or it is forcibly transmitted. But how could this work mechanistically?

It is possible that there is a threshold before maternal GCs are transferred to the embryo ('the threshold theory'), in an attempt to prevent short-term fluctuations in environmental conditions influencing embryonic and then possibly future development and survival. Alternatively, females could actively control the amount of GCs they transfer to their developing offspring ('the environmental regulation theory'), so that when conditions become difficult, they 'choose' to pass this information on to prepare

their offspring for a ‘tricky’ environment. The possible mechanisms involved for either the ‘threshold’ or ‘environmental regulation’ theories are unclear and need to be studied further. One possible mechanism that could be involved in both cases is CBG (corticosteroid-binding globulin), which binds and transports biologically active, unconjugated corticosteroids in plasma, potentially preventing them crossing the blood / follicle barrier until free (unbound) corticosteroids exceed CBG capacity (Breuner & Orchinik, 2002). We already know from studies in mammals, that use the placenta to constantly interact with the developing embryo, that there is an enzyme that regulates GC transmission (in addition to CBG being present in the blood and its possible role) known as 11ß-hydroxysteroid-dehydrogenase type 2 (11ß-HSD2). Under normal / basal conditions, 11ß-HSD2 is involved in regulating the exposure of a low dose of GCs to the foetus (Benediktsson *et al*., 1997). However, chronic elevation of maternal GCs (Otten *et al*., 2004) or the suppression of 11ß-HSD2 (Staud *et al*., 2006) can result in increased exposure of the foetal compartment to these maternal GCs. This method would allow females to confer information about the environment into which their offspring are born, but only when alterations in development or offspring ‘expectations’ are needed or, more likely, unavoidable. It is possible that CBG and/or 11ß-HSD2 exist (or similar compounds to them) in the likes of yolk, allowing GCs passed from mother to offspring to be limited in their capacity to diffuse into the embryo during the early stages of maturation. So, if maternal GCs do not necessarily correlate with those transferred to the offspring, can we use, for example, yolk GC measurements to tell us about the maternal stress state?

8.3. MATERNALLY DERIVED STRESS HORMONES – AN EFFICIENT MEASURE OF ENVIRONMENTAL STRESS?

In this thesis, we chose to sample yolks as they provide immediate nutrition of the embryo and are high in lipids, thus provide a likely depot for CORT accumulation. Given that the egg also contains a large proportion of albumen, which is deposited into the egg over a much shorter timeframe, we also characterised the concentrations of CORT in the albumen. In doing so, we limited ourselves to measuring CORT in simply the embryonic environment. In the chapters following **Chapter 3**, we focussed on measuring CORT in the yolks of wild birds rather than captive-bred animals. As discussed previously in this thesis, taking blood samples from wild birds, while not altering their reproductive behaviour or disturbing other individuals in close proximity (especially in colonial breeding animals), is difficult. It is however difficult to confirm the exact stressors present (and those that are causing a stress response) and the interactions between these environmental factors when dealing with wild and even human populations, as researchers cannot control the surrounding environment as would be more feasible in a controlled, laboratory environment (Monaghan, 2008). However, laboratory experiments have the downside that they provide less realistic measures of the effects that environmental conditions early in life have on fitness and their correlates (Monaghan, 2008). Although we will not have information about the maternal stress condition, we can still ask and attempt to answer questions relating to the effects stressors can have on changing the embryonic environment and the potential life-history and evolutionary effects thereafter.

Our results from the work on the gulls (**Chapters 4 & 5**) and the eiders (**Chapter 6**) suggest that CORT concentrations in the yolk of birds may not be strongly influenced by the maternal environment. Interestingly, similar results have been shown in another oviparous species from a separate taxon, the green anole (*Anolis carolinensis*). It has been shown in this species that yolk CORT in their eggs does not vary according to laying order (as seen consistently throughout this thesis), nor did a stressor (in this case diet) impact on yolk CORT concentrations (although it did on testosterone) (Lovern & Adams, 2008). In the Lovern & Adams study, as with our study on human disturbances, it may simply be that the stressors vary in their impact on the activation of the HPA axis and the subsequent effects transmitted to the developing offspring. Studies on humans have found that the World Trade Centre attack had a significant impact on foetal development (by reducing growth) in mothers at or close to the disaster (Berkowitz *et al*., 2003), whereas a major earthquake only resulted in an early, but not premature, birth date (not seen after the terrorist attack) (Glynn *et al*., 2001). Significantly, it has been shown previously that increased maternal cortisol concentrations are responsible (at least in part) for lower birth weights in humans (Seckl & Meaney, 2004). These results add to increasing evidence that the activation of the stress response and the transmission of this information to the offspring can be unpredictable and the effects not always as expected.

Irrespective of experimental conditions, it was interesting to note the potential for differences between species that could have implications for broad generalisations across taxa. We found, for example, that average yolk CORT concentrations in the Eiders were high compared to the gulls and finches (1.5ng/ml compared to an average

of 0.5ng/ml), although there was some overlap across species. In the gulls, a trend for the herring gull yolks to have higher CORT in the 2nd clutches suggests there may be differences in the stress responses between the species. Furthermore, there is the potential for differences between altricial and precocial birds due to their differences in their development, hatching and care needs (Monaghan, 2008) that could be influenced by maternally derived hormones. Several studies have shown that the sexes can respond differently to negative early life environmental conditions in birds (Nager *et al*., 1999; Martins, 2004), including the stress response (Lormee *et a*l., 2003; Spencer & Verhulst, 2007; Madison *et al*., 2008; Wada *et al*., 2008), but differences in the stress response between sexes have also been found in the likes of guinea pigs (offspring - Lingas & Matthews, 2006), rats (adults - Knuth & Etgen, 2005; offspring - Kapoor *et al*., 2006), humans (Kajantie & Phillips, 2006) and elasmobranches (Manire *et al*.,2007). Differences between viviparous and oviparous species are another obvious area where, despite the HPA axis being relatively similar morphologically and/or functionally across taxa in adult vertebrates (Carsia, 1990; Norris, 1997; Wingfield & Ramenofsky, 1999), the communication between embryo and mother are substantially different. I have already briefly discussed some of the mechanisms that are/may be in place to regulate continual embryo/mother contact during gestation. Oviparous mothers (and fathers) have their own tools for further influencing the development of their embryos after laying though, with changes in incubation behaviour potentially altering development. Concentrations of hormones in eggs change over embryonic development (most work having focused on androgens so far – see Navara & Mendonça, 2008), with some in chickens diffusing into the embryo as early as 2 days into incubation (when blood circulation begins) (Romanoff, 1960). Others may be metabolised into other

compounds (similar to the placenta 11ß-HSD2 enzyme regulation) and when the HPA axis begins to develop (the pituitary gland beginning development after approximately 1 day and the adrenal gland after 4-5 days in chickens) (Elf & Fivizzani, 2002; Jenkins & Porter, 2004), the embryo could begin synthesising GCs (with notable increases by day 14 in chickens – Wise & Frye, 1973), in preparation for hatching. Furthermore, the ability to 'detect' GCs could be severely hampered by receptor levels, which are known to significantly increase with the increases in GCs in the later stages of development (Porter *et al*., 2007), but less is known about their activity in the earlier stages of development. It would make sense to delay receptor development until later in the developmental sequence, so as to prevent high GC concentrations in the yolk or those crossing the placenta to have major, detrimental effects on development. It also appears that the ratio between the two main types of receptors, Glucocorticoid Receptors (GR) & Mineralocorticoid Receptors (MR), could be important in regulating the effects of pre- and post-natal stress (as well as throughout an individual's lifespan), as MR have a higher affinity for GCs and can buffer an individual from the damaging effects of chronic GC concentrations. (Lai *et al*., 2007). Over-activation of GR by consistently high levels of circulating glucocorticoid can make neurones vulnerable to neurotoxins (Reagan & McEwen, 1997; Sapolsky, 2000), disrupting neuroendocrine control mechanisms and impairing cognition and behaviour. Predominant MR activation, as opposed to activation of MR and GR simultaneously, can trigger distinct and even opposite responses (de Kloet *et al*., 1999). Therefore, a higher MR:GR ratio could provide greater protection against chronically elevated concentrations of GCs. Finally, the role that fathers could have on hormone transfer has so far lacked investigation.. There is evidence in the literature that females can manipulate investment in their

offspring depending on the quality of their partners (Sheldon, 2000; Kolm, 2001; Saino *et al*., 2001; Rutstein *et al*., 2004), with changes in the activation of the maternal HPA axis possible depending on the quality of care, supervision and protection a mate may give. Subsequently, alterations in maternal deposition of GCs may also occur.

One issue that has recently become of interest to those studying GCs is the potential for the secondary stress hormones (i.e. cortisol in those where CORT is the primary hormone, and vice versa) to play an active role in early development. There is a contradiction present, as although GCs have profound effects on embryonic development, circulating levels are low neonatally (Schmidt & Soma, 2008). It has been found that the thymus and bursa of Fabricius in chickens (a primary immune organ in birds, with the functional equivalent in mammals being the bone marrow (Cooper *et al*., 1966; Abdou & Abdou, 1972)) have the potential to synthesise cortisol from cholesterol in areas outwith the adrenals (Lechner *et al*., 2001). This could allow the chicken immune system to produce cortisol locally (rather than coming from the adrenal gland and through the bloodstream), meaning that cortisol may be more important than initially thought in the development and function of certain tissues (Schmidt & Soma, 2008). Schmidt & Soma measured baseline CORT and cortisol concentrations in the plasma of developing zebra finches, as well as in the immune organs (bursa of Fabricius, thymus, spleen) and certain brain regions. Although CORT was the predomiant GC measured in plasma, cortisol was the predominant GC in the immune tissues (and decreased with age, compared to CORT that increased with age in plasma). Cortisol concentrations were also higher in the immune organs compared to plasma. In the brain, CORT and cortisol concentrations were similar (and low), suggesting the

zebra finch brain does not have the ability to synthesise these hormones. These results indicate that local production of GCs in immune tissues could allow GCs to regulate the immune system (at least in part), while buffering against high GC concentrations during development (and the potential detrimental effects). In a sense, “stressed tissue in a calm organism” (Breuner, 2008). This could have serious implications for research in this field, with the role of stress hormones in the blood being under- or over-emphasised.

8.4 FUTURE STUDIES

It is clear from some of the discussion above that there are fundamental gaps in our knowledge regarding the roles of GCs according to sex, age, taxa, stressors, previous experience, receptors and their synthesis in the tissues. In addition, there are other areas that could be of importance in understanding GCs and their maternal transmission.

Firstly, it is still unclear if there is a direct, correlated relationship between maternal GC concentrations in the blood and those transferred to the developing offspring. Experimental studies using various stressors (predators, diet, isolation, handling, temperature and sexual and same-sex competition) should be used to assess the basal and stress-induced plasma concentrations of mothers during the laying period, and if using birds or reptiles, compare these to concentrations in yolk. As mentioned in this thesis, this methodology does have the problem of the effect of blood sampling during the egg laying period. It may be necessary to acclimatise the experimental animals to human contact and blood sampling to attempt to minimise any effects.

Alternatively, the use of blood-sucking bugs has offered a novel, non-invasive blood sampling technique (Arnold *et al*., 2008). Various degrees of stressors could also be used to test if the 'threshold theory' (see **Section 8.2**) is indeed correct. This research could be carried on to then study effects during development by measuring various body condition correlates to investigate patterns of growth and development. It may also be possible to use whole embryo in situ hybridisation techniques (as brains may be too small in many species) to investigate the effects on receptor development and balance throughout the different stages of embryonic development. CORT injections into yolk could then also be used to examine the effects on development through the stages of maturation. Using birds would also allow investigators to manipulate incubation conditions to investigate how parents could alter development by changing the incubation strategy.

A recent pilot study as part of an undergraduate project I ran in collaboration with Dr Karen Spencer investigated the changes in CORT concentrations in different layers of yolk. In birds, yolk is laid down in concentric layers over the period of approximately 3-10 days (depending on the species) (King, 1973), but these layers begin to break down once incubation is under way. However, if these eggs are sampled prior to incubation, there is the potential to identify if GC concentrations vary in the layers according to the daily GC concentrations in the maternal plasma (Adkins-Regan *et al*., 1995; Lipar *et al.*, 1999; Bowden *et al*., 2001). Varying GC concentrations in the layers could have effects on development, for example, high concentrations in the internal layers where the embryo is situated. Our pilot study, although limited in sample size, suggested that concentrations did not vary significantly according to layer

(internal, intermediate and external). If this studied was to be repeated with a larger sample size and perhaps experimentally controlled stressors on different days, it could provide evidence of fluctuations in maternally derived GC concentrations directly related to individual stressors and if females can manipulate CORT concentrations according to the layer formation. It could simply be that our birds did not receive different stressors on the various days of yolk production and hence we could not see any differences according to layers. It should be noted that the layers needed to be able to detect sufficient GC concentrations may be too small in many species, so large-egg species would be advantageous. This could limit the use of captive-bred animals for such a study however. Turtles may provide an interesting model species, as their yolks are laid down over much longer periods than birds (perhaps as much as 10 months in the painted turtle, *Chrysemys picta;* Congdon & Tinkle, 1982), giving investigators more scope for identifying correlations between individual stressors and maternal GC deposition. In addition, all follicles in a given clutch are yolked simultaneously (Congdon & Tinkle, 1982), meaning each egg should be exposed to the same maternal hormone environment. Differences between eggs could hint at mothers selectively altering the GC deposition into yolk.

In addition to (and possibly interacting with) CORT, other hormones, nutrients and immune agents could be used to transfer information about the maternal environment to the embryo. In **Chapter 7**, we examined the relationship between CORT and lysozyme (an innate antimicrobial) concentrations. Although we found no correlation, it may still be possible that activation of the HPA axis in females can directly impact on the concentrations of immune agents transferred to offspring.

Lysozyme is just one of many anti-microbial proteins transferred from mother to embryo. For example, Avidin combines with biotin (essential for cell/bacterial growth) and Apoprotein with riboflavin (essential for basic functions of the cell metabolism), making both these compounds unavailable for use by microbes (Board & Fuller, 1974; Fassbinder *et al*., 2000). It may require all these anti-microbial proteins to be analysed along with albumen CORT to decipher if there is indeed a relationship between innate immunity and stress. Furthermore, this study provided tentative evidence for a trend in differences in albumen CORT over the laying order. As CORT is laid down in the albumen over the last 24 hours before egg laying, albumen CORT measures could be used to provide more accurate measures of the effects of individual stressors on maternal transmission of CORT. Hence, albumen may provide a better source for identifying the relationship between maternal GC concentrations and those transferred to the egg/offspring, although yolk CORT is more likely to influence the development of the embryo. There could also be effects of stress on the transfer of antibodies to developing offspring, but this could be much more difficult to study due to the effects of previous exposure to disease (resulting in antibody production in the mothers).

CHAPTER 9

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