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# Corticosterone responses to different stimuli and phenotypic plasticity in corticosterone responses in the kororā (little penguin, *Eudyptula minor*)

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## Abstract

The overall aims of the research presented in this thesis were to compare in little penguins (*Eudyptula minor*) corticosterone concentrations following exposure to a variety of stimuli with corticosterone responses to handling, to investigate the relationship between corticosterone response to handling and corticosterone concentrations after exposure to different stimuli, and to examine plasticity in corticosterone responses. The study involved measurement of corticosterone concentrations in blood samples collected after a 15 min stimulus. Handling resulted in a greater mean plasma corticosterone concentration than all other stimuli. Plasma corticosterone concentrations in little penguins in nestboxes after the presentation of a novel object were greater than concentrations after the sound of dog barking and the sound of human talking. Corticosterone concentrations were higher in birds that were exposed to penguin alarm calls than birds exposed to human talking. Differences between mean corticosterone concentrations after human talking and dog barking, and between mean concentrations after the penguin alarm call and the novel object, were not significant. The mean concentration after human talking was the lowest and the mean concentration after novel object was the highest relative to responses to handling. Corticosterone concentrations after handling and concentrations after exposure to other stimuli were not correlated. A reaction norm revealed the existence of plasticity in corticosterone concentrations in little penguins. The reaction norm approach used to determine plasticity in this study did not allow for the quantification of the degree of plasticity.

The findings of the present study have provided information about the way little penguins respond to different type of stimuli, whether responses to handling are similar to responses to other stimuli in the same bird, and show that the use of a reaction norm approach can provide useful information about plasticity in corticosterone responses to different stimuli in birds.

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## Chapter 1: Introduction

Animals live in complex environments. They are exposed to a wide variety of stimuli, some of which may be threatening. Stimuli that are perceived to be threatening activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased secretion of glucocorticoids from the adrenal gland. The primary glucocorticoid in birds is corticosterone, whereas in most mammals the primary glucocorticoid is cortisol. Stimuli that activate the HPA axis are called stressors, and animals are said to experience stress when the HPA axis is activated in response to a stressor. Corticosterone responses in birds have been widely studied to provide information about responses of birds to stressors.

A stimulus used frequently by researchers to induce corticosterone responses in birds is capture and restraint, often called capture and handling. This stimulus has been applied to free-living birds, to birds that have been captured and held in captivity, and to domesticated birds. For example, corticosterone responses have been measured in species such as the Japanese quail (*Coturnix japonica*; Malisch *et al.*, 2010), chicken (*Gallus gallus*; Ericsson *et al.*, 2014), Eurasian tree sparrow (*Passer montanus*; Li *et al.*, 2017), and North Island saddleback (*Philesturnus rufusater*; Adams *et al.*, 2010). A blood sample is collected as soon as possible after birds are captured or picked up, then further blood samples are collected over periods that are usually up to 30 minutes, and sometimes up to 60 minutes after the time of capture. It is generally assumed that capture and restraint is analogous to a natural stressor such as an attack by a predator (Wingfield *et al.*, 1992), and that the corticosterone response to capture and restraint is a measure of the responsiveness of a bird to natural stressors. However, this is only an assumption and very few studies have considered corticosterone responses of birds to stimuli other than capture and restraint.

Phenotypic plasticity is the capacity of a given genotype to have different phenotypes in different environmental conditions, so plasticity in a trait is the capacity of the trait to alter as environmental conditions change (Cockrem, 2013a). Corticosterone responses of animals can differ between environments or at different times of the year, so the responses show phenotypic plasticity. For example, faecal corticosterone concentrations in greylag geese (*Anser anser*) were higher in geese exposed to high environmental temperatures than in geese exposed to low environmental temperatures (Frigerio *et al.*, 2004). One method of measuring plasticity in corticosterone responses is to generate reaction norms, which are graphical representations of corticosterone concentrations measured in different environmental situations. To date, no studies have been conducted to investigate the plasticity of corticosterone responses of birds to different stimuli.

Corticosterone responses to capture and handling have been measured in a range of species of penguins such as Magellanic penguin (*Spheniscus magellanicus*; Hood *et al.*, 1998; Walker *et al.*, 2005), Adelie penguin (Pygoscelis adeliae; Vleck *et al.*, 2000; Cockrem *et al.*, 2008b; Cockrem *et al.*, 2009a), kororā (*Eudyptula minor*; Chilvers *et al.*, 2016; Cockrem *et al.*, 2017b), and King penguin (*Aptenodytes patagonicus*; Viblanc *et al.*, 2016). Cockrem *et al.* (2017b) measured corticosterone responses to capture and restraint in a large sample size of kororā. There have not been any studies of responses of any species of penguin to more than one stimulus. In the present study, corticosterone concentrations were measured after little penguins were exposed to a range of stimuli, and these responses were compared to the response to the standard capture and restraint protocol stimulus. This literature review provides an overview of stress, corticosterone, and the mechanisms involved in physiological responses to stressors. It also evaluates studies of corticosterone responses in birds to capture

and handling, and to other stimuli. Finally, it discusses the concept of phenotypic plasticity and how reaction norms may be used to measure plasticity in corticosterone responses.

## 1.1 Stress in animals

### 1.1.1 What is stress?

Stress is a term that is widely used and rarely defined. Stress is defined here as a situation in which the HPA axis is activated with increased secretion of glucocorticoids in response to a stimulus that is perceived to be a threat (Cockrem, 2013a). Although the word stress has a negative connotation to it, a stress response is actually a mechanism to help an animal adjust to the situation (Herman and Cullinan, 1997). Whilst animals may change their behaviour when they experience stress, the measurement of stress requires the measurement of glucocorticoids. When a stress response is initiated, there is activation of the sympathetic nervous system with adrenaline and noradrenaline release which induces immediate changes in bodily functions such as an increase in heart rate to facilitate quick responses to the stressor (e.g. fight or flight; Carravieri *et al.*, 2016). The HPA axis and the sympathetic nervous system are activated together at the beginning of a stress response (Fischer and Romero, 2016).

### 1.1.2 What is a stressor?

A stimulus that triggers HPA axis activation is termed a stressor. Stressors include both stimuli from an animal's environment and stimuli from within the body. They can be classified as physical or emotional stressors (Table 1.1). Physical stressors can be internal stimuli such as a reduction in blood glucose concentrations, or external stimuli such as a change in air temperature. Emotional stressors are stimuli such as predator vocalisations that are perceived as threatening once information from the environment has been assessed in

relation to learned or inherited information (Cockrem, 2013a; Serrats *et al.*, 2017). Stressors stimulate the activity of various areas of the brain that interact with the HPA axis to produce a stress response (Dayas *et al.*, 2001).

Table 1. 1. Examples of physical and emotional stressors.

| Species | Physical | Emotional | Reference |
|---|---|---|---|
| Rat | | Maternal defence | (Neumann *et al.*, 2001) |
| Rat | Fasting, temperature manipulation (cold/heat) | Crowding | (Djordjevic *et al.*, 2003) |
| Chicken | | Blue ball point pen (novel object), restraint | (Fraisse and Cockrem, 2006) |
| European starling | | Human talking, radio playback, cage rolling, bag restraint, cage tapping | (Cyr *et al.*, 2007) |

## 1.2 The hypothalamic-pituitary-adrenal (HPA) axis

The hypothalamus, anterior pituitary, and adrenal gland are the three structures that make up the HPA axis, the key system responsible for stress responses in animals (Fig.1.1).

The initial process that happens when the HPA axis is activated by a stressor is the release into the pituitary portal blood of corticotrophin-releasing factor (CRF) which is synthesised in neurons in the hypothalamic paraventricular nucleus (PVN; Aguilera, 1998). Arginine vasotocin (AVT) in birds (Castro *et al.*, 1986) and vasopressin (VP) in mammals (Aguilera, 1998) are also released from neuronal fibres that terminate in the median eminence (Aguilera, 1998; Cornett *et al.*, 2013).

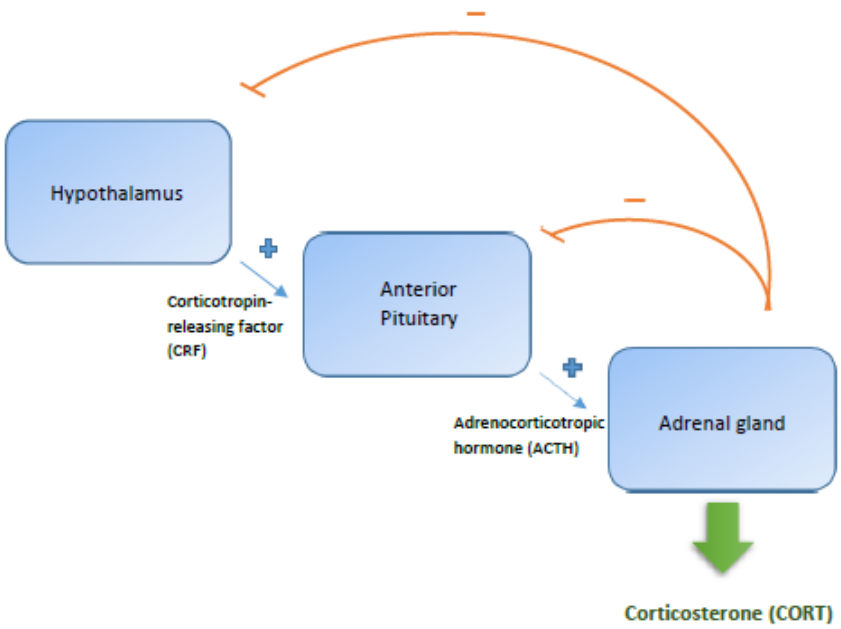



Fig. 1. 1. A schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis. The blue arrows point to the type of tissue the secretory products affect. The stimulatory action of the secretory products is indicated by (+), whereas the inhibitory feedback by the adrenal glucocorticoids is indicated by (−).

CRF binds to receptors (CRF receptor 1 and 2) on cells of the pituitary called corticotrophs, stimulating to synthesis of proopiomelanocortin (POMC; Shipp *et al.*, 2015; Gallo-Payet, 2016; Harris and Carr, 2016). Post-translational processing of POMC leads to the release of adrenocorticotropic hormone (ACTH) into the blood (Stevens and White, 2010). AVT and VP are also ACTH secretagogues (Castro *et al.*, 1986; Aguilera, 1998). ACTH from the pituitary gland binds to receptors in adrenocortical cells and initiates the synthesis and secretion of corticosterone or cortisol. The glucocorticoids have negative feedback on the HPA axis, in particular at the level of the hypothalamus (Aguilera, 2011).

## 1.3 Glucocorticoids

### 1.3.1 Actions of glucocorticoids

Glucocorticoids are corticosteroids which have metabolic actions and also influence a wide variety of other processes (e.g. reproduction or migration; Angelier and Wingfield, 2013). The primary function of corticosterone is to increase blood glucose levels, both in response to fasting and also to support immediate survival in acute situations such as a predatory encounter (Munck *et al.*, 1984).

#### 1.3.1.1 Effects on physiology

Reproductive function can be inhibited when corticosterone secretion increases. Glucocorticoids have been proposed to be involved in the timing of breeding in birds that leads to offspring being reared in a 'food-rich' environment (Lattin *et al.*, 2016). Elevated corticosterone can reduce reproductive activity by influencing yolk synthesis, decreasing the likelihood of laying eggs in birds (Salvante and Williams, 2003), and by suppression of responses of ovarian cells to reproductive hormones in rats (Hsueh and Erickson, 1978). Corticosterone can have direct effect on gonadal cells (Hsueh and Erickson, 1978) rather than altering the level of reproductive hormones (Astheimer *et al.*, 2000).

Glucocorticoids influence the immune system by both stimulating and reducing immune responses (Schmidt *et al.*, 2009). Glucocorticoid effects are dependent on glucocorticoid concentrations and duration of exposure (BrougHolub and Kraal, 1996). Corticosterone can reverse inflammation by stimulating the production of proteins that have anti-inflammatory effects during periods of infection or disease (Carnuccio *et al.*, 1987; Knowles *et al.*, 1990). Corticosterone also participates in reducing the release of free-radicals such as nitric oxide from macrophages (BrougHolub and Kraal, 1996), and promotes immune reactions by

bringing leukocytes to target tissues (Dhabhar *et al.*, 1996). In some cases, corticosterone can have suppressive effect on antibody generation (Stier *et al.*, 2009).

A glucocorticoid rise in blood is helpful to animals as it promotes appropriate responses towards a stressor. There is also the general notion that increased corticosterone over prolonged periods can be detrimental (Sapolsky *et al.*, 2000) and can negatively affect functions such as immune activity and growth (Fowles *et al.*, 1993). Intravenous treatment with dexamethasone (DEX), an imitator of glucocorticoid activity, has been used to study the harmful effects of extended exposure to corticosterone in birds (Birrenkott and Wiggins, 1984). For example, in ducks (*Anas platyrhynchos*), a small dose of DEX given for a month had a dampening effect on immunity, as indicated by lowered antibody levels detected in the blood (Fowles *et al.*, 1993). A period of four weeks of DEX treatment markedly reduced the body weight of ducks (Fowles *et al.*, 1993). In another study using chickens, birds that received DEX several times within 33 days had reduced growth (Wideman and Pevzner, 2012). It is difficult to study effects of high corticosterone concentrations in birds using DEX however, because firstly, it is a synthetic glucocorticoid which may not induce the same physiological responses as corticosterone, and secondly, DEX's half-life is longer than corticosterone (e.g. 36 min for DEX and 22 min for corticosterone in chickens; Birrenkott and Wiggins, 1984) indicating that DEX can have longer impact on the body compared to corticosterone. Furthermore, in nature, birds generally do not have persistently high glucocorticoid concentrations so it is not certain that these deleterious effects will occur in free-living birds.

#### 1.3.1.2 Effects on behaviour

Corticosterone can influence chick behaviours such as begging (Loiseau *et al.*, 2008) and aggression (Quillfeldt *et al.*, 2006). Corticosterone levels can rise when chicks are exposed to stressors such as a lack of food (Kitaysky *et al.*, 2003). Offspring may also receive glucocorticoids in yolk from their mother which can affect the way the offspring will respond to stressors throughout their life (Hayward and Wingfield, 2004). Chicks that received corticosterone tended to beg more compared to their counterparts that did not receive corticosterone treatment (Loiseau *et al.*, 2008). Begging calls were also produced in greater frequency in nestlings that secreted more corticosterone than those that secreted less (Quillfeldt *et al.*, 2006). Another behaviour chicks show after hatching is sibling rivalry (Tarlow *et al.*, 2001) where one offspring may kill a sibling in order to gain more access to food provided by their parents (Tarlow *et al.*, 2001). This type of aggression has been found in chicks with high concentrations of corticosterone (Ferree *et al.*, 2004).

Corticosterone can suppress behaviours associated with reproduction such as parental care. For example, parent black-legged kittiwakes (*Rissa tridactyla*) treated with corticosterone decreased their time spent at the nest and took longer to return to the nest (Angelier *et al.*, 2009). Adelie penguins with artificially elevated corticosterone were more likely to cease incubation (Spee *et al.*, 2011). These effects may be facilitated by the changes in circulating prolactin concentrations induced by the corticosterone treatment (Angelier *et al.*, 2009; Spee *et al.*, 2011). On the other hand, enhanced provision of food to chicks has been linked to high levels of corticosterone in blood, as shown by improved chick growth (Crossin *et al.*, 2012). Nevertheless, high HPA axis activity during the breeding season may result in parent birds investing in their own survival as oppose to survival of their chicks, leading to delays in

reproduction (Angelier and Wingfield, 2013; Krause *et al.*, 2015b) and negative effects on breeding success (Angelier *et al.*, 2009).

Foraging behaviour seems to be, at least in part, mediated by corticosterone which is a hormone that can promote behaviours to efficiently seek food whilst minimising energy use. High concentrations of corticosterone have been found in seabirds that dive more frequently and put more effort in finding prey (Angelier *et al.*, 2008; Crossin *et al.*, 2012). Corticosterone treated Adelie penguins had increased speeds of locating and hunting prey, signifying an efficient foraging tactic (Cottin *et al.*, 2014). In addition, there is evidence that birds with higher corticosterone concentrations made longer trips and covered larger foraging areas (Angelier *et al.*, 2007).

### 1.3.2 Corticosterone synthesis

ACTH has transcriptional effects on the production of steroidogenic enzymes (Gallo-Payet, 2016; Ruggiero and Lalli, 2016). These enzymes catalyse the conversion processes leading to the synthesis of corticosterone (Fig. 1.2). In all vertebrates, endoplasmic P450scc (P450 side chain cleavage) cleaves cholesterol to form pregnenolone, although the subtype of this enzyme vary between reptiles, birds and mammals (Hess, 2002; Gallo-Payet, 2016). Progesterone is produced from pregnenolone by 3β-hydroxysteroid dehydrogenase in the mitochondria (Hess, 2002). Progesterone undergoes hydroxylation via the activity of 21- and 11β-hydroxylase to form 11- and 11β-deoxycorticosterone, respectively. The final step involves the conversion of these deoxycorticosterones to corticosterone.

Fig. 1. 2. Adrenal cortical biosynthesis of corticosterone (from Hess, 2002).

### 1.3.3 Delivery of corticosterone

Corticosterone, like other steroids, is present in the blood as free steroid and also bound to binding proteins. Corticosterone in birds binds to corticosteroid-binding globulin (CBG, also known as transcortin) and to albumin (Hess, 2002). The affinity of CBG for corticosterone is greater than the affinity of albumin for corticosterone. There are differences between species of birds in the affinity of CBG for corticosterone (Malisch and Breuner, 2010), and affinity

appear to be stronger in birds than in mammals (Delehanty *et al.*, 2015). Bound corticosterone cannot enter cells, so corticosterone must dissociate from binding proteins before crossing cell membranes and activating intracellular receptors (Malisch and Breuner, 2010; Delehanty *et al.*, 2015).

### 1.3.4 Glucocorticoid receptors

Glucocorticoids bind to mineralocorticoid (MR) and glucocorticoid (GR) receptors. The binding affinity of glucocorticoids to MR is greater than the binding affinity to GR (Zimmer and Spencer, 2014). Within the avian HPA axis, GR are primarily expressed in the hypothalamic paraventricular nucleus and the pituitary gland. MR are also found in the hypothalamus but expressed at a lower level than GR (Zimmer and Spencer, 2014). Both receptor types are important in mediating the negative feedback of corticosterone within the HPA axis (see section 1.2), and high MR and GR numbers may indicate enhanced feedback mechanism and recovery from stress (Zimmer and Spencer, 2014). Receptor numbers and density vary between individuals, and may vary seasonally (Krause *et al.*, 2015a). Exposure to prenatal or postnatal stressors can affect GR numbers in adulthood. For example, brain GR were increased in adult quail that had been exposed to stressors just before or after hatching (Zimmer and Spencer, 2014).

## 1.4 The central nervous system (CNS) and the perception of stimuli

Responses to emotional stressors begin in the central nervous system (CNS) where perception of stimuli occurs (Capoccia *et al.*, 2013). During perception, an animal receives information as stimuli through sensory receptors, processes this information in the brain, and determines whether or not the stimulus will be harmful (Fernandez-Juricic *et al.*, 2004). Perception is therefore an important neural process that affects an animal's survival. Brain responses to

stimuli are linked to the HPA axis through pathways that control the activity of CRF neurons. It was proposed that there was no specificity in the effects of stressors on neuronal pathways and that central responses to all stressors are similar (Selye, 1936). However, more recent data suggest that different stressors are processed in different brain regions and lead to the activation of different neurochemical pathways to activate the stress axis. A key structure in stimulus perception is the amygdala, an area believed to be able to distinguish between types of stressors. Appreciating the neural circuits implicated in the production of a hormonal stress response is important to understanding how animals respond to different stressors.

Stressors activate neural pathways that lead to the hypothalamic PVN via projecting fibres from various parts of the brain. Responses to stressors involve the activation or inhibition of neurochemical pathways within certain regions of the brain, as well as the interaction between these brain regions via the activity of neurotransmitters (Arroyo *et al.*, 2016). Neurochemical pathways implicated in the control of the HPA axis activity include the noradrenergic (noradrenaline; Leibowitz *et al.*, 1989), adrenergic (adrenaline; Labrie *et al.*, 1984), serotonergic (5-HT; Liposits *et al.*, 1987)), and GABA-ergic (g-aminobutyric acid; Gaston *et al.*, 2017) pathways. In rats, a dense supply of excitatory catecholaminergic (Liposits and Paull, 1989) and serotoninergic neurons provides input into the PVN through axons extending into the PVN which make contact with CRF neurons arising from this nucleus in the parvocellular region (Sawchenko *et al.*, 1983; Liposits *et al.*, 1987; Jorgensen *et al.*, 2002). These pathways are well described in the mammalian brain, primarily in laboratory rats, whereas they are less clear in birds. Nevertheless, brain structures in birds that correspond with structure in mammals may serve analogous functions during responses to stressors.

There is strong evidence that when an animal is exposed to an emotional stressor the limbic system, especially the amygdala, an emotion-related area, receives input and sends processed information about the stressor to the PVN (Dayas *et al.*, 1999; Radley and Sawchenko, 2015). For example, 15 minutes of restraint (an emotional stressor) enhanced neuronal activity within the amygdala of rats, as indicated by intense activation of c-fos gene (Dayas *et al.*, 1999). On the other hand, physical stressors (e.g. histamine treatment) can activate both the amygdala (Ulrich-Lai and Herman, 2009) and also the brainstem cells (Lachuer *et al.*, 1991). The brain has the ability to determine whether a stressor is physical or emotional (Dayas *et al.*, 2001).

The bed nucleus of the stria terminalis (BNST) and the nucleus of solitary tract (NST) act as intermediary relay sites for inputs from the amygdala and outputs to the parvocellular region of the PVN (Fig. 1.3). It is thought that sensory input processed in the sensory thalamus reaches the amygdala (Ressler, 2010). Evaluation of a stimulus in relation to memory of previous situations occurs within the basolateral amygdala (BLA), leading to discernment of whether or not the stimulus is a threat (BLA; Ressler, 2010), whereas the brain stem integrates stimuli that require immediate responses (e.g. pain; Ulrich-Lai and Herman, 2009). In the rat brain, the medial (MeA) and central (CeA) nuclei of the amygdala respond to emotional and physical stressors respectively via glutamatergic fibres from the BLA. Innervation from the MeA then projects to the BNST of the forebrain and the CeA initiates responses by both the BNST and the NST. The BLA is also involved in stimulating the BNST. The BNST in turn innervates the PVN via fibres leading to CRF neurons (Ulrich-Lai and Herman, 2009). Noradrenergic fibres connect the NST to the PVN and provide stimulatory input (Ulrich-Lai and Herman, 2009). The CeA also excites the brain stem leading to stimulation of the dorsal raphe nucleus which in turn promotes PVN activation

possibly via serotonergic neurons (Ressler, 2010). Indirect stimulatory pathways from other limbic regions such as the infralimbic cortex (IL) innervate the CeA and NST (Ulrich-Lai and Herman, 2009). Inhibition of the PVN originates from the BNST through GABA-ergic neurotransmission.

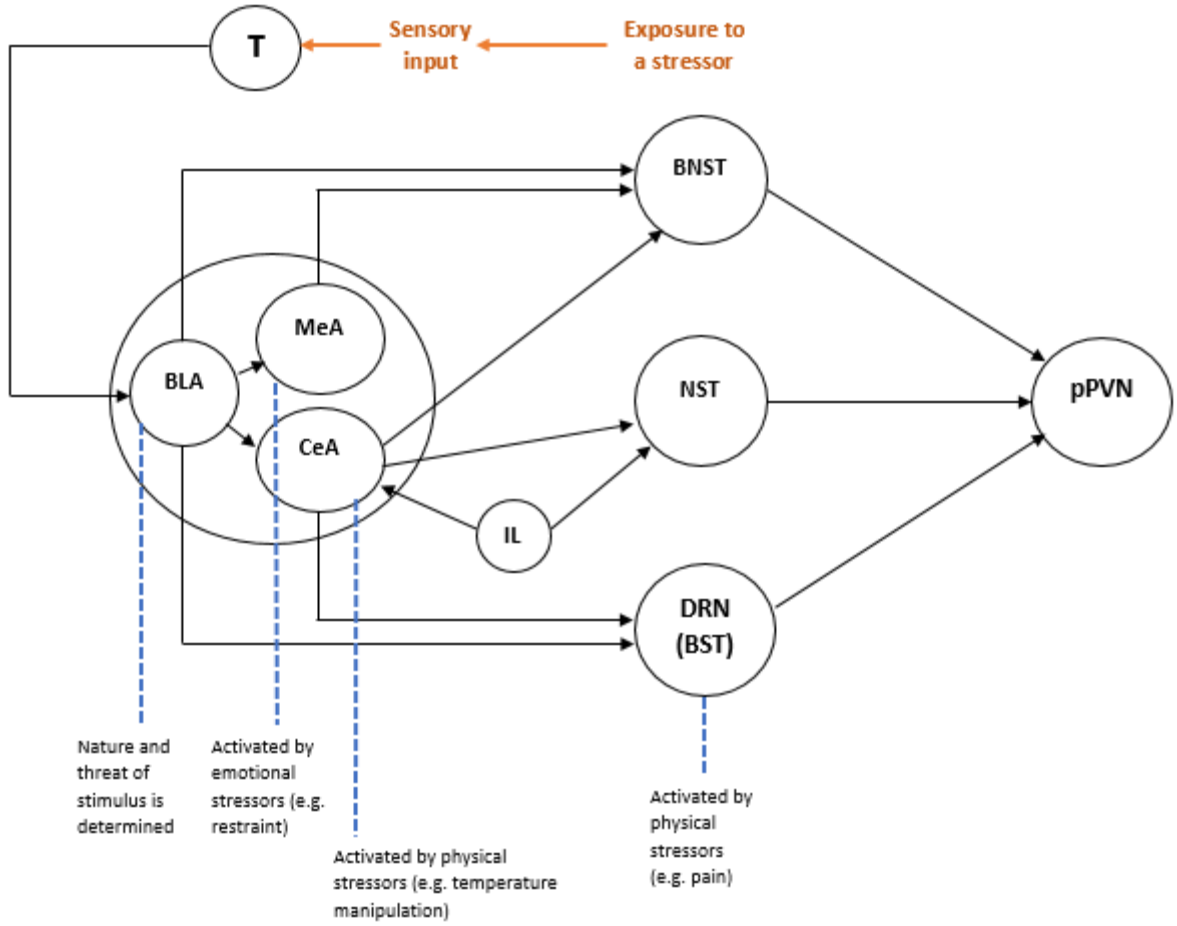



Fig. 1. 3. Representation of neurochemical inputs to the PVN during a stress response. The bed nucleus of the stria terminalis (BNST) and nucleus of the solitary tract (NST) act as intermediary relay sites for stimulation of the PVN. Emotional stressors activate the medial nucleus of the amygdala (MeA) whereas physical stressors activate the central nucleus of the amygdala (CeA). The MeA innervates the BNST and the CeA innervates both the BNST and NST. Noradrenergic neurons project from the BNST and NST to the PVN. Some physical stressors (e.g. pain) also directly activate the brain stem (BST). Dorsal raphe nucleus serotonergic neurons innervate the PVN. Arrows point towards the brain region they innervate. Terms: thalamus (T), basolateral amygdala (BLA), infralimbic cortex (IL). Modified from Ulrich-Lai and Herman (2009) and Ressler (2010).

There is evidence that the relationship between the level of neurochemical release and the level of plasma corticosterone secretion depends on the type of stressor. Pacak *et al.* (1998) exposed rats to several stressors and found that the release of PVN noradrenaline differed depending on the stressor and the level of discomfort or metabolic demand involved with the stimulus. For example, insulin injections which lower blood glucose concentrations resulted in higher concentrations of ACTH and corticosterone in the blood than of extracellular PVN noradrenaline, and formaldehyde treatment (which induces tissue irritation) induced a greater increase in PVN noradrenaline than in plasma corticosterone. Rats subjected to immobilisation (which can cause discomfort as well as increase energy expenditure during struggling) had corticosterone responses that were similar to noradrenaline responses. Pacak *et al.* (1998) proposed that the release of hypothalamic noradrenaline may not be the predominant prerequisite to the secretion of ACTH by the pituitary gland when an animal is exposed to a stimulus that increases metabolism but does not cause discomfort. The authors also commented that the assumption made by Selye (1936) that brain pathways responds in the same manner to all stressors is not correct. However, some pathways (e.g. striatal 5-HT) does not seem to have major involvement in the activation of the HPA axis and the release of corticosterone. A study by Kirby *et al.* (1997) showed that striatal 5-HT in rats increased markedly above baseline after exposure to a forced swim (0.5 hr) and to tail pinch (5 min) test, but blood samples revealed that a significant rise in plasma corticosterone concentration occurred only after a forced swim. Other stressors such as immobilisation and forced locomotion did not significantly increase 5-HT in the striatum, even though they stimulated a greater corticosterone response compared to tail pinch. Although the results of this study indicate that striatal 5-HT does not participate in stress-induced corticosterone response, 5-HT system in other regions of the brain may be associated with the HPA axis.

Some studies have injected noradrenaline and 5-HT in animals to examine the effect these neurochemicals on CRF expression in the PVN. For instance, injections of noradrenaline directly into the PVN of rats promoted CRF pre-mRNA expression even up until 120 min had elapsed from the time of injection, suggesting enhanced transcription of CRF (Itoi *et al.*, 1994). The same study also reported that ACTH concentrations in the blood had risen. Furthermore, Leibowitz *et al.* (1989) investigated whether plasma corticosterone in rats would change when noradrenaline was injected into the PVN and found that plasma corticosterone concentrations increased in proportion to the dose of noradrenaline. Leibowitz *et al.* (1989) also reported that injections of 5-HT into the PVN of conscious rats lead to a rise in plasma corticosterone. Similar results have been found in a study of broiler chicks, where 5-HT directly injected into the cerebral lateral ventricles increased circulating corticosterone levels and a stronger endocrine response was observed when 5-HT was injected with CRF (Zhang *et al.*, 2004). These results suggests that noradrenaline and 5-HT in the brain influence glucocorticoid responses indirectly through CRF activation at the hypothalamic level.

## 1.5 Corticosterone responses

### 1.5.1 How do we measure corticosterone responses?

The magnitude of the corticosterone response of a bird to a stressor reflects the sensitivity of the bird to stimuli from its immediate environment (Cockrem, 2007). The standard method for the measurement of corticosterone responses involves capture of free-living birds or picking up of captive and domesticated birds followed by the collection of a blood sample as quickly as possible. After the collection of the first blood sample the bird is placed into a bag or box, with further blood samples collected until 30 or sometimes 60 minutes after the time

of capture. The bird is then released. Whilst corticosterone concentrations can be measured in other sample types, for example faeces (Fraisse and Cockrem, 2006), corticosterone responses over 30 to 60 minutes can only be measured in plasma or serum.

The corticosterone concentration in the first blood sample taken after a bird is captured or picked up is often called the baseline corticosterone concentration. Romero and Reed (2005) considered results from a variety of studies and suggested that corticosterone concentrations in blood samples collected within three minutes of the time of capture could be considered to reflect concentrations in undisturbed birds. The great majority of studies of corticosterone in birds use this assumption. However, there is good evidence that in many cases corticosterone concentrations increase within three minutes of capture, and mean corticosterone concentrations in initial samples that are reported in many studies are not true baseline corticosterone concentrations. Baugh *et al.* (2013) collected blood from great tits (*Parus major*) within three minutes of capture in a net and compared plasma corticosterone concentrations of birds that were sampled before two minutes and birds that were sampled near three minutes. This study found a greater mean corticosterone concentration in the blood collected around three minutes than before two minutes. A more extensive study by Small *et al.* (2017) assessed the rate of corticosterone increase during the few minutes after the initial capture in Florida scrub-jays (*Aphelocoma coerulescens*). Blood collected up to three times in less than 3 min post-capture in the same bird revealed that it only takes two minutes to detect a rise in corticosterone. Furthermore, birds that produced a greater glucocorticoid response to 30 min confinement in a cloth bag showed a faster elevation in circulating corticosterone levels during the first two to three minutes after initial capture than birds with a smaller glucocorticoid response. This indicates that not only there is evidence against the three minute rule, plasma corticosterone concentration of birds actually changes at

different rates within this short period. Corticosterone concentrations in the first blood sample collected in studies of corticosterone responses of birds would be better termed initial concentrations rather than the baseline concentrations.

### 1.5.2 Corticosterone responses of birds

Corticosterone responses have been measured in many species of birds (Cockrem, 2013c). Mean plasma corticosterone concentrations are relatively low in the first blood sample. Mean concentrations generally increase when birds are sampled for up to 30 minutes, and either continue to increase or remain relatively constant when sample collection is continued for up to 60 minutes after the time of the first sample. For example, mean corticosterone concentration in common diving petrels (*Pelecanoides urinatrix*) increased from ~10 ng/ml at 0 min to 40 ng/ml after 30 min (Smith *et al.*, 1994). The magnitude of corticosterone responses can vary between sexes (Small and Schoech, 2015), lines of birds selected for different behaviour characteristics (Baugh *et al.*, 2012), groups of birds that have experienced different times in captivity (Owen-Ashley *et al.*, 2006), life-history phases (Krause *et al.*, 2015b), and seasons (Wingfield *et al.*, 1992).

Corticosterone responses of domesticated birds such as chickens and quail are lower than responses of wild birds. For example, mean peak corticosterone concentrations in Japanese quail handled for 15 min and then confined in a cardboard box were ~8 to 14 ng/ml (Cockrem *et al.*, 2010), and corticosterone concentrations in individual birds were generally less than 20 ng/ml. In contrast, mean corticosterone concentrations of up to 200 ng/ml have been reported in free-living birds (Adams *et al.*, 2005). Domesticated birds have been selected over many generations and it is to be expected that animals selected for production traits have lower corticosterone responses to contact with people than do wild birds.

There is considerable variation between individual birds in the magnitude of their corticosterone response to a stressor. Examples of individual variation in corticosterone responses in domesticated (chickens; Littin and Cockrem, 2001) and free-living birds (grey-faced petrels; Adams *et al.*, 2005) are shown in Fig. 1.4. The grey faced petrels were chicks sampled shortly before the time of fledging. The wide range of responses of these birds shows that individual variation in corticosterone responses is present in birds before they fledge. Individual variation in corticosterone responses has a genetic basis, arising from differences in experiences before and after hatching (Cockrem, 2013c), and indicates that birds differ in how they perceive a stressor (Cockrem *et al.*, 2009a). Birds with relatively high corticosterone responses are said to have high levels of awareness of and sensitivity to stimuli from their immediate environment (Cockrem *et al.*, 2009a).

Fig. 1. 4. Corticosterone responses of individual chickens (A) and grey-faced petrels (B). Graphs retrieved from Littin and Cockrem (2001) and Adams *et al.* (2005), respectively.

Corticosterone responses are usually measured when animals are exposed to a single stressor. The intensity or duration of a stressor has been varied and birds have been exposed to a stressor on more than one occasion in just a few studies. For example, different frequencies of flickering light have been applied to European starlings (*Sturnus vulgaris*; Maddocks *et al.*, 2001), and quail were restrained for durations ranging from 10 to 120 minutes. Corticosterone concentrations in quail increased after 10 minutes of restraint and did not change when restraint was continued for up to 120 minutes (Hazard *et al.*, 2008). Most studies of corticosterone responses of animals to different intensities of a single stressor have been conducted in rats that have used stressors likely to cause pain or discomfort such as electric foot-shock (Rabasa *et al.*, 2011), temperature (Abel, 1993), or noise (Chandralekha *et al.*, 2005). Stressors have also been applied repeatedly for several weeks. For example, quail were repeatedly induced into tonic immobility or were mechanically restrained (Jones *et al.*, 2000; Hazard *et al.*, 2008). Repeated exposure to a stressor generally leads to birds becoming accustomed to the stimulus and to reductions in the size of the corticosterone response to the stressor. For example, corticosterone responses declined in quail that were restrained repeatedly over a 4 day period (Jones *et al.*, 2000) and declined in American kestrels (*Falco sparverius*) that were repeatedly restrained (Love *et al.*, 2003). Handling on a single occasion was reported not to affect responsiveness of birds to handling at a subsequent occasion (Longordo *et al.*, 2011). Responses of individual birds to different stressors are considered in section 1.5.3.

#### 1.5.2.1 Corticosterone responses of penguins

Corticosterone responses of penguins have been measured in the little penguin (Carroll *et al.*, 2016; Cockrem *et al.*, 2017b), Adelie penguin (Cockrem *et al.*, 2008b; Cockrem *et al.*, 2009a), emperor penguin (Cockrem *et al.*, 2008b), king penguin (Viblanc *et al.*, 2016),

Magellanic penguin (Hood *et al.*, 1998; Walker *et al.*, 2005) and gentoo penguin (*Pygoscelis papua*; Holberton *et al.*, 1996). Mean corticosterone concentrations increased over sampling periods of up to 30 minutes (Cockrem, 2013c), with individual variation in corticosterone responses (e.g. Adelie and emperor penguins; Cockrem *et al.* (2008b). Corticosterone concentrations after one hour of restraint varied from 20.3 to 288.2 ng/ml in 96 little penguins (Cockrem *et al.*, 2017b). Corticosterone responses of penguins can also differ between breeding stages. For example, responses of king penguins brooding chicks were lower than responses of penguins during incubation (Viblanc *et al.*, 2016).

### 1.5.3 Corticosterone responses to different stimuli

Animals encounter many stimuli in their natural environment, and the ability to respond to varying environments is beneficial in terms of adapting to change (Cockrem, 2015). The standard protocol of Wingfield *et al.* (1992) for the measurement of corticosterone responses to capture and handling has been used in many studies of birds. A variety of other stimuli have also been used in studies of corticosterone responses of birds and mammals, and different methods of handling and restraint have also been used. Comparisons can be made between corticosterone responses to stimuli other than the standard handling and restraint protocol with responses to the standard protocol. The following sections review corticosterone responses to different restraint techniques and to various other stimuli.

#### 1.5.3.1 Capture and handling methods

Corticosterone responses of birds to capture and handling using different techniques have been measured. The majority of studies of corticosterone responses of chickens have measured responses to handling procedures frequently encountered during translocation and husbandry practices (e.g. weighing, crating, and transfer). These handling protocols differed

in the method of capture of birds (e.g. by hand or machine) and in the degree of roughness during handling (e.g. soft or coarse). Studies on free-living bird are conducted outdoors with birds generally subjected to one type of handling stimulus or restraint method before they are put inside a bag or a box for confinement. Birds that live in high latitudes have trouble regulating their body temperature so birds have been put into open mesh bags rather than the cloth bags used for studies of birds in temperate latitudes (Cockrem *et al.*, 2009a). There are no studies of corticosterone responses to different handling or restraint techniques in free-living birds.

Delezie *et al.* (2006) reported that chickens caught by a machine and bled 15 min following the beginning of the capture process had higher mean corticosterone concentration than hand-caught birds (19.72 ng/ml c.f. 15.91 ng/ml). However, towards the end of the capture process, mean corticosterone concentration of 14.72 ng/ml in mechanically caught chickens were lower than the concentration of 16.07 ng/ml in those that were hand-caught. These results suggest an association between the method of capture and the magnitude of endocrine response. It is worth considering that the birds were exposed to the sound produced by the machine before they were mechanically caught and sampled. It is possible that the chickens initiated corticosterone responses before mechanical capture.

Corticosterone responses of chickens have been compared between different methods of handling (Fig. 1.5; Broom et al. (1986) unpublished data cited in Knowles and Broom, 1990). After capture, one group of birds were held with their body upright and the other group of birds were held with their body inverted, which were considered as soft and coarse method of handling, respectively (Broom, 1990). Birds were then placed in a crate for two minutes, and were bled 5 min and 0.5 hr after capture. Corticosterone concentrations were markedly

higher at 5 min in birds that had received coarse handling compared with birds that had received soft handling (Broom et al. (1986) unpublished data cited in Knowles and Broom, 1990). Corticosterone concentration in birds that receive coarse handling continued to increase up to 30 min but the opposite was observed in birds that were handled softly. This study showed that the two handling techniques induced different corticosterone responses in the birds. Another study reported that chickens handled in a 'face-down' position had higher corticosterone concentrations than birds that were handled in an upright standing position (Kannan and Mench, 1996). The magnitude of corticosterone responses of chickens appears to be related to the nature of the handling process. This is likely to be the case for free living birds as well, even though the capture and restraint of free-living birds is generally considered to be a standard stressor. When a hood was placed over the head of Adelie penguins corticosterone after 30 minutes of manual restraint was lower than in birds without hoods (15.7 cf. 28.3 ng/ml; (Cockrem *et al.*, 2008b). Evidently corticosterone responses can vary between different restraint techniques.

Overall, in domesticated birds, variations in handling techniques can affect the magnitude of corticosterone responses. Corticosterone responses in birds may be considerably higher when birds are handled in a more aggressive way, and differences in responses may also arise due to variation in capture methods. The above studies demonstrate that technique is clearly an important aspect of capture-handling stressors used in the measurement of corticosterone responses in birds.

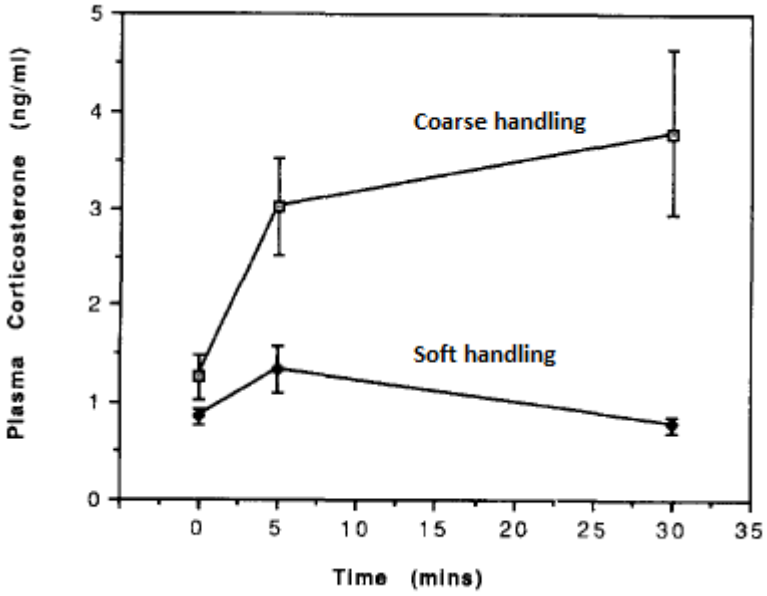



Fig. 1. 5. Corticosterone responses induced by coarse (□) and soft (●) handling in chickens. After handling, the birds were subjected to crating (2 min). Collection of blood took place before capture, 5 min, and 0.5 h following capture. Graph taken and modified from (Broom et al. (1986) unpublished data cited in Knowles and Broom, 1990).

### 1.5.3.2 Other stimuli

There are many stimuli that stimulate glucocorticoid release in animals other than capture and restraint (Table 1.2). There are more studies of mammalian than avian corticosterone responses to different stimuli, with most studies conducted on laboratory rodents. Several rat and mice strains have been subjected to various physical and emotional stressors. In rats, a forced swim lasting 15 min elevated circulating corticosterone markedly above baseline concentrations (Abel, 1993). Stimuli such as immobilisation, elevated-plus maze (Rodgers *et al.*, 1999), heat stress (Gong *et al.*, 2015), electric footshock (Rabasa *et al.*, 2011), isolation in

an open field (Gentsch *et al.*, 1981) and a novel environment (Hennessy, 1991) also increase glucocorticoid concentrations in rodents.

In mice, corticosterone responses to different stimuli have been compared to restraint-induced responses. For example, corticosterone responses of mice to a standard restraint stressor were greater than responses to other stimuli (e.g. cold environment, isolation, and swim stress; Bowers *et al.*, 2008). Mean corticosterone responses to a cold environment and swim stress (340 and 310 ng/ml) were lower than responses to restraint (410 ng/ml). In contrast, corticosterone following isolation was substantially lower (120 ng/ml), indicating that isolation was a weaker stressor than the other stressors used in the study. However, the duration of the stressors differed between stimuli, so comparisons between stimuli were confounded by the differences in stimulus duration.

Table 1. 2. Corticosterone responses to different stimuli in birds and mammals. C = corticosterone or cortisol concentration (ng/ml).

| Species | Stressors | Results | Reference |
|---|---|---|---|
| **Birds** | | | |
| European starling | Light, music, 'non-novel' human (caretaker), novel human, experimenter handled the birds and implanted transmitters | Individual bird exposed to one stressor.<br>C elevated above baseline for all stressors.<br>Baseline 4 ng/ml, light 8 ng/ml, music 10 ng/ml, 'non-novel' human (caretaker) 9.5 ng/ml, novel human 10 ng/ml, experimenter 12 ng/ml | (Nephew *et al.*, 2003) |
| Japanese quail | TI, restraint (cage), balloon (novel object), isolation ('non-novel' environment), novel environment, cagemate swap | Individual bird exposed to one stressor.<br>C elevated above baseline for all stressors except cagemate swap. | (Hazard *et al.*, 2008) |
| Japanese quail | Mating, social, restraint (crush cage) | Restraint 12 ng/ml, female-female interaction 5.8 ng/ml | (Rutkowska *et al.*, 2011) |
| Chicken | 2 min of shackling (hung upside down), 3.5 h of heat stress & 2 min shackling | Control 5.59 ng.ml<br>Shackling 8.5 ng/ml<br>Heat & shackling 10.8 ng/ml<br>Sig. difference in C between stressors. | (Debut *et al.*, 2005) |

| Species | Stressors | Results | Reference |
|---|---|---|---|
| | | | |
| Pied flycatcher | Male intruder, woodpecker decoy (predator), weasel decoy (predator), restraint (net) | Individual bird exposed to one stressor.<br>Control 15.2 ± 1.9 ng/ml, Restraint 94 ± 12.7 ng/ml, weasel 46.9 ± 11 ng/ml, woodpecker 12.4 ± 3.8 ng/ml, intruder 44.5 ± 6.9 ng/ml<br>No sig. increase in C after woodpecker stimulus.<br>Weasel = intruder | (Silverin, 1998) |
| Great tit | Owl decoy (predator), moving box, brambling decoy (non-predator) | Individual bird exposed to one stressor.<br>C at 30 min sig. greater in owl than box or brambling.<br>No sig. difference in C between box and brambling. | (Cockrem and Silverin, 2002a) |
| European stonechat | Restraint (cloth bag), restraint (cage), inside cage with live owl (predator) | Individual bird exposed to one stressor.<br>C at 30 min restraint (cloth bag) ~10 ng/ml, restraint (cage) ~14 ng/ml, cage + owl ~16 ng/ml | (Canoine *et al.*, 2002) |
| Starling | Restraint (cloth bag), cage disturbance, crowding, radio music (auditory), tube (space restraint), rollercart | Individual birds exposed to all 6 stressors.<br>C at 30 min crowding < music < disturbance < rollercart < restraint < tube | (Rich and Romero, 2005) |

| Species | Stressors | Results | Reference |
|---|---|---|---|
| | | | |
| European starling | 4 or 30 min chronic stimulus including all of the following stressors:<br>radio, predator vocalisation, novel object, toy snake and other predator decoys, cage disturbances, human talking and restraint inside bag | Did not test corticosterone responses to different stimuli within the same birds. | (Cyr and Romero, 2007) |
| **Mammals** | | | |
| Rat | Forced swim, tail pinch, immobilisation (100 min), cold stress (2h), forced locomotion (0.5 h) | Individual rats exposed to one stressor.<br>Control 44.8 ng/ml < Tail pinch 156.8 ng/ml = cold stress 243.2 ng/ml < immobilisation 446.4 ng/ml = forced locomotion 518.4 ng/ml < forced swim 747.2 ng/ml<br>Sig. C increase following all stressors. | (Kirby *et al.*, 1997) |
| Rat | Immobilisation, intense foot shock, mild foot shock | Individual rats exposed to one stressor.<br>All stressors sig. increased C above basal levels. | (Rabasa *et al.*, 2011) |
| Rat | 2 s x 6 foot shock, social defeat | Individual rats exposed to one stressor.<br>C after 24 h<br>Foot shock 1958.7 ± 284.6 ng/ml > social stress 887.3 ± | (Kavushansky *et al.*, 2009) |

| Species | Stressors | Results | Reference |
|---|---|---|---|
| | | 161.8 ng/ml = control 1082.6 ± 200.9 ng/ml | |
| Rat | Handling (5 sec), novel environment (3 min) | C after 15 min handling 116 ± 12 ng/ml<br>15 min novel environment 217 ± 8 ng/ml<br>Sig. difference in C between the two stressors. | (Seggie and Brown, 1975) |
| Rat | Prolonged restraint, 'mash-up' stress (consisting of numerous different stressors).<br>Bled after novel environment stress of 0.5 h | Control 225 ng/ml, restraint 286 ng/ml, 'mash-up' stress 317 ng/ml<br>Sig. increase in C after all stressors. | (Marin *et al.*, 2007) |
| Rat | All stressors 75 min<br>Confinement in chamber + predator (cat), confinement in chamber + no predator, home cage | Confinement in chamber + predator (conscious cat) > confinement in chamber + no predator = home cage | (Mesches *et al.*, 1999) |
| Mouse | Restraint (plexiglass), forced swim, isolation, low room temperature (4 $^{o}$C), handling | Restraint > low temp > forced swim > isolation > handling | (Bowers *et al.*, 2008) |
| Mouse | 6 days of stress (once/per day). Escapable footshock, inescapable footshock, psychological stimulus (sight, sound, and odour of mice subjected to inescapable footshock) | Individual rats exposed to one stressor.<br>Control 90.5 ng/ml<br>Inescapable footshock > escapable footshock = psychological stimulus | (Palermo-Neto *et al.*, 2003) |

| Species | Stressors | Results | Reference |
|---|---|---|---|
| | | | |
| Mouse | Isolation, grouped housing, regrouping, noise, tail pinch | Individual mice exposed to one stressor.<br>Isolation 48 ng/ml, grouped 59 ng/ml, regrouping 304 ng/ml, noise 106 ng/ml, tail pinch 76 ng/ml | (Gibb *et al.*, 2008) |
| Mouse | 11 h once/day for 15 days<br>Handling, electric shock 1, 3, or 15 times | Handling = 53 ng/ml<br>Electric shock 1 = 195 ng/ml, 3 = 195 ng/ml, 15 = 172 ng/ml.<br>Sig. increase in C after all stressors. | (Irwin *et al.*, 1986) |
| Mouse | Handling and acute pain elicited by capsaicin injection, carrageenan injection, chronic inflammatory pain injection, partial sciatic nerve lesion, intrathecal injection | Individual mice exposed to one stressor.<br>Sig. increase in C after all stressors.<br>Did not compare corticosterone responses between stimuli. | (Benedetti *et al.*, 2012) |
| Mouse | Rat (predator), toy (novel object), empty cage | C at 15 min.<br>Cage 79 ± 16 ng/ml = Toy 114 ± 20 ng/ml < Rat 223 ± 44 ng/ml<br>Sig. increase in C only after predator stimulus. | (Santana Amaral *et al.*, 2010) |

| Species | Stressors | Results | Reference |
|---|---|---|---|
| Guinea pig | Unfamiliar environment (0.5 h), social stress (10 min) | Animal exposed to both stressors (15 guinea pigs per group).<br>Sig. increase in C after both stressors.<br>Did not compare C between stressors. | (Sachser, 1987) |
| Dog | 60 s of sound blasts, electric shock, falling bag, opening umbrella, restraint by hand, restraint by collar | Dogs exposed to all 6 stressors (10 dogs per group)<br>Sig. increase in C after falling bag, shock, sound blast.<br>Other stimuli failed to change C | (Beerda *et al.*, 1998) |

Note: The concentration of all results has been converted into ng/ml.

Corticosterone concentrations following restraint have also been compared to concentrations found after exposure to other stimuli in birds. Rich and Romero (2005) found that plasma corticosterone in starlings was markedly higher after 30 minutes of restraint inside cloth bags (mean level of 30 ng/ml) than after 30 minutes of an auditory stimulus (music playback; 10 ng/ml) or 30 minutes of other stimuli (crowding (several birds put inside the same cage), disturbance (tapping and shaking cage) and rollercart (rolling cage side to side); corticosterone < 22 ng/ml for the stimuli). Similarly, corticosterone responses of Japanese quail to 10 minutes of restraint were greater than responses to isolation or to a novel object (e.g. balloon), while there were no significant increase in corticosterone after the introduction of a new quail to the cage (Hazard *et al.*, 2008). Furthermore, elevated plasma corticosterone concentrations in restrained birds continued to stay at high levels for 120 minutes after capture, whereas there was a rapid drop in plasma corticosterone back down to initial concentrations after exposure to isolation or to a novel object. The results from starlings and quail indicate that some stimuli are stronger stressors than others, and that restraint elicited greater corticosterone responses than did other stimuli used in the studies.

It is important to consider the validity of the widely held assumption that corticosterone responses of birds to capture and restraint are equivalent to responses to natural stressors such as an attack by a predator. One approach to testing this assumption is to compare the magnitude of corticosterone responses to natural stimuli with the magnitude of responses to capture and restraint. Corticosterone was greater in captive European stonechats (*Saxicola rubicola*) after 30 minutes of exposure to a live owl situated 1.5 metres from their cage than after restraint using a standard cloth bag method (16 ng/ml versus 10 ng/ml; Canoine *et al.* (2002). The study showed that the corticosterone response to the sight of a natural predator

was greater than the response to restraint. In a different study, glucocorticoid levels in free-living pied flycatchers (*Ficedula hypoleuca*) was greater after 10 minutes of restraint than after 10 minutes of exposure to a stuffed predator (weasel or woodpecker) in the vicinity of their nest site (Silverin, 1998). The corticosterone results show that pied flycatchers perceived the stuffed predator to be less threatening than the experience of capture and restraint. The placement of a stuffed predator in the vicinity of a nest was different from a predatory attack as the stuffed predator did not move, unlike a live owl. These situations are quite different from seeing a live predator actually pursuing another bird. In fact, the sight of a predator (e.g. raptor) attacking a conspecific induces similar corticosterone responses to capture and restraint in European starlings (35.01 ng/ml and 32.92 ng/ml, respectively; Jones *et al.*, 2016). In this study, not only did the raptors fly during an attack, they also achieved physical contact with the conspecific bird that produced alarm calls. Therefore, there was a combined effect of more than one stimulus including the sound of the alarm call and sight of the attack. Also, the starlings were placed inside small observation cages that did not allow much space for the birds to escape or fly away. Indeed, there are great differences in corticosterone responses between free-living great tits that have the freedom to fly away from a stuffed predator and birds that are restricted to the aviary (Cockrem and Silverin, 2002a). These studies did not address whether handling is analogous to natural stressors such as predator encounters, but do show that corticosterone responses to a predator stimulus can differ from responses to restraint.

Corticosterone responses of birds to auditory stimuli have occasionally been reported. Predator calls or alarm calls produced by conspecifics have been recorded and played to chicks, which have fully developed responses to stressors like that of adults in some species. Ibanez-Alamo *et al.* (2011) played magpie calls to one-week-old common blackbird (*Turdus*

*merula*) chicks in nests and the birds were bled 30 minutes after the end of the 180 minute stimulus. They found that the sound did not induce a marked increase in corticosterone concentrations when compared to concentrations measured in response to silent control. The authors suggested that elevated corticosterone is a potential mechanism to dampen conspicuous behaviour such as begging during an exposure to predator sounds nearby. However, nestling vocalisations were not measured during this experiment and the correlation between corticosterone concentration and begging frequency was not determined. The authors also noted that the sound of magpies alone does not signal danger to the chicks as opposed to an attack where birds must respond in ways that will help with their immediate survival. Indeed, capture and handling, a situation similar to an attack by a predator, stimulated high level of corticosterone secretion in blackbird chicks. Dufty and Crandall (2005) conducted a similar study where they played the sound of alarm calls produced by American kestrel adults to chicks and found that corticosterone responses of chicks to the alarm call were comparable to responses to control sound consisting of heterospecific songs. Rivers *et al.* (2011) also exposed parent alarm calls to chicks up to eight days old and bled the chicks four minutes after the end of the playback, and again after one hour. Corticosterone concentrations in both the first and second blood samples indicated that the sound of parent alarm calls was not a stressor and did not lead to increased plasma corticosterone. However, handling increased corticosterone concentrations markedly above initial levels, indicating that being handled by the experimenter was a stressor while the sound of alarm calls was not. Contrasting results were reported in a 2010 study where taped distress calls of pied flycatcher chicks were played to other chicks for 60 minutes then the chicks were bled immediately afterwards (Tilgar *et al.*, 2010). Corticosterone concentrations in birds that heard distress calls were greater than in control birds that heard songs of other species. It is important to note that this study exposed the chicks to an hour long playback

repeatedly throughout a five day period whereas Dufty and Crandall (2005) and Rivers *et al.* (2011) used playback durations of only 10 minutes or less for a single day.

There are several limitations when investigating corticosterone responses to different stimuli in birds. Firstly, it is challenging to apply different stimuli to each bird, particularly in free-living birds, due to the difficulty in capturing the same bird more than once. Secondly, stimuli used to induce stress responses in experiments are not natural. Free-living birds experience natural stressors such as climatic disturbances, predators, social conflicts, fluctuations in food levels (Vleck *et al.*, 2000) and it is hard to mimic these stimuli in the field or laboratory. Finally, in a bird's natural habitat, it usually takes seconds for predators to attack (Mesches *et al.*, 1999). However, predator decoys used in studies are presented for longer durations (> 5 minutes) and the decoys do not attack the bird. Predator decoys are still simulated stressors and therefore cannot be fully comparable to a 'real-life' predator encounter in the wild.

## 1.6 Phenotypic plasticity and reaction norms

### 1.6.1 What is phenotypic plasticity?

Phenotypic plasticity is the capacity of a given genotype to have different phenotypes in different environmental conditions, so plasticity in a trait is the capacity of the trait to alter as environmental conditions change (Cockrem, 2013a). For example, plasticity can be seen in the pattern of plumage colour of free-living pied flycatchers where the white patch on the wings becomes larger during times of greater compared to less rainfall (Jarvisto *et al.*, 2016). Another example is changes in calls made by zebra finches (*Taeniopygia guttata*) during pair formation compared with after pair formation (D'Amelio *et al.*, 2017). Plastic responses to changes in environmental conditions enable animals to withstand or adapt to changes that are

not in their control (Via *et al.*, 1995). Traits differ in their plasticity, and quantification of plasticity in a trait will depend on the environment that is studied (Pemac and Tucic, 1998). The measurement of plasticity can provide information on the effect that environment conditions have on the trait of interest, the ability of an animal to adapt to changes in the environment, and the differences in trait expression between individuals and populations (Via *et al.*, 1995). Therefore, it is important to quantify plasticity of phenotypic traits in order to determine the way animals react physiologically and behaviourally when their environment changes (Valladares *et al.*, 2006b).

### 1.6.2 The measurement of plasticity

Although there have been many debates over the best approach or model to assess trait plasticity, reaction norms are commonly used to measure plasticity and to demonstrate how phenotypic traits respond when environments change. Phenotypic plasticity can be quantified from the slope of a reaction norm (Valladares *et al.*, 2006a), where a reaction norm is the range of phenotypes that individuals within a population can produce in a given set of environments (Dingemanse *et al.*, 2010). Reactions norms can show the different phenotypes that an animal expresses in different environments (Pemac and Tucic, 1998). The two main methods used to quantify plasticity in animals are the polynomial approach and the character state approach (Via *et al.*, 1995). Both approaches use reaction norms, with the former approach often applied in environments that are continuous such as climatic temperature (Charmantier and Gienapp, 2014) whereas the latter approach is said to be suitable in an environment that is fixed such as the range of predator species for a particular animal (Vantienderen and Koelewijn, 1994; Bryant *et al.*, 2006). Both approaches are frequently used in studies of mammals whereas experiments on birds tend to use the polynomial

approach to describe environmentally induced changes in physiological responses. The two approaches will be examined in the following sections.

#### 1.6.2.1 Polynomial approach

A polynomial approach considers average values of phenotypic traits in relation to environmental variables that are continuous rather than discrete (Bryant *et al.*, 2006). The simplest form of polynomial reaction norm is the linear model where the environmentally-driven phenotypic change is measured by a slope which is the regression line formed by the phenotypic response to the environment (Pemac and Tucic, 1998; Valladares *et al.*, 2006b). This method was used in a study of blue tits (*Cyanistes caeruleus*) in which mean laying dates were plotted against mean breeding period air temperatures (Porlier *et al.*, 2012). Plasticity was observed in all groups of birds studied, with a linear relationship between laying dates and temperature. The simplest version of the linear model to measure trait plasticity is to use just two levels of the environmental variable (Noach *et al.*, 1997; Perez and Garcia, 2002), thus making the polynomial linear approach useful in fixed environments as well (Rocha and Klaczko, 2012). For example, the mean response of the phenotypic trait of interest (e.g. laying date) could be measured at two ambient temperature and a line drawn between the two mean phenotypic value points. The line represents a 'two point' reaction norm, with the slope of the line a measure of the plasticity of laying date across different temperatures. However, it is not ideal to use this approach when there are three or more discrete environmental levels (Valladares *et al.*, 2006b).

Reaction norms from linear models can be compared between individuals or populations. As an example, assume that there are four different populations of a species each of which is represented by a slope (Fig.1. 6) on a graph of the mean value of a phenotypic variable versus

the level of an environmental variable. The slopes may be the same indicating that difference in trait plasticity does not exist among populations (Fig. 1.6A). In other cases, there may be variation in the slopes indicating that there is variation in trait plasticity with some populations having more or less plasticity (Fig. 1.6B; Rocha and Klaczko, 2012). A slope that is steeper means more plasticity compared to a shallower slope (Fig. 1.7; Hau and Goymann, 2015). If each of the slopes represents an individual then differences between slopes will signify variation in trait plasticity between individuals.

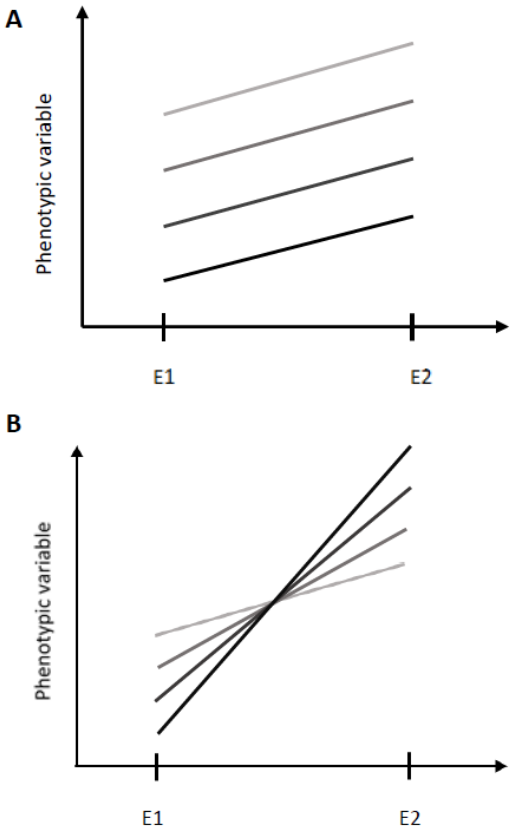



Fig. 1. 6. Variation in the slopes between individuals (A) or populations (B) signifying differences in plasticity of their phenotypic trait. Graph modified from Rocha and Klaczko (2012).

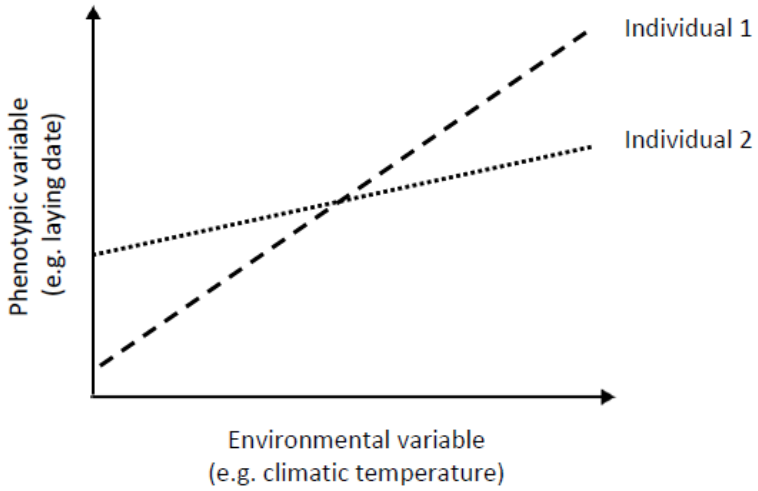



Fig. 1. 7. Example of reaction norms for a phenotypic variable (e.g. laying date) versus an environmental variable (e.g. climatic temperature). The steeper slope of individual 1 compared with individual 2 indicates that individual 1 is more plastic than individual 2. Graph modified from Hau and Goymann (2015).

Reaction norms of linear models are important in evolutionary studies. Plasticity in a trait is heritable and can be under selection pressures (Brommer *et al.*, 2005). In addition, steeper slopes (i.e. greater plasticity) are reported to be selected (Brommer *et al.*, 2005). Certainly, being plastic and having the ability to modify physiological or behavioural responses when environments change can be advantageous in terms of adapting to new situations and the study of trait plasticity is important (Gladalski *et al.*, 2016).

### 1.6.2.2 Character state approach

The character state approach to describing flexibility in responses of traits involves measurement of average trait responses in several distinct environments or different levels of a continuous environmental variable (Via *et al.*, 1995; Bryant *et al.*, 2006). In this approach,

distinct environments such as species of predators are plotted randomly along the x-axis, producing reaction norms that may not be linear (Via *et al.*, 1995). In cases where the environments are different levels of a continuous variable, the environment levels can be ordered by the mean phenotype (Via *et al.*, 1995). The way in which phenotypic plasticity is measured in this approach is less complicated than using the slopes of the polynomial linear models; the phenotypic change is the difference between the two means when there are two distinct environments or two levels of a continuous environmental variable (Via *et al.*, 1995). However, quantification of plasticity is challenging when the environment has several different levels (Schlichting, 1986). An example of the use of this approach was a study of milk production responses in cattle in different environments. Menendez-Buxadera *et al.* (2016) reported that cows with high levels of milk production in a 'food-rich' environment had inadequate milk production in an environment with moderate level of pasture. This approach can be an alternative to the polynomial approach in cases where a reaction norm is not linear and there is a more complicated response profile (Gomulkiewicz and Kirkpatrick, 1992; Via *et al.*, 1995).

### 1.6.3 Plasticity in corticosterone responses in birds

Endocrine responses are an example of plastic traits that can adjust continuously to environmental alterations (Charmantier and Gienapp, 2014). For example, more corticosterone is secreted on average following 1 hour after capture in Lapland longspurs (*Calcarius lapponicus*) that were in a snowstorm (i.e. environment with a higher % of snow cover) than without a snowstorm (i.e. environment with a lower % of snow cover) for 60 min (Krause *et al.*, 2016). This is an example of plasticity in corticosterone responses in birds. The degree of plasticity in corticosterone response of a bird can differ from another bird. Lendvai *et al.* (2014) showed this by subjecting house sparrows (*Passer domesticus*) to two

alternating diets: one week of restricted food (low % of daily intake) then one week of control diet (usual % of daily intake) within a five week period. Birds were bled on the last day of every week to determine plasma corticosterone concentrations and levels of oxidative stress. This study revealed that mean corticosterone concentrations were significantly different between birds on restricted and control diets, and that reaction norms for corticosterone responses (i.e. plasticity) differed between birds. There was some evidence that the differences in changes in body mass could have attributed to differences in plasticity among individuals because the loss of body mass due to food restriction may be greater in larger birds compared to smaller birds leading to more corticosterone secretion in larger birds. Although antioxidant capacity was lower during food restriction, antioxidant capacity was not associated with corticosterone concentrations, indicating that oxidative stress cannot be used to explain the variation in plasticity in corticosterone responses between birds. However, this experiment involved blood measurements to find indications of oxidative stress and did not include measurements in other areas of the body (e.g. tissue or liver) where oxidative stress parameters may be more prominent.

#### 1.6.3.1 Reaction norms to a variety of different stimuli

Reaction norms can be generated for corticosterone responses of animals to different stressors. If corticosterone responses are recorded in birds that are subjected to different stimuli, the average responses can be plotted to give a reaction norm (Cockrem, 2013a). For example, the reaction norm of corticosterone responses in starlings exposed to two different stimuli is presented in Fig. 1.8 (Nephew *et al.*, 2003). Clearly, there is plasticity of corticosterone responses of starlings to different stressors. It is challenging to compare plasticity of corticosterone responses between individuals as each bird must be exposed to at least two stimuli (Cockrem, 2013a). The amount of plasticity shown by individual birds is

valuable information as it provides an indication of how well individuals may be able to cope with different stressors that they may encounter naturally.

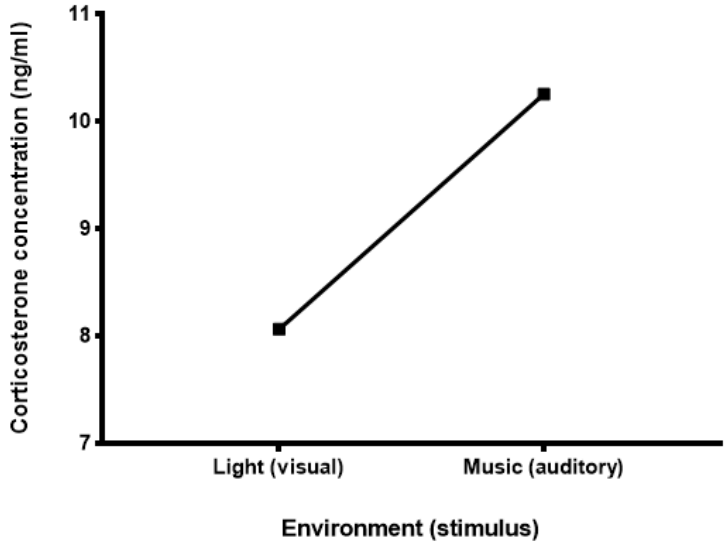



Fig. 1. 8. Reaction norm showing mean corticosterone responses to two stimuli. Graph was produced using data from Nephew *et al.* (2003).

## 1.7 Outline of thesis

The present study was conducted to investigate corticosterone responses of little penguins to a range of stimuli and to determine whether birds show plasticity in their corticosterone responses. A study of corticosterone responses of little penguins at the Oamaru blue penguin colony is described in chapter two of the thesis. Corticosterone concentrations were measured in penguins that were restrained and in penguins that were exposed to other stimuli (novel object, penguin alarm call, dog barking and human talking). A reaction norm for phenotypic plasticity of corticosterone responses of little penguins to a range of stimuli was determined. The last chapter of the thesis provides a discussion of the results of the study and offers recommendations for future studies.

# Chapter 2: Corticosterone in kororā (little penguins; *Eudyptula minor*) exposed to handling and restraint and to other stimuli

## 2.1 Abstract

Physiological and behavioural responses generally differ between birds exposed to different stimuli. Corticosterone responses to capture and handling are useful to detect stress, but they do not tell us how animals will respond to other types of stimuli. The relationship between corticosterone responses to handling and the responses of the same animals to another stressor have not previously been studied. In this experiment, free-living little penguins (*Eudyptula minor*) were exposed to five different stimuli: handling, novel object (toy car), penguin alarm call, dog barking, and human talking. They were sampled after 15 min of stimulus for the measurement of corticosterone concentrations. Simultaneously, behaviours during each treatment were recorded to further understand the responses of these birds to the stimuli. Birds showed a clear corticosterone response to handling. The mean corticosterone response to handling was 465, 267, 220, and 125% higher than mean responses to human talking, dog barking, penguin alarm call and novel object. Mean corticosterone concentrations after presentation of a novel object were greater than mean concentrations after exposure to the dog barking and to the human talking but not the penguin alarm call. The mean concentration after the penguin alarm call was greater than the mean concentration after human talking. There was no significant difference between the mean concentrations after human talking and dog barking, and no significant difference between mean concentrations after the penguin alarm call and the novel object. Correlations between corticosterone concentrations after 15 min of handling and concentrations after exposure of the same birds to other stimuli were not significant. From the present results, it can be concluded that the corticosterone responses of little penguins differ between stimuli and the

magnitude of corticosterone responses to handling may not be similar to the magnitudes of responses to other stimuli in the same bird.

## 2.2 Introduction

Animals are constantly receiving stimuli from their environment. When stimuli are perceived to be threatening the stimuli trigger important physiological processes and are known as stressors. Stressors activate the hypothalamic-pituitary-adrenal (HPA) axis which responds by secreting glucocorticoids (Cockrem, 2013c). Glucocorticoids promote changes in the body that assist in producing suitable reactions towards the stressful situation and overcoming it (Cyr *et al.*, 2007). Secretion from the adrenal gland of the avian glucocorticoid, corticosterone, begins within one or two minutes when the HPA axis is activated in response to a stressor. A standard stressor for birds is capture and restraint. When birds are captured and restrained corticosterone concentrations in the blood increase and generally reach a peak after approximately 30 min. While glucocorticoid responses to a single stressor have been examined, few studies have attempted to evaluate the responsiveness of the HPA axis to different stressors. The typical capture and handling protocol has been applied to many species of birds and their corticosterone responses measured (Kannan and Mench, 1996; Mueller *et al.*, 2006; Cockrem *et al.*, 2010). Whilst this standard protocol is widely considered to mimic natural stressors encountered in the wild (Silverin, 1998), there is limited evidence to support this assumption. Some attempts have been made to investigate the reactions of birds to other stressors but due to the variation in the type of stressors used among studies, comparison of responses within a species is challenging (Bowers *et al.*, 2008). In birds, the magnitude of corticosterone responses can vary between stimuli (Rich and Romero, 2005). This also raises the question: do birds that show relatively high corticosterone response to capture and handling similarly respond highly to a different type of stressor? It would be valuable to know whether responses to other stimuli are similar to capture and handling, a known stressor in birds.

Little penguins inhabiting the Oamaru harbour in the South Island of New Zealand have been previously studied by Cockrem *et al.* (2017b) who measured corticosterone responses of penguins to capture and handling. Penguins had typical avian corticosterone responses and individuals were generally consistent in their responses. Corticosterone responses to capture and restraint have also been measured in other species of penguin (eg. Magellanic penguins, Hood *et al.*, 1998; Adelie penguins, Vleck *et al.*, 2000; Walker *et al.*, 2005; king penguins, Viblanc *et al.*, 2016). Corticosterone responses to stimuli other than capture and restraint have not been measured in penguins. In the present study corticosterone responses of little penguins to a variety of stimuli were measured to determine relationships between responses to simulated natural stressors and responses to the standard capture and restraint protocol.

Physiological traits may show phenotypic plasticity, where a trait response can vary when environments change (Gladalski *et al.*, 2016). For example, basal corticosterone concentrations can vary between years, clearly showing plasticity (Doody *et al.*, 2008). An approach to measure plasticity of a phenotypic trait is to produce a reaction norm which is a graphical representation of the change in trait response across different conditions of the environment. Little is known regarding the plasticity of corticosterone responses in free-living birds.

The main objective of this study was to examine the effect of different stimuli on plasma corticosterone concentrations in little penguins. The study used four stimuli (human talking, dog barking, penguin alarm call, and novel object) as well as the standard stressor known to activate the HPA axis in little penguins (handling; Cockrem *et al.*, 2017b). Some birds were sampled twice, once after handling and once after exposure to a different stimulus, to examine the relationship between corticosterone response to handling and corticosterone

concentrations after exposure to different stimuli. The study also used a reaction norm approach to examine plasticity of corticosterone concentrations of little penguins after exposure to different stimuli.

## 2.3 Methods

### 2.3.1 Study site

The study was conducted at Oamaru (45° 07' S, 170° 58' E) on the east coast of the South Island of New Zealand. There are more than 400 nestboxes at the quarry and creek colonies at Oamaru. The penguins feed off the north Otago coastline. The quarry colony is visited by tourists who can walk along boardwalks during the day and sit in a viewing stand to watch birds come ashore at night. The creek colony is a conservation area that is visited only for research purposes. The quarry and creek colonies are protected by fences so there are no roaming predators or unregulated human movements. The birds at the quarry colony are exposed to low levels of sound from visitor conversation during the day and in the early evening. The nestboxes are of a standard design for little penguins and are located on open grass and underneath trees and shrubs. All chicks are banded for individual identification with numbered metal bands shortly before fledging (Cockrem *et al.* 2017; Agnew *et al.* 2014). All nestboxes are checked weekly and the presence of birds, eggs, and chicks recorded (Agnew *et al.* 2014).

### 2.3.2 Blood sampling

Free-living little penguins of both sexes were sampled over four weeks during the prelaying stage of breeding in September and October 2016. Nestboxes at the quarry and creek colonies were checked and birds sampled in nestboxes that did not contain eggs. Sexes of birds were determined from records in the penguin colony database.

Blood samples were collected following our standard protocol (handling; Cockrem *et al.*, 2017b). Two researchers quietly approached a nestbox then one person opened the nestbox lid and picked up a bird whilst the other person started a timer. The bird was quickly carried to the sampling site located a few metres away where one person held the penguin on their lap by restraining the bird with their hands. The other person collected a blood sample from the brachial vein on the flipper using a heparinised 25 g needle and 1 ml syringe or into capillary tubes after puncture of the vein with a 25 g needle. Brachial veins on both flippers were used to collect blood samples. The volume of blood collected was up to 1 ml. After collection of the sample a cotton swab was applied to the collection site then blood samples were transferred to heparinised tubes and placed in a container filled with ice. Samples were kept cool for up to 2 h then centrifuged for 5 min. Plasma was removed from the sample using a fixed needle glass syringe (500µl, Hamilton 750N), placed in a labelled tube and frozen at -20 °C. Beak dimensions (length, width, and depth) were measured using calipers and birds were weighed after blood samples were collected. The study was conducted under permits from the Massey University Animal Ethics Committee and the Department of Conservation.

### 2.3.3 Study design

Blood samples were collected from 119 birds in nestboxes for the measurement of corticosterone concentrations after birds were exposed to different stimuli. 60 birds were handled for 15 min following our standard protocol for the measurement of corticosterone response in little penguins (handling; Cockrem et al., 2017b) and other species (Cockrem et al., 2008a; Cockrem, 2013b). 32 of the 60 penguins were sampled again from one to four weeks later. Blood samples were collected after 15 min of exposure in their nestbox to a

novel object (toy car), the sound of a dog barking, the sound of penguin alarm calls or to human talking for 15 min. A further 59 birds that had not previously been sampled were sampled after exposure to one of the four stimuli for 15 min.

#### 2.3.3.1 Handling

The 15 min handling stimulus followed our standard protocol for the collection of blood samples for the measurement of corticosterone responses in penguins. Birds were picked up from their nestbox and held by hand whilst an initial (0 min) blood sample was collected. Birds were then placed in a cloth bag which was held by hand and lifted and inverted at 30 sec intervals until 15 min had elapsed from the time when each bird was first picked up. A second blood sample was then collected.

#### 2.3.3.2 Novel object

A red toy car (25 cm length x 14 cm width x 7 cm height) containing a battery powered electric motor which turned the rear wheels of the car was used as a novel object stimulus. The car was placed inside the nestbox through the entrance. A remote control was used to switch the electric motor on and off so that rotation of the wheels of the car could be started and stopped remotely. The wheels were rotated for periods of 15 sec with intervals of 5 to 30 sec between periods of wheel rotation. The duration of the stimulus sequence was three min, with 1.5 min of wheel rotation and 1.5 min of quiet within the sequence (see Table 2.1). The three min sequence was repeated five times to create a stimulus of 15 min duration. A blood sample was collected after the 15 min stimulus. 9 birds that had previously been sampled after the handling stimulus and 15 birds not previously sampled were exposed to the novel object stimulus. It is important to note that the novel object provided both visual and

auditory stimuli to penguins in nestboxes, and that the visual stimulus included both the sight of the car and the sight of the turning wheels of the car.

Table 2. 1. Sequence of periods of toy car wheels turning and wheels not turning.

| Time (min:sec) | Start and stop | Duration of wheels turning (sec) | Duration of wheels not turning (sec) |
|---|---|---|---|
| 0:00 | Start | | |
| 0:15 | Stop | 15 | |
| 0:20 | Start | | 5 |
| 0:35 | Stop | 15 | |
| 1:05 | Start | | 30 |
| 1:20 | Stop | 15 | |
| 1:25 | Start | | 15 |
| 1:40 | Stop | 15 | |
| 1:55 | Start | | 15 |
| 2:10 | Stop | 15 | |
| 2:35 | Start | | 15 |
| 2:50 | Stop | 15 | |
| 3:00 | End | | 10 |

#### 2.3.3.3 Penguin alarm call

Alarm calls made by penguins at the time they were picked up from their nestboxes were recorded from three penguins. Audacity 2.1.2 (2016) software was used to create a three min

sequence of periods of penguin calls and periods of silence, with the sequence repeated five times to create a stimulus of 15 min duration. The sequence contained 1.5 min sound and 1.5 min quiet and consisted of:

15 sec penguin call #1

5 sec quiet

15 sec penguin call #2

30 sec quiet

15 sec penguin call #3

5 sec quiet

15 sec penguin call #1

15 sec quiet

15 sec penguin call #2

25 sec quiet

15 sec penguin call #3

10 sec quiet

The calls were played through a wireless Bluetooth speaker (Iluv Aud Mini Portable Ultraslim Bluetooth Speaker) which was placed on top of the nestbox so the sound was played through a gap between two boards that made up the roof of the nestbox. A blood sample was collected after the 15 min stimulus. 5 birds that had previously been sampled after the handling stimulus and 17 birds not previously sampled were exposed to the novel object stimulus.

The sound intensity of the penguin alarm calls was recorded by placing a microphone inside a nestbox and playing the calls from the wireless Bluetooth speaker placed at the nestbox

entrance. The recording was played back to ensure that sounds would be audible to a penguin inside the nestbox. The mean loudness of the three min sound sequence was determined using the Audacity software. The recording and sound measurement process was repeated for the other two sound stimuli (dog barking and human talking, see below), and the loudness of each sound file adjusted so that the sound level inside the nestbox was similar for each of the three sound stimuli.

#### 2.3.3.4 Dog barking

Recordings of barking sounds made by three breeds of dog (Yorkshire terrier, Shih tsu and Chihuahua) were used as a dog barking stimulus. A three min sequence of barking of the three dogs was created following the same sequence of periods of sound and periods of quiet used for the penguin alarm call stimulus. The sequence was played five times over 15 min following same procedure used for the penguin alarm calls then a blood sample was collected. 9 birds that had previously been sampled after the handling stimulus and 13 birds not previously sampled were exposed to the dog barking stimulus.

#### 2.3.3.5 Human talking

A 15 min human talking stimulus was presented to birds in nestboxes following the same procedure that was used for the penguin alarm call and dog barking stimuli. Recordings of three people talking during radio interviews were used to create a three min sequence that was repeated five times over 15 min. 9 birds that had previously been sampled after the handling stimulus and 14 birds not previously sampled were exposed to the human talking stimulus.

### 2.3.4 Behaviour of penguins during exposure to stimuli

Vocal responses of birds in nestboxes were noted during the 15 min stimulus periods. Occurrences of pecking of the novel object (toy car) were also noted. The observations provided a qualitative assessment of the behavioural responses of the birds to the stimuli.

### 2.3.5 Corticosterone radioimmunoassay

The radioimmunoassay procedure described in Cockrem *et al.* (2009b) and Cockrem *et al.* (2017a) was used for measurement of plasma concentrations of corticosterone. Plasma samples were diluted in phosphate buffered saline with gelatine (PBSG) and were assayed in duplicate. 10µl of PBSG-diluted plasma was incubated for 2 h at room temperature with iodinated corticosterone and anti-corticosterone antiserum (MP Biomedicals, USA). A second antibody precipitant solution (MP Biomedicals, USA) containing starch to increase adhesion of the pellet to the tube was added and each sample vortexed thoroughly, incubated for 15 min at room temperature to separate bound and free corticosterone, then centrifuged for 15 min at 2 000 g at 4$^{o}$C. Supernatants were removed by aspiration and the pellets counted on a PerkinElmer Wallac Wizard 1470 gamma counter for 5 min. The assay was validated for the measurement of corticosterone in little penguin plasma by Cockrem *et al.* (2017a). The assay limit of detection, expressed as ng corticosterone per ml plasma, was 0.65 ng/ml. Solutions of corticosterone in PBSG were used as low and high controls in every assay. The intra-assay coefficients of variation were 8.4 and 7.2%, and inter-assay coefficients of variation were 14.6 and 16.1%.

### 2.3.6 Statistics

Data analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc). Relationships between the time taken to collect blood samples and corticosterone

concentrations were determined using linear regression. ANCOVA was used to compare y axis intercepts of linear regression lines. One-way ANOVA with post hoc comparisons made with Tukey's multiple comparisons tests was used to compare mean corticosterone concentrations between stimuli. Unpaired *t* tests were used to compare mean corticosterone concentrations between sexes and to compare corticosterone concentrations between birds that had previously been handled and birds that had not previously been handled for each stimulus. Pearson correlation was used to examine correlations between corticosterone responses to handling and responses to other stimuli for birds that had previously been handled. A reaction norm to quantify plasticity in corticosterone responses of little penguins to different stimuli was determined by plotting mean corticosterone concentrations after exposure of penguins to 15 min of five stimuli. Results of statistical tests were considered to be significant when *p* values were less than 0.05. Data are presented as individual values or as mean ± S.E.

## 2.4 Results

### 2.4.1 Behaviour

Birds made occasional vocalisations during the human talking and dog barking stimuli. Vocalisations were much more frequent and continued for longer when recordings of penguin alarm calls were played. Most birds performed a ‘growl’ as oppose to an alarm type call. Some birds vocalised for the whole duration of the 15 min stimulus. Interestingly, some birds in adjacent nestboxes also vocalised during the penguin alarm call treatment. When the novel object was presented, the majority of birds responded by pecking at the toy car aggressively. Some birds briefly vocalised with a warning call when the toy car was placed in their nestbox while others were more frequent in their vocalisations towards the stimulus. Birds did not call when they experienced the handling stimulus.

### 2.4.2 Corticosterone in relation to time taken to collect blood samples

The time taken to collect each blood sample was recorded in minutes and seconds starting from the time when each bird was picked up in its nestbox. The times, converted to decimal values, ranged from 1.1 to 12.3 min (mean 4.6 $\pm$ 0.2 min) due to the challenges of collecting blood from inactive penguins. There were significant linear relationships between corticosterone concentrations and sample collection time for the human talking, dog barking and penguin alarm call stimuli ($r^2 = 0.450$, $p = 0.0006$; $r^2 = 0.292$, $p = 0.0095$; $r^2 = 0.239$, $p = 0.0245$; see Appendix Fig. 1). The y intercepts of the linear regressions (the predicted corticosterone concentrations at 0 min) were 1.20 ± 2.78, 2.39 ± 5.75 and 7.58 ± 5.56 ng/ml. The mean sample collection time differed between the five stimuli (one way ANOVA; $F_{4,140} = 16.46$, $p < 0.0001$; see Appendix Fig. 2), was longer for the handling stimulus than for the other stimuli ($p < 0.001$ for all comparisons of handling with other stimuli), and did not differ between the other stimuli.

### 2.4.3 Corticosterone concentrations after exposure of penguins to different stimuli

Corticosterone concentrations of all individual birds are presented in Fig. 2.1. Plasma corticosterone concentrations after exposure to a stimulus for 15 min varied from 4.29 to 63.20, 2.56 to 30.99, 6.71 to 46.18, 5.35 to 62.14, and 18.45 to 130.90 ng/ml following the stimuli of dog barking, human talking, penguin alarm call, novel object, and handling, respectively.

Mean plasma corticosterone concentrations after exposure of penguins to different stimuli are shown in Fig. 2.2. There were significant differences between stimuli in mean corticosterone

concentrations (see Table 2.2 for statistics). The mean corticosterone concentrations after 15 min handling (63.24 ± 3.79 ng/ml) was greater than the concentrations after exposure of penguins to the other stimuli (mean concentrations all <30 ng/ml; $p < 0.0001$ for all comparisons). The mean concentration after exposure to the novel object (28.05 ± 3.10 ng/ml) was greater than the mean concentration after the dog barking (17.23 ± 2.91 ng/ml) and to the human talking (11.18 ± 1.67 ng/ml). The mean concentration after the penguin alarm call (19.73 ± 2.67 ng/ml) was greater than the mean concentration after human talking. There was no significant difference between the mean concentrations after human talking and dog barking, and no significant difference between mean concentrations after the penguin alarm call and the novel object (see Table 2.2).

Long (2017) collected blood samples from penguins in the Oamaru colony over a four week period immediately before the current study. Corticosterone concentrations in these samples can be considered to be initial, 0 min concentrations for comparison with concentrations in samples collected after penguins were exposed to stimuli for 15 min. Mean corticosterone concentrations in initial samples collected by Long (2017) were 20.12 ± 1.96 ng/ml ($n = 58$). The mean time taken to collect these samples was 6.3 ± 0.7 min. There was a significant linear regression relationship between corticosterone concentrations and time taken to collect initial samples ($r^2 = 0.286$, $p < 0.001$). The slope of the regression line was 3.04 ± 0.64 ng/ml.min and the y intercept was 0.87 ± 4.40 ng/ml. The y axis intercepts of linear regression lines of corticosterone concentrations versus time taken to collect the blood samples were 1.20 ± 2.77, 2.39 ± 5.75 and 8.33 ± 5.58 for human talking, dog barking and penguin alarm call. The slopes of the regression lines were 2.77 ± 0.68, 3.93 ± 1.37 and 4.80 ± 1.98 ng/ml.min respectively. The regression was not significant for the novel object. ANCOVA analyses to statistically compare y axis intercepts of linear regression lines

between initial samples and each of the three stimuli for which there were significant regressions showed that there was no difference between the intercepts for initial samples and human talking and no difference between the intercepts for initial samples and dog barking ($F_{1,77}$ = 0.0636, $p$=0.8016; $F_{1,77}$ = 2.297, $p$=0.1337). The difference between the intercepts for human talking and for dog barking was not quite significant ($F_{1,41}$ = 3.821, $p$=0.0575). The intercept for penguin alarm calls was significantly greater than the intercept for initial samples ($F_{1,75}$ = 9.817, $p$=0.0025).

### 2.4.4 Sex differences in corticosterone responses to different stimuli

Mean corticosterone concentrations in males were higher than concentrations in females following exposure to the novel object ($t_{22}$ = 3.125, $P$ = 0.0049) and to handling ($t_{36}$ = 4.414, $P$ < 0.0001), and did not differ significantly between sexes in birds exposed to human talking, dog barking, and penguin alarm calls (Fig.2.3).

### 2.4.5 Comparison between previous handling and no previous handling

Mean corticosterone concentrations of birds handled or not handled before other stimulus treatments are presented in Fig. 2.4. There was no significant difference in the mean corticosterone concentration after dog barking between birds that had previously been handled and birds that had not been handled ($t_{20}$= 0.544, $p$ = 0.592). Similarly, mean corticosterone concentrations did not differ between birds previously handled or not previously handled for the human talking ($t_{21}$ = 0.211, $p$ = 0.835), penguin alarm call ($t_{20}$ = 0.499, $p$ = 0.624), and novel object ($t_{22}$ = 1.039, $p$ = 0.310) stimuli.

### 2.4.6 Correlations between corticosterone concentrations after handling and after exposure to other stimuli

There were no significant correlations between corticosterone concentrations after handling and concentrations after exposure to other stimuli (see Appendix Fig. 3). Pearson correlations between concentrations after handling and concentrations after exposure to other stimuli were human talking $r^2 = 0.327$ ($p = 0.1386$), dog barking $r^2 = 0.102$ ($p = 0.4011$), penguin alarm call $r^2 = 0.097$ ($p = 0.6091$), novel object $r^2 = 0.218$ ($p = 0.2048$) and for all four stimuli combined $r^2 = 0.003$ ($p = 0.7829$).

### 2.4.7 Reaction norm to different stimuli

Mean corticosterone responses to different stimuli are presented as a reaction norm in Fig. 2.5. The environmental variable was not continuous so a linear regression analysis cannot be performed on these data.

Table 2. 2. Statistical analysis for comparison of mean corticosterone concentrations after exposure to different stimuli.

| Groups | *F* | Df | *p* |
|---|---|---|---|
| All 5 stimuli | 50.38 | 4,145 | <0.0001 |
| *Post-hoc test* | | | |
| Human vs Dog | | | 0.1939 |
| Human vs Penguin | | | 0.0109 |
| Human vs Novel object | | | <0.0001 |
| Human vs Handling | | | <0.0001 |
| Dog vs Penguin | | | 0.8004 |
| Dog vs Novel object | | | 0.0199 |
| Dog vs Handling | | | <0.0001 |
| Penguin vs Novel object | | | 0.2927 |
| Penguin vs Handling | | | <0.0001 |
| Novel object vs Handling | | | <0.0001 |

Note: The first row shows the results of a one-way ANOVA for the comparison between group means. The remaining rows show *post-hoc* comparisons of different stimuli.

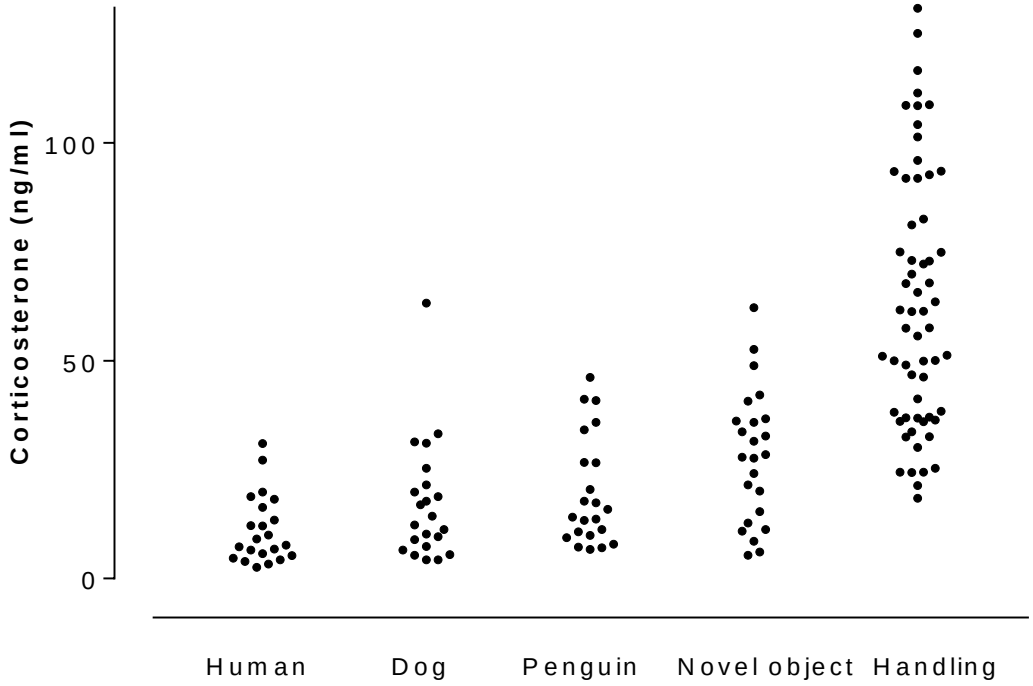



Fig. 2. 1. Corticosterone concentrations of individual birds exposed to different stimuli.

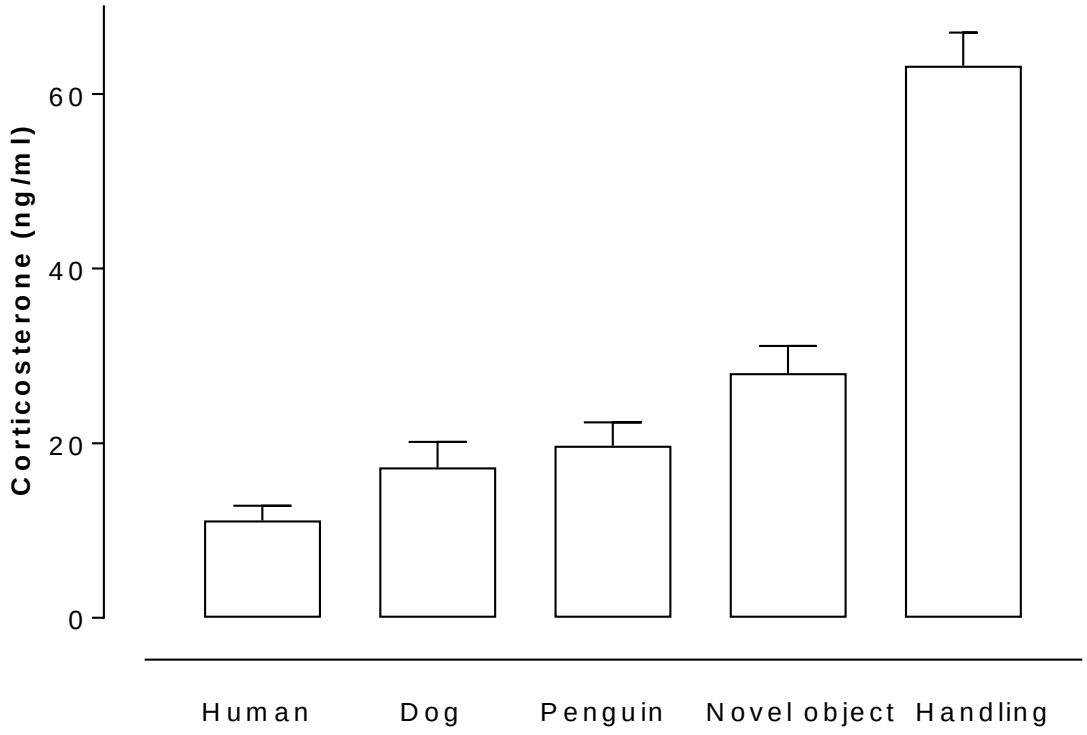



Fig. 2. 2. Mean (± S.E.) plasma corticosterone concentrations in birds following exposure to different stimuli for 15 min. The number of birds per group was as follows: n = 22 for human talking; n = 22 for dog barking; n = 22 for penguin alarm call; n = 24 for novel object; and n = 58 for handling.

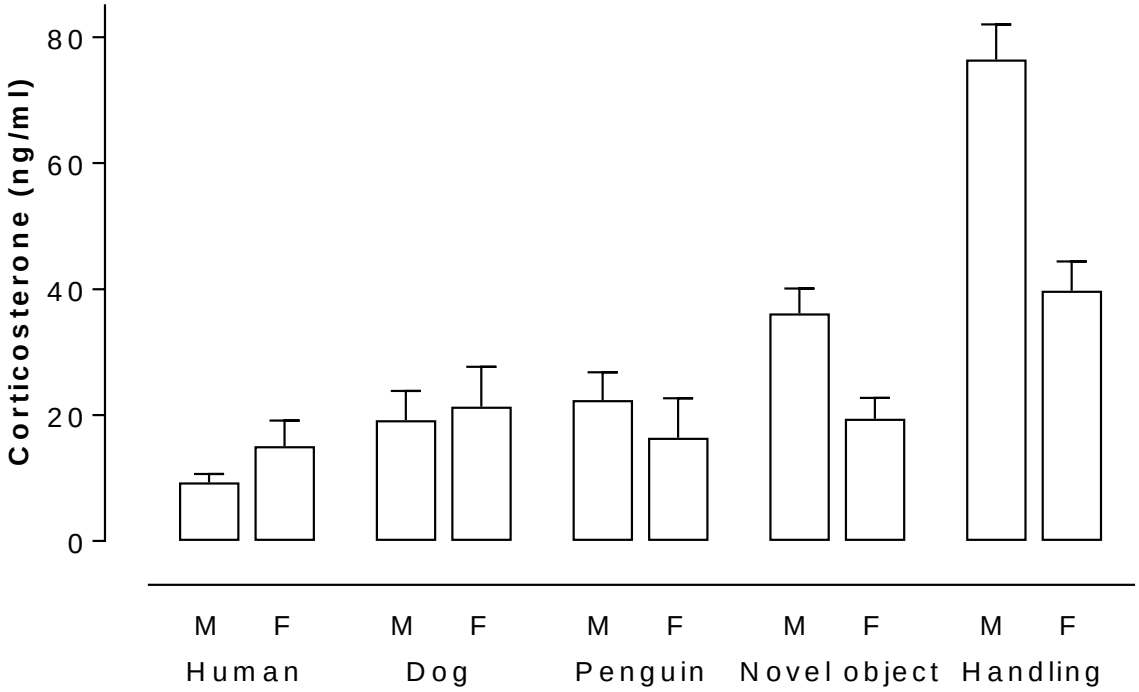



Fig. 2. 3. Mean (± S.E.) corticosterone concentrations in male and female little penguins after exposure to different stimuli. M = male and F = female.

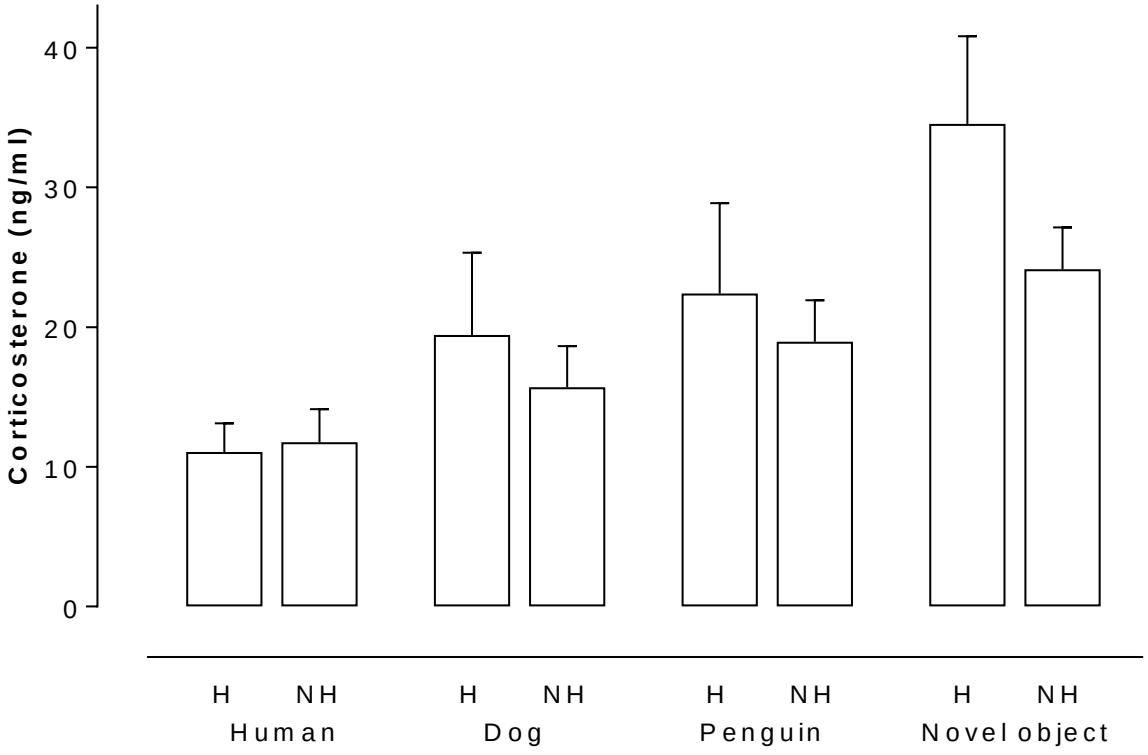



Fig. 2. 4. Mean (± S.E.) corticosterone concentrations in birds that were previously handled (H) and birds that were not handled (NH) before exposure to other stimuli.

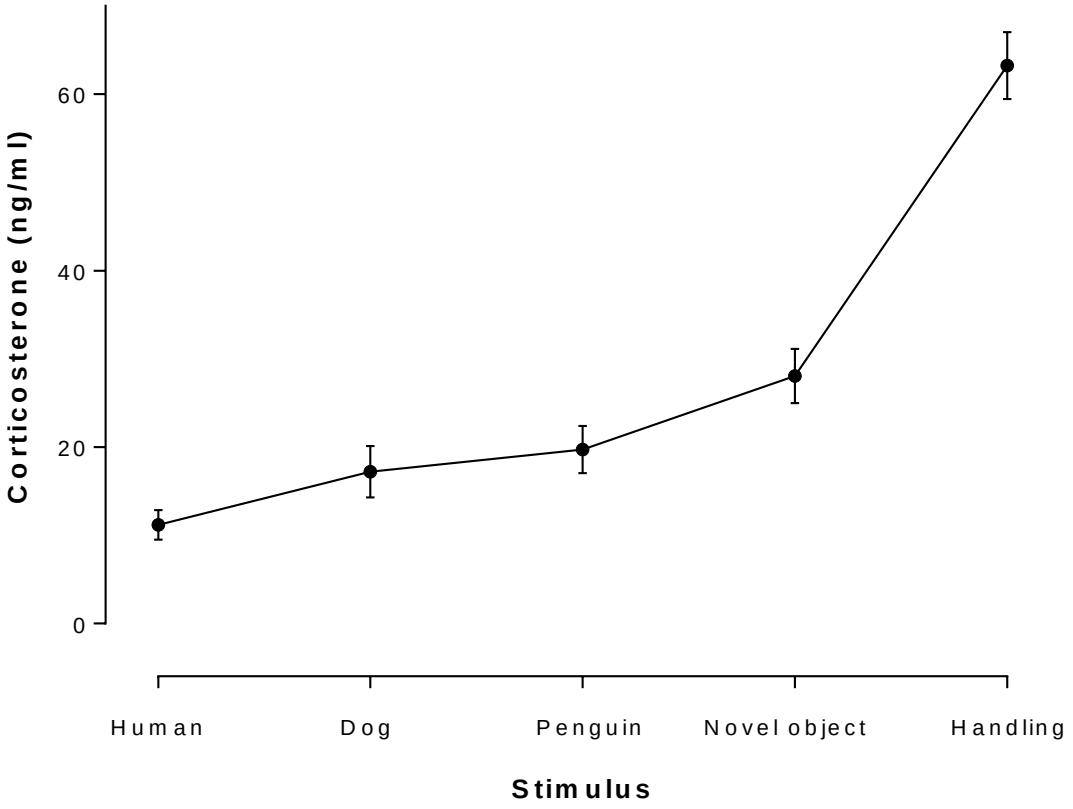



Fig. 2. 5. Reaction norm for mean (± S.E.) corticosterone concentrations in little penguins exposed to different stimuli.

## 2.5 Discussion

This is the first study of corticosterone responses of any species of penguin to different stimuli. Corticosterone responses to 15 min of handling were greater than responses of penguins in nestboxes to auditory and visual stimuli that did not include handling. Presentation of a novel object in nestboxes induced larger corticosterone responses than auditory stimuli. The response to a novel object was greater than responses to human talking and dog barking, and not significantly different from responses to penguin alarm calls. Mean corticosterone responses differed between stimuli, indicating plasticity in the corticosterone responses of little penguins. A reaction norm was used to represent this plasticity.

### 2.5.1 Behaviour

This is the first study of little penguins in which observations of behaviour, as well as measurement of corticosterone responses, were made when birds were exposed to a variety of stimuli. Penguins made few vocalisations in response to human talking and to dog barking, with brief calls during human talking and vocalisations when the dog barking stimulus began but not thereafter. Whilst dogs are predators of little penguins, penguins will also nest underneath houses where there is a dog present. When dogs attack and kill penguins it is likely that the attack would be brief as penguins would not be able to escape from dogs. There are no reports of the behaviour of free-living penguins when they are attacked by a dog. Behavioural responses to penguin alarm calls were more intense than responses to the other two auditory stimuli, with many birds calling in response to the penguin alarm call throughout the 15 min period of exposure to the stimulus. When birds were presented with a novel object in their nestbox they vocalised and also pecked at the object. Pecking behaviour has also been described in free-living great tits introduced to a pink figurine attached to a perch (Baugh *et al.*, 2017). The author suggested that the an encounter with an unfamiliar

object on the perch could be analogous to a situation where the bird experiences an intrusion; however, the behaviour of tits induced by an actual intruder tend to be more intense (Verbeek *et al.*, 1996). Other behavioural responses of birds towards a novel object include a report by Richard *et al.* (2008) who found that quail showed avoidance behaviour when a PVC cylinder was introduced into their home cage. The quail spent time away from the object for a longer duration than birds in the control group. This contrasts to the behaviour seen in the penguins in the present study where the birds approached and pecked at the object almost immediately after presentation. In the present study an object was presented once for 15 min whereas the experiment by Richard *et al.* (2008) involved five sessions of object presentation over a period of 30 min where most birds did not approach the object. Birds repeatedly exposed to a novel object within a short time frame may show habituation, not necessarily towards the object but towards the repeated act of presentation. Indeed, ravens spend more time in the area of the first object introduced compared with following novel objects (Stoewe *et al.*, 2006).

The present study showed that responses of penguins to auditory stimuli may differ from responses to stimuli with several components. Most studies of responses of animals to visual or auditory stimuli have been studies of mammals exposed to a single stimulus (e.g. Blumstein *et al.*, 2000; Christensen *et al.*, 2005). However, stimuli that activate multiple sensory modes may induce different behaviours than stimuli with a single component (Hale *et al.*, 2017). For instance, food that is yellow accompanied by a specific chemical odour was frequently avoided by domestic chicks, but colour or odour alone did not induce any response (Rowe and Guilford, 1996), suggesting that there was a compound effect of more than one discrete components. Whether pecking behaviour, only observed in the novel object test of the present study, was an innate response to a foreign object inside the bird's nest or was a

response to the effect of a combination of sight, movement, and sound (turning of wheels) of the toy car requires further study. On the other hand, the greater vocalisations in the auditory tests compared to the novel object test indicate that multi-component stimuli may be perceived differently from auditory stimuli alone in penguins.

### 2.5.2 Corticosterone responses to different stimuli

One of the goals of the present study was to determine the relative magnitude of corticosterone responses of birds to different stimuli in comparison with responses to capture and handling. As expected, compared to corticosterone response to handling, the responses at 15 min to all other stimuli were much lower, indicating that handling was the strongest stressor; the mean corticosterone response to handling was 465, 267, 220, and 125% greater than mean responses to human talking, dog barking, penguin alarm call and novel object. In previous studies, corticosterone responses to capture followed by handling, or to restraint in captive animals, have also been measured and compared to responses to other stressors in a range of species of birds and mammals (Seggie and Brown, 1975; Silverin, 1998; Canoine *et al.*, 2002; Rich and Romero, 2005; Bowers *et al.*, 2008; Hazard *et al.*, 2008; Rutkowska *et al.*, 2011). In an experiment with European starlings, Rich and Romero (2005) exposed birds to a variety of stimuli for 30 min and found that confinement in a tube with four other birds (where birds could walk around) induced a higher mean corticosterone response than the classic method of restraint in a bag (40 c.f. 30 ng/ml). The mean corticosterone response to handling was at least 50% higher than mean responses to predator decoys (weasel or woodpecker) or a territorial intruder used in the same study in nest-building male pied flycatchers (Silverin, 1998). These studies clearly show that there are contrasting effects of different stimuli on the HPA axis of birds. Corticosterone responses of laboratory rodents also depend on the type of stimulus. For example, mean corticosterone concentrations

increased by 105% from mean baseline corticosterone in handled mice whereas they increased by 1085% and 940% in individuals exposed to restraint or low temperature, respectively (Bowers *et al.*, 2008).

In another study, handling in rats induced a significantly lower corticosterone response than placement in a novel environment (116 ± 12 c.f. 217 ± 8 ng/ml, Seggie and Brown, 1975). However, both of these studies applied handling to the animals very briefly (≤30 sec) whilst the durations of exposure to other stimuli were much longer, with some reaching up to 2 hours (e.g. restraint or low temperature, see Bowers *et al.*, 2008). Comparisons of corticosterone responses to different stimuli cannot be made when the durations of exposure to the stimuli differ. Thus, in the present experiment, corticosterone was measured after the same durations of handling and of exposure to other stimuli.

Although the mean initial corticosterone concentration measured in little penguins at the Oamaru Blue Penguin Colony sampled over several weeks before the current study (Long, 2017) can be used for comparison with concentrations after exposure to various stimuli, it is important to note that initial levels do not reflect true measure of baseline corticosterone concentration in an undisturbed bird. Whilst it has been widely accepted that corticosterone measured in samples collected up to 3 min from the time of capture is a reliable estimate of the concentration that would be observed in birds before exposure to a stressor (Small *et al.*, 2017), corticosterone concentrations in birds often increase within 3 min of capture. In Florida scrub-jays, the first indication of corticosterone elevation was at 1.5 min from the time of capture (Small *et al.*, 2017). In great tits, mean plasma corticosterone level measured within 3 min was markedly below levels induced after 0.5 hour from the time of capture, but because the onset of the increase was before 3 min, this initial concentration was not the same

concentrations in undisturbed birds (Baugh *et al.*, 2013). Furthermore, other studies have reported a relationship between the sample collection time and initial plasma concentrations in samples collected <3 min after capture (Rensel *et al.*, 2010; Newman *et al.*, 2017). Based on these results, it is clear that the view that corticosterone in samples collected up to 3 min reflect concentrations in undisturbed birds is not correct. For the little penguins, it took up to 6 min on average to collect initial samples, the mean initial corticosterone concentration was relatively high, and corticosterone concentrations measured in these initial samples did not represent concentrations in undisturbed penguins (baseline concentrations).

The human talking and dog barking stimuli did not elicit significant corticosterone responses in little penguins at Oamaru. Statistical comparisons of y intercepts of linear regressions of corticosterone concentrations versus time taken to collect the blood samples showed that penguins did not have any corticosterone response to human talking or to dog barking, whereas they did have a response to the penguin alarm call. The y intercepts were estimates of corticosterone concentrations in birds after 15 min of stimulus without the confounding effect of corticosterone responses induced by the sampling procedure. The intercepts for initial samples (collected by Long (2017)), human talking, and dog barking were all low (<2.5 ng/ml) and were not significantly different, suggesting that birds in boxes did not have significant corticosterone responses to the human talking and dog barking stimuli. The results also suggest that some birds initiated a corticosterone response when they heard the dog barking; mean corticosterone concentrations after the dog barking were higher than concentrations after the human talking although this difference was not significant. These results indicate that on average the birds secure in boxes did not perceive the sound of a barking dog to be a threat.

The presence of a human, live predator, or a predator decoy can initiate a corticosterone response in some species of birds (Silverin, 1998; Cockrem and Silverin, 2002a; Mueller *et al.*, 2006). However, research regarding the corticosterone responses of birds to predator calls is limited. Common blackbird chicks did not show significant increases in their corticosterone concentrations in response to playback of calls produced by magpies, a predator species (Ibanez-Alamo *et al.*, 2011). The authors suggested that, to the chicks, the sound of the playbacks would have appeared as if the predator is near and that the chicks respond by decreasing begging behaviour (as the chicks also increased testosterone levels) to avoid signalling the site of their nest to the potential predator. Butler *et al.* (2009) exposed parent black-capped (*Vireo atricapilla*) and white-eyed (*Vireo griseus*) vireos to a crow or to a human standing near their open-nest with crow predatory calls and human voice playbacks around the nest, and the vireos did not increase corticosterone concentrations. The vireos were exposed to stimuli over several days whereas in the current study penguins were exposed to a stimulus once only, Santana Amaral *et al.* (2010) showed that plasma corticosterone concentrations in mice placed in an apparatus that contained a rat in an adjacent chamber increased within 5 min and reached peak concentrations of 142.3 $\pm$ 21.4 ng/ml around 15 min following the start of the exposure in comparison with concentrations of 57.03 $\pm$ 12.3 ng/ml in control mice. Magellanic penguins can increase corticosterone concentrations after seeing a human approaching and standing around their nest (Fowler, 1999). Free-living great tits that were able to escape from a predator (stuffed owl) did not have a significant corticosterone response whereas there was a significant corticosterone response in great tits in an aviary that could not move away from a stuffed owl placed in the aviary (Cockrem and Silverin, 2002a). The HPA axis is activated when an animal perceives a stimulus to be a threat. The results from different studies show how corticosterone concentrations indicate whether or not a stimulus is a threat and show how it cannot be

assumed that a stimulus that in some situations is a threat to an animal is always perceived to be a threat.

Several previous studies have investigated the responses of birds to sound of alarm calls of conspecifics (Kleindorfer *et al.*, 1996; Dufty and Crandall, 2005; Tilgar *et al.*, 2010). Tilgar *et al.* (2010) found that 1 hour playback of distress calls of chicks resulted in an elevation in corticosterone concentrations in 9-day-old pied flycatcher chicks when compared with control birds that received adult singing recordings. By contrast, exposure to adult alarm calls for 3 min had no significant effect on corticosterone concentrations in American kestrel chicks (Dufty and Crandall, 2005). These contrasting results may be due to differences between species or could be related to the duration of playback; however, further data are needed to distinguish between these possibilities. The current study found that adult little penguins had corticosterone responses to the sound of penguin alarm calls, indicating that birds in nestboxes perceived penguin alarm calls as an indication of the presence of a threat. The penguin alarm calls had been recorded when birds were picked up from their nestboxes, so the sound of the penguin alarm call may have been interpreted as indicating a predation event at a nearby nest. Another study showed that young Belding's ground squirrels (*Urocitellus beldingi*) increased mean plasma cortisol concentrations to 86.31 $\pm$ 31.56 ng/ml after hearing trills (conspecific's alarm call) recordings in comparison with concentrations of 45.52 $\pm$ 2.57 ng/ml in controls (Mateo, 2010). This response to trills in the absence of any direct threat from a predator, and without seeing conspecifics in danger or escaping, was analogous to the corticosterone response of the little penguins to the sound of penguin alarm calls.

Alarm calls are important for conspecifics to signal danger and to promote anti-predator responses from the receiver (see Kleindorfer *et al.*, 1996). In the current study, birds in nestboxes into which alarm calls were played responded by calling. It is also important to note that birds in other nestboxes often called in response to the calls made by the birds that heard the recorded alarm calls. The birds that called in the other nestboxes might have initiated corticosterone responses. Tilgar *et al.* (2010) reported that parents from other pied flycatcher breeding pairs as well as birds of five other species nesting nearby responded to playback of chick distress calls with mobbing behaviour, indicating that alarm calls can be recognised by birds of other species. However, this type of response increases the risk of detection by predators by indicating nest location. Birds may avoid confrontation with a predator by behaving in a passive way, for example, younger nestlings that are limited in their ability to fly away typically freeze and produce fewer vocalisations when hearing distress calls (Tilgar *et al.*, 2010). Birds may show an active response such as the carrion crows (*Corvus corone*) that have been reported to fly away after hearing conspecific alarm calls regardless of the situation (predator or non-predator enclosure; Bila *et al.*, 2017). Alarm calls also elicited calling in these crows (Bila *et al.*, 2017).

It has been hypothesized that novelty is not a threatening experience but it evokes changes in behaviour of birds due to the unpredictable nature of the stimulus which could explain the moderate increase in corticosterone reported in many of previous studies of responses to novel objects (Richard *et al.*, 2008; Baugh *et al.*, 2017). The present findings regarding responses to a novel object indicate that the penguins perceived the presence of the novel object inside the nestbox to be a threat. However, the corticosterone response to the novel object was much lower than the response to 15 min of handling and restraint (28.05 $\pm$ 3.10 cf. 63.24 $\pm$ 3.79 ng/ml). Similarly, great tits showed a smaller elevation in corticosterone in

response to a novel object (e.g. pink panther figurine attached to a perch; Baugh *et al.*, 2017) than the response to handling-restraint (Cockrem and Silverin, 2002b). Hazard *et al.* (2008) also found that 10 min presentation of a novel object (balloon) induced corticosterone responses in Japanese quail that were markedly smaller than those produced after restraint. The results from these studies indicate that birds perceive handling and restraint to be a greater threat than the sight of a novel object.

Male little penguins had greater corticosterone responses to handling and to exposure to a novel object than female little penguins, with no sex differences in corticosterone concentrations after exposure of birds to penguin alarm calls, dog barking or human talking. Cockrem *et al.* (2017b) also found higher corticosterone 15 min after handling in male little penguins, although there were no sex differences after 30 and 60 min of handling. Sex differences in corticosterone responses of birds are reported in some studies and not others, and there may be a sex difference at one stage of breeding and not at other stages. For example, female but not male quail of a line selected for low tonic immobility had significant corticosterone responses after exposure to a red balloon (e.g. novel object; Hazard *et al.*, 2008). There were no sex differences in corticosterone responses to capture in breeding Adelie penguins (Vleck *et al.*, 2000) or in non-breeding European starlings (Nephew *et al.*, 2003) and European stonechats (Canoine *et al.*, 2002) which may be due to the birds being sampled during the non-breeding season or once the breeding season had terminated (Carroll *et al.*, 2016). Responses of white-crowned sparrows (*Zonotrichia leucophrys*) differed between males and females when the birds were in the incubating or territorial establishment stage, but did not differ at the prebasic molting phase (Krause *et al.*, 2015b). Generalisations cannot be made about sex differences in corticosterone responses in birds.

The absence of a difference in corticosterone concentrations between birds that had been previously handled and not handled before exposure to another stimulus indicates that the differences in corticosterone responses between stimuli resulted from the effect of stimuli and were not related to whether or not birds had previously been handled. In some studies, previous handling experience has affected corticosterone responses of birds. For example, as the number of capture and restraint episodes increased in quail the magnitude of corticosterone responses reduced (Jones *et al.*, 2000). It was also found that corticosterone concentrations of great tits following capture then handling were slightly, but not significantly, higher when they were first sampled compared to when they were sampled a third time (Cockrem and Silverin, 2002b). Carroll *et al.* (2016) reported that corticosterone concentrations 30 min after capture and handling began were higher in little penguins that had experienced research handling in the past than in penguins that had not been handled. Comparison of the results of the present study and those of other studies should be done with care because the other species were subjected to several episodes of handling whereas the little penguins in our study were handled only once before another stimulus was applied. This is supported by the finding by Longordo *et al.* (2011) that mice did not significantly increase corticosterone concentrations in blood after only one handling session, but they did show increases when they received handling for six consecutive days. It should be also noted that while other studies used only handling for both previous and subsequent stressor, the present study used handling as a prior stimulus but a completely different stimulus for the second sampling.

There were no significant correlations between corticosterone concentrations in samples collected in the current study from little penguins after 15 min of handling and concentrations after exposure of the same birds to penguin alarm calls or to a novel object (which elicited

significant corticosterone responses) or after exposure to the sound of human talking or dog barking (which did not elicit significant responses). It was predicted that a bird that had a corticosterone response to handling that was relatively high in comparison to other birds would also have a relatively high response to a different stimulus. The measurement of corticosterone concentrations in the current study was confounded by the challenges of blood sample collection which meant that the measured concentrations were affected by increases in corticosterone between the time when a bird was picked up from its nestbox and the time when a blood sample was collected. We did not have a true measure of corticosterone after 15 min of exposure to a stimulus (penguin alarm or novel object), nor did we have a true measure of corticosterone after 15 minutes of exposure to handling, so we cannot draw firm conclusions about whether or not the corticosterone response to handling in an individual bird is related to its corticosterone response to other stimuli.

### 2.5.3 Reaction norms

Reaction norms are graphical representations of the change in a trait response across different conditions of the environment. If an environmental variable is continuous, for example temperature, then a linear regression allows the estimation of the slope which directly measures the level of plasticity an individual or a population shows in the phenotypic trait of interest (steeper slope means greater plasticity; Hau and Goymann, 2015). Reactions norms for variables that are not continuous, for example methods of handling birds, can usefully be represented graphically (Cockrem, 2013a) although they do not provide quantitative measures of plasticity.

Examples of reaction norms for corticosterone responses to different stimuli in captive European stonechats, European starlings and great tits and in domesticated Japanese quail are

shown in Fig 2.6. In birds, both basal corticosterone and corticosterone responses have been found to differ when environmental situations change; for example, baseline corticosterone before leaving for a foraging trip tended to be higher than after the trip in free-living Adelie penguins (Angelier *et al.*, 2008), and Krause *et al.* (2016) found that restraint-induced mean corticosterone concentrations in Lapland longspurs were higher 0.5 and 1 hour after capture during a snowstorm than when birds were caught when snow was not falling. The ability to respond to changing environments is likely to be advantageous for survival and plasticity in birds, although that requires further investigation. In the present study, the reaction norm indicates that little penguins at Oamaru show plasticity in their corticosterone responses. This is the first description of a reaction norm for corticosterone in penguins.

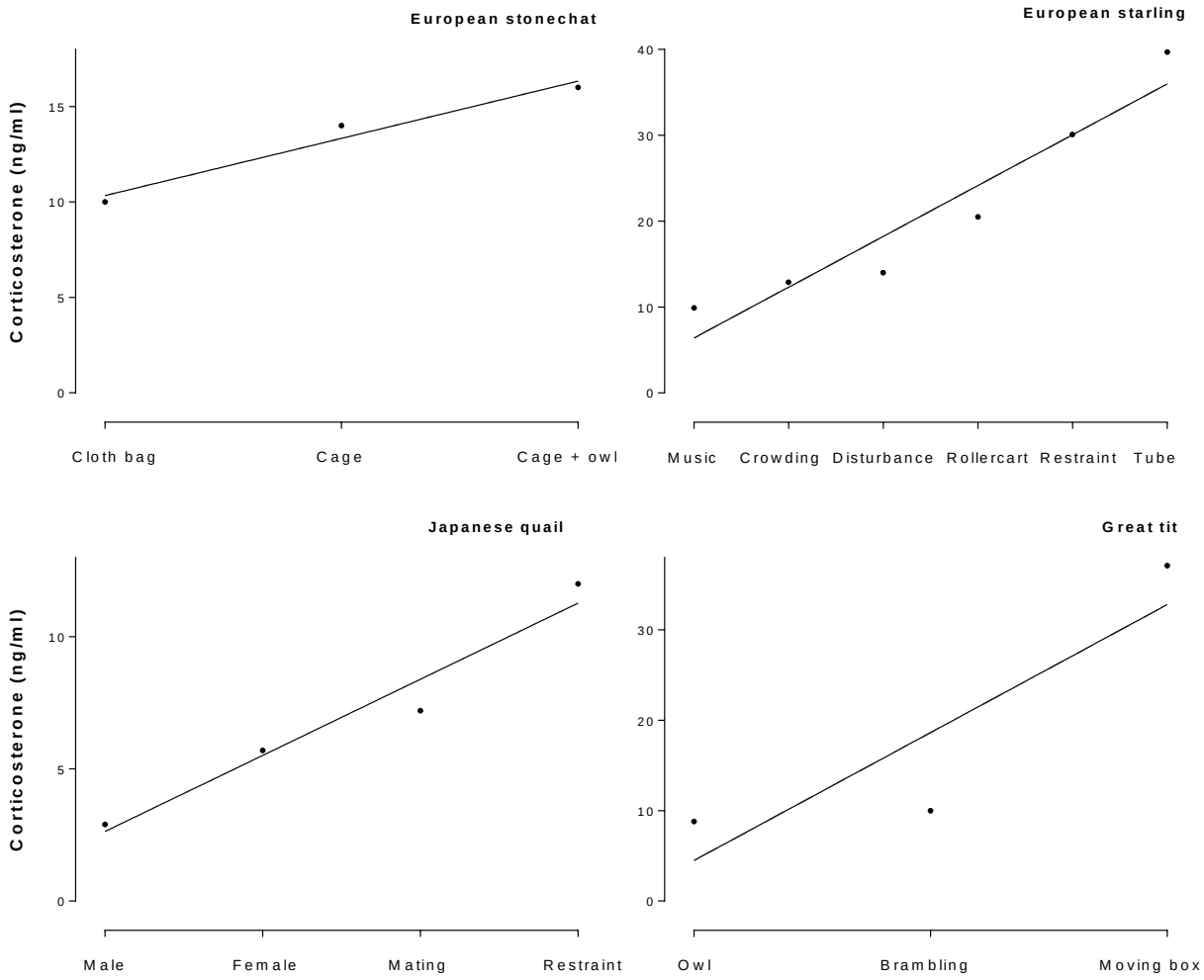



Fig. 2. 6. Reaction norms of corticosterone responses to different stimuli in captive and free-living birds. European stonechats were restrained inside a cloth bag, placed in a cage, or put inside a cage that contained an owl (data from Canoine *et al.*, 2002). Captive European starlings were sampled after exposure to 0.5 h of restraint inside a cloth bag, confinement in tube, music playback, crowding, cage disturbance, or rolling cage (data from Rich and Romero, 2005). Female quail were placed in a cage that faced a male, put in a cage with another female, put in a cage with a male that mated with the female, or restrained (data from Rutkowska *et al.*, 2011). Great

tits were placed in an aviary and presented with a stuffed owl, a stuffed brambling, or a moving box (data from Cockrem and Silverin, 2002a).

## 2.6 Conclusion

The corticosterone results from the current study indicate that little penguins in nestboxes did not perceive the sound of human talking or the sound of dog barking to be threats. Penguin alarm calls did elicit corticosterone responses and hence were perceived to be threatening. The placement of a novel object in the nestboxes also elicited a corticosterone response which was larger than the response to the sound of penguin alarm calls. The responses to alarm calls and to a novel object were both markedly smaller than the response to handling and restraint. Limitations of this sampling protocol associated with the difficulty of collection of blood samples meant that the relationship between corticosterone responses to handling and responses to other stimuli could not be determined. This study has also demonstrated that little penguins show plasticity in their corticosterone responses, with further investigation required to define the degree of such plasticity.

## Chapter 3: General discussion

Two previous studies showed that capture and handling is a stressor that initiates corticosterone responses in little penguins (Carroll *et al.*, 2016; Cockrem *et al.*, 2017b)**.** However, corticosterone responses of little penguins, or indeed any penguin species, to other stimuli that could be stressors have not been measured. The current study was conducted to examine corticosterone responses of little penguins to a range of stimuli and to investigate relationships between responses to the widely used capture and handling stressor and responses to other stimuli.

### 3.1 Major conclusions

The current study provides novel data on corticosterone responses in a free-living bird species and addressed important questions associated with the responsiveness of the avian HPA axis to different stimuli. The main aim of this research was to examine the effect of a variety of stimuli on plasma corticosterone concentrations in little penguins. The plasticity of corticosterone responses was also examined by taking a reaction norm approach. The key findings of the study were:

1. Corticosterone responses to other stimuli were compared to the responses to standardised capture and handling in little penguins. As seen in other species of birds, little penguins show distinct differences in corticosterone responses to different types of stimuli. Exposure to a novel object for 15 min elicited greater corticosterone responses than exposure to 15 min of dog barking or human talking. Mean responses to penguin alarm calls did not differ from mean responses to a novel object. 15 min of handling stimulated greater corticosterone responses than exposure to any other stimuli used in the present study. The results show that exposure to stimuli that do

not include handling initiates corticosterone responses that are smaller than responses to handling, and that stimuli that might have been thought to initiate corticosterone responses, such as the sound of human talking, are not necessarily perceived by penguins as stressors.

2. Male little penguins had greater corticosterone responses to handling and to exposure to a novel object than female little penguins, with no sex differences in corticosterone concentrations after exposure of birds to penguin alarm calls, dog barking or human talking.

3. The absence of a difference in corticosterone concentrations between birds that had been previously handled and not handled before exposure to another stimulus indicates that the differences in corticosterone responses between stimuli resulted from the effect of stimuli and were not related to whether or not birds had previously been handled.

4. There were no significant correlations between corticosterone concentrations after 15 min of handling and concentrations after exposure of the same birds to other stimuli. The measurement of corticosterone concentrations in the current study was confounded by the challenges of blood sample collection, and firm conclusions cannot be drawn about whether or not the corticosterone response to handling in a little penguin is related to its corticosterone response to other stimuli.

5. Plasticity in corticosterone responses can be described by a reaction norm. Corticosterone responses in little penguins show plasticity as their responses to different stimuli vary, but the full extent of this plasticity is yet to be determined.

### 3.2 Future directions

There is a lack of information available on how free-living birds respond to different stressors. The present study, the first to describe corticosterone responses to stimuli other than capture and handling in a free-living penguin species, has shown that responses can markedly differ between stimuli. These findings prompt the need for further investigation to better understand how birds perceive different stimuli variation, and to understand how plasticity in corticosterone responses could be beneficial to the birds.

Perception of the degree to which a stimulus is a threat determines the size of corticosterone responses in birds. The current study indicates that handling is perceived by little penguins as a stronger stressor than any the other stimuli used in the study, with human talking and the sound of dog barking not perceived as threatening by little penguins in nestboxes. Penguin alarm calls did elicit corticosterone responses and hence were perceived as threatening, so auditory stimuli can be stressors for penguins. Whilst responses to emotional stressors such as a predator encounter or handling have been extensively studied, little is known about corticosterone response of birds to physical stressors (e.g. insulin challenge). Physical stressors evoke corticosterone responses by different neural pathways to emotional stressors and do not involve the process of perception. Further research may be able to determine whether the responsiveness of the HPA axis differs between physical and emotional stressors. It could be useful to measure brain levels of catecholamine as well as corticosterone concentration following exposure to each stimulus to see if there is a correlation between the

two responses. This would give an insight to whether changes in brain amine activity are associated with changes in HPA axis activity.

It would be valuable to measure the full time course of corticosterone responses of little penguins to the stimuli that were tested in the current study. Long (2017) collected blood samples from little penguins at various times after they were returned to their nestbox at the end of a standard 60 min period of restraint in a box. The study showed that mean corticosterone concentrations returns to initial values within two hours. An equivalent protocol could be used to determine how long it takes for corticosterone concentrations to return to initial values in little penguins after exposure to a stimulus for 15, 30, or 60 min. Such a study could find the increase in corticosterone levels in response to a stimulus relative to a different stimulus and provide information on both the peak and duration of corticosterone response to each stimulus. Furthermore, it would be useful to determine if there are distinct behavioural responses associated with the corticosterone response to each stimulus. A correlation analysis between behavioural scores and corticosterone concentration could identify any association.

Further study with larger sample sizes of birds are required to determine if there is a relationship between responses to handling and responses to other stimuli. The current study did not show any correlation between the two responses which could be a result of very small sample sizes. It is not known whether responses to two different stimuli will be found to be related in little penguins if a larger sample size is used or if stimuli other than those used in the present study are applied. Only an experiment involving sampling of sufficient number of birds could confirm if individuals that respond highly to handling also respond highly to

other stimuli and those that are low responders to handling are also low responders to a different stimulus.

The current study provides novel information on the phenotypic plasticity of corticosterone response in little penguins, but more analysis is needed to determine the extent to which this species can show plasticity in this trait. The current study took a reaction norm approach to describe plasticity in corticosterone responses across different stimuli. The slope of a reaction norm could not be determined as different stimuli were used rather than an environmental variable that was continuous such as different durations of a stimulus. Therefore, in future work, corticosterone responses of little penguins to a continuous environmental variable could be measured in a similar fashion to the experiment described in (Charmantier *et al.*, 2008).

Comparison of reaction norms between different populations of penguins or between different species of penguins could be useful in identifying differences in plasticity of physiological traits. It would be interesting to determine reaction norms for other species of penguins and compare these to the reaction norm produced in the present study to see if they would respond similarly to little penguins when exposed to identical stressors. Furthermore, a reaction norm may be useful in future studies for identifying individuals with relatively higher or lower plasticity in stress responses to see if which birds are more suitable for a particular environment (e.g. a more constant rather than unpredictable environment). The results of the present study show that little penguins are plastic in their corticosterone responses at a population level. This is likely to arise from plasticity pattern from individual level (Porlier *et al.*, 2012), thus it could be that little penguins show differences in individual

plasticity. Corticosterone responses to different stimuli of one bird could be compared to another bird to determine if variation in plasticity exists between individuals.

To summarise, this thesis includes both relevant background and results of a novel experiment that provides important information about the corticosterone responses of little penguins. In New Zealand, great efforts are made to conserve this free-living species and therefore studies on their responsiveness to stressors, particularly human disturbances and potential predators, can assist in the management of little penguins. An important finding of this study was that corticosterone responses of birds to standardised handling can be markedly different from the responses to other stimuli. It is generally assumed that relative magnitude of corticosterone responses to the standard capture and handling protocol reflects the responsiveness of individual birds to natural stimuli. Whilst the validity of this assumption was tested in the current study, larger sample sizes are needed to be able to draw conclusions about the assumption. Another valuable finding is that little penguins are plastic in their corticosterone responses and hence can respond differently to different environmental stimuli. The current study provides a basis for more research on little penguins and other species of penguins.

## References

Abel, E. L. (1993). Physiological correlates of the forced swim test in rats. Physiology & Behavior 54: 309-317.

Adams, N. J., Cockrem, J. F., Taylor, G. A., Candy, E. J. and Bridges, J. (2005). Corticosterone responses of hand-reared and parent-reared grey-faced petrel chicks (*Pterodroma macroptera gouldi*). Zoo Biology 24: 283-290.

Adams, N. J., Parker, K. A., Cockrem, J. F., Brunton, D. H. and Candy, E. J. (2010). Corticosterone responses and post-release survival in translocated North Island Saddlebacks (*Philesturnus rufusater*) in New Zealand. Emu 110: 296-301.

Aguilera, G. (1998). Corticotropin releasing hormone, receptor regulation and the stress response. Trends in Endocrinology and Metabolism 9: 329-336.

Aguilera, G. (2011). HPA axis responsiveness to stress: Implications for healthy aging. Experimental Gerontology 46: 90-95.

Angelier, F., Bost, C. A., Giraudeau, M., Bouteloup, G., Dano, S. and Chastel, O. (2008). Corticosterone and foraging behavior in a diving seabird: The Adelie penguin, *Pygoscelis adeliae*. General and Comparative Endocrinology 156: 134-144.

Angelier, F., Clement-Chastel, C., Welcker, J., Gabrielsen, G. W. and Chastel, O. (2009). How does corticosterone affect parental behaviour and reproductive success? A study of prolactin in black-legged kittiwakes. Functional Ecology 23: 784-793.

Angelier, F., Shaffer, S. A., Weimerskirch, H., Trouve, C. and Chastel, O. (2007). Corticosterone and foraging behavior in a pelagic seabird. Physiological and Biochemical Zoology 80: 283-292.

Angelier, F. and Wingfield, J. C. (2013). Importance of the glucocorticoid stress response in a changing world: Theory, hypotheses and perspectives. General and Comparative Endocrinology 190: 118-128.

Arroyo, L., Carreras, R., Valent, D., Pena, R., Mainau, E., Velarde, A., Sabria, J. and Bassols, A. (2016). Effect of handling on neurotransmitter profile in pig brain according to fear related behaviour. Physiology & Behavior 167: 374-381.

Astheimer, L. B., Buttemer, W. A. and Wingfield, J. C. (2000). Corticosterone treatment has no effect on reproductive hormones or aggressive behavior in free-living male tree sparrows, *Spizella arborea*. Hormones and Behavior 37: 31-39.

Baugh, A. T., Schaper, S. V., Hau, M., Cockrem, J. F., de Goede, P. and van Oers, K. (2012). Corticosterone responses differ between lines of great tits (*Parus major*) selected for divergent personalities. General and Comparative Endocrinology 175: 488-494.

Baugh, A. T., van Oers, K., Naguib, M. and Hau, M. (2013). Initial reactivity and magnitude of the acute stress response associated with personality in wild great tits (Parus major). General and Comparative Endocrinology 189: 96-104.

Baugh, A. T., Witonsky, K. R., Davidson, S. C., Hyder, L., Hau, M. and van Oers, K. (2017). Novelty induces behavioural and glucocorticoid responses in a songbird artificially selected for divergent personalities. Animal Behaviour 130: 221-231.

Beerda, B., Schilder, M. B. H., van Hooff, J., de Vries, H. W. and Mol, J. A. (1998). Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. Applied Animal Behaviour Science 58: 365-381.

Benedetti, M., Merino, R., Kusuda, R., Ravanelli, M. I., Cadetti, F., dos Santos, P., Zanon, S. and Lucas, G. (2012). Plasma corticosterone levels in mouse models of pain. European Journal of Pain 16: 803-815.

Bila, K., Berankova, J., Vesely, P., Bugnyar, T. and Schwab, C. (2017). Responses of urban crows to con- and hetero-specific alarm calls in predator and non-predator zoo enclosures. Animal Cognition 20: 43-51.

Birrenkott, G. P. and Wiggins, M. E. (1984). Determination of dexamethasone and corticosterone half-lives in male broilers. Poultry Science 63: 1064-1068.

Blumstein, D. T., Daniel, J. C., Griffin, A. S. and Evans, C. S. (2000). Insular tammar wallabies (*Macropus eugenii*) respond to visual but not acoustic cues from predators. Behavioral Ecology 11: 528-535.

Bowers, S. L., Bilbo, S. D., Dhabhar, F. S. and Nelson, R. J. (2008). Stressor-specific alterations in corticosterone and immune responses in mice. Brain Behavior and Immunity 22: 105-113.

Brommer, J. E., Merila, J., Sheldon, B. C. and Gustafsson, L. (2005). Natural selection and genetic variation for reproductive reaction norms in a wild bird population. Evolution 59: 1362-1371.

Broom, D. M. (1990). Effects of handling and transport on laying hens. Worlds Poultry Science Journal 46: 48-50.

BrougHolub, E. and Kraal, G. (1996). Dose- and time-dependent activation of rat alveolar macrophages by glucocorticoids. Clinical and Experimental Immunology 104: 332-336.

Bryant, J. R., Lopez-Villalobos, N., Pryce, J. E., Holmes, C. W. and Johnson, D. L. (2006). Reaction norms used to quantify the responses of New Zealand dairy cattle of mixed breeds to nutritional environment. New Zealand Journal of Agricultural Research 49: 371-381.

Butler, L. K., Bisson, I.-A., Hayden, T. J., Wikelski, M. and Romero, L. M. (2009). Adrenocortical responses to offspring-directed threats in two open-nesting birds. General and Comparative Endocrinology 162: 313-318.

Canoine, V., Hayden, T. J., Rowe, K. and Goymann, W. (2002). The stress response of European stonechats depends on the type of stressor. Behaviour 139: 1303-1311.

Capoccia, S., Berry, A., Bellisario, V., Vacirca, D., Ortona, E., Alleva, E. and Cirulli, F. (2013). Quality and timing of stressors differentially impact on brain plasticity and neuroendocrine-immune function in mice. Neural plasticity 2013: 971817-971817.

Carnuccio, R., Dirosa, M., Guerrasio, B., Iuvone, T. and Sautebin, L. (1987). Vasocortin: a novel glucocorticoid-induced antiinflammatory protein. British Journal of Pharmacology 90: 443-445.

Carravieri, A., Muller, M. S., Yoda, K., Hayama, S.-i. and Yamamoto, M. (2016). Dominant parasympathetic modulation of heart rate and heart rate variability in a wild-caught seabird. Physiological and Biochemical Zoology 89: 263-276.

Carroll, G., Turner, E., Dann, P. and Harcourt, R. (2016). Prior exposure to capture heightens the corticosterone and behavioural responses of little penguins (*Eudyptula minor*) to acute stress. Conservation Physiology 4

Castro, M. G., Estivariz, F. E. and Iturriza, F. C. (1986). The regulation of the corticomelanotropic cell activity in Aves. II. Effect of various peptides on the release of ACTH from dispersed, perfused duck pituitary cells. Comparative Biochemistry and Physiology a-Physiology 83: 71-75.

Chandralekha, G., Jeganathan, R., Viswanathan and Charan, J. C. (2005). Serum leptin and corticosterone levels after exposure to noise stress in rats. The Malaysian journal of medical sciences : MJMS 12: 51-6.

Charmantier, A. and Gienapp, P. (2014). Climate change and timing of avian breeding and migration: evolutionary versus plastic changes. Evolutionary Applications 7: 15-28.

Charmantier, A., McCleery, R. H., Cole, L. R., Perrins, C., Kruuk, L. E. B. and Sheldon, B. C. (2008). Adaptive phenotypic plasticity in response to climate change in a wild bird population. Science 320: 800-803.

Chilvers, B. L., Finlayson, G., Candy, E. J., Sriram, A., Morgan, K. J. and Cockrem, J. F. (2016). Corticosterone stress hormone responses in oil rehabilitated and non-rehabilitated little penguins. Marine Pollution Bulletin 113: 312-315.

Christensen, J. W., Keeling, L. J. and Nielsen, B. L. (2005). Responses of horses to novel visual, olfactory and auditory stimuli. Applied Animal Behaviour Science 93: 53-65.

Cockrem, J. F. (2007). Stress, corticosterone responses and avian personalities. Journal of Ornithology 148: S169-S178.

Cockrem, J. F. (2013a). Corticosterone responses and personality in birds: Individual variation and the ability to cope with environmental changes due to climate change. General and Comparative Endocrinology 190: 156-163.

Cockrem, J. F. (2013b). Individual variation in glucocorticoid stress responses in animals. Gen. Comp. Endocrinol. 181: 45-58.

Cockrem, J. F. (2013c). Individual variation in glucocorticoid stress responses in animals. General and Comparative Endocrinology 181: 45-58.

Cockrem, J. F. (2015). Individual variation in corticosterone responses and adaptability to environmental change in birds. Integrative and Comparative Biology 55: E32-E32.

Cockrem, J. F., Agnew, P., Barrett, D. P., Candy, E. J. and Potter, M. A. (2017a). Individual variation of corticosterone responses of little penguins (*Eudyptula minor*) sampled in two successive years at Oamaru, New Zealand. Gen. Comp. Endocrinol. 244: 86-92.

Cockrem, J. F., Barrett, D. P., Candy, E. J. and Potter, M. A. (2009a). Corticosterone responses in birds: Individual variation and repeatability in Adelie penguins (*Pygoscelis adeliae*) and other species, and the use of power analysis to determine sample sizes. General and Comparative Endocrinology 163: 158-168.

Cockrem, J. F., Barrett, D. P., Candy, E. J. and Potter, M. A. (2009b). Corticosterone responses in birds: individual variation and repeatability in Adelie penguins (*Pygoscelis adeliae*) and other species, and the use of power analysis to determine sample sizes. Gen. Comp. Endocrinol. 163: 158-168.

Cockrem, J. F., Candy, E. J., Barrett, D. P., Agnew, P. and Potter, M. A. (2017b). Individual variation and repeatability of corticosterone responses of little penguins (*Eudyptula minor*) sampled in two successive years at Oamaru, New Zealand. General and comparative endocrinology 244: 86-92.

Cockrem, J. F., Candy, E. J., Castille, S. A. and Satterlee, D. G. (2010). Plasma corticosterone responses to handling in Japanese quail selected for low or high plasma corticosterone responses to brief restraint. British Poultry Science 51: 453-459.

Cockrem, J. F., Potter, M. A., Barrett, D. P. and Candy, E. J. (2008a). Corticosterone responses to capture and restraint in emperor and Adelie penguins in Antarctica. Zool. Sci. 25: 291-298.

Cockrem, J. F., Potter, M. A., Barrett, D. P. and Candy, E. J. (2008b). Corticosterone responses to capture and restraint in emperor and Adelie penguins in Antarctica. Zoological Science 25: 291-298.

Cockrem, J. F. and Silverin, B. (2002a). Sight of a predator can stimulate a corticosterone response in the great tit (*Parus major*). General and Comparative Endocrinology 125: 248-255.

Cockrem, J. F. and Silverin, B. (2002b). Variation within and between birds in corticosterone responses of great tits (*Parus major*). General and Comparative Endocrinology 125: 197-206.

Cornett, L. E., Kang, S. W. and Kuenzel, W. J. (2013). A possible mechanism contributing to the synergistic action of vasotocin (VT) and corticotropin-releasing hormone (CRH) receptors on corticosterone release in birds. General and Comparative Endocrinology 188: 46-53.

Cottin, M., MacIntosh, A. J. J., Kato, A., Takahashi, A., Debin, M., Raclot, T. and Ropert-Coudert, Y. (2014). Corticosterone administration leads to a transient alteration of foraging behaviour and complexity in a diving seabird. Marine Ecology Progress Series 496: 249-262.

Crossin, G. T., Trathan, P. N., Phillips, R. A., Gorman, K. B., Dawson, A., Sakamoto, K. Q. and Williams, T. D. (2012). Corticosterone predicts foraging behavior and parental care in Macaroni penguins. American Naturalist 180: E31-E41.

Cyr, N. E., Earle, K., Tam, C. and Romero, L. M. (2007). The effect of chronic psychological stress on corticosterone, plasma metabolites, and immune responsiveness in European starlings. General and Comparative Endocrinology 154: 59-66.

Cyr, N. E. and Romero, L. M. (2007). Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. General and Comparative Endocrinology 151: 82-89.

D'Amelio, P. B., Trost, L. and ter Maat, A. (2017). Vocal exchanges during pair formation and maintenance in the zebra finch (*Taeniopygia guttata*). Frontiers in Zoology 14

Dayas, C. V., Buller, K. M., Crane, J. W., Xu, J. and Day, T. A. (2001). Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. Eur. J. Neurosci. 14: 1143-1152.

Dayas, C. V., Buller, K. M. and Day, T. A. (1999). Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. European Journal of Neuroscience 11: 2312-2322.

Debut, M., Berri, C., Arnould, C., Guemene, D., Sante-Lhoutellier, V., Sellier, N., Baeza, E., Jehl, N., Jego, Y., Beaumont, C. and Le Bihan-Duval, E. (2005). Behavioural and physiological responses of three chicken breeds to pre-slaughter shackling and acute heat stress. British Poultry Science 46: 527-535.

Delehanty, B., Hossain, S., Jen, C. C., Crawshaw, G. J. and Boonstra, R. (2015). Measurement of free glucocorticoids: quantifying corticosteroid-binding globulin binding affinity and its variation within and among mammalian species. Conservation Physiology 3

Delezie, E., Lips, D., Lips, R. and Decuypere, E. (2006). Is the mechanisation of catching broilers a welfare improvement? Animal Welfare 15: 141-147.

Dhabhar, F. S., Miller, A. H., McEwen, B. S. and Spencer, R. L. (1996). Stress-induced changes in blood leukocyte distribution - Role of adrenal steroid hormones. Journal of Immunology 157: 1638-1644.

Dingemanse, N. J., Kazem, A. J. N., Reale, D. and Wright, J. (2010). Behavioural reaction norms: animal personality meets individual plasticity. Trends Ecol. Evol. 25: 81-89.

Djordjevic, J., Cvijic, G. and Davidovic, V. (2003). Different activation of ACTH and corticosterone release in response to various stressors in rats. Physiological Research 52: 67-72.

Doody, L. M., Wilhelm, S. I., McKay, D. W., Walsh, C. J. and Storey, A. E. (2008). The effects of variable foraging conditions on common murre (*Uria aalge*) corticosterone concentrations and parental provisioning. Hormones and Behavior 53: 140-148.

Dufty, A. M. and Crandall, M. B. (2005). Corticosterone secretion in response to adult alarm calls in American Kestrels. Journal of Field Ornithology 76: 319-325.

Ericsson, M., Fallahsharoudi, A., Bergquist, J., Kushnir, M. M. and Jensen, P. (2014). Domestication effects on behavioural and hormonal responses to acute stress in chickens. Physiology & Behavior 133: 161-169.

Fernandez-Juricic, E., Erichsen, J. T. and Kacelnik, A. (2004). Visual perception and social foraging in birds. Trends in Ecology & Evolution 19: 25-31.

Ferree, E. D., Wikelski, M. C. and Anderson, D. J. (2004). Hormonal correlates of siblicide in Nazca boobies: support for the Challenge Hypothesis. Hormones and Behavior 46: 655-662.

Fischer, C. P. and Romero, L. M. (2016). The use of alpha- or beta-blockers to ameliorate the chronic stress of captivity in the house sparrow (*Passer domesticus*). Conservation physiology 4: cow049-cow049.

Fowler, G. S. (1999). Behavioral and hormonal responses of Magellanic penguins (*Spheniscus magellanicus*) to tourism and nest site visitation. Biological Conservation 90: 143-149.

Fowles, J. R., Fairbrother, A., Fix, M., Schiller, S. and Kerkvliet, N. I. (1993). Glucocorticoid effects on natural and humoral immunity in mallards. Developmental and Comparative Immunology 17: 165-177.

Fraisse, F. and Cockrem, J. F. (2006). Corticosterone and fear behaviour in white and brown caged laying hens. British Poultry Science 47: 110-119.

Frigerio, D., Dittami, J., Mostl, E. and Kotrschal, K. (2004). Excreted corticosterone metabolites co-vary with ambient temperature and air pressure in male Greylag geese (*Anser anser*). General and Comparative Endocrinology 137: 29-36.

Gallo-Payet, N. (2016). Adrenal and extra-adrenal functions of ACTH. Journal of Molecular Endocrinology 56: T135-T156.

Gaston, M. S., Cid, M. P. and Salvatierra, N. A. (2017). Bicuculline, a GABA(A)-receptor antagonist, blocked HPA axis activation induced by ghrelin under an acute stress. Behavioural Brain Research 320: 464-472.

Gentsch, C., Lichtsteiner, M. and Feer, H. (1981). Locomotor activity, defecation score and corticosterone levels during an openfield exposure: a comparison among individually and group-housed rats, and genetically selected rat lines. Physiology & Behavior 27: 183-186.

Gibb, J., Hayley, S., Gandhi, R., Poulter, M. O. and Anisman, H. (2008). Synergistic and additive actions of a psychosocial stressor and endotoxin challenge: Circulating and brain cytokines, plasma corticosterone and behavioral changes in mice. Brain Behavior and Immunity 22: 573-589.

Gladalski, M., Banbura, M., Kalinski, A., Markowski, M., Skwarska, J., Wawrzyniak, J., Zielinski, P. and Banbura, J. (2016). Effects of extreme thermal conditions on plasticity in breeding phenology and double-broodedness of Great Tits and Blue Tits in central Poland in 2013 and 2014. International Journal of Biometeorology 60: 1795-1800.

Gomulkiewicz, R. and Kirkpatrick, M. (1992). Quantitative genetics and the evolution of reaction norms. Evolution 46: 390-411.

Gong, S., Miao, Y.-L., Jiao, G.-Z., Sun, M.-J., Li, H., Lin, J., Luo, M.-J. and Tan, J.-H. (2015). Dynamics and Correlation of Serum Cortisol and Corticosterone under Different Physiological or Stressful Conditions in Mice. Plos One 10

Hale, R., Piggott, J. J. and Swearer, S. E. (2017). Describing and understanding behavioral responses to multiple stressors and multiple stimuli. Ecology and Evolution 7: 38-47.

Harris, B. N. and Carr, J. A. (2016). The role of the hypothalamus-pituitary-adrenal/interrenal axis in mediating predator-avoidance trade-offs. General and Comparative Endocrinology 230: 110-142.

Hau, M. and Goymann, W. (2015). Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions. Frontiers in Zoology 12

Hayward, L. S. and Wingfield, J. C. (2004). Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. General and Comparative Endocrinology 135: 365-371.

Hazard, D., Couty, M., Richard, S. and Guemene, D. (2008). Intensity and duration of corticosterone response to stressful situations in Japanese quail divergently selected for tonic immobility. General and Comparative Endocrinology 155: 288-297.

Hennessy, M. B. (1991). Sensitization of the plasma corticosterone response to novel environments. Physiology & Behavior 50: 1175-1179.

Herman, J. P. and Cullinan, W. E. (1997). Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. Trends in Neurosciences 20: 78-84.

Hess, L. (2002). Corticosteroid synthesis and metabolism in birds. Seminars in Avian and Exotic Pet Medicine 11: 65-70.

Holberton, R. L., Helmuth, B. and Wingfield, J. C. (1996). The corticosterone stress response in Gentoo and King penguins during the non-fasting period. Condor 98: 850-854.

Hood, L. C., Boersma, P. D. and Wingfield, J. C. (1998). The adrenocortical response to stress in incubating Magellanic Penguins (*Spheniscus magellanicus*). Auk 115: 76-84.

Hsueh, A. J. W. and Erickson, G. F. (1978). Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. Steroids 32: 639-648.

Ibanez-Alamo, J. D., Chastel, O. and Soler, M. (2011). Hormonal response of nestlings to predator calls. General and Comparative Endocrinology 171: 232-236.

Irwin, J., Ahluwalia, P., Zacharko, R. M. and Anisman, H. (1986). Central norepinephrine and plasma corticosterone following acute and chronic stressors: influence of social isolation and handling. Pharmacology Biochemistry and Behavior 24: 1151-1154.

Itoi, K., Suda, T., Tozawa, F., Dobashi, I., Ohmori, N., Sakai, Y., Abe, K. and Demura, H. (1994). Microinjection of norepinephrine into the paraventricular nucleus of the hypothalamus stimulates corticotropin-releasing factor gene expression in conscious rats. Endocrinology 135: 2177-2182.

Jarvisto, P. E., Calhim, S., Schuett, W., Sirkia, P. M., Velmala, W. and Laaksonen, T. (2016). Carry-over effects of conditions at the wintering grounds on breeding plumage signals in a migratory bird: roles of phenotypic plasticity and selection. Journal of Evolutionary Biology 29: 1569-1584.

Jones, B. C., Smith, A. D., Bebus, S. E. and Schoech, S. J. (2016). Two seconds is all it takes: European starlings (*Sturnus vulgaris*) increase levels of circulating glucocorticoids after witnessing a brief raptor attack. Hormones and Behavior 78: 72-78.

Jones, R. B., Satterlee, D. G., Waddington, D. and Cadd, G. G. (2000). Effects of repeated restraint in Japanese quail genetically selected for contrasting adrenocortical responses. Physiology & Behavior 69: 317-324.

Jorgensen, H., Knigge, U., Kjaer, A., Moller, M. and Warberg, J. (2002). Serotonergic stimulation of corticotropin-releasing hormone and pro-opiomelanocortin gene expression. Journal of Neuroendocrinology 14: 788-795.

Kannan, G. and Mench, J. A. (1996). Influence of different handling methods and crating periods on plasma corticosterone concentrations in broilers. British Poultry Science 37: 21-31.

Kavushansky, A., Ben-Shachar, D., Ritcher-Levin, G. and Klein, E. (2009). Physical stress differs from psychosocial stress in the pattern and time-course of behavioral responses, serum corticosterone and expression of plasticity-related genes in the rat. Stress 12: 412–425.

Kirby, L. G., ChouGreen, J. M., Davis, K. and Lucki, I. (1997). The effects of different stressors on extracellular 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. Brain Research 760: 218-230.

Kitaysky, A. S., Kitaiskaia, E., Piatt, J. and Wingfield, J. C. (2003). Benefits and costs of increased levels of corticosterone in seabird chicks. Hormones and Behavior 43: 140-149.

Kleindorfer, S., Hoi, H. and Fessl, B. (1996). Alarm calls and chick reactions in the moustached warbler, Acrocephalus melanopogon. Animal Behaviour 51: 1199-1206.

Knowles, R. G., Salter, M., Brooks, S. L. and Moncada, S. (1990). Antiinflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. Biochemical and Biophysical Research Communications 172: 1042-1048.

Knowles, T. G. and Broom, D. M. (1990). The handling and transport of broilers and spent hens. Applied Animal Behaviour Science 28: 75-91.

Krause, J. S., McGuigan, M. A., Bishop, V. R., Wingfield, J. C. and Meddle, S. L. (2015a). Decreases in Mineralocorticoid but not Glucocorticoid Receptor mRNA Expression During the Short Arctic Breeding Season in Free-Living Gambel's White-Crowned Sparrow (*Zonotrichia leucophrys gambelii*). Journal of Neuroendocrinology 27: 66-75.

Krause, J. S., Meddle, S. L. and Wingfield, J. C. (2015b). The Effects of Acute Restraint Stress on Plasma Levels of Prolactin and Corticosterone across Life-History Stages in a Short-Lived Bird: Gambel's White-Crowned Sparrow (*Zonotrichia leucophrys gambelii*). Physiological and Biochemical Zoology 88: 589-598.

Krause, J. S., Perez, J. H., Chmura, H. E., Meddle, S. L., Hunt, K. E., Gough, L., Boelman, N. and Wingfield, J. C. (2016). The stress response is attenuated during inclement weather in parental, but not in pre-parental, Lapland longspurs (*Calcarius lapponicus*) breeding in the Low Arctic. Hormones and Behavior 83: 68-74.

Labrie, F., Giguere, V., Proulx, L. and Lefevre, G. (1984). Interactions between CRF, epinephrine, vasopressin and glucocorticoids in the control of ACTH secretion. Journal of Steroid Biochemistry and Molecular Biology 20: 153-160.

Lachuer, J., Gaillet, S., Barbagli, B., Buda, M. and Tappaz, M. (1991). Differential early time course activation of the brainstem catecholaminergic groups in response to various stresses. Neuroendocrinology 53: 589-596.

Lattin, C. R., Breuner, C. W. and Romero, L. M. (2016). Does corticosterone regulate the onset of breeding in free-living birds?: The CORT-Flexibility Hypothesis and six potential mechanisms for priming corticosteroid function. Hormones and Behavior 78: 107-120.

Leibowitz, S. F., Diaz, S. and Tempel, D. (1989). Norepinephrine in the paraventricular nucleus stimulates corticosterone release. Brain Research 496: 219-227.

Lendvai, A. Z., Ouyang, J. Q., Schoenle, L. A., Fasanello, V., Haussmann, M. F., Bonier, F. and Moore, I. T. (2014). Experimental Food Restriction Reveals Individual Differences in Corticosterone Reaction Norms with No Oxidative Costs. Plos One 9

Li, Y., Sun, Y., Krause, J. S., Li, M., Liu, X., Zhu, W., Yao, Y., Wu, Y. and Li, D. (2017). Dynamic interactions between corticosterone, corticosteroid binding globulin and testosterone in response to capture stress in male breeding Eurasian tree sparrows. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology 205: 41-47.

Liposits, Z. and Paull, W. K. (1989). Association of dopaminergic fibers with corticotropin releasing hormone (CRH)-synthesizing neurons in the paraventricular nucleus of the rat hypothalamus. Histochemistry 93: 119-127.

Liposits, Z., Phelix, C. and Paull, W. K. (1987). Synaptic interaction of serotonergic axons and corticotropin releasing-factor (CRF) synthesizing neurons in the hypothalamic paraventricular nucleus of the rat: a light and electron-microscopic immunocytochemical study. Histochemistry 86: 541-549.

Littin, K. E. and Cockrem, J. F. (2001). Individual variation in corticosterone secretion in laying hens. British Poultry Science 42: 536-546.

Loiseau, C., Sorci, G., Dano, S. and Chastel, O. (2008). Effects of experimental increase of corticosterone levels on begging behavior, immunity and parental provisioning rate in house sparrows. General and Comparative Endocrinology 155: 101-108.

Long, K. (2017). Complete time course of corticosterone responses to handling in kororā (little penguins, *Eudyptula minor*). MSc thesis, Massey University, Palmerston North

Longordo, F., Fan, J., Steimer, T., Kopp, C. and Luthi, A. (2011). Do Mice Habituate to "Gentle Handling"? A Comparison of Requesting Behavior Corticosterone Levels and Synaptic Function in Handled and Undisturbed C57BL/6J. Sleep 34: 679-681.

Love, O. P., Shutt, L. J., Silfies, J. S. and Bird, D. M. (2003). Repeated restraint and sampling results in reduced corticosterone levels in developing and adult captive American kestrels (*Falco sparverius*). Physiological and Biochemical Zoology 76: 753-761.

Maddocks, S. A., Goldsmith, A. R. and Cuthill, I. C. (2001). The influence of flicker rate on plasma corticosterone levels of European starlings, *Sturnus vulgaris*. General and Comparative Endocrinology 124: 315-320.

Malisch, J. L. and Breuner, C. W. (2010). Steroid-binding proteins and free steroids in birds. Molecular and Cellular Endocrinology 316: 42-52.

Malisch, J. L., Satterlee, D. G., Cockrem, J. F., Wada, H. and Breuner, C. W. (2010). How acute is the acute stress response? Baseline corticosterone and corticosteroid-binding globulin levels change 24 h after an acute stressor in Japanese quail. General and Comparative Endocrinology 165: 345-350.

Marin, M. T., Cruz, F. C. and Planeta, C. S. (2007). Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. Physiology & Behavior 90: 29-35.

Mateo, J. M. (2010). Alarm calls elicit predator-specific physiological responses. Biology Letters 6: 623-625.

Menendez-Buxadera, A., Palacios-Espinosa, A., Luis Espinosa-Villavicencio, J. and Guerra-Iglesias, D. (2016). Genotype Environment interactions for milk production traits in Holstein and crossbred Holstein-Zebu cattle populations estimated by a character state multibreed model. Livestock Science 191: 108-116.

Mesches, M. H., Fleshner, M., Heman, K. L., Rose, G. M. and Diamond, D. M. (1999). Exposing rats to a predator blocks primed burst potentiation in the hippocampus in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience 19: RC18-RC18.

Mueller, C., Jenni-Eiermann, S., Blondel, J., Perret, P., Caro, S. P., Lambrechts, M. and Jenni, L. (2006). Effect of human presence and handling on circulating corticosterone levels in breeding blue tits (*Parus caeruleus*). General and Comparative Endocrinology 148: 163-171.

Munck, A., Guyre, P. M. and Holbrook, N. J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Reviews 5: 25-44.

Nephew, B. C., Kahn, S. A. and Romero, L. M. (2003). Heart rate and behavior are regulated independently of corticosterone following diverse acute stressors. General and Comparative Endocrinology 133: 173-180.

Neumann, I. D., Toschi, N., Ohl, F., Torner, L. and Kromer, S. A. (2001). Maternal defence as an emotional stressor in female rats: correlation of neuroendocrine and behavioural

parameters and involvement of brain oxytocin. European Journal of Neuroscience 13: 1016-1024.

Newman, A. E. M., Hess, H., Woodworth, B. K. and Norris, D. R. (2017). Time as tyrant: The minute, hour and day make a difference for corticosterone concentrations in wild nestlings. General and Comparative Endocrinology 250: 80-84.

Noach, E. J. K., DeJong, G. and Scharloo, W. (1997). Phenotypic plasticity of wings in selection lines of *Drosophila melanogaster*. Heredity 79: 1-9.

Owen-Ashley, N. T., Turner, M., Hahn, T. P. and Wingfield, J. C. (2006). Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*). Hormones and Behavior 49: 15-29.

Pacak, K., Palkovits, M., Yadid, G., Kvetnansky, R., Kopin, I. J. and Goldstein, D. S. (1998). Heterogeneous neurochemical responses to different stressors: a test of Selye's doctrine of nonspecificity. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 275: R1247-R1255.

Palermo-Neto, J., Massoco, C. D. and de Souza, W. R. (2003). Effects of physical and psychological stressors on behavior, macrophage activity, and Ehrlich tumor growth. Brain Behavior and Immunity 17: 43-54.

Pemac, D. and Tucic, B. (1998). Reaction norms of juvenile traits to light intensity in Iris pumila (Iridaceae): a comparison of populations from exposed and shaded habitats. Plant Systematics and Evolution 209: 159-176.

Perez, A. and Garcia, C. (2002). Evolutionary responses of *Drosophila melanogaste*r to selection at different larval densities: changes in genetic variation, specialization and phenotypic plasticity. Journal of Evolutionary Biology 15: 524-536.

Porlier, M., Charmantier, A., Bourgault, P., Perret, P., Blondel, J. and Garant, D. (2012). Variation in phenotypic plasticity and selection patterns in blue tit breeding time: between- and within-population comparisons. Journal of Animal Ecology 81: 1041-1051.

Quillfeldt, P., Masello, J. F., Strange, I. J. and Buchanan, K. L. (2006). Begging and provisioning of thin-billed prions, *Pachyptila belcheri*, are related to testosterone and corticosterone. Animal Behaviour 71: 1359-1369.

Rabasa, C., Munoz-Abellan, C., Daviu, N., Nadal, R. and Armario, A. (2011). Repeated exposure to immobilization or two different footshock intensities reveals differential adaptation of the hypothalamic-pituitary-adrenal axis. Physiology & Behavior 103: 125-133.

Radley, J. J. and Sawchenko, P. E. (2015). Evidence for involvement of a limbic paraventricular hypothalamic inhibitory network in hypothalamic-pituitary-adrenal

axis adaptations to repeated stress. Journal of Comparative Neurology 523: 2769-2787.

Rensel, M. A., Wilcoxen, T. E. and Schoech, S. J. (2010). The influence of nest attendance and provisioning on nestling stress physiology in the Florida scrub-jay. Hormones and Behavior 57: 162-168.

Ressler, K. J. (2010). Amygdala Activity, Fear, and Anxiety: Modulation by Stress. Biological Psychiatry 67: 1117-1119.

Rich, E. L. and Romero, L. M. (2005). Exposure to chronic stress downregulates corticosterone responses to acute stressors. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 288: R1628-R1636.

Richard, S., Wacrenier-Cere, N., Hazard, D., Saint-Dizier, H., Arnould, C. and Faure, J. M. (2008). Behavioural and endocrine fear responses in Japanese quail upon presentation of a novel object in the home cage. Behavioural Processes 77: 313-319.

Rivers, J. W., Martin, L. B., Liebl, A. L. and Betts, M. G. (2011). Parental alarm calls of the white-crowned sparrow fail to stimulate corticosterone production in nest-bound offspring. Ethology 117: 374-384.

Rocha, F. B. and Klaczko, L. B. (2012). Connecting the dots of nonlinear reaction norms unravels the threads of genotype-environment interaction in drosophila. Evolution 66: 3404-3416.

Rodgers, R. J., Haller, J., Holmes, A., Halasz, J., Walton, T. J. and Brain, P. F. (1999). Corticosterone response to the plus-maze: High correlation with risk assessment in rats and mice. Physiology & Behavior 68: 47-53.

Romero, L. M. and Reed, J. M. (2005). Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 140: 73-79.

Rowe, C. and Guilford, T. (1996). Hidden colour aversions in domestic clicks triggered by pyrazine odours of insect warning displays. Nature 383: 520-522.

Ruggiero, C. and Lalli, E. (2016). Impact of ACTH signaling on transcriptional regulation of steroidogenic genes. Frontiers in Endocrinology 7

Rutkowska, J., Place, N. J., Vincent, S. and Adkins-Regan, E. (2011). Adrenocortical response to mating, social interaction and restraint in the female Japanese quail. Physiology & Behavior 104: 1037-1040.

Sachser, N. (1987). Short-term responses of plasma norepinephrine, epinephrine, glucocorticoid and testosterone titers to social and nonsocial stressors in male guinea pigs of different social status. Physiology & Behavior 39: 11-20.

Salvante, K. G. and Williams, T. D. (2003). Effects of corticosterone on the proportion of breeding females, reproductive output and yolk precursor levels. General and Comparative Endocrinology 130: 205-214.

Santana Amaral, V. C., Gomes, K. S. and Nunes-de-Souza, R. L. (2010). Increased corticosterone levels in mice subjected to the rat exposure test. Hormones and Behavior 57: 128-133.

Sapolsky, R. M., Romero, L. M. and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21: 55-89.

Sawchenko, P. E., Swanson, L. W., Steinbusch, H. W. M. and Verhofstad, A. A. J. (1983). The distribution and cells of origin of serotonergic inputs to the paraventricular and supraoptic nuclei of the rat. Brain Research 277: 355-360.

Schlichting, C. D. (1986). The evolution of phenotypic plasticity in plants. Annual Review of Ecology and Systematics 17: 667-693.

Schmidt, K. L., Chin, E. H., Shah, A. H. and Soma, K. K. (2009). Cortisol and corticosterone in immune organs and brain of European starlings: developmental changes, effects of restraint stress, comparison with zebra finches. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 297: R42-R51.

Seggie, J. A. and Brown, G. M. (1975). Stress response patterns of plasma corticosterone, prolactin, and growth-hormone in rat, following handling or exposure to novel environment. Canadian Journal of Physiology and Pharmacology 53: 629-637.

Selye, H. (1936). A syndrome produced by diverse nocuous agents. Nature 138: 32-32.

Serrats, J., Grigoleit, J. S., Alvarez-Salas, E. and Sawchenko, P. E. (2017). Pro-inflammatory immune-to-brain signaling is involved in neuroendocrine responses to acute emotional stress. Brain Behavior and Immunity 62: 53-63.

Shipp, S. L., Yi, J. Q., Dridi, S., Gilbert, E. R. and Cline, M. A. (2015). The central anorexigenic mechanism of adrenocorticotropic hormone involves the caudal hypothalamus in chicks. Neuropeptides 53: 29-35.

Silverin, B. (1998). Behavioural and hormonal responses of the pied flycatcher to environmental stressors. Animal Behaviour 55: 1411-1420.

Small, T. W., Bebus, S. E., Bridge, E. S., Elderbrock, E. K., Ferguson, S. M., Jones, B. C. and Schoech, S. J. (2017). Stress-responsiveness influences baseline glucocorticoid levels: Revisiting the under 3 min sampling rule. General and Comparative Endocrinology 247: 152-165.

Small, T. W. and Schoech, S. J. (2015). Sex differences in the long-term repeatability of the acute stress response in long-lived, free-living Florida scrub-jays (*Aphelocoma*

*coerulescens*). Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 185: 119-133.

Smith, G. T., Wingfield, J. C. and Veit, R. R. (1994). Adrenocortical response to stress in the common diving petrel, *Pelecanoides urinatrix*. Physiological Zoology 67: 526-537.

Spee, M., Marchal, L., Lazin, D., Le Maho, Y., Chastel, O., Beaulieu, M. and Raclot, T. (2011). Exogenous corticosterone and nest abandonment: A study in a long-lived bird, the Adelie penguin. Hormones and Behavior 60: 362-370.

Stevens, A. and White, A. (2010). ACTH: cellular peptide hormone synthesis and secretory pathways. Results and problems in cell differentiation 50: 63-84.

Stier, K. S., Almasi, B., Gasparini, J., Piault, R., Roulin, A. and Jenni, L. (2009). Effects of corticosterone on innate and humoral immune functions and oxidative stress in barn owl nestlings. Journal of Experimental Biology 212: 2084-2090.

Stoewe, M., Bugnyar, T., Loretto, M.-C., Schloegl, C., Range, F. and Kotrschal, K. (2006). Novel object exploration in ravens (*Corvus corax*): Effects of social relationships. Behavioural Processes 73: 68-75.

Tarlow, E. M., Wikelski, M. and Anderson, D. J. (2001). Hormonal correlates of siblicide in Galapagos Nazca boobies. Hormones and Behavior 40: 14-20.

Tilgar, V., Saag, P., Kulavee, R. and Mand, R. (2010). Behavioral and physiological responses of nestling pied flycatchers to acoustic stress. Hormones and Behavior 57: 481-487.

Ulrich-Lai, Y. M. and Herman, J. P. (2009). Neural regulation of endocrine and autonomic stress responses. Nature Reviews Neuroscience 10: 397-409.

Valladares, F., Sanchez-Gomez, D. and Zavala, M. A. (2006a). Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. J. Ecol. 94: 1103-1116.

Valladares, F., Sanchez-Gomez, D. and Zavala, M. A. (2006b). Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. Journal of Ecology 94: 1103-1116.

Vantienderen, P. H. and Koelewijn, H. P. (1994). Selection on reaction norms, genetic correlations and constraints. Genetical Research 64: 115-125.

Verbeek, M. E. M., Boon, A. and Drent, P. J. (1996). Exploration, aggressive behavior and dominance in pair-wise confrontations of juvenile male great tits. Behaviour 133: 945-963.

Via, S., Gomulkiewicz, R., Dejong, G., Scheiner, S. M., Schlichting, C. D. and Vantienderen, P. H. (1995). Adaptive phenotypic plasticity: consensus and controversy. Trends in Ecology & Evolution 10: 212-217.

Viblanc, V. A., Gineste, B., Robin, J.-P. and Groscolas, R. (2016). Breeding status affects the hormonal and metabolic response to acute stress in a long-lived seabird, the king penguin. General and Comparative Endocrinology 236: 139-145.

Vleck, C. M., Vertalino, N., Vleck, D. and Bucher, T. L. (2000). Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adelie Penguins. Condor 102: 392-400.

Walker, B. G., Boersma, P. D. and Wingfield, J. C. (2005). Physiological and behavioral differences in Magellanic Penguin chicks in undisturbed and tourist-visited locations of a colony. Conservation Biology 19: 1571-1577.

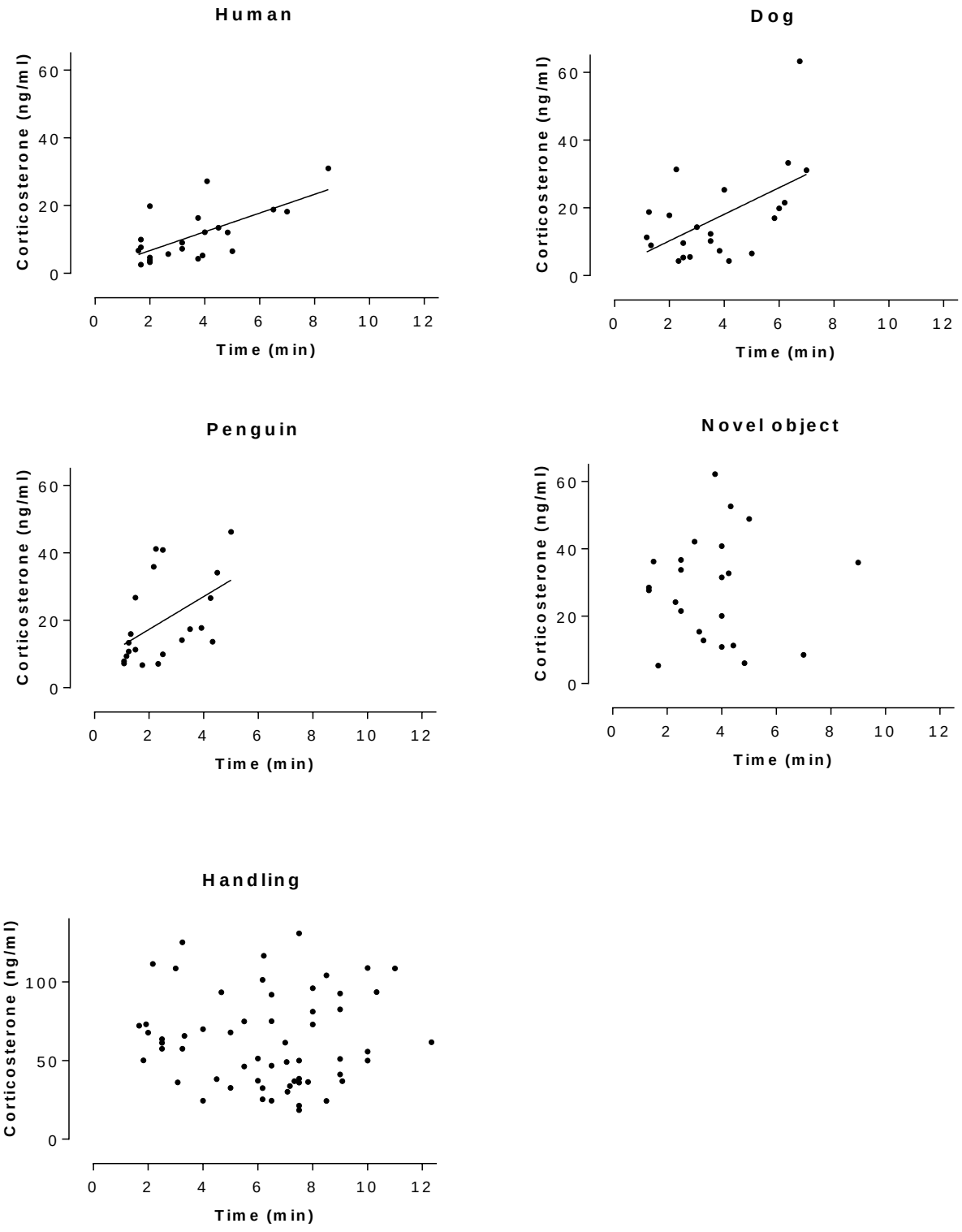
Wideman, R. F. and Pevzner, I. (2012). Dexamethasone triggers lameness associated with necrosis of the proximal tibial head and proximal femoral head in broilers. Poultry Science 91: 2464-2474.

Wingfield, J. C., Vleck, C. M. and Moore, M. C. (1992). Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. Journal of Experimental Zoology 264: 419-428.

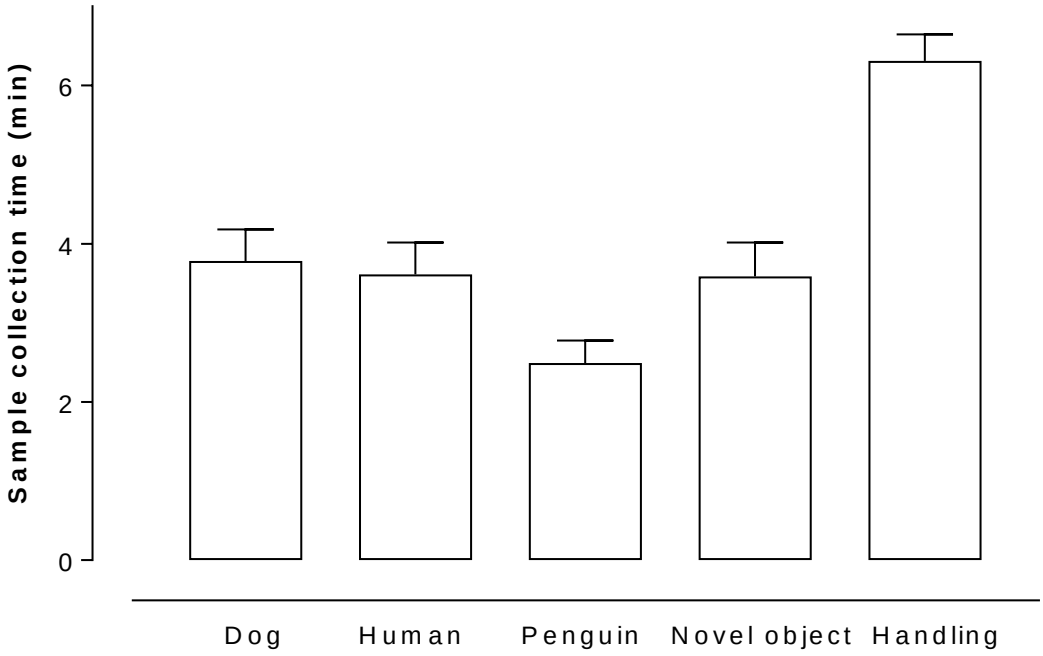
Zhang, R., Tachibana, T., Takagi, T., Koutoku, T., Denbow, D. M. and Furuse, M. (2004). Serotonin modifies corticotropin-releasing factor-induced behaviors of chicks. Behavioural Brain Research 151: 47-52.

Zimmer, C. and Spencer, K. A. (2014). Modifications of glucocorticoid receptors mrna expression in the hypothalamic-pituitary-adrenal axis in response to early-life stress in female Japanese quail. Journal of Neuroendocrinology 26: 853-860.

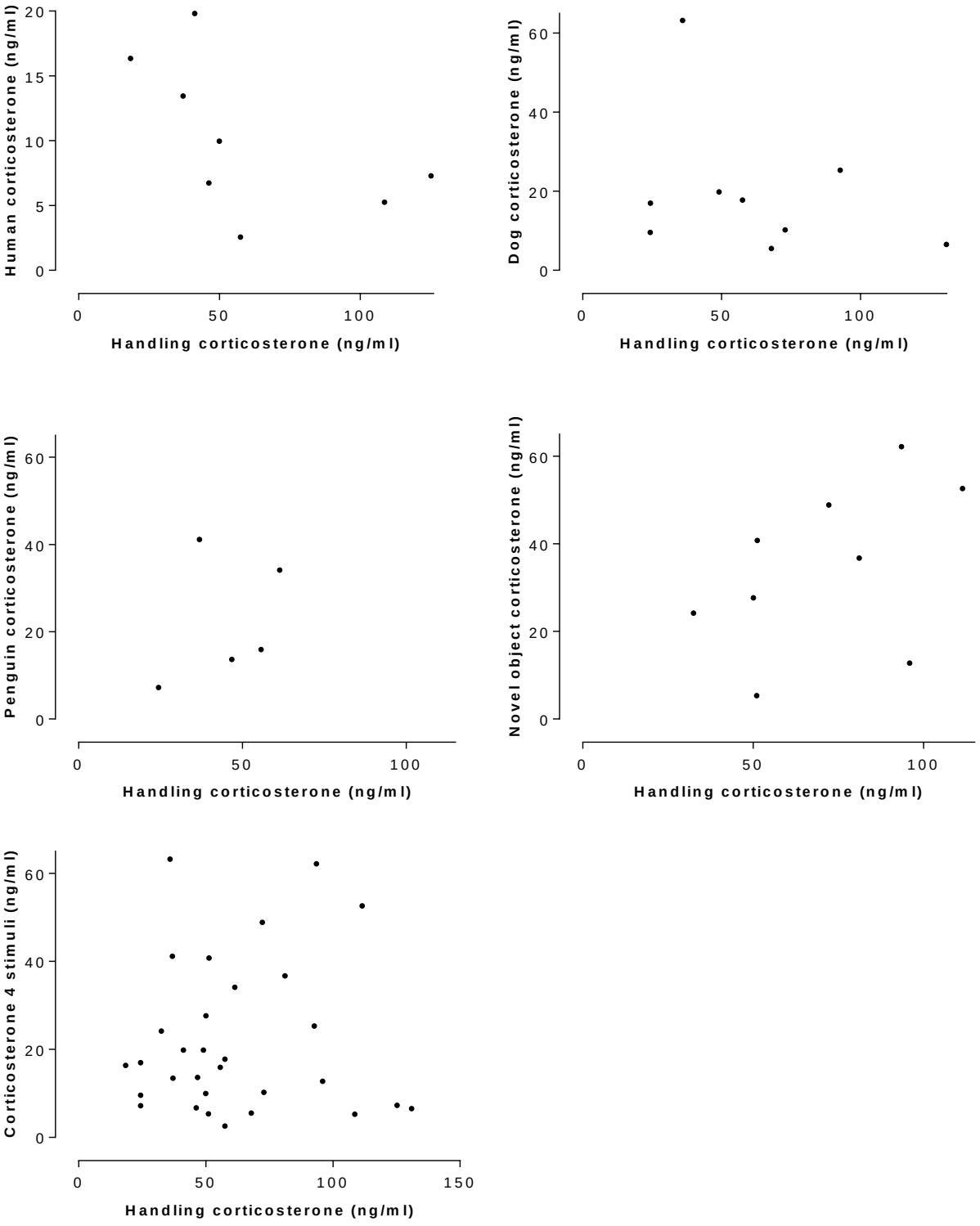
## Appendix




Appendix Fig. 1. Relationships between corticosterone concentrations and sample collection times.




Appendix Fig. 2. Mean sample collection times (mean ± S.E.) for the five different stimuli.




Appendix Fig. 3. Correlations between corticosterone responses to handling and responses to other stimuli.