The time course of corticosterone responses in kororā (little penguin, *Eudyptula minor*)

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Abstract

When birds and other vertebrates perceive a situation to be threatening the hypothalamo-pituitary-adrenal (HPA) axis is activated and glucocorticoid hormones are secreted from the adrenal gland. Activation of the HPA axis in response to a stimulus perceived to be threatening is called a stress response. The main glucocorticoid hormone in birds is corticosterone. Corticosterone responses of birds are typically measured by the collection of an initial blood sample when a bird is captured or picked up, then the collection of further blood samples until 30 to 60 minutes has elapsed, at which time the bird is released. Whilst this standard sampling protocol provides information on the size of the corticosterone response, it does not provide any indication of how long it takes for corticosterone concentrations to return to initial values. The main objective of this thesis was to characterise the total duration of the corticosterone response of free-living kororā (little penguins, *Eudyptula minor*).

Little penguins at Oamaru were picked up from their nestboxes and initial blood samples collected. Birds were handled and then restrained by being placed in a box. Further blood samples were collected 15, 30 and 60 min after the birds were first picked up. Birds were then returned to their nest boxes and an additional blood sample collected 15, 30, 60, 120, 240, or 360 min later. Mean corticosterone concentrations declined to initial values two hours after birds were returned to nest boxes. The rates at which corticosterone concentrations increased when a stressor was present and then decreased when the stressor was no longer present were positively correlated. Seasonal changes in corticosterone responses in little penguins were also investigated in this study. Mean corticosterone responses were similar in winter and in the pre-laying period, whereas mean responses were lower in birds during early chick rearing. Corticosterone responses during the pre-laying

period were greater in male than female little penguins. The current study is the first to document the complete corticosterone responses of free-living penguins and provides information about changes of corticosterone concentrations after a stressor is removed from the free-living individuals. It is also the first to reveal that free-living penguins with relatively high corticosterone responses to a stressor had relatively high rates of corticosterone decline.

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Chapter 1: General introduction

1.1 Introduction

When birds and other vertebrates perceive a situation to be threatening the hypothalamic-pituitary-adrenal (HPA) axis is activated and glucocorticoid hormones are secreted from the adrenal gland (Herman *et al.*, 2016). Activation of the HPA axis in response to a stimulus perceived to be threatening is called a stress response. The main glucocorticoid in most mammals and fish is cortisol and the main glucocorticoid in rodents, birds, amphibians and reptiles is corticosterone (Carsia and Harvey, 2000). Glucocorticoids are predominantly metabolic hormones that have multiple effects and can also act on a wide range of organ systems and tissues, including the immune and circulatory systems. When glucocorticoids are released during a stress response they act to help the animals adjust to the situation.

Plasma concentrations of glucocorticoids provide an indication of the degree of stress experienced by an animal in response to a stimulus. An increase in plasma corticosterone concentrations when an animal is exposed to a stressor is called a corticosterone response, and corticosterone responses have been measured in many species of animals. Corticosterone responses are one indicator of an animal's ability to cope with changes in the environment. Glucocorticoid responses to the same stressor vary among species and individuals (Cockrem, 2013a). For example, corticosterone responses to a standard handling protocol vary among different species of birds (Cockrem *et al.*, 2009). The magnitude of corticosterone responses depends on the nature of the stressor and can vary seasonally (Romero, 2002). For example, corticosterone responses of great tits (*Parus major*) exposed to a predator stimulus were greater than responses to a moving box (Cockrem and Silverin, 2002).

Corticosterone is necessary for survival, as it prepares animals to cope with stressors and assists individuals to react accordingly to the situation. It is often said that prolonged corticosterone elevation can be harmful and negatively affect the health of an animal (Romero, 2004; Saino *et al.*, 2005; Shini *et al.*, 2009). However, statements such as this hardly define prolonged or elevation, and there is limited evidence from free-living animals for negative effects of glucocorticoids on health. Another main function of corticosterone is to send negative feedback to the HPA axis to prevent further secretion of corticosterone. This could also be considered a defence mechanism to prevent adverse effects of corticosterone on the body. The measurement of the efficacy of negative feedback may indicate an animals' ability to recover from stress (Romero, 2004).

The measurement of corticosterone responses in free-living birds contributes to understanding of the coping strategy of free-living birds to changes in their environment (Cockrem, 2007; Koolhaas *et al.*, 2010; Grace and Anderson, 2014). Corticosterone responses have been measured in many species of birds, both domestic and free-living. A common method to measure corticosterone responses of animals is to collect a series of blood samples after a bird is captured. The collection of blood from flying birds can be challenging as the birds must be captured. Some free-living birds, such as penguins, are easier to approach and capture than the other free-living species. Penguins are marine animals that breed on land in colonies. Corticosterone responses to capture and restraint have been measured in 10 of the 19 species of penguins: Adelie (*Pygoscelis adeliae*; Cockrem *et al.*, 2008), emperor (*Aptenodytes forsteri*; Cockrem *et al.*, 2008), Magellanic *(Spheniscus magellanicus*; Walker *et al.*, 2015a), Humboldt (*Spheniscus humboldti*; Walker *et al.*, 2015a), rockhopper (*Eudyptes chrysocome;* Walker *et al.*, 2015a), Galapagos (*Spheniscus mendiculus*; Walker *et al.*, 2015a), gentoo (*Pygoscelis papua*; Holberton *et al.*, 1996), king

(*Apetenodytes patagonicus*; Holberton *et al*., 1996), yellow-eyed (*Megadytes antipodes*; Ellenberg *et al.*, 2007) and little penguins (Cockrem *et al.*, 2016).

Corticosterone responses of birds are reviewed here. The review begins with definitions of stress, stressor stress responses and an overview of the stress system, especially the HPA axis. The following sections consider corticosterone responses of birds, the negative feedback function of glucocorticoids and the efficacy of negative feedback in birds. The last section reviews studies of corticosterone responses of penguins.

1.2 Stress in animals

Standard terms in stress physiology are stress, stressor and stress responses. However, these terms are not defined in many studies, and there is no agreed definition of these commonly used terms. Definitions of these terms used in the current review are presented below.

1.2.1 Stress

The term ‘stress' has been widely used across diverse fields of literature, and its definition in articles ranges from broad to specific. Stress has been broadly defined as a “state of threatened or perceived as threatened homoeostasis” (Charmandari *et al.*, 2005). Cockrem (2007) suggested a more precise definition, in which stress is a state “when the hypothalamus-pituitary-adrenal (HPA) axis is activated with increased secretion of glucocorticoids in response to stressor”. This definition will be used throughout this review.

1.2.2 Stressor

The term stressor refers to any stimuli that provoke stress responses of animals and activate the HPA axis. Stressors are divided into two main types; (1) physiological and (2)

psychological. Physiological stressors could be physical injuries or disturbed internal environments of animals, such as dehydration or infection (Pacak and Palkovits, 2001; Cockrem, 2007; Ulrich-Lai and Herman, 2009). Changes in the external environment, such as temperature drop, noise and vibration, may be considered to be physical stressors (Pacak and Palkovits, 2001) if they activate the HPA axis. Psychological stressors are stimuli that are perceived to be threatening, with perception depending both on innate recognition of stimuli and also experience and learning by animals (Pacak and Palkovits, 2001; Cockrem, 2007). Pacak and Palkovits (2001) suggested that social interactions among individuals could be a kind of stressor in some situations. The duration and intensity of stressors affect the magnitude of responses of animals (Pacak and Palkovits, 2001; Cockrem *et al.*, 2010). Novel stressors also elicit larger stress responses than repeated stimuli (Creel, 2001). It is important to note that individuals from the same population may perceive the same stressor differently (Cockrem, 2013b).

1.2.3 Stress system

Stress stimuli are conveyed to the brain of animals through viscerosensory and somatosensory pathways, subsequently activating the stress system (Pacak and Palkovits, 2001). The stress system coordinates the responses of an animal to stressors. It is mainly made up of the peripheral and central components of the nervous system. The central components are corticotropin- releasing hormone (CRH) and vasopressin (AVP) neurons in the paraventricular nucleus (PVN) of the hypothalamus, and noradrenergic neurones in the locus coeruleus (NE; Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015). The peripheral components comprise the HPA axis and the autonomic sympathetic nervous system (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015). The HPA axis is a vital neuroendocrine component of the stress system. Activation of the HPA axis results in glucocorticoid

secretion. Glucocorticoids mediate most of the stress responses to stress stimuli and also regulate activity of the HPA axis. This review focuses on the HPA axis and on actions glucocorticoids in the stress system.

1.2.4 Stress responses

In broad terms, stress responses can be defined as responses of animals to stressors (Cockrem, 2007). The responses can be divided into physiological and behavioural responses. The stress system regulates the stress responses so that an animal can adjust to the new situation and increase its chances of survival in a short period. Physiologically, animals release glucocorticoids in response to a stressor. In terms of behaviour, a stressor may trigger flight-or-fight responses.

It has been proposed that physical and behavioural adaptations are shown by animals when they experience stress (see Table 1.1). Animals become more focused and alert, whereas digestion, reproductive, and feeding behaviours are inhibited when the stress system is activated (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015). The stress system redirects oxygen and nutrients to the central nervous system and body sites that need extra resources (Pacak and Palkovits, 2001; Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015). There are also increases in respiratory rate, gluconeogenesis and lipolysis to provide energy, and changes in cardiovascular tone, blood pressure and heart rate (Pacak and Palkovits, 2001; Charmandari *et al.*, 2005). A restraining mechanism is also switched on to prevent over-response of the stress system (Ulrich-Lai and Herman, 2009; Nicolaides *et al.*, 2015). The outcome of these adaptations is said to be re-establishment of homeostatic balance and preparation of animals for flight-or-fight responses (Pacak and Palkovits, 2001).

Table 1.1. Behavioural and physical adaptations when animals are under stress. Table from Charmandari *et al.* (2005).

Behavioural adaptations: adaptive redirection of behavior	Physical adaptations: adaptive redistribution of energy
Increased arousal and alertness	Oxygen and nutrients directed to the CNS and stressed body sites
Increased cognition vigilance and focused attention	Altered cardiovascular tone, increased blood pressure and heart rate
Excitement or depression	Increased respiratory rate
Heightened analgesia	Increased gluconeogenesis and lipolysis
Increased temperature	Detoxification
Suppression of appetite and feeding behavior	Inhibition of growth and reproduction
Suppression of reproductive activity	Inhibition of digestion-stimulation of colonic motility
Containment of the stress response	Containment of the immunity response

1.3 Hypothalamic-pituitary-adrenal (HPA) axis

1.3.1 Overview

The HPA axis plays an important role in the stress system, and it regulates the stress responses of an animal to a stressor. It is made up of three major components; the hypothalamus, the pituitary gland and the adrenal gland (Fig. 1.1). There are multiple interactions, including stimulatory and negative feedback mechanisms, among the three components via neural and hormone stimuli (Nicolaides *et al.*, 2015). The final products of the HPA axis are glucocorticoids secreted from the adrenal gland. Glucocorticoids bind to glucocorticoid receptors throughout the body and have metabolic actions (Herman *et al.*, 2016). Glucocorticoids re-establish the animals' homoeostasis, a state of equilibrium in an organism, and assist the animal to adapt or escape from the stressor (Nicolaides *et al.*, 2015; Herman *et al.*, 2016). The main functions of glucocorticoids are to redistribute energy resources and to regulate activity of HPA axis by acting on the hypothalamus and pituitary gland via the negative feedback loop. The components and activities of HPA axis are similar throughout vertebrates.

1.3.2 The hypothalamus

Stressors activate the HPA axis and trigger the paraventricular nucleus (PVN) of the hypothalamus to release corticotrophin-releasing hormone (CRH) and arginine vasotocin (AVT, in birds and non-mammal vertebrates; Carsia and Harvey, 2000). CRH and AVT are synthesised in the parvocellular neurones of the PVN and released into the hypothalamic-pituitary portal circulation (Herman *et al.*, 2016). PVN is considered to be the activation site of stress responses of HPA axis as both hormones (PVN and AVT) are synthesised in the PVN.

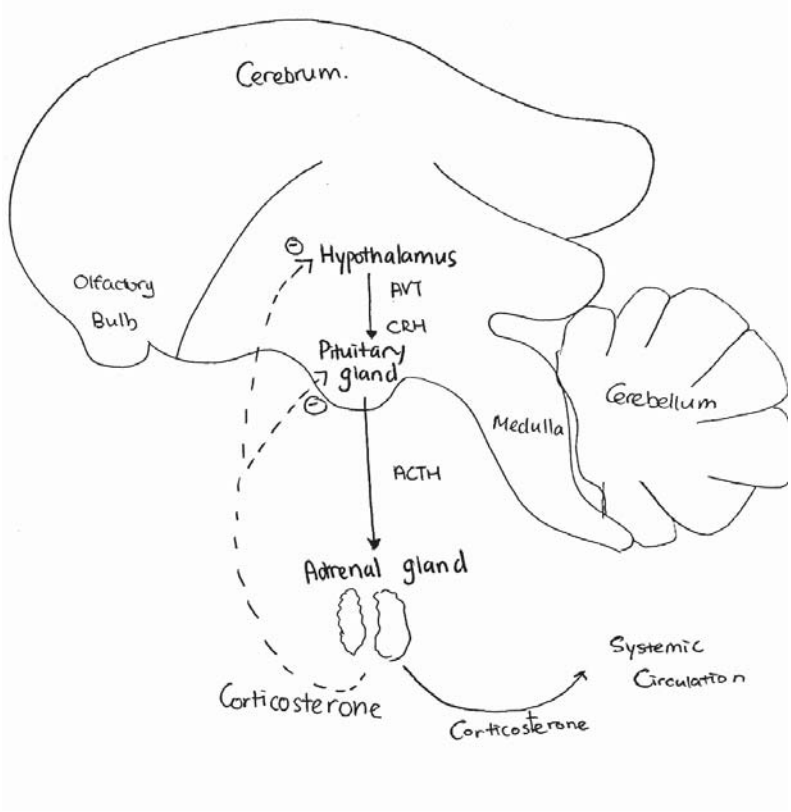


Fig. 1.1. The hypothalamus-pituitary-adrenal axis in birds. The black arrow indicates the release pathways for hormones, and the dotted arrows indicate negative feedback of corticosterone.

1.3.3 The pituitary gland

CRH and AVT stimulate secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into the general blood circulation (Carsia and Harvey, 2000). CRH binds to CRH1 and CRH2 (CRH receptors; Herman *et al.*, 2016). It stimulates the transcription of the pro-opiomelanocortin (POMC) gene, and then the synthesis of ACTH (Herman *et al.*, 2016). AVT binds to the vasopressin VT2 receptor (VT2R) and also stimulates the secretion of ACTH (Sharma *et al.*, 2009). However, CRH is the main peptide that stimulates secretion of ACTH. AVT is thought to maintain the stimulation effect of CRH on ACTH (Sharma *et al.*, 2009).

1.3.4 The adrenal gland

Avian adrenal glands are located craniomedial to both the kidneys and the gonads (Carsia and Harvey, 2000). The adrenal parenchyma is made up of adrenocortical cells and chromaffin cells, in the mammals (Carsia and Harvey, 2000), but the two cell types are not distinctly separated into two layers (outer cortex and inner medulla) as they are in the mammalian adrenal gland. Instead, the two cell types intermingle, with the higher portion of chromaffin cells at the central vascular portion of adrenal glands (Carsia and Harvey, 2000).

ACTH stimulates the production and secretion of glucocorticoids from the adrenal cortices. ACTH binds to melanocortin two receptors (MC2R) and induces the synthesis of glucocorticoids (Herman *et al.*, 2016). The details of synthesis process will be explained in the next section. Glucocorticoids are released into the systemic blood circulation from the adrenal gland.

1.3.5 Glucocorticoids

Glucocorticoids are the final product of the HPA axis. They are also known as the stress hormones as they regulate the stress responses of animals to a stressor. In most mammals and fish, cortisol is the prominent glucocorticoid. In birds, rodents, amphibians and reptiles, the main glucocorticoid is corticosterone.

1.3.5.1 Synthesis of corticosterone

Cortisol and corticosterone are synthesised cholesterol via a series of steroid conversions catalysed by enzymes. The primary structure of the enzymes is homologous in birds and mammals, but the pathways can differ between vertebrate groups (Carsia and Harvey, 2000).

The binding of ACTH to MC2R at the adrenal glands initiates the synthesis of glucocorticoids from cholesterol. This process is also known as the steroidogenic biosynthesis pathway (Fig. 1.2). Cholesterol is transferred to the inner mitochondrial membrane via steroidogenic acute regulatory protein (StAR) and then converted to pregnenolone by mitochondrial cytochrome P-450$_{SCC}$, a cholesterol side-chain cleavage enzyme (Carsia and Harvey, 2000). Pregnenolone is then converted to progesterone by the microsomal dehydrogenase-isomerase enzyme (Carsia and Harvey, 2000). Next, the enzyme microsomal P-450$_{C21}$ converts progesterone to 11-deoxycorticosterone. 11-deoxycorticosterone is also one of the hormones secreted by the avian adrenal gland, though corticosterone remains the dominant glucocorticoid in birds (Carsia and Harvey, 2000). Lastly, in birds the mitochondrial enzyme, P-450$_{11\beta}$, catalyses the conversion of 11-deoxycorticosterone to corticosterone (Carsia and Harvey, 2000).

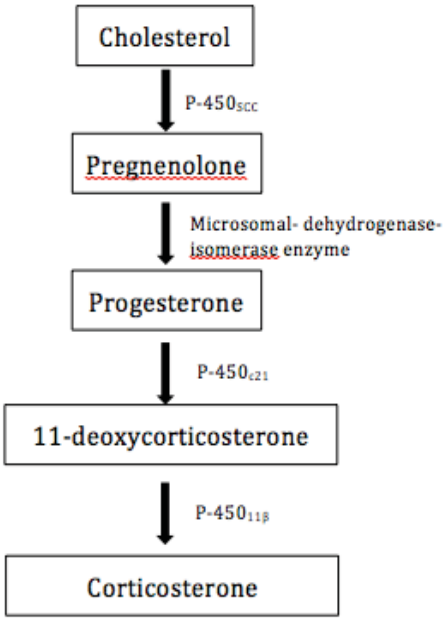


Fig. 1.2. The steroidogenic biosynthesis pathway in the avian adrenal gland. Figure adapted from Carsia and Harvey (2000).

1.3.5.2 Glucocorticoid receptors

Corticosterone travels through blood circulation and binds to glucocorticoid (GR) and mineralocorticoid (MR; Herman *et al.*, 2016). The receptors differ in their binding affinity to glucocorticoids, with GRs being low-affinity receptors and MRs being high-affinity receptors. GRs are occupied and activated when glucocorticoid concentrations increase when animals are responding to a stressor. In contrast, glucocorticoids at basal concentrations activate high-affinity MR (Herman *et al.*, 2016). GRs are thought to be the main receptor that regulates stress responses of animals to stressors.

Corticosterone crosses cell membranes to bind to the receptors in target cells. First, corticosterone binds to the receptor-binding domain of these receptors and enters the nucleus of target cells. Then, the receptor-ligand complex binds with another complex (homodimerization), and interact with the transcription initiation complex (Nicolaides *et al.*, 2015) leading to the transcription of genes and translation of mRNA.

The abundance and distributions of receptors in tissues determine the effects of glucocorticoids on tissues (Senft *et al.*, 2016). GR and MR are distributed in the peripheral system and in the brain. In particular, both receptors are found in the hippocampus and the hypothalamus of the central nervous system (Senft *et al.*, 2016). In the peripheral system, GR and MR are located in the liver, muscle, lungs and other tissues (Herman *et al.*, 2016). Glucocorticoids also mediate negative feedback via binding to receptors at the hypothalamus and pituitary gland (Sharma *et al.*, 2009; Myers *et al.*, 2012). It has recently been shown that glucocorticoids also bind to membrane-bound glucocorticoid receptors (mGR). These receptors are mainly responsible for rapid behavioural effects of glucocorticoids, as the

process does not involve genomic actions. The membrane-associated receptors are also involved in the inhibition of HPA axis activities (Myers *et al.*, 2012).

Glucocorticoids travel in the blood as free hormones and are also bound to binding proteins, especially corticosteroid binding globulin (CBG). Only free glucocorticoids can cross the cell membranes and bind to glucocorticoid receptors. Corticosterone that has bound to CBG is unable to cross the cell membranes and exert actions. The quantity and binding capacity of corticosterone binding globulins (CBG) influence the effects of glucocorticoids (Carsia and Harvey, 2000; Landys *et al.*, 2006).

1.3.5.3 Actions of glucocorticoids

Glucocorticoids have diverse actions on both physiological and behaviour processes of animals. They influence multiple systems, including the immune and circulatory systems. This section reviews the actions of glucocorticoids.

1.3.5.3.1 Physiological actions

Animals usually have additional energy demands when a stressor is present. Glucocorticoids redirect energy and nutrients tissues that need extra energy (Landys *et al.*, 2006). Glucocorticoids are catabolic hormones that stimulate protein and lipid catabolism and hence stimulate the release of energy from complex molecules (Peckett *et al.*, 2011). At the same time, glucocorticoids promote lipogenesis and fat deposition in the liver as a backup energy source, and stimulate glycogenolysis and gluconeogenesis leading to increased blood glucose concentrations (a process of producing sugar; Uchoa *et al.*, 2014). These are metabolic processes that promote energy provisioning and help animals adjust to stressors.

Glucocorticoids act on the immune system, in particular on cell-mediated immune responses and the production of antibodies (Chrousos, 1995; Carsia and Harvey, 2000; Uchoa *et al.*, 2014). Glucocorticoids can suppress the production of cytokines and reduce the number of circulating leucocytes (Uchoa *et al.*, 2014). Similarly, the anti-inflammatory effect of glucocorticoids is achieved via inhibiting the expression of inflammatory genes (Chrousos, 1995; Uchoa *et al.*, 2014). The primary role of glucocorticoids in negative feedback will be reviewed in a later section.

1.3.5.3.2 Behavioural actions

Glucocorticoids can influence behaviour of animals. A stressor may trigger the flight-or-fight response in an animal. Corticosterone can increase locomotor activity and hence promote the movement of an animal away from a stressor (Landys *et al.*, 2006). The actions of corticosterone on behaviour have been studied by administering dexamethasone which is a synthetic glucocorticoid. Dexamethasone treatment stimulated locomotor activity in white-crowned sparrows and white-throated sparrows (Breuner and Hahn, 2003; Landys *et al.*, 2006). In contrast to activity, feeding and sexual behaviour of animals are said to be suppressed by glucocorticoids (Sapolsky *et al.*, 2000).

Effects of glucocorticoids on behaviour may vary according to situations. Captive Gambel's white-crowned sparrows with corticosterone implants had increased activity when food was absent, whereas the activity levels decreased when food was available (Breuner and Hahn, 2003). Similarly, while the treatments of GR antagonists suppress food intake of white-crowned sparrows during hyperphagia (an intense feeding state for the preparation of migration), the same treatment did not affect wintering birds (Landys *et al.*, 2006).

1.4 The avian corticosterone response

Corticosterone responses of birds have been measured in many situations. For example, the variation of corticosterone responses across life history stages has been measured in many species of birds (Romero, 2002; Romero, 2006; Lattin *et al.*, 2016). The corticosterone responses of birds have been compared among species, individuals, populations, and also between genders (Cockrem *et al.*, 2009; Walker *et al.*, 2015a; Cockrem *et al.*, 2016). Corticosterone responses of birds to a range of stimuli have been measured, with stimuli including handling, the presence of predators, and alarm calls (Cockrem and Silverin, 2002; Dufty Jr and Crandall, 2005).

A standard protocol is often used to measure corticosterone responses in birds (Wingfield et al., 1994; as cited in Walker *et al.*, 2015a), with handling and restraint being this standard stressor. A bird is captured or is removed from a cage or nest and an initial blood sample is collected as quickly as possible. The corticosterone concentration in the first blood sample, if sample is collected within three min, is often considered to represent the corticosterone concentration in an undisturbed bird (Romero and Reed, 2005). However, recent studies revealed that corticosterone concentrations actually begin to rise within one or two min. For example, the plasma corticosterone concentrations begin to increase 2 min post-capture in Florida scrub-jays (*Aphelocoma coerulescens*; Small *et al.*, 2017). Furthermore, the rate of increase of plasma corticosterone varies depending on the phenotype of stress responses of individuals (Small *et al.*, 2017). Individuals showed higher corticosterone responses to a stressor also had higher corticosterone concentrations in the initial sample. Similarly, in the nestlings of Savannah sparrows (*Passerculus sandwichensis*), the time taken to collect blood samples is highly correlated to the concentrations levels within the first three min post-

capture (Newman *et al.*, 2017). Therefore, blood samples taken within first three min may not indicate the corticosterone concentrations of an undisturbed bird.

After the first blood sample is collected the bird is placed in a bag or a box, with further blood samples taken at various times up to 30 or 60 min after the bird was first picked up (Li *et al.*, 2011; Walker *et al.*, 2015a; Cockrem *et al.*, 2016). The intervals between sampling blood samples are usually 15 to 30 min but vary among studies (Walker *et al.*, 2015a). Corticosterone responses are sometimes measured for no more than 15 min (Ellenberg *et al.*, 2007; Walker *et al.*, 2015a). There are only a few studies that have measured corticosterone responses of birds for more than 60 min (Adams, 2000; Cockrem and Silverin, 2002; Hazard *et al.*, 2008). Whilst corticosterone can be measured in samples collected noninvasively, for example, faecal samples (Cockrem *et al.*, 2012), corticosterone responses can only be measured in samples that are collected by catching and holding birds.

Corticosterone concentrations generally increase rapidly after the initial blood sample is collected in free-living birds, usually continue to increase up to 30 min after the initial sample. If samples are collected out to 60 min, then mean corticosterone concentrations generally do not increase significantly between 30 and 60 min. Concentrations may continue to increase, remain relatively constant, or decline in individual birds (Cockrem *et al.*, 2009). The magnitude of the corticosterone responses varies among species. For example, corticosterone responses of great tits (*Parus major*) are lower than grey-faced petrels (*Pterodroma gouldi*; Cockrem *et al.*, 2009). In domesticated birds, corticosterone responses are generally much smaller than responses of free-living birds. For example, corticosterone responses of Japanese quail are low in comparison with great tits and grey-faced petrels (Fig. 1.3). Mean corticosterone responses in quail increased between zero and 15 min, decreased

from 15 to 30 min, then remained low (Cockrem *et al.*, 2009). The birds were handled for the first 15 min, and then placed in a cardboard box. They thus experienced a stressor of rather greater magnitude in the first 15 min compared with the remaining 45 min (Graph 1; Cockrem *et al.*, 2009).

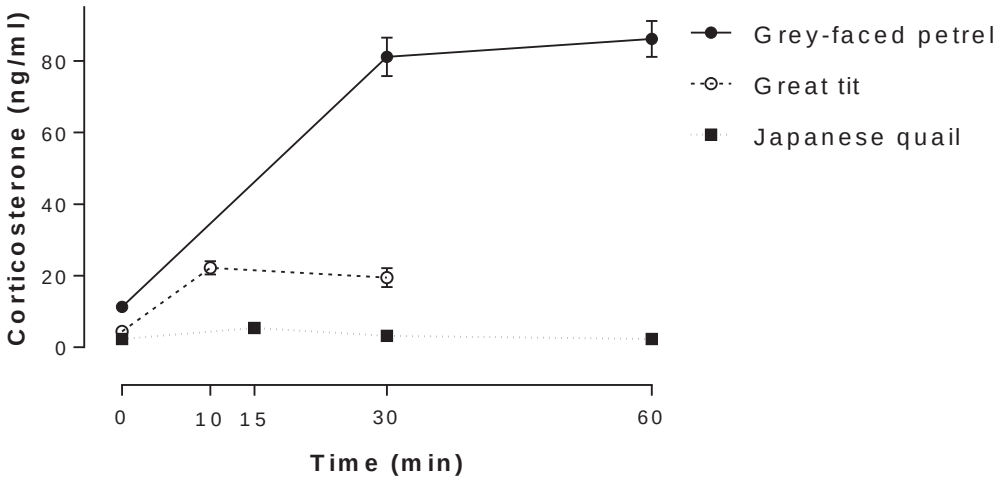


Fig. 1.3. Mean corticosterone responses of three species of birds. Figure from Cockrem *et al.* (2009).

1.4.1 Individual variation and repeatability of corticosterone responses

In most of the literature, corticosterone responses are usually presented as the mean responses of a group of animals, with little or no attention paid to individual responses. Whilst individual variation in glucocorticoid responses of animals has received little attention until recent years, the importance of this variation is now well recognised (Cockrem, 2013a). In birds, individual corticosterone responses have been shown in laying hens (Littin and Cockrem, 2001), great tits (Fig. 4; Chua and Cockrem, 2002), zebra finches (*Taeniopygia guttata*; Wada *et al.*, 2008) and many other species. The corticosterone response of an individual can be quite different from the mean corticosterone response of a group. Some

animals respond greatly to a stressor that triggers a small response in other individuals, and such variation indicates that each individual responds to stressors differently. For example, in zebra finch nestlings, the maximum corticosterone concentration was 29-fold higher than the minimum corticosterone concentration at 30 min (Wada *et al.*, 2008). Coefficients of variation can be used to compare variation between groups of animals with quite different absolute glucocorticoid concentrations (Cockrem, 2013b), and great tits had a high coefficient of variation in their corticosterone responses (Fig. 4; Chua and Cockrem, 2002).

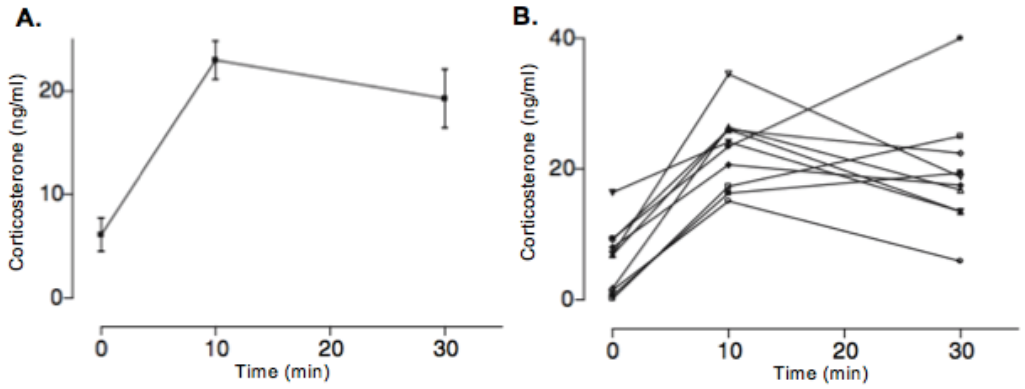


Fig. 1.4. Mean and individual corticosterone responses of great tits to handling and restraint Figure retrieved from Cockrem (2007).

Corticosterone responses indicate the ability of animals to cope with environmental changes, and such responses are consistent over time. Individuals that were repeatedly sampled at different times produced similar corticosterone responses on each occasion to the same stressor (Wada *et al.*, 2008; Rensel and Schoech, 2011; Grace and Anderson, 2014). The corticosterone responses of individuals are therefore considered as one of the trait characteristics of coping styles. “Coping style” is a coherent set of trait characteristics, which are a set of constant behavioural and physiological responses of animals to stressors across time and occasions (Cockrem, 2007; Koolhaas *et al.*, 2010). It is the same concept as animal

personalities. Animals can be considered to have proactive or reactive personalities. The personalities of birds have been well studied, with many behavioural and physiological phenotypes of reactive and proactive birds recorded (Jones *et al.*, 1992; Jones *et al.*, 2000; Groothuis and Carere, 2005; Aplin *et al.*, 2013; Grace and Anderson, 2014). Proactive animals are bold, aggressive, and less fearful with low corticosterone responses. In contrast, reactive animals are considered as shy, cautious, and more fearful with high corticosterone responses (Cockrem, 2007). The study of personalities or coping styles provides insight into the characteristics of successful animals in certain environments.

1.4.2 Seasonal changes in corticosterone responses

Corticosterone responses of birds can vary seasonally. Seasonal changes of corticosterone levels (both baseline and stress-induced) are well studied in birds (see reviewed by Romero, 2002). This review focuses on the corticosterone responses to a stressor instead of baseline corticosterone concentrations.

The majority of species considered by Romero (2002) have seasonal changes in corticosterone responses. For example, in white-crowned sparrows (*Zonotrichia lecophrys*) corticosterone responses are substantially higher during the breeding season in May than at other times of year (Fig. 1.5). During the pre-basic moult after the breeding season, the corticosterone responses are relatively low compared to other seasons. In the winter, corticosterone responses are intermediate (Romero, 2002). In contrast, house sparrows did not have clear seasonal differences in corticosterone responses, except concentrations during late breeding were lower than during breeding (Lattin *et al.*, 2012). On the other hand, in Eurasian tree sparrows (*Passer montanus*), corticosterone levels were higher during late breeding and wintering stages than those in pre-basic moult and early breeding life history

stages (Li *et al.*, 2011). The seasonal pattern of responses vary among species but, in general, corticosterone responses to stressors are lowest during moult in most species (Romero, 2002).

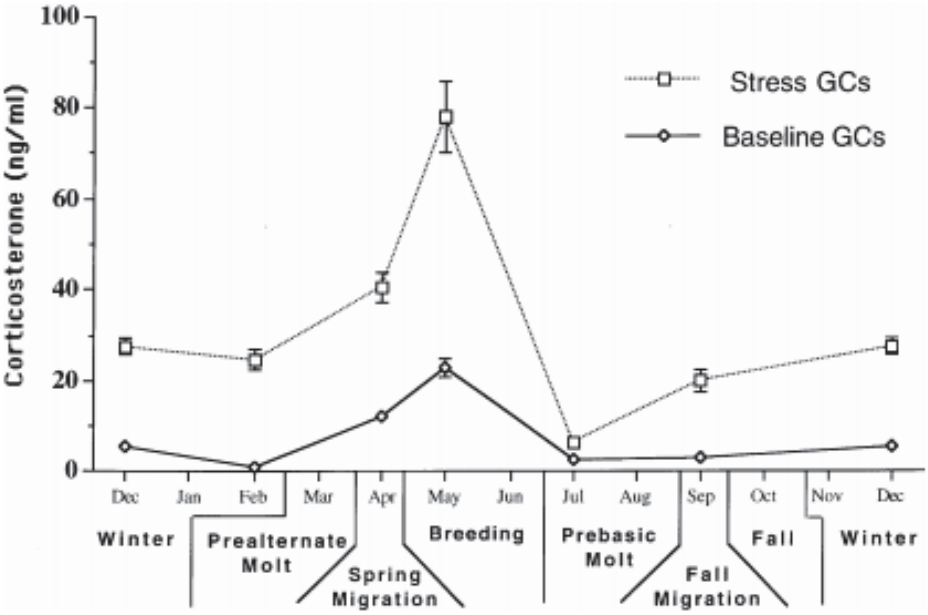


Fig. 1.5. Seasonal changes in stress-induced and baseline glucocorticoid concentrations in white-crowned sparrows. Figure retrieved from Romero (2002).

Corticosterone responses of birds during wintering (non-breeding), pre-breeding and breeding seasons are considered in detail below. Lattin *et al.* (2016) considered results from studies of corticosterone responses in some species of birds during the pre-breeding period in comparison with the breeding season. Most species considered in the review, including Equatorial stone chats (*Saxicola torquata rubicola*), house sparrows (*Passer domesticus*), mountain white- crowned sparrows (*Zonotrichia leucophrys* oriantha) and rufous-winged sparrows (*Peucaea* carpalis), had similar corticosterone responses before and during breeding. Corticosterone responses were higher during pre-breeding than breeding in great tits (*Parus major*), red knots (*Calidris canutus islandica*), dusky flycatchers (*Empidona obersholseri*), snow buntings (*Plectrophenax nivalis*), Lapland longspurs (*Calcarius*

lapponicus) and tree swallows (*Tachycineta bicolor)*. While not included in the review, the Atlantic puffin (*Fratercula arctica*) also had higher responses during pre-breeding (Rector *et al.*, 2012). Only one species in the review, grey-faced petrels (*Pterodroma macroptera gouldi*), have lower pre-breeding than breeding (late incubation) responses. Some species higher corticosterone responses during winter than in the breeding seasons Romero (2002). Some of these species live in extreme environments, such as redpolls (*Carduelis flammea*) in Alaska and cactus wrens (*Campylorhynchus brunneicapillus*), curve-billed thrashers (*Toxostoma curvirostre*) and Abert's towhees (*Amphispiza bilineata*) in the desert. However, willow tits (*Poecile montanus*) from a more temperate environment also showed the same pattern of seasonal variation. In contrast, white-crowned sparrow and house sparrow showed lower corticosterone responses during winter than during breeding (Romero, 2002). However, the review compared corticosterone responses between breeding and post-breeding (considered as wintering) in birds, and the breeding season was not further categorised into multiple stages. The two reviews provided evidence that avian corticosterone responses vary seasonally and that seasonal patterns differ between species.

Several hypotheses have been proposed to explain seasonal changes in corticosterone responses. Hypotheses include the Energy Mobilization Hypothesis (Dallman *et al.*, 1993), Preparative Hypothesis (Sapolsky *et al.*, 2000), and Behaviour Hypothesis (Wingfield *et al.*, 1998). The Energy Mobilization Hypothesis proposes that glucocorticoid concentrations are highest during an energetically costly period of the year, as glucocorticoids play an important role in redirecting and mobilising energy. The Preparative Hypothesis suggests that animals have increased corticosterone responses at the time of the year when there are high chances of occurrence of unfavourable conditions. Lastly, the Behaviour Hypothesis suggests that

corticosterone concentrations are regulated according to the requirement of expressing glucocorticoid-mediated behaviour seasonally (Romero, 2002).

Lattin *et al.* (2016) proposed that all three hypotheses could explain high corticosterone responses during pre-breeding in certain birds. Pre-breeding is an energetically demanding period for the animals. Birds perform behaviours such as territorial defence, sexual display and competition between conspecifics. These behaviours are relatively high cost, given that they could cause injuries or increase exposure of the animals to predators. Enhanced corticosterone responses could prepare animals for adverse conditions and for energy-demanding activities. Birds could also be more sensitive to environmental stimuli due to enhanced corticosterone concentrations and would be able to initiate breeding at appropriate timing via regulating the reproductive behaviour and physiology.

In addition to corticosterone responses, HPA axis activity varies seasonally, such as the sensitivity of the pituitary tissue to CRF and AVT, adrenal tissue to ACTH and the efficacy of negative feedback of corticosterone (Romero *et al.*, 1998; Romero, 2006; Lattin *et al.*, 2012). The binding capacity of glucocorticoids receptors and corticosteroid binding globulins vary seasonally too (Breuner and Orchinik, 2001; Lattin and Romero, 2013; Lattin and Romero, 2015).

1.4.3 Other factors that affect corticosterone responses

Many factors affect the magnitude of corticosterone responses. The duration and type of stimuli may change the stress responses of birds significantly. Individuals exposed to handling and restraint may respond differently to predator stimuli. Corticosterone responses vary between genders in many species (Astheimer *et al.*, 1994; Li *et al.*, 2011).

Environmental factors also may affect corticosterone responses greatly, and the same species could have different corticosterone responses at different locations (Li *et al.*, 2011). Likewise, migratory and non-migratory birds could have distinct corticosterone responses to a stressor (Astheimer *et al.*, 1995; Landys *et al.*, 2006). Physiological state, health, and body condition may influence the degree of stress responses of birds (Sockman and Schwabl, 2001; Landys *et al.*, 2006). Capture of animals and maintenance of them in captivity could affect corticosterone responses (Dickens *et al.*, 2009; Lattin *et al.*, 2012). In addition to corticosterone responses, the overall function of HPA axis can also be affected at levels including the efficacy of negative feedback of corticosterone, the sensitivity of adrenal and pituitary tissues to stimulatory hormones, and the binding capacity of corticosteroid binding globulins or receptors (Lattin *et al.*, 2012; Lattin and Romero, 2014).

1.5 Negative feedback of corticosterone

Corticosterone secretion increases when animals respond to a stressor. Whilst it is assumed that corticosterone concentrations return to initial values after the end of the period of exposure to a stressor, concentrations are almost invariably only measured during the presence of the stressor. The return of corticosterone concentrations to initial values results both from a reduction in corticosterone secretion and from the clearance of corticosterone from the plasma. The reduction in corticosterone secretion can be considered to result from a combination of a reduction in the activity of CRH neurons when the stimuli from the stressor are no longer present, together with negative feedback of glucocorticoids. Corticosterone negative feedback is said to prevent excessive secretion of corticosterone (Myers *et al.*, 2012), and it is often said that prolonged elevation of corticosterone may negatively affect health and fitness of animals (Romero, 2004). For examples, laying hens that were repeatedly exposed to a stressor produced low quality eggs with lower chances of hatching compare to

control hens (Saino *et al.*, 2005). The nestlings were smaller and showed slower plumage development than the control nestlings too (Saino *et al.*, 2005). Long-term administration of exogenous corticosterone can also cause elevations of glucose and cholesterol concentrations, along with changes in body and organ weight (Shini *et al.*, 2009). High corticosterone concentrations have been reported to inhibit reproduction and to reduce feather quality during moulting (Romero *et al.*, 2005). The following sections described the physiological mechanisms of glucocorticoid negative feedback. The majority of the information comes from mammals, especially rats and mice. Information on birds is presented after the information from mammals.

1.5.1 Physiological mechanism

The negative-feedback mechanisms of corticosterone that are currently known can be divided broadly into delayed and rapid feedback mechanisms. Rapid feedback can result in a fast inhibition effect on the HPA axis. On the other hand, the effect of delayed feedback is not immediate and occurs over a longer time frame than rapid feedback (Myers *et al.*, 2012; Uchoa *et al.*, 2014). Both types of feedbacks are regulated by intracellular- and membrane-bound receptors (Feldman and Weidenfeld, 1999; Uchoa *et al.*, 2014). Glucocorticoid receptors (GRs) are involved in the delayed feedback mechanism. On the other hand, there is increasing evidence that membrane-associated receptors are involved in the rapid feedback mechanism (Di *et al.*, 2003; Evanson *et al.*, 2010; Perez *et al.*, 2013).

1.5.1.1 Rapid feedback mechanism

The rapid feedback mechanism does not involve genomic actions, and the effect of rapid feedback may happen within seconds to min. The administration of glucocorticoid receptor antagonists partially reversed rapid inhibitory effects of synthetic corticosterone (Feldman

and Weidenfeld, 1999). Such evidence suggests that the feedback effect is not entirely via corticosterone binding to the intracellular glucocorticoid receptors (Feldman and Weidenfeld, 1999; Hinz and Hirschelmann, 2000). Instead, corticosterone binds to the membrane-associated glucocorticoid receptors (mGR) to induce fast negative feedback on the HPA axis. The mGRs are located in the central nervous system but fast negative feedback on HPA axis occurs mainly on the parvocellular neurons in the PVN (Evanson *et al.*, 2010). Corticosterone binds to mGRs, induces synthesis of endocannabinoids (eCBs) and activates a retrograde signalling pathway (Evanson *et al.*, 2010). Retrograde signalling refers to the process in which a retrograde messenger is released from the post-synaptic neuron and binds to receptors at the axon terminal of the presynaptic neuron (Di *et al.*, 2003). In this case, the retrograde messenger could be a type of endocannabinoid. The eCBs bind to the type-1-cannabinoid receptors (CB1) at the presynaptic glutamate neuron. The action inhibits the release of glutamate (GLU), an excitatory neurotransmitter, from the presynaptic glutamate neuron to the post-synaptic terminal, the parvocellular neurosecretory neurons (Di *et al.*, 2003). The action inhibits the excitation of the parvocellular secretory neurons and any subsequent activities in the PVN, such as secretion of CRH (Fig. 1.6). This process is also known as GC-induced suppression of excitation (GSE; Myers *et al.*, 2012).

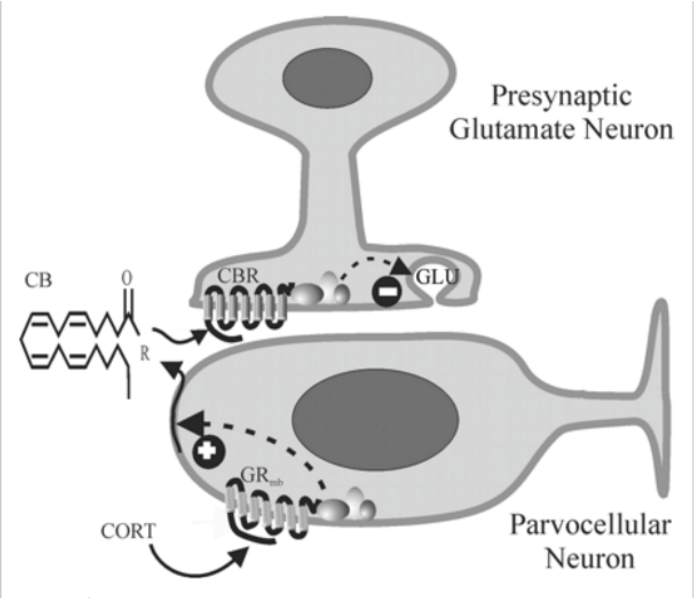


Fig. 1.6. This diagram shows a model of rapid glucocorticoids actions in parvocellular neurons of the PVN. Glucocorticoids binds to membrane-associated glucocorticoid receptors and activates an intracellular signalling pathway (the dashed-arrow). They induce endocannabinoid synthesis (+), and then endocannabinoid binds to the endocannabinoid receptors on pre-synaptic glutamate terminals. This process inhibits the secretion of glutamate (GLU) onto the post-synaptic PVN neuron (-). The neuronal activity and hormone secretion of PVN is inhibited too. Figure from Di *et al.* (2003).

1.5.1.2 Delayed feedback mechanism

The delayed feedback mechanism includes a series of genomic actions following the binding of corticosterone to glucocorticoid receptors (GRs). Genomic actions include transcription of genes and translation of mRNA (Nicolaides *et al.*, 2015). The inhibitory effect of delayed feedback mechanism takes hours or even days to happen due to the genomic actions. In the

brain, GRs are densely expressed in the hippocampus and the pre-frontal cortex (Herman *et al.*, 2012). It is thought that these regions play a major role in the negative feedback mechanism. Lesions of these regions result in delayed inhibition of the HPA axis (Sapolsky *et al.*, 1991; Feldman and Weidenfeld, 2001). In addition, the knockout of receptors in these regions also results in decreased efficacy of negative feedback (Herman *et al.*, 2012). This suggests that corticosterone binds to the receptors in these regions, where it mediates negative feedback on the HPA axis.

The delayed feedback mechanisms are complicated, with corticosterone influencing neurocircuitry and signalling mechanisms in the brain. Corticosterone prevents the secretion of CRH from PVN through binding to receptors in per-limbic prefrontal cortex (plPFC; Radley *et al.*, 2009). This region has little or no contact with the PVN (Myers *et al.*, 2012). Instead, there are intermediary neurons that relay the influence of corticosterone to the PVN (Myers *et al.*, 2012). The bed nucleus of the stria terminalis (BST) is one of the relay sites, connecting the plPFC to the PVN (Myers *et al.*, 2012). The BST receives excitatory input, which are glutamate neurons, from the pre-limbic prefrontal cortex (Choi *et al.*, 2007). The BST also contains a GABAergic population of neurons that project to PVN (Choi *et al.*, 2007). GABAergic neurons produce GABA, a kind of inhibitory neurotransmitter, at the PVN. The intermediary synapses inhibit the activities at PVN via sending the inhibitory neurotransmitter (GABA; Myers *et al.*, 2012). The inhibition of PVN activities leads to suppression of the synthesis and secretion of CRH and AVP, and hence to suppression of the secretion of corticosterone (Fig. 1.7). The hippocampal formation, especially the ventrical subiculum (vSUB), like the plPFC, also inhibits PVN activity through the same mechanism (Fig. 7; Radley *et al.*, 2009).

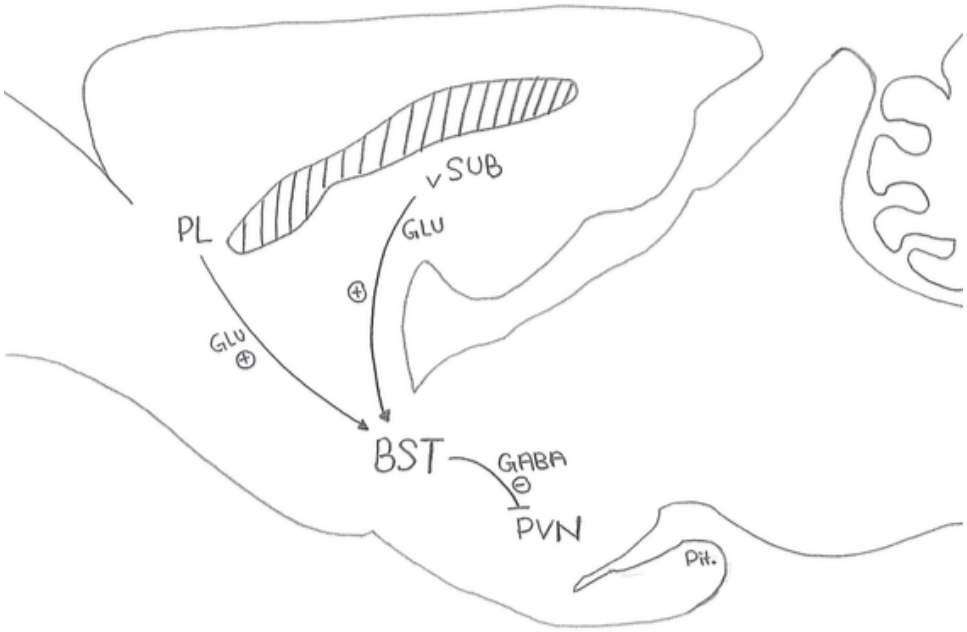


Fig. 1.7. This figure shows the bed nucleus of the stria terminalis (BST) as a relay site between the pre-limbic pre-frontal cortex (PL) and the PVN, and between the vSUB and the PVN in the brain of rats. Corticosterone binds to receptors in the PL and vSUB and triggers the activity of GLU neurones that project to the BST. The BST receives excitatory input (GLU) from the PL and vSUB, and GABAergic neurons towards from the BST towards the PVN in the hypothalamus. The GABAergic neurons inhibit the synthesis and secretion of CRH from the PVN. (+) is excitatory input and (–) is inhibitory input. Abbreviations: PL-pre-limbic; vSUB- ventrical subiculum; Glu- glutamate; BST- Bed of stria terminalis; GABA- gamma-aminobutyric acid; PVN- paraventricular nuclues; Pit. - Pituitary gland.

1.5.1.3 Other mechanisms

Corticosterone may also inhibit the activity of the HPA axis via enhancing the degradation of mRNA in neuron signalling molecules. In the brain, the medial parvocellular PVN receives

synaptic innervation from stress-regulatory neurons (Uchoa *et al.*, 2014). These neurons project from the nucleus of the solitary tract (NTS) directly to the sub-region of the PVN that contains CRH neurons (Larsen *et al.*, 1997; Myers *et al.*, 2012; Uchoa *et al.*, 2014). The neurons contain excitatory-peptide neuromodulators, glucagon-like peptide-1 (GLP-1), that stimulate the synthesis and release of CRH (Larsen *et al.*, 1997). Stress-induced corticosterone can inhibit the excitatory effect of GLP-1 (Zhang *et al.*, 2009). The precursor of GLP-1 is preproglucagon (PPG). In the NTS, the administration of exogenous glucocorticoids results in decreased PPG mRNA expression (Zhang et al., 2009), decreased fiber density and reductions in the stimulatory effect of GLP-1. At the same time, glucocorticoids did not affect the transcription process of the gene. Instead, injection of glucocorticoid triggered transcription of the PPG gene (Zhang *et al.*, 2009). With the decreased expression of PPG mRNA due to glucocorticoids injection, glucocorticoids may mediate negative feedback via temporarily destabilizing the PPG mRNA (Li *et al.*, 2011; Myers *et al.*, 2012). The destabilizing of the PPG mRNA suppresses the output of GLP-1 to the PVN, and also the synthesis of CRH (Fig. 1.8).

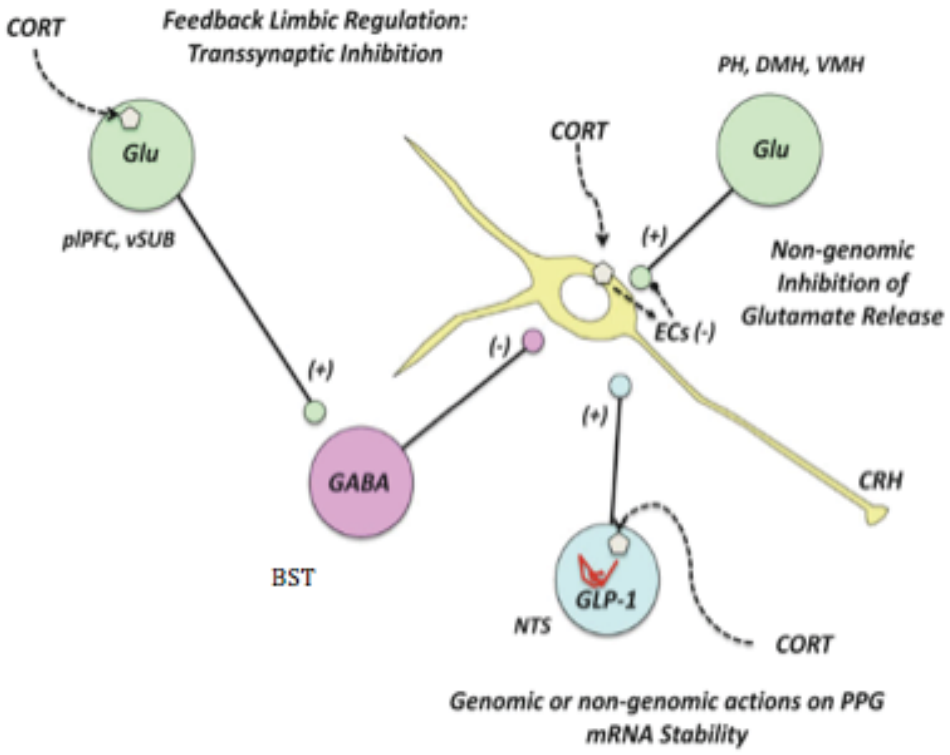


Fig. 1.8. The three main negative feedback pathways of glucocorticoids. Glucocorticoids induce rapid feedback via non-genomic inhibition of glutamate release to the PVN (Upper left). Glucocorticoids also induce delayed feedback via genomic inhibition, binding to the receptors at plPFC and vSUB (right). These regions have little contact with PVN, and the BST act as a synapse relay. It receives excitatory inputs from the plPFC and vSUB, and has inhibitory GABA projections to the PVN. Lastly, glucocorticoids could affect PPG mRNA stability, subsequently affecting the output of PLG-1 from the NTS to the PVN (bottom). (+) is excitatory inputs and (-) is inhibitory inputs. Abbreviations: CORT- corticosterone; plPFC- pre-limbic pre-frontal cortex; vSUB- ventral subiculum; BST- bed nucleus of stria terminalis; GABA- gamma-aminobutyric acid; NTS- nucleus of the solitary tract; GLP-1 – glucagon-link peptide-1; EC- endocannabinoid; VMH- entromedial hypothalamus; PH- posterior hypothalamus; DMH- dorsomedial hypothalamus. Figure retrieved from Myers *et al.* (2012).

1.5.2 The efficacy of negative feedback

The efficacy of negative feedback is an important critical function of the HPA axis that influences the magnitude of responses of animals to stressors. Measurements of the efficacy of negative feedback would inform us about the ability of animals to cope with stressors and to reduce corticosterone concentrations efficiently (Romero, 2004). There are two main methods that are have been used to measure negative feedback in the HPA axis (Romero, 2004). The first measures the duration of corticosterone responses of animals to stressors (Romero, 2004). Measuring the duration of corticosterone responses could be informative for field studies. It provides information on both the efficacy of negative feedback and the total amount of corticosterone released by animals in response to stressors. The other method, more commonly used in birds, is to experimentally measure negative feedback by injecting synthetic glucocorticoids (Romero, 2004). The injection of synthetic glucocorticoids (usually dexamethasone) initiates negative feedback on the HPA axis.

1.5.2.1 Total duration of corticosterone responses

The total length of corticosterone responses starts from when an animal is first exposed to a stressor and finishes when corticosterone concentrations have returned to initial values. Few studies have described the full duration of corticosterone responses in birds. Free-living birds would have to be recaptured after they had been released at the end of a period of exposure to a stressor, and it is usually very difficult to recapture a free-living bird. In domestic birds, such as Japanese quail and laying hens, corticosterone concentrations returned to basal levels within 60 min while the birds are still exposed to the stressors (Littin and Cockrem, 2001; Cockrem *et al.*, 2009).

Most studies that have measured the total duration of corticosterone responses were conducted in rats. For example, the total duration of corticosterone responses in two strains of rats, Lewis and Fischer rats, was reported by (Grota *et al.*, 1997). The rats were taken out from the home cage, an initial blood sample was collected, then rats were returned to the home cage. Blood samples were subsequently collected using a catheter. Corticosterone concentrations increased following exposure to a stressor and peaked 15 to 30 min after the first sample (Grota *et al.*, 1997). Corticosterone levels returned to baseline 60 to 90 min after the initial sample (Grota *et al.*, 1997). Rats that were exposed to handling stimuli showed a slightly different response. Their corticosterone concentrations started to decline slowly after the peak, and remained higher than baseline at the end of sampling (90 minutes after the first sample; Grota *et al.*, 1997). These animals were reared in the laboratory, and studies on wild animals are limited.

Measurement of the total duration of corticosterone responses in birds involves the collection of samples following the standard protocol, after which birds are returned to their home cage, released, or returned to their nest site. A single further blood sample is collected at a later time (Adams, 2000; Cockrem and Silverin, 2002; refer to Appendix Table 1; Hazard *et al.*, 2008). When different birds are sampled at different times mean corticosterone concentrations at various times after the end of the stressor can be calculated. Multiple samples cannot be collected from the same bird once a stressor has ended because there would be a confounding effect of sample collection. The total duration of stress responses has been measured in only few species of free-living birds due to the practical difficulties of recapturing a bird after the end of a period of handling and restraint. In the study that measured the duration of corticosterone responses of northern brown kiwi, it was not practical to return free-living individuals to their burrows, so they were held in boxes instead

(Adams, 2000). However, restraining animals could continue to induce a stress response, as shown in Japanese quail (Hazard *et al.*, 2008). Most of the studies that measured that measure the duration of corticosterone responses have been done on captive animals.

The corticosterone responses of Japanese quail have been measured for more than 60 min. Japanese quails were bled, placed in a crush cage for 10 min, bled again and then returned to their home cage (Hazard *et al.*, 2008). Corticosterone concentrations increased and peaked after quails were exposed to the stressor (Hazard *et al.*, 2008). Corticosterone concentrations in quails returned to initial sampling level within 30 min after the birds were returned to their home cages. The corticosterone levels remained at initial sampling level 110 min after the birds were returned to home cages (Hazard *et al.*, 2008).

The total duration of corticosterone responses of captive great tits to stressors was recorded in a study of corticosterone responses of great tits to different stimuli (Cockrem and Silverin, 2002). Birds in an aviary were exposed to a stimulus for 30 min and then a single blood sample was collected 30 or 60 min or three or six after the birds were first exposed to the stimuli (Cockrem and Silverin, 2002). A stuffed owl was used as a predator stimulus, and it induced corticosterone responses in great tits. The corticosterone concentrations increased following exposure to stressor and remained high after an hour. Corticosterone concentrations had returned to initial concentrations three hours later and remained at baseline six hours after birds were first exposed to the stuffed owl (Fig. 9; Cockrem and Silverin, 2002). The study revealed that corticosterone in the great talents returned to initial concentrations within two hours from the end of exposure to a stressor.

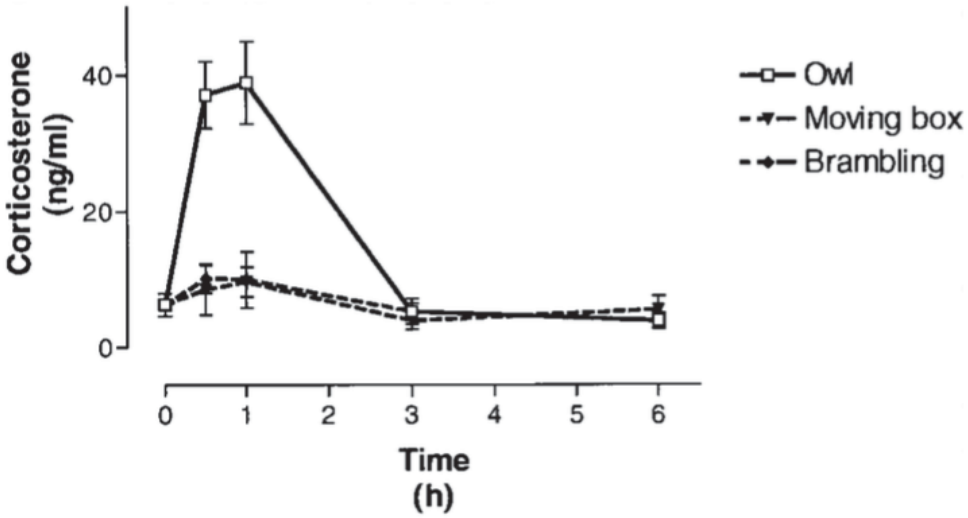


Fig. 1.9. Corticosterone responses of great tits in response to an owl (predator stimulus), moving box or brambling (from Cockrem and Silverin (2002)).

The total duration of corticosterone responses to handling and restraint have also been measured in wild and captive northern brown kiwi (*Apteryx mantelli*). The birds were captured and placed in a plastic box, with blood samples collected 0, 30, and 60 min after the bird was picked up. Birds were then returned to their burrows or nestboxes. Some of the free-living birds were kept in plastic boxes instead of being returned to their burrows. Birds were picked up and sampled again two, four, six or eight hours after they were returned to their habitats (Adams, 2000).

Corticosterone concentrations in birds in a nocturnal house returned to initial concentrations two hours after the birds were returned to their nestbox and remained low two hours later (Adams, 2000). Corticosterone concentrations in birds held in outdoor pens were significantly lower than peak concentrations seven hours after the birds were returned to

nestboxes. Concentrations at this time remained higher than initial concentrations (Adams, 2000).

In general, corticosterone concentrations of domestic birds have been reported to return to initial concentrations within one hour after the end of a period of exposure to a stressor, whereas it has been reported that free-living birds have taken longer for corticosterone to return to initial concentrations. Corticosterone in free-living birds can return to initial concentrations within two hours. In the studies mentioned above, the duration of corticosterone responses was not the main focus and information about the birds other than their corticosterone concentrations was not recorded.

1.5.2.2 Administration of dexamethasone (DEX)

A method used to measure the efficacy of negative feedback of corticosterone on HPA axis is via the administration of dexamethasone. Dexamethasone (DEX) is a synthetic exogenous glucocorticoid that has the same effect of corticosterone or cortisol (Lattin *et al.*, 2012). DEX binds to GRs at the hypothalamus and pituitary gland and inhibits the continuous secretion of glucocorticoids from the adrenal glands (Feldman and Weidenfeld, 1999). It is used to determine the efficacy of negative feedback by observing how quickly the HPA axis ceases secretion of corticosterone. The negative feedback function could be heavily dependent on the dosage of DEX, with higher dosages potentially inducing a higher negative feedback rate and longer suppression time (Westerhof *et al.*, 1994; Dickens *et al.*, 2009). In house sparrows an injection of DEX at 1mg/kg body weight can induce negative feedback on the HPA axis (Lattin *et al.*, 2012). Corticosterone concentrations in DEX-injected birds reduced rapidly after injection to become lower than concentrations in control birds (Fig. 10; Lattin *et al.*, 2012).

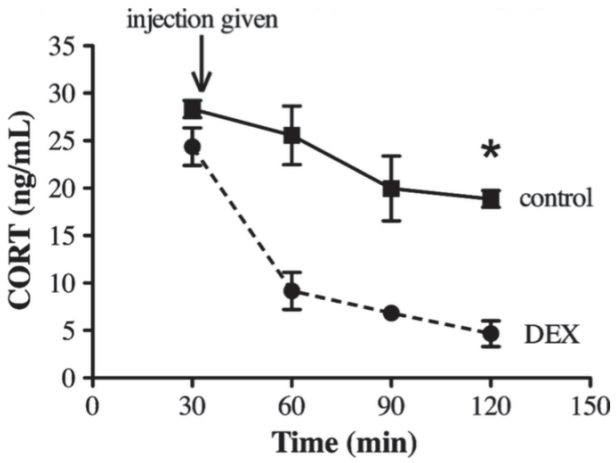


Fig. 1.10. Corticosterone concentrations of control and DEX-injected birds after receiving injections. The birds were exposed to stressors at 0 min, and 30 min later, the birds were injected with Ringers' solution (control) or DEX. The * implies there is significant difference between the control and DEX-injected birds. Figure retrieved from Lattin *et al.* (2012).

To test the negative feedback of the HPA axis, birds are first exposed to a stressor to induce corticosterone response. DEX is then injected into the animals at the time of the corticosterone peak which is usually between 30 to 60 min after the birds have been exposed to the stressor. Final blood samples are taken later to determine the corticosterone concentrations in response to DEX (Lattin *et al.*, 2012). The efficacy of feedback is calculated by comparing the corticosterone concentrations of control birds and DEX-injected birds (Lattin *et al.*, 2012). Lattin *et al.* (2012) calculated the percentage by which corticosterone concentrations decreased after the injection of DEX. The higher the percentage of corticosterone concentration decreases following the injection of DEX, the higher the efficacy of negative feedback on the HPA axis. In other words, a 100% decrease of

corticosterone concentrations after DEX injection implies a complete shut off of the HPA axis due to glucocorticoids. This direct observation of efficacy of negative feedback can be performed in a shorter period of time than measurement of the total duration of corticosterone responses. The response of birds to a DEX injection does not, however, provide information on how long it takes for corticosterone concentrations to return to initial levels in birds once a stressor has ended.

There are few studies on the efficacy of negative feedback using DEX in birds. The strength of negative feedback can vary between situations, just as corticosterone responses vary between situations in birds. For example, the efficacy of negative feedback can differ between free-living and captive birds. Negative feedback function in wild chukars (*Alectoris chukar*) and white-crowned sparrows (*Zonotrichia leucophrys*) had changed after five days of captivity (Dickens *et al.*, 2009; Lattin *et al.*, 2012). In the chukar the efficacy of negative feedback decreased by the 5th day of captivity. The HPA axis had failed to respond to DEX injection and inhibit the secretion corticosterone effectively. However, from the 9th day of captivity onwards, the efficacy of negative feedback returned to levels consistent of those on the 1st day of captivity (Dickens *et al.*, 2009). In contrast, the negative feedback efficacy of white-crowned sparrows increased after being kept in the captivity for five days, regardless of seasons (Lattin *et al.*, 2012). Negative feedback in white-crowned sparrows can also change seasonally, with the efficacy of negative feedback lower during pre-laying compare to late winter and breeding (Lattin *et al.*, 2012). The seasonal changes of negative feedback function may vary among species or populations. In house sparrows, there were no changes in negative feedback function between breeding and molting seasons (Liebl *et al.*, 2013). The flexibility of negative feedback could help the animals reduce corticosterone concentrations according to the circumstances or the animals' needs.

The stress responses of animals are mediated by the regulatory elements of the HPA axis. As the elements are functionally related, they could be correlated to each other. The correlation of the regulatory elements is a relatively new research area, and there are few studies that address this question. Liebl *et al.* (2013) tested the covariation of regulatory elements, particularly the relationship between negative feedback and corticosterone responses to stressors. The efficacy of negative feedback was positively correlated with the corticosterone responses to stressors in house sparrows (Liebl *et al.*, 2013). Individuals that released higher concentrations in response to stressors were able to inhibit the release of corticosterone to a greater degree after injection of DEX. At the same time, this study showed individual variation in the responses to stressors and the ability to cope with stressors (Liebl *et al.*, 2013). However, the study was confounded by the fact that the experiment lacked of a control group and the relationship between the two factors was low. There are limited studies on the co-variation of regulatory elements of the HPA axis. More research in this area would provide better understanding of the regulation of HPA axis function.

1.6 Corticosterone responses of penguins

Penguins are flightless marine birds, and most species breed in colonies. Penguins are easier to approach and capture than most avian species, and hence can be convenient species for the study of corticosterone responses. Responses of penguins to stimuli from their environment have been studied using a variety of methods including measurements of heart rate, behavioural observations, and measurements of corticosterone responses. Behavioural observations do not provide information about the magnitude of the stress responses of birds. Whilst heart rates has been measured in penguins and has been sent to indicate the degree of stress that the birds are experiencing, heart rate as a function of the activity of the

sympathetic nervous system and heart rate, can increase when animals do not perceive a situation to be threatening. Heart rate has been measured in penguins using a dummy egg placed in the nest (Culik *et al.*, 1989). Corticosterone secretion increase when birds perceive a stimulus as threatening, and corticosterone responses can be considered to be the stress responses in penguins.

Corticosterone responses have been measured in 10 species of penguins, including the Adelie (*Pygoscelis adeliae*), emperor (*Aptenodytes forsteri*; Cockrem *et al.*, 2008), Magellanic (*Spheniscus magellanicus*), Humboldt (*Spheniscus humboldti*), rockhopper (*Eudyptes chrysocome*), Galapagos (*Spheniscus mendiculus*; Walker *et al.*, 2015a), gentoo (*Pygoscelis papua*), king (*Apetenodytes patagonicus*; Holberton *et al.*, 1996), yellow-eyed (*Megadytes antipodes*; Ellenberg *et al.*, 2007) and the kororā (Little penguins; *Eudyptula minor*; Cockrem *et al.*, 2016). In all studies, handling and restraint in a box or bag has been used as a stressor (Cockrem *et al.*, 2008; Cockrem *et al.*, 2016) and sampling has followed the standard protocol for the measurement of corticosterone responses in birds. Birds were captured, and an initial blood sample was collected, and then further samples were collected over periods of up to 30 or 60 min. The birds were kept in a box or a bag between samples. Free-living birds were studied, and sample sizes in some studies were small (e.g. yellow-eyed penguins (n=9 and n=7; Ellenberg *et al.*, 2007), gentoo penguins (n=6; Holberton *et al.*, 1996) and king penguins (n=6; Holberton *et al.*, 1996).

The general pattern of the corticosterone responses of penguins is similar to the pattern of responses of other free-living birds. Corticosterone concentrations in penguins increase rapidly in response to a stressor and remain elevated at the end of 30 or 60 min sampling (Holberton *et al.*, 1996; Cockrem *et al.*, 2008; Walker *et al.*, 2015a). There is variation

between the penguin species in the size of their corticosterone responses, just as there is variation between species in other groups of birds (see Fig. 1.11). Little penguins have the most pronounced responses to handling and restraint among all penguin species. The mean corticosterone concentration of little penguins 30 min after exposure to a stressor was 114.2 ng/ml (Cockrem *et al.*, 2016). Galapagos penguins had the lowest corticosterone concentrations 30 min after exposure to stressors (12.5 ng/ml; (Walker *et al.*, 2015a).

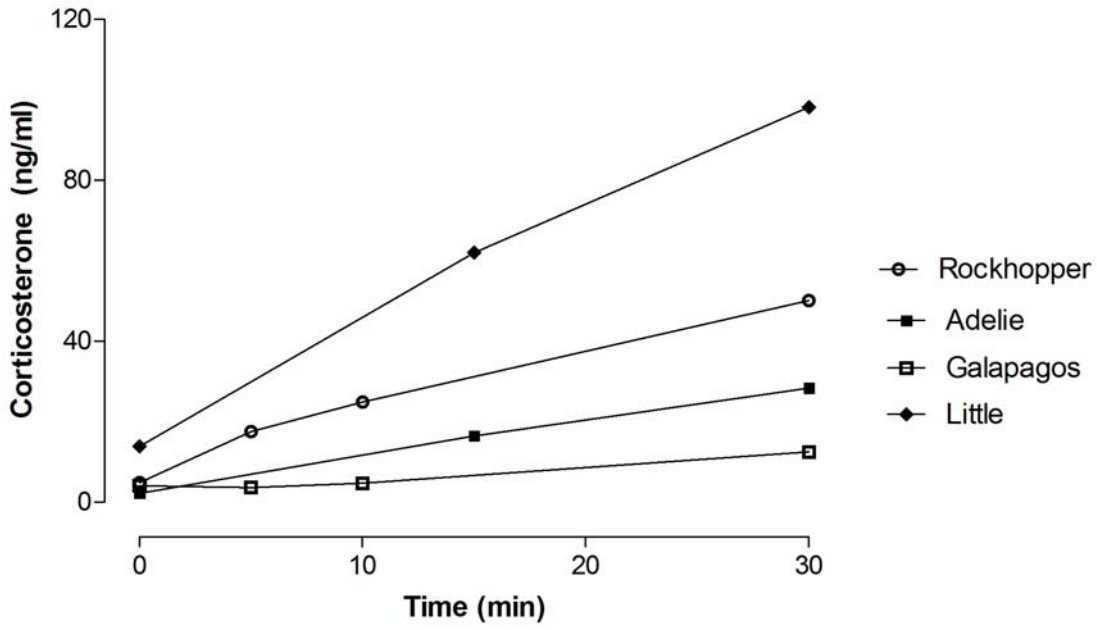


Fig. 1.11. Corticosterone responses of four species of penguins. The results are from four different studies. The results of little penguins are from Cockrem *et al.* (2016); individual were handled for 15 min and kept in a box in between sampling intervals. The results of Gapapagos and Rockhopper penguins are from Walker *et al.* (2015a); capture and restraint were used as stressors in the study. The results of Adelie penguins are from Cockrem *et al.* (2008); the penguins were captured and restrained within a box in between sampling intervals.

There is also considerable individual variation in corticosterone responses in penguins. In little penguins, capture and restraint induced a broad range of corticosterone responses. Some birds had higher corticosterone responses, whilst others showed relatively small responses. Corticosterone concentrations at 60 min of birds with high responses were 14-fold higher than concentrations in birds low responses (Cockrem *et al.*, 2016). In Adelie penguins, corticosterone concentrations varied from 0.76 to 10.82 ng/ml at initial sampling, and from 9.67 to 78.70 ng/ml at 30 min (Cockrem *et al.*, 2009). In addition, the corticosterone responses of the two species were repeatable. Adelie penguins were sampled on three separate occasions during the breeding season, with four-day intervals between each sampling occasion. In general, corticosterone concentration at 30 min and the total integrated corticosterone responses were repeatable (Cockrem *et al.*, 2009). Little penguins were sampled in the year 2012 and 2013 in winter, and 23 individuals were sampled in both years (Cockrem *et al.*, 2016). The corticosterone responses of the individuals were ranked, and the ranks of corticosterone responses showed significant repeatabilities (Cockrem *et al.*, 2016). In contrast to both studies, the corticosterone responses of Magellanic penguins during the incubation period were lower in 2001 than 1999 (Walker *et al.*, 2015a). However, the sample size in 2001 was small in this study. The results of little penguins and Adelie penguins are consistent with the results of other free-living birds, such as great tits (Chua and Cockrem, 2002). The corticosterone responses of penguins vary among individuals, and the responses are repeatable on different occasions.

Seasonal variation in corticosterone responses has been measured only in Magellanic penguins. As in other groups of birds, corticosterone responses of Magellanic penguins were lower during the moulting season (Villanueva *et al.*, 2014). However, in contrast to many

species of birds, the magnitude of corticosterone responses of Magellanic penguins did not change during the breeding season. The corticosterone responses of male Magellanic penguins were measured during settlement, incubation and chick rearing. The settlement period was considered as the pre-breeding period. There were no differences in the corticosterone responses among the three periods (Walker et al., 2014).

Corticosterone responses have also been compared between colonies of penguins. There was no difference in corticosterone responses between two colonies of Magellanic penguins (Walker *et al.*, 2015a). There were no also differences in the corticosterone responses of little penguins at two colonies at Oamaru (Cockrem *et al.*, 2016). One colony is visited by people as a tourist attraction, whereas the other colony is not visited by tourists. It was reported that yellow-eyed penguins in an area visited by tourists had higher corticosterone responses than birds at a non-tourist site (Ellenberg *et al.*, 2007). However, this there was a small sample size in the study and the non-tourist site was a predator-free offshore island, whereas the tourist site was located on the mainland where predators are present (Ellenberg *et al.*, 2007). The authors' conclusion that the presence of tourists had an influence on corticosterone responses of yellow-eyed penguins was not therefore not warranted, as a comparison between colonies was a comparison between mainland and island colonies as well as a comparison between colonies with different levels of tourist visits (Cockrem *et al.*, 2016).

Other studies have compared the corticosterone responses of penguins at different ages, sex, and also rehabilitated birds and control birds (Walker *et al.*, 2015a; Chilvers *et al.*, 2016; Cockrem *et al.*, 2016). The responses of individuals to different types and durations of stressors have been measured too (Cockrem *et al.*, 2008). These studies contribute to the understanding of the stress physiology of penguins. However, many studies have had small

sample sizes, and other regulatory elements of the HPA axis and the efficacy of negative feedback have not been measured in penguins.

1.7 Outline of thesis

The overall aim of the current study was to determine the total duration of the corticosterone responses of free-living little penguins, and to determine the relationship between the rate of increase of corticosterone concentrations whilst birds were experiencing a stressor, and the rate of decrease of corticosterone concentrations once the stressor had ended. This first chapter has been a general introduction. The study of corticosterone responses in little penguins as described in detail in chapter two. The complete duration of corticosterone responses of little penguins was measured. Responses of little penguins were measured during the pre-breeding period and also during early chick rearing. These responses were compared with those of penguins sampled in winter in a previous study. The third chapter provides a general discussion of the findings from the experimental study and directions for future research.

Chapter 2: Experimental studies of the corticosterone responses of kororā

2.1 Introduction

Birds respond to a stressor with activation of the hypothalamic-pituitary-adrenal (HPA) axis and the secretion of corticosterone (Carsia and Harvey, 2000). Corticosterone regulates both physiological and behavioural responses to stressors and is often called a stress hormone. Secretion of corticosterone ceases with removal of the stressor. Corticosterone in circulation is cleared or metabolised mainly in the liver and kidneys (Carsia and Harvey, 2000). It is vital that corticosterone levels return to baseline after removal of the stressor because extended elevation of corticosterone concentrations may be harmful (Romero *et al.*, 2005; Saino *et al.*, 2005; Shini *et al.*, 2009).

The secretion of corticosterone in response to a stressor is known as a corticosterone response (Cockrem, 2007). Corticosterone responses have been measured in various bird species, including both wild and domestic birds (Cockrem and Silverin, 2002; Jones *et al.*, 2002; Cockrem *et al.*, 2009; Grace and Anderson, 2014). The standard protocol for measuring corticosterone responses uses handling and restraint as a stressor, with blood samplings collected at 0 min, then at intervals which are commonly 15 or 30 min, with sample collection continuing for up to one hour (Wingfield et al., 1994; as cited in Walker *et al.*, 2015a). In free-living birds, corticosterone concentrations usually increase rapidly and typically peak 30 or 60 min after the initial exposure to the stressor and remain elevated at the end of one-hour sampling (Cockrem *et al.*, 2009). Whilst there have been many studies of corticosterone responses of birds, few studies in free-living birds have investigated changes in corticosterone concentrations once the stressor of restraint has ended. The only descriptions in free-living birds of the complete time course of corticosterone responses from

the initiatin of the response to the return of corticosterone concentrations to initial values are those of the northern brown kiwi and great tits (Adams, 2000; Cockrem and Silverin, 2002). The small number of studies reflects the difficulties associated with recapturing free-living birds after they have been released at the end of a period of restraint.

The measurement of full corticosterone responses in a range of species will be valuable as it is important to know how long corticosterone takes to return to initial levels once a stress is no longer present. For example, corticosterone concentrations returned to initial sampling levels within two hours post-handling in great tits and northern brown kiwis (Adams, 2000; Cockrem and Silverin, 2002). Previous studies have found that corticosterone responses to a stressor vary among individuals (Cockrem, 2013b) and individuals may also vary in the rate at which corticosterone concentrations return to initial levels. Liebl *et al.* (2013) reported that house sparrows with greater responses to a stressor were also more efficient in bringing down corticosterone concentrations. However, the study measured the efficacy of negative feedback of corticosterone on HPA axis via injection of synthetic glucocorticoids, dexamethasone. To our knowledge, no study has investigated the correlation of the elevation and reduction of corticosterone concentrations through measuring the complete corticosterone responses.

The complete corticosterone responses of free-living little penguins (*Eudyptula minor*) using artificial nest boxes can be measured, as individuals remain in their nestboxes during the day so can be returned to their nestbox after a period of restraint and then sampled at various times after restraint has ended. The aim of the current study was to characterise the total duration of the corticosterone responses of free-living little penguins. Based on a small number of previous studies, plasma corticosterone concentrations of little penguins were

expected to return to initial levels within two hours after penguins were returned to their nest box (Adams, 2000; Cockrem and Silverin, 2002). We also determined whether the rates of increase and decrease of corticosterone concentrations are correlated in little penguins. We predicted that the elevation of corticosterone would be correlated with the reduction of corticosterone concentrations, based on the study by Liebl *et al.* (2013). Many studies reported that the corticosterone responses of birds change across breeding season (Lattin *et al.*, 2016). The current study additionally compared the corticosterone responses of pre-breeding and breeding little penguins. It was expected the corticosterone responses of little penguins would differ between the two periods based on the summary of studies in the review by Lattin *et al.* (2016). Finally, this study compared the corticosterone responses of male and female little penguins during pre-breeding.

2.2 Materials and methods

2.2.1 Study site and animals

The study was carried out at Oamaru on the east coast of the South Island of New Zealand. There are two little penguin colonies in Oamaru, the Quarry and the Creek colony, and the current study was conducted in both colonies. The Quarry colony is run as a tourist facility with approximately 300 nest boxes on site. Visitors can walk around the colony during the day and can watch the little penguins come ashore in the evening. The Creek colony is 1km away from the Quarry colony and has around 250 nest boxes. There are approximately 160 breeding pairs in each colony. Both colonies are protected breeding habitats of free-living little penguins. All penguins at both colonies are banded shortly before fledging with an individually numbered metal band on the right flipper or inserted with an individually numbered microchip to the back of the neck. Adult birds that arrive from another colony are banded or have a transponder inserted when they are first found at the Creek or Quarry

colony. Little penguins go out to the sea during daytime and come ashore in the evening, though some penguins stay in the nest boxes during the daytime too.

The 2016 breeding season started in late August. Non-breeding adult penguins were sampled in September 2016 for a study of the duration of corticosterone responses, and adults with chicks from one to two weeks old were sampled in late October and early November 2016. Blood samples were collected from nonbreeding birds when they were removed from nestboxes (initial sample) and 15, 30 and 60 min later, whereas initial, 15 and 30 min samples were collected from birds with chicks. Only penguins that were found in the nest boxes during daytime were sampled. Corticosterone response data from penguins sampled during the winter by Cockrem *et al.* (2016) were used for comparisons of corticosterone responses at different times of year. The study was conducted under permits from the Massey University Animal Ethics Committee and the Department of Conservation.

2.2.2 Blood sampling

Blood samples were collected following the protocol for little penguins at Oamaru used Cockrem *et al.* (2016). The penguins were removed from the nest boxes and initial blood samples were collected as soon as possible. Blood was collected from the brachial vein of the flippers using a 25 g needle and 1ml heparinised syringe or into capillary tubes after the vein was punctured using a 25 g needle. The band number of the penguin, the number of the nest box and the time taken to complete each blood sampling were recorded. Then, the penguin was then weighed with a Pesola balance, bill length, depth and width were measured with callipers and the flipper length was measured using a ruler. After the measurements, the bird was held by hand until 15 min after the bird was picked upped, and then a second blood sample was collected. The birds were then placed in an opaque plastic white box (29 x 39 x29

cm) that had a lid with holes for ventilation. Further blood samples were collected 30 and 60 min after the time of capture then the bird was returned to its nestbox. Blood samples were kept cool on ice until centrifuged. Plasma was withdrawn and kept in the freezer at a temperature of -20 °C until corticosterone concentrations.

2.2.3 Study design

The standard protocol used for measuring corticosterone responses in birds is to collect an initial blood sample and then to collect further blood samples from the bird whilst it is confined or restrained within a bag. This protocol does not provide any information on the full duration of the response; rather, the protocol only provides information on the response of the bird whilst it is experiencing stressor. The full duration of the corticosterone response of little penguins was measured in the current study by collecting an additional blood sample from a penguin at 15, 30, 60, 120, 180, or 360 min after the bird had been returned to its nestbox at the end of the standard 0, 15, 30 and 60 min sampling protocol. The additional samples were thus collected at varying times after the bird was no longer experiencing the stressor of confinement in a plastic box. The times of the last sample collections were 75, 90, 120, 180, 240 and 420 min after the initial 0 min sample.

Corticosterone responses of penguins sampled during the pre-laying stage of breeding were compared with responses of non-breeding birds sampled in 2012 during the winter (data from Cockrem *et al.* (2016)) and with responses of penguins sampled during early chick rearing in late October and early November 2016. Birds sampled during early chick rearing (chicks one to two weeks old) were bled at 0, 15 and 30 min after being picked up following the same protocol that was used for birds during the pre-laying stage of breeding, so comparisons of

corticosterone responses between stages of breeding were made for corticosterone concentrations at 0, 15 and 30 min.

2.2.4 Corticosterone radioimmunoassay

Corticosterone concentration in plasma diluted in phosphate buffered saline with gelatine (PBSG) were measured by radioimmunoassay using the method of Cockrem *et al.* (2016). The corticosterone radioimmunoassay kit was from MP Biomedicals, USA. The sensitivity of the corticosterone assay was determined as the hormone concentration at the mean -2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity is expressed as ng corticosterone per ml plasma, and it was 0.65ng/ml. Solutions of corticosterone in PBSG were used as low and high controls in every assay. The coefficients of variation for intra-assay were 8.4% and 7.2%, while for inter-assay were 14.6% and 16.1%.

2.2.5 Statistics

Data analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc). The relationship between the time taken to collect the first blood sample and initial corticosterone concentrations was determined using linear regression. All ANOVA analyses were performed on log transformed data. One way repeated measures ANOVAs, with post hoc comparisons made with Sidak's multiple comparisons tests, were used to compare mean corticosterone concentrations between times for each of the six groups of birds (standard sampling protocol then further sample at 15, 30, 60, 120, 180, or 360 min after the bird had been returned to its nestbox). One way repeated measures ANOVA, with post hoc comparisons made with Sidak's multiple comparisons tests, was used to compare mean corticosterone concentrations between times for data from all six groups combined for the 0, 15, 30 and 60 min standard

sampling times. One way ANOVA, with post hoc comparisons made with Sidak's multiple comparisons tests, was used to compare mean corticosterone concentrations between times for the final samples. Relationships between corticosterone concentrations in the last sample and corticosterone concentrations at 60 min, and between the ranks of corticosterone concentrations in the last sample and corticosterone concentrations at 60 min, were determined using linear regression. Mean corticosterone concentrations were compared between times and breeding stages using repeated measures two way ANOVA, with post hoc comparisons made with Tukey's multiple comparisons tests. Mean corticosterone concentrations were compared between times and sexes using repeated measures two way ANOVA, with post hoc comparisons made with Sidak's multiple comparisons tests.

The rate of change in corticosterone concentrations after birds were returned to their nestbox following the 60 min samples was calculated using the formula:
Rate of change = (corticosterone concentration in last sample - 60 min corticosterone concentration at 60 min)/ time elapsed between 60 min sample and last sample.
Relationships between the rate of change in corticosterone concentrations after birds were returned to their nestbox and corticosterone concentrations at 60 min were determined using linear regression.

The total area under each corticosterone response curve was determined in Prism using the trapezoid rule and was termed the integrated corticosterone response (Cockrem and Silverin, 2002). Relationships between the integrated corticosterone response from the time each bird was returned to its nestbox at 60 min and the last sample and the integrated corticosterone response from 0 to 60 min were determined using linear regression.

Data are presented as individual values or as mean ± S.E.

2.3 Results

2.3.1 Corticosterone concentrations in initial samples

The time from capture until the completion of blood sampling was recorded in min and seconds then converted to a decimal value. This time ranged from 1.7 to 12.3 min (mean 6.3 ± 0.7 min). There was a significant linear regression relationship between corticosterone concentrations and initial blood samples and the time taken to collect the samples ($r^2 = 0.286$, $p < 0.001$; Fig. 2.1). The y intercept of the linear regression (the predicted corticosterone concentration at 0 min) was 0.87 ± 4.40 ng/ml. The corticosterone concentrations in the first samples are therefore called initial concentrations and are not considered to represent corticosterone concentrations in undisturbed penguins.

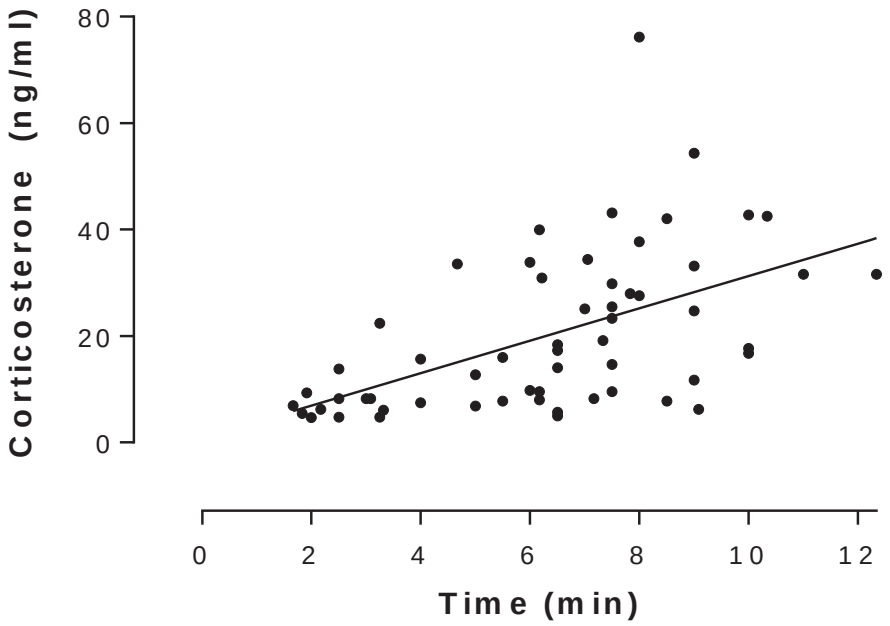


Fig. 2.1. Linear regression of corticosterone concentrations in initial blood samples versus time taken to collect the samples. $n = 58$.

2.3.2 Individual variation in corticosterone responses to 60 min handling and restraint followed by return of birds to their nestbox

There was a wide range of corticosterone responses to 60 min handling and restraint in little penguins (Fig. 2.2). Initial corticosterone concentrations varied from 4.69 to 54.32 ng/ml, except for one bird with a very high concentration of 76.12 ng/ml. The widely ranging and high initial concentrations in samples that took up to 12.3 min to collect indicate that these values reflect variation between birds in their responsiveness to the stressor of handling. Corticosterone concentrations increased from the initial sample to 15 min in all birds except the bird that had a very high corticosterone concentration and its first sample. Concentrations continued to increase from 15 to 30 min in most birds, then from 30 to 60 min increased further, remained relatively constant or decreased. Corticosterone concentrations at 60 min varied widely and ranged from 15.34 to 210.80 ng/ml.

Corticosterone concentrations had increased in eight of 10 birds 15 min after they were returned to their nestbox (Fig. 2.2A), whereas concentrations remained relatively constant or decreased in nine of 10 birds 30 min after return to their nestbox (Fig. 2.2B) and had declined in all birds sampled 60 or more min after return to the nestbox (Figs. 2.2C, D, E and F). The range of concentrations 30 min after return to nestbox was 22.44 to 181.2 ng/ml, whilst the range 60 min after return was 5.02 to 114.00 ng/ml and the range after 120 min was 4.37 to 51.60 ng/ml. Corticosterone concentrations 360 min after return to the nestbox were from 5.83 to 26.74 ng/ml and were within the range of initial concentrations.

2.3.3 Duration of corticosterone responses

Mean plasma corticosterone concentrations changed over time in all groups of penguins (Fig. 2.3; see Table 2.1 for statistics and Appendix Tables 2 for mean values). Mean plasma corticosterone concentrations significantly increased from 0 min to 15 min in all groups, did

not change significantly from 15 to 30 and from 30 to 60 min in four groups and increased from 15 to 30 min and not from 30 to 60 min in two groups. Mean corticosterone concentrations 15 and 30 min after birds were returned to their nestbox were not significantly different from concentrations at 60 min, whilst mean concentrations 60 min and longer after birds were returned to nestbox had declined significantly from concentrations at 60 min. Mean corticosterone concentrations 120 min or longer after birds were returned to their nestbox were not significantly different from initial corticosterone concentrations.

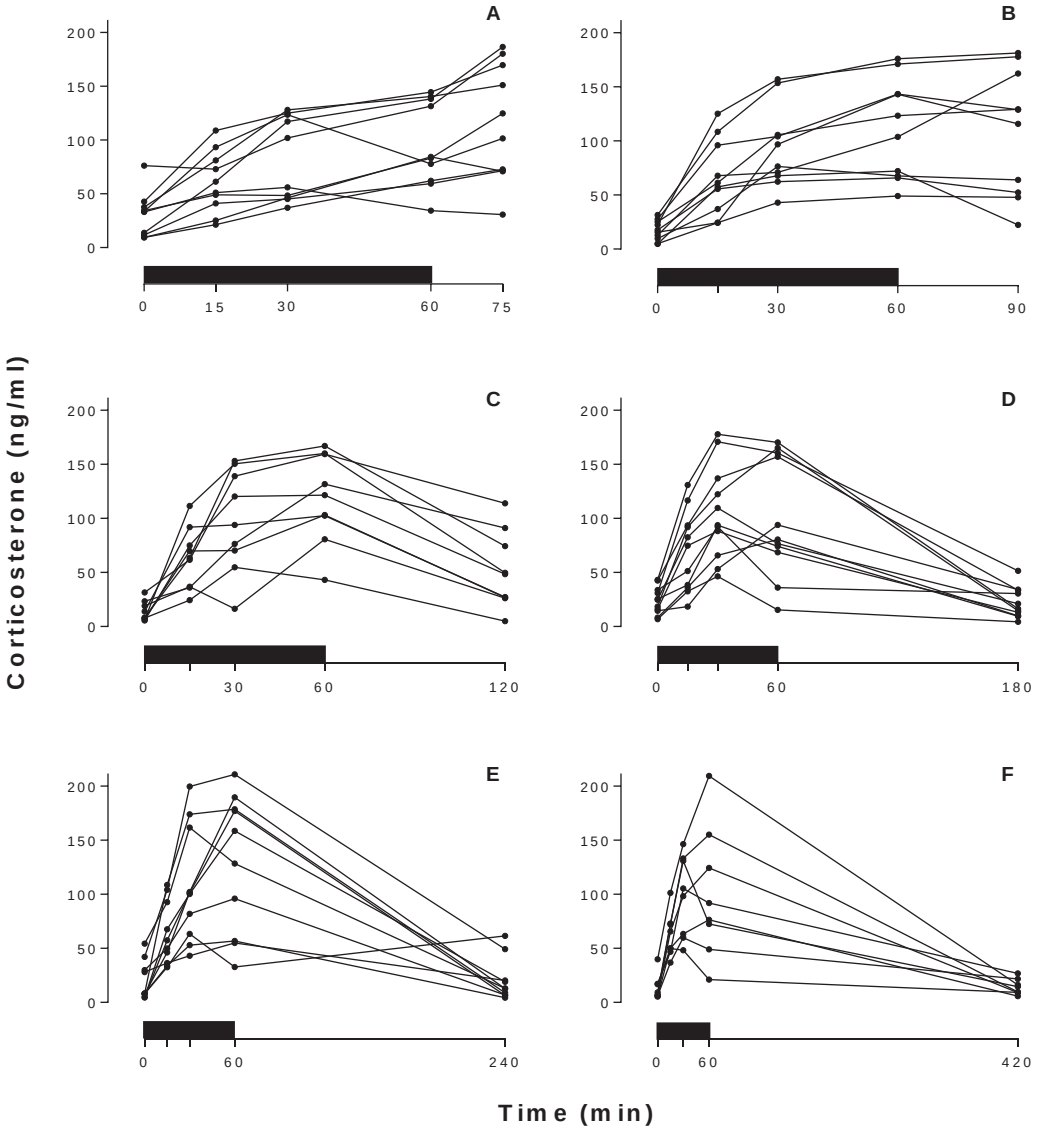


Fig. 2.2. Individual plasma corticosterone responses of little penguins. The black bar on the x-axis represents the 60 min duration of handling and restraint. Birds were returned to nestboxes and a final sample collected 15 (A), 30 (B), 60 (C), 120 (D), 240 (E) or 360 (F) min later. Sample sizes were 10, 10, 9, 11, 10 and 8 respectively.

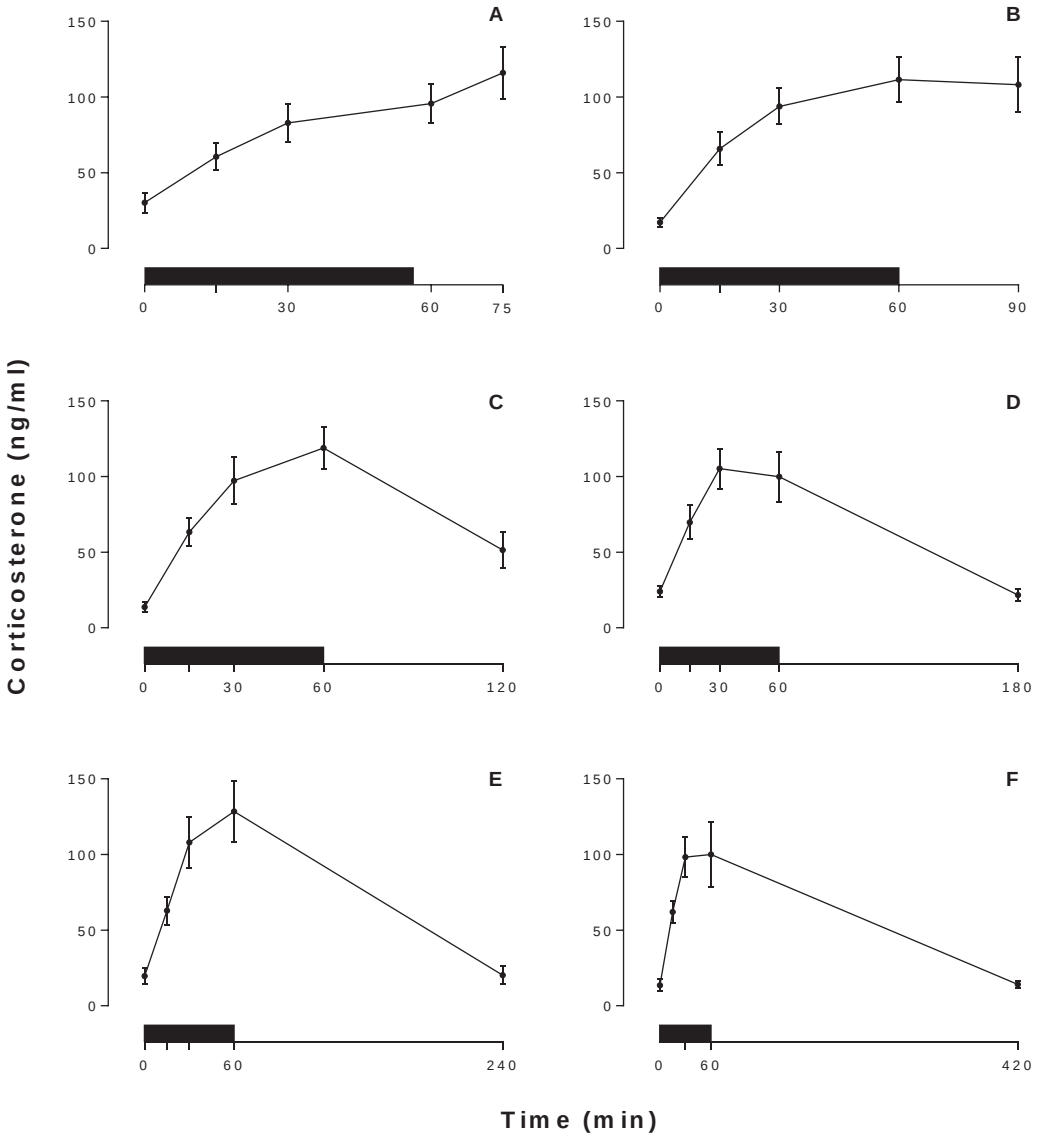


Fig. 2.3. Mean (± SE) plasma corticosterone responses of little penguins. The black bar on the x-axis represents the 60 min duration of handling and restraint. Birds were returned to nestboxes and a final sample collected 15 (A), 30 (B), 60 (C), 120 (D), 240 (E) or 360 (F) min later. Sample sizes were 10, 10, 9, 11, 10 and 8 respectively.

Table 2.1. Statistical analyses of plasma corticosterone concentrations in little penguins sampled 15, 30, 60, 120, 180 or 240 min after returned to their nestbox following a standard 60 min corticosterone response sampling protocol.

	F	Degrees of freedom	*p*
Time of last sample after return to nestbox			
15 min	25.18	4, 36	<0.0001
30 min	47.44	4, 36	<0.0001
60 min	24.58	4, 32	<0.0001
120 min	42.35	4, 40	<0.0001
180 min	26.96	4, 36	<0.0001
240 min	42.14	4, 28	<0.0001
Comparisons between times for each group. Note that the times are time after the initial sample.			
Last sample 15 min after return to nestbox			
0 vs. 15 min		1, 36	<0.0001
15 vs. 30 min		1, 36	Ns
30 vs. 60 min		1, 36	Ns
60 vs. 75 min		1, 36	Ns
0 vs 75 min		1, 36	<0.0001
Last sample 30 min after return to nestbox			
0 vs. 15 min		1, 36	<0.0001
15 vs. 30 min		1, 36	Ns
30 vs. 60 min		1, 36	Ns
60 vs. 90 min		1, 36	Ns
0 vs 90 min		1, 36	<0.0001
Last sample 60 min after return to nestbox			
0 vs. 15 min		1, 36	<0.0001
15 vs. 30 min		1, 36	Ns
30 vs. 60 min		1, 36	Ns
60 vs. 120 min		1, 36	<0.001
0 vs 120 min		1, 36	<0.001
Last sample 120 min after return to nestbox			
0 vs. 15 min		1, 36	<0.0001
15 vs. 30 min		1, 36	<0.05
30 vs. 60 min		1, 36	Ns
60 vs. 180 min		1, 36	<0.0001
0 vs 180 min		1, 36	Ns

	F	Degrees of freedom	*p*
Last sample 180 min after return to nestbox			
0 vs. 15 min		1, 36	<0.0001
15 vs. 30 min		1, 36	Ns
30 vs. 60 min		1, 36	Ns
60 vs. 240 min		1, 36	<0.0001
0 vs 240 min		1, 36	Ns
Last sample 360 min after return to nestbox			
0 vs. 15 min		1, 36	<0.0001
15 vs. 30 min		1, 36	<0.05
30 vs. 60 min		1, 36	Ns
60 vs. 420 min		1, 36	<0.0001
0 vs 420 min		1, 36	Ns

Note: The first six rows show the results of one way repeated measures ANOVA for the effects of time on plasma corticosterone concentration in each group. The remaining rows show the results of Sidak's comparisons of times in each group.

Mean plasma corticosterone concentrations of all penguins are shown in Fig. 2.4. The 0 to 60 min data are for birds in all six groups shown in Fig. 2.3 combined, whilst mean concentrations thereafter are for bird shown in each of the six panels in Fig. 2.3. The figure shows the full duration of the corticosterone response of penguins to 60 min of handling and restraint. Plasma corticosterone concentrations had not changed significantly 15 and 30 min after birds were returned to their nestbox, declined after 60 min and continued to decline to reach concentrations not significantly different from initial concentrations 120 min after birds were returned to their nestbox. Corticosterone concentrations did not change significantly between 120 and 360 min after birds were returned to their nestbox.

The mean corticosterone concentrations for the duration of the corticosterone response are expressed as percentages of mean concentrations at 60 min in Fig. 2.5. Mean corticosterone concentrations 15 min after birds were returned to their nestbox had increased increased to

118.7% of concentrations at 60 min. Mean concentrations decreased to 94.2% at 30 min, 39.3% at 60 min and ≤30.1% of concentrations at 60 min from 120 min onwards after birds were returned to the nestboxes.

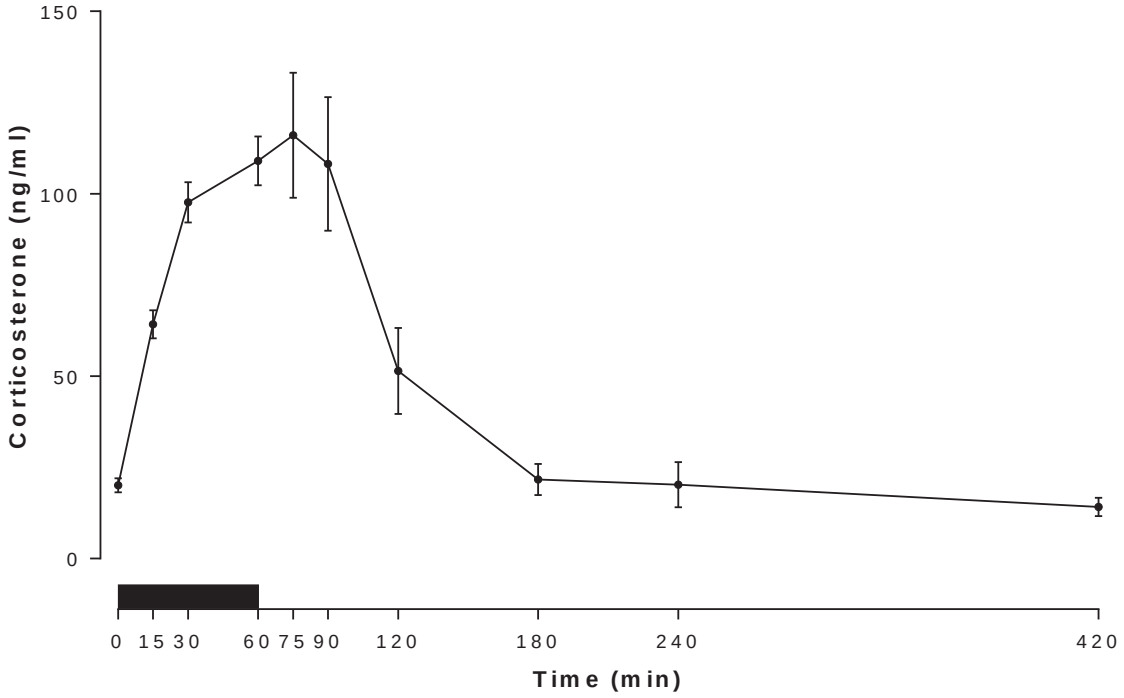


Fig. 2.4. Mean (±SE) plasma corticosterone responses of little penguins returned to their nestbox after 60 min of handling and restraint (the black bar on the x-axis represents this 60 min period). Birds were returned to nestboxes and a final sample collected 15 to 360 min later. Sample sizes were 0 to 60 min, *n* = 58; 75 min, *n* = 10; 120 min, *n* = 9; 180 min, *n* = 11; 240 min, *n* = 10; 420 min, *n* = 8.

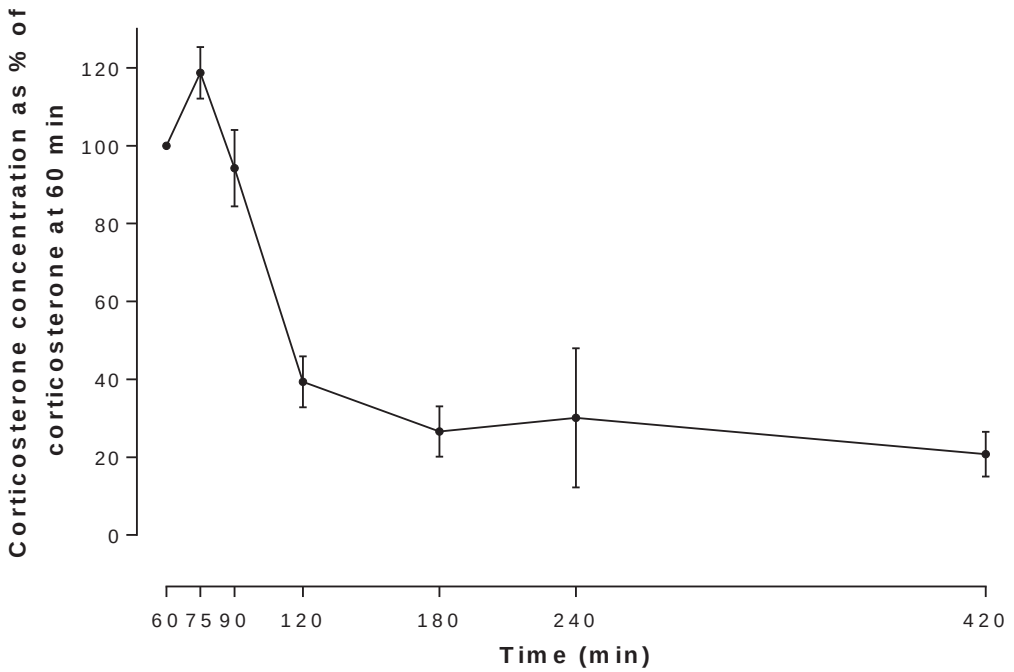


Fig. 2.5. Mean corticosterone concentration as % of mean corticosterone concentrations at 60 min.

2.3.4 Corticosterone in last sample in relation to corticosterone at 60 min

There were significant correlations between corticosterone concentrations in the last sample and corticosterone at 60 min for birds sampled 15, 30 and 60 min after return to their nestbox (r^2=0.893, p<0.0001; r^2=0.772, p=0.0008; r^2=0.644, p=0.0093; see Appendix Fig. 1). Corticosterone concentrations in the last sample and corticosterone at 60 min were not related for birds sampled 120, 180 and 360 min after return to their nestbox. Similarly, there were significant relationships between the ranks of corticosterone in the last sample and ranks of corticosterone at 60 min for birds sampled 15, 30 and 60 min (r^2=0.611, p=0.0075; r^2=0.669, p=0.0038; r^2=0.723, p=0.0037; see Appendix Fig. 2) but not 120, 180 and 360 min after return to their nestbox.

2.3.5 Rate of change of corticosterone and integrated corticosterone responses

There were significant correlations between the rate of change of corticosterone between 60 min and the time of the last sample and corticosterone concentrations at 60 min for birds sampled 120, 180 and 360 min (r^2=0.936, p<0.0001; r^2=0.925, p<0.0001; r^2=0.987, p<0.0001; see Fig. 2.6) but not 15, 30 or 60 min after return to their nestbox.

There were significant correlations between integrated corticosterone responses from 0 to 60 min and integrated corticosterone responses between 60 min and the time of the last sample for birds sampled 15, 30, 60, 120, 180 and 360 min after return to their nestbox (r^2=0.746, p=0.0013; r^2=0.854, p=0.0001; r^2=0.672, p=0.0069; r^2=0.820, p=0.0001; r^2=0.789, p=0.0006; r^2=0.910, p=0.0002; see Fig. 2.7).

2.3.6 Breeding stages

A two-way repeated measures ANOVA showed that there were significant differences between breeding stages and between times for plasma corticosterone concentrations at 0, 15 and 30 min in winter nonbreeding, prelaying and early chick rearing birds (Fig. 2.8, see Table 2.2 for statistics). Mean corticosterone concentrations at 0 min were higher in prelaying birds than in nonbreeding birds (20.12 $\pm$ 1.96 cf. 13.91 $\pm$ 0.90 ng/ml). Whilst corticosterone concentrations at 15 min appeared to be lower in early chick rearing birds than in birds at other breeding stages there was no significant difference between stages. Mean corticosterone concentrations at 30 min were lower in higher in early chick rearing birds than in nonbreeding birds and prelaying birds (54.31 $\pm$ 6.85, 98.25 $\pm$ 4.34 and 97.68 $\pm$ 5.59 ng/ml), and did not differ between nonbreeding and prelaying birds.

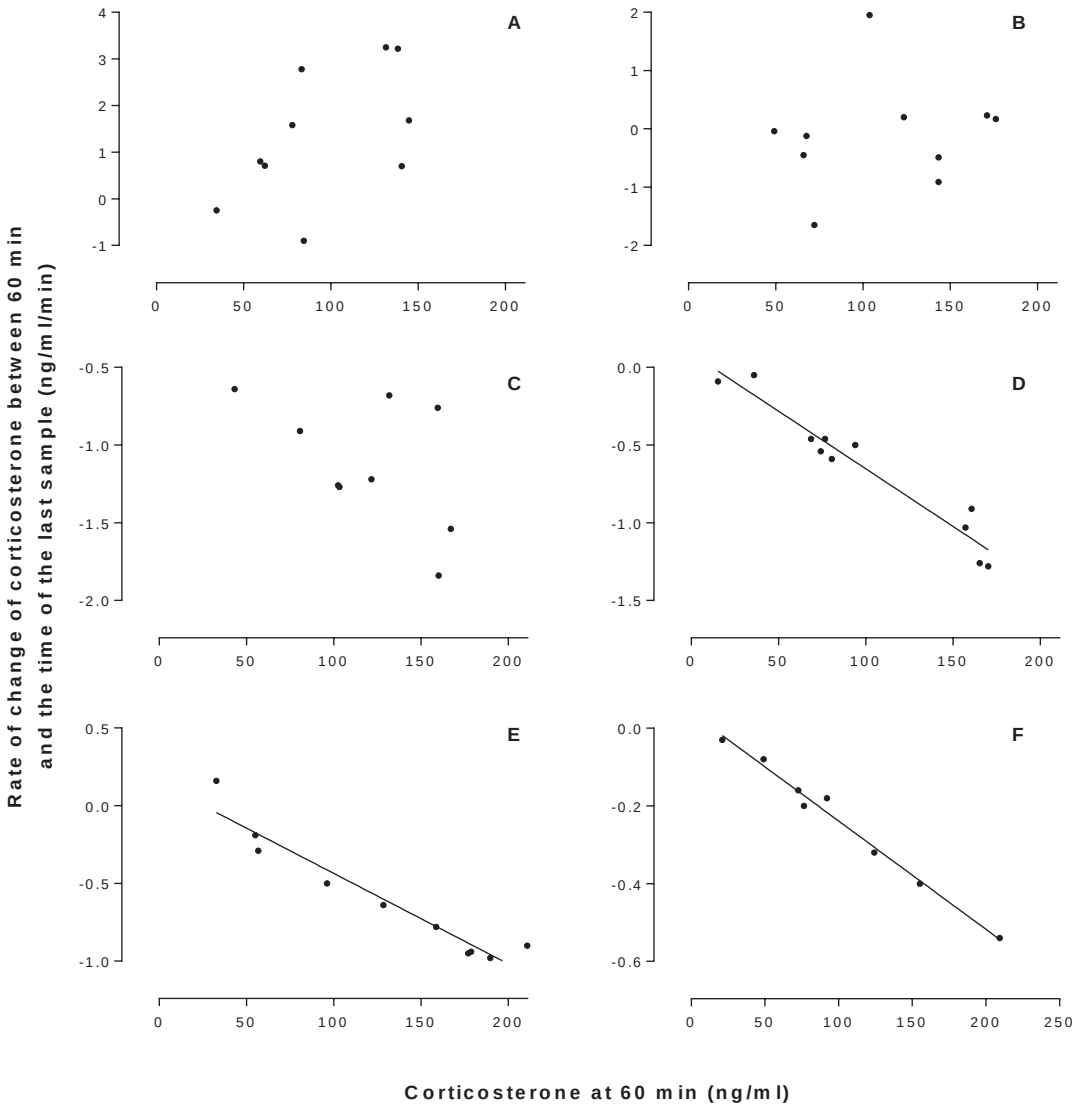


Fig. 2.6. Relationships between the rate of change of corticosterone between 60 min and the time of the last sample and corticosterone concentrations in the last sample. Last samples were collected 15 (A), 30 (B), 60 (C), 120 (D), 240 (E) or 360 (F) min after birds were returned to their nestbox.

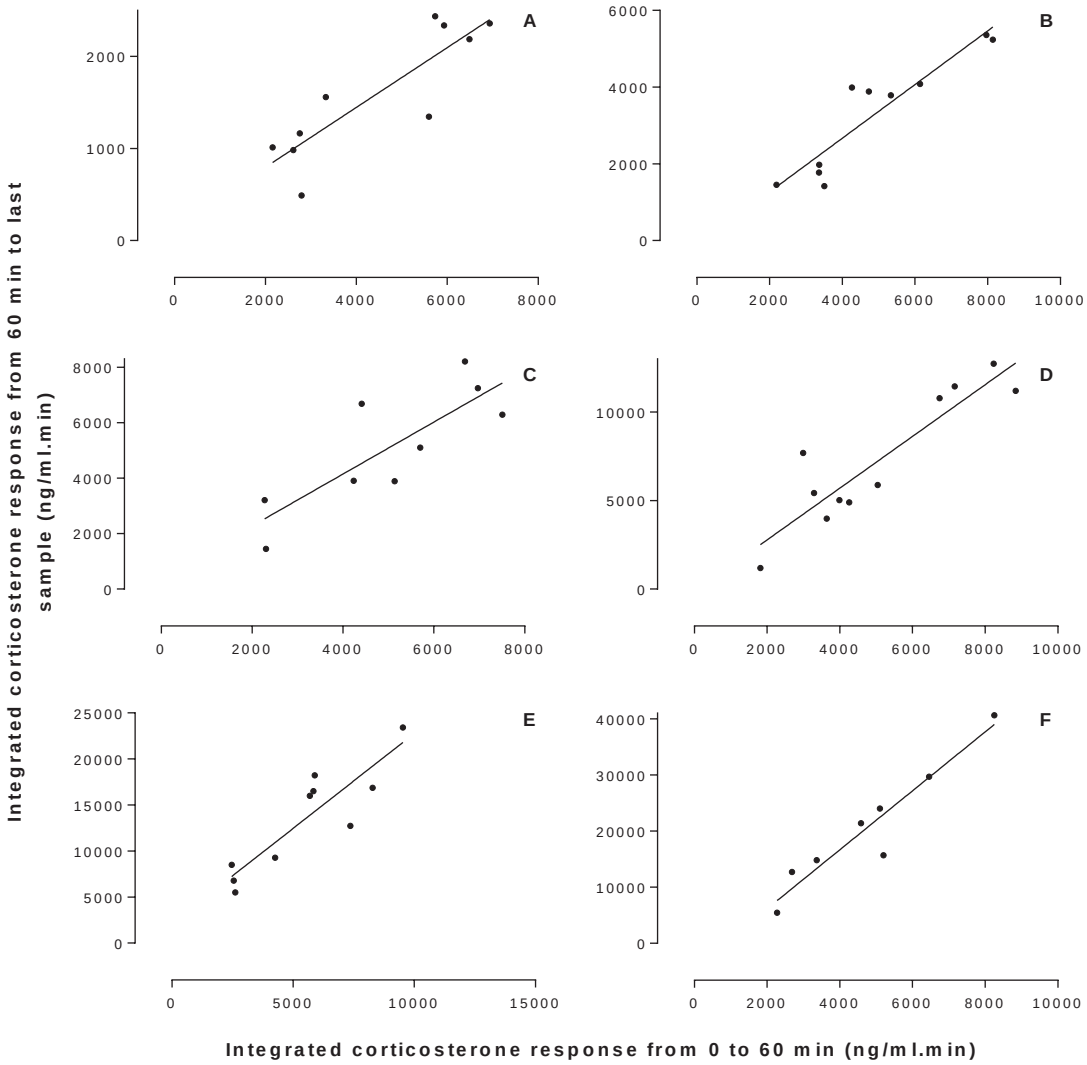


Fig. 2.7. Relationships between integrated corticosterone responses from 0 to 60 min and integrated corticosterone responses between 60 min and the time of the last sample. Last samples were collected 15 (A), 30 (B), 60 (C), 120 (D), 240 (E) or 360 (F) min after birds were returned to their nestbox.

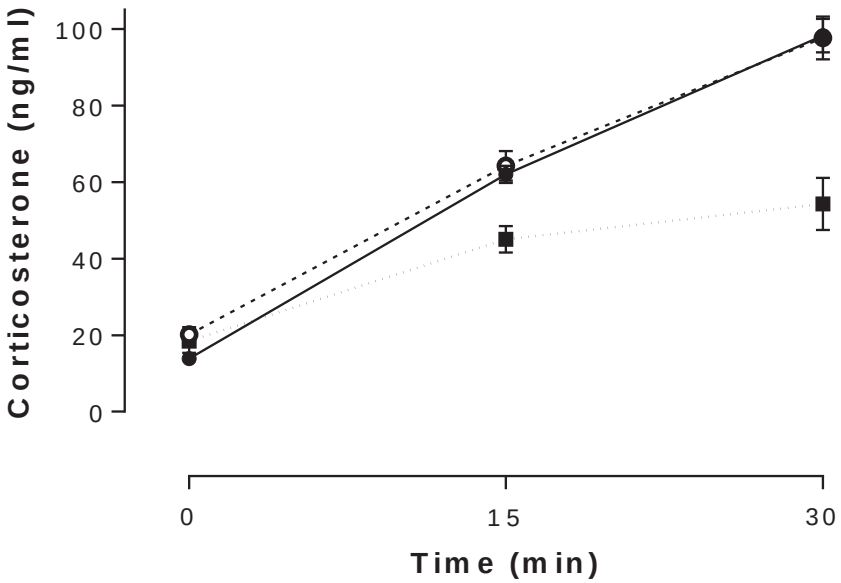


Fig. 2.8. Mean (± S.E.) plasma corticosterone responses of little penguins at three stages of breeding at Oamaru (nonbreeding in winter — ● — ; prelaying --- ○---; early chick rearing ··· ■···). Sample sizes were 96, 58 and 14 for nonbreeding in winter, prelaying and early chick rearing. Winter nonbreeding data are from Cockrem *et al.* (2016).

Table 2.2. Statistical analyses of plasma corticosterone responses in little penguins sampled at three stages of breeding.

	F	Degrees of freedom	*p*
Breeding stage	3.414	2, 165	0.0353
Time	405.8	2, 330	<0.0001
Interaction of breeding stage and time	8.815	4, 330	<0.0001
Comparisons between breeding stages times for each time.			
0 min			
Nonbreeding vs. prelaying		1, 495	0.0028
Nonbreeding vs early chick rearing		1, 495	0.2655
Prelaying vs early chick rearing		1, 495	0.9318
15 min			
Nonbreeding vs. prelaying		1, 495	0.9842
Nonbreeding vs early chick rearing		1, 495	0.1229
Prelaying vs early chick rearing		1, 495	0.1740
30 min			
Nonbreeding vs. prelaying		1, 495	0.9533
Nonbreeding vs early chick rearing		1, 495	0.0001
Prelaying vs early chick rearing		1, 495	0.0004

Note: The first three rows show the results of two way repeated measures ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc tukey's multiple comparisons between breeding stages for each time.

2.3.7 Corticosterone responses of male and female penguins

A two-way ANOVA showed that that were significant overall effects of both sex ($F_{1,50}$=19.39, p<0.0001) and time ($F_{3,50}$=167.50, p<0.0001) on plasma corticosterone concentrations. Mean corticosterone concentrations in male and female penguins were 25.24± 2.76 and 12.54 ± 2.32 ng/ml at 0 min, 73.84± 5.20 and 45.29± 3.91 ng/ml at 15 min, 109.76 ±7.48 and 71.18± 6.28 ng/ml at 30 min, and 121.97± 8.75 and 78.34± 9.19 ng/ml at

60 min (Fig. 2.9). There were significant differences between male and female penguins in corticosterone concentrations at 0, 15 and 60 min, whilst the difference at 30 min was not significant (p<0.0001, p=0.0192, p=0.0560 and p=0.0217 respectively).

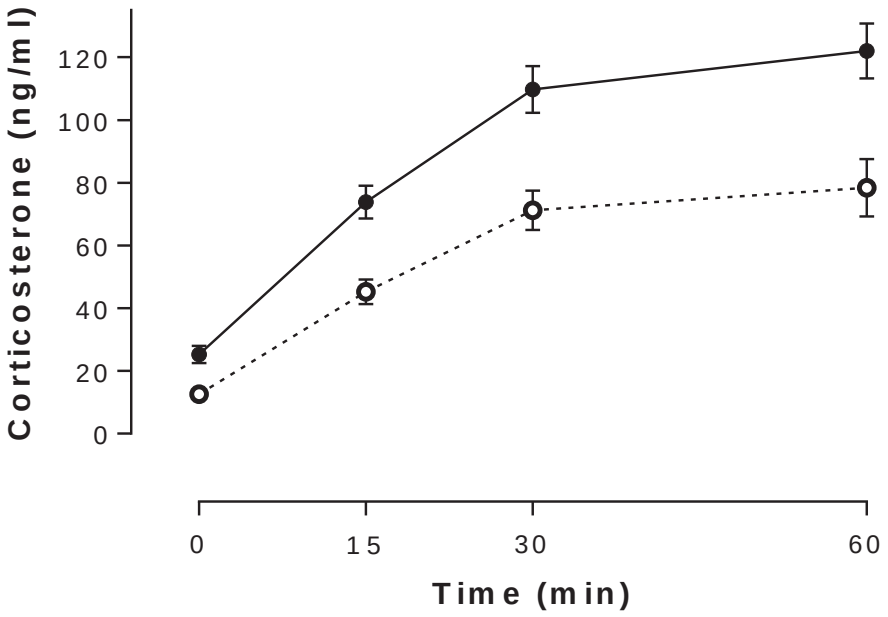


Fig. 2.9. Mean (± S.E.) plasma corticosterone responses of male (— ● —) and female (--- ○---) little penguins at Oamaru. Sample sizes were 24 and 18.

2.4 Discussion

This is the first study to report the total duration of the corticosterone responses of free-living birds. It is also the first to examine the relationship between the rates of increase and decline in plasma corticosterone concentrations in response to a stressor in birds. The study also described corticosterone responses of pre-breeding and breeding, and female and male little penguins. While there was substantial individual variation in the corticosterone responses of the penguins, mean plasma corticosterone concentrations had returned to near initial sampling levels 120 min after the stressors were removed. Furthermore, the rate of increase of corticosterone concentrations in response to a stressor was significantly correlated with the rate of decrease of corticosterone concentrations after removal of the stressor. Corticosterone responses of little penguins were greater in pre-breeding male than female penguins, and were greater in winter and pre-laying penguins compared with early chick rearing penguins.

2.4.1 Sampling time and initial corticosterone concentrations

It is common in the corticosterone literature for authors to refer to corticosterone concentrations in the first samples as baseline corticosterone, where baseline corticosterone is said to be the corticosterone concentration in an undisturbed bird. For little penguins, Carroll *et al.* (2016) considered plasma corticosterone levels in blood samples collected within four min of first handling reflect baseline levels for this species. Romero and Reed (2005) suggested that blood samples need to be taken within three min of capture to obtain baseline plasma concentrations of corticosterone because concentrations started to increase about three

min after initial handling. However, it has become apparent that corticosterone concentrations often increase within 2 min of the time when a bird is first disturbed, and corticosterone concentrations in the first blood sample collected from birds almost invariably reflect not the concentration in the undisturbed bird but instead include the beginnings of a corticosterone response to the stressor of being picked up. This view is supported by studies such as Baugh *et al.* (2013), Newman *et al.* (2017) and Small *et al.* (2017).

The mean time taken to obtain initial samples in the current study was 6 min 20 seconds and there was a significant relationship between corticosterone concentrations and time taken to collect the first blood sample. We therefore refer to plasma corticosterone concentrations in the first samples as initial corticosterone rather than baseline corticosterone, and note that these concentrations are higher than concentrations would have been before the birds were captured. In addition to the extended sampling time, the penguins may have been aware of our approach before they were removed from their nest boxes. This may have caused them to initiate a corticosterone response before they were first handled. Corticosterone concentrations in the last samples from some birds, particularly at 180, 240 and 420 min from the time of the first sample, were lower than corticosterone concentrations in initial samples in some birds. This observation indicates that the initial samples in these birds included responses of little penguins to the stressor of being handled for blood sampling.

2.4.2 Total duration of corticosterone responses of little penguins

The main aim of this study was to determine the total duration of corticosterone responses in little penguins and to determine changes in corticosterone concentrations after removal of the stressor. Mean plasma corticosterone concentrations increased rapidly from initial samples (0 min) to 15 min and from 15 to 30 min after penguins were removed from their nestboxes, then did not change significantly from 30 to 60 min. Plasma corticosterone concentrations in most birds had increased 15 min after they were returned to their nestboxes. Concentrations in most birds sampled 30 min after returned to their nestbox were similar to concentrations in the sample at the end of the 60 min handling and restraining. Corticosterone concentrations declined in all birds sampled 60 min or more after return to their boxes (see Fig. 2.2). Mean corticosterone concentrations of birs returned to the nestboxes for 60 min or longer were lower than mean concentrations in samples at the end of the 60 min stressor, and did not differ from mean concentrations in initial samples (Fig. 2.3). After penguins had been back in their nestboxes for 60 min the mean corticosterone concentrations had decreased to <40% of peak levels (Fig. 2.5). Plasma corticosterone concentrations for most of the birds had returned to initial sampling levels within 120 min of having been returned to their nest boxes and remained low thereafter (Fig. 2.2; Fig. 2.4). The total duration of corticosterone responses was thus two hours after the end of the 60 min stressor.

Complete corticosterone responses have been recorded in two other species of birds held in captivity (the northern brown kiwi (Apteryx mantelli) and the great tit (Parus major); Adams,

2000; Cockrem and Silverin, 2002). Kiwis were sampled after a handling stressor and great tits were sampled after exposure to the sight of a predator. Mean corticosterone concentrations were similar to initial concentrations two hours after the end of the stressor in both studies. The first samples collected after the end of the stressor were taken after two hours, so it is possible that mean corticosterone concentrations had returned to initial concentrations within two hours. It is nonetheless interesting that in all three species for which data is available (little penguins, kiwis and great tits) mean corticosterone concentrations had returned to initial concentrations at two hours after the end of the stressor.

Substantial variation in the corticosterone responses exists across different species of birds (Cockrem *et al.*, 2009). The magnitude of the corticosterone responses of little penguins, great tits, and northern brown kiwi are quite different (Fig. 2.10). Mean corticosterone responses of great tits were smaller than responses of the other two species. The great tits were exposed to a different stimulus (sight of a predator; Cockrem and Silverin, 2002) from the restraint experienced by the little penguins and northern brown kiwi. The type of stressor may influence the magnitude of mean corticosterone responses of birds (Cockrem and Silverin, 2002). Also, ecological and environmental factor may affect the magnitude and patterns of corticosterone responses of animals (Dickens *et al.*, 2009).

There is also extensive variation between wild and domestic species. In contrast to free-living birds, the corticosterone responses of domestic birds are smaller (Adams, 2000; Cockrem and Silverin, 2002; Cockrem *et al.*, 2009; Cockrem *et al.*, 2016). For example, the size of corticosterone responses of Japanese quail is smaller compared to free-living penguins, with peak corticosterone concentrations of Japanese quails being less than 25 ng/ml in the study by Hazard *et al.* (2008). Corticosterone concentrations in quail restrained for 10 min had returned to initial concentrations 50 min later (Hazard *et al.*, 2008).

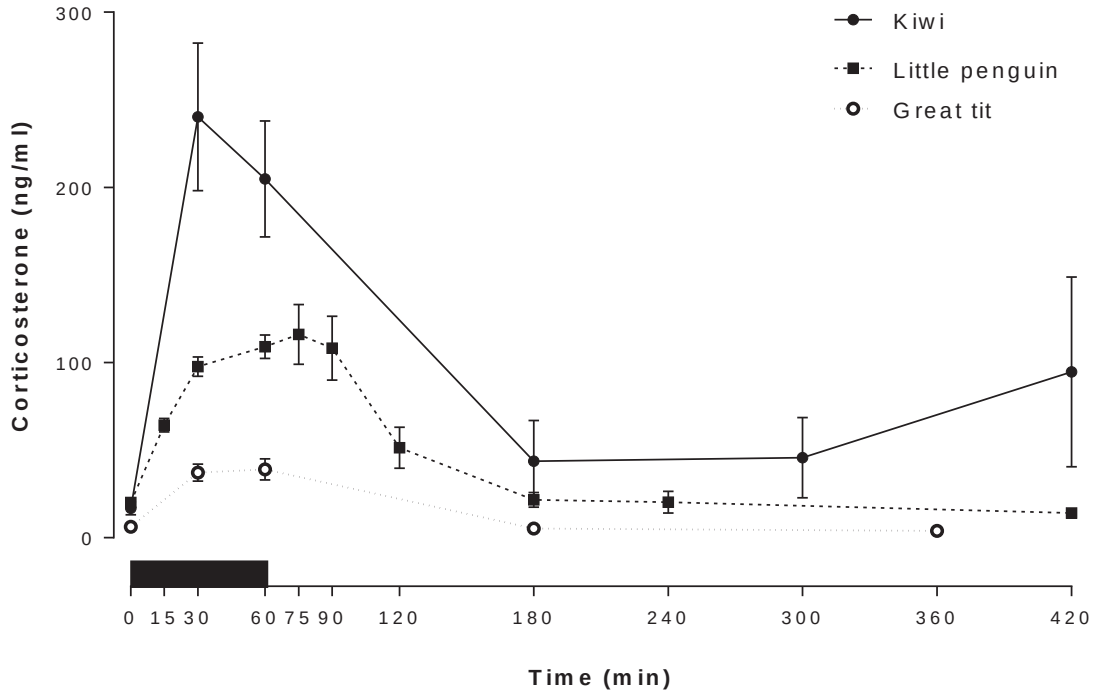


Fig. 2.10. The complete corticosterone responses of three species of birds. Black bars indicate the time length of birds being exposed to the stressors. Data for northern brown kiwi are from Adams (2000), and data for great tits are from Cockrem and Silverin (2002).

2.4.3 Individual variation in corticosterone responses

Previous studies have focused primarily on the mean corticosterone response of a population; but in recent years individual variation in corticosterone responses has received greater attention. The corticosterone response of an individual can differ markedly from the mean corticosterone response of a population. In the current study, although mean corticosterone concentrations had returned to initial levels 120 min after birds were returned to their nestboxes (Fig. 2.4), corticosterone remained elevated in some birds (Fig. 2.2). Variation between birds was also apparent when birds were returned to their nest boxes for 15 or 30 min (Fig. 2.2), with corticosterone concentrations continuing to increase in some birds while started to decline in others. Additionally, our results highlight the variation among individuals in the rate in which plasma corticosterone concentrations decline.

These findings are consistent with those of Cockrem *et al.* (2016) who measured corticosterone responses of penguins in the same population at Oamaru. Individual variation in corticosterone responses has also been shown in laying hens (Littin and Cockrem, 2001), great tits (Chua and Cockrem, 2002), zebra finches (Wada *et al.*, 2008), Adelie penguins (Cockrem *et al.*, 2009) and many other species. Many factors could contribute to individual variation in the corticosterone responses of birds, including genetic factors and pre- and post-hatching experience (Cockrem, 2013b).

2.4.4 The rate of decline of corticosterone concentrations

One of the primary objectives of our study was to determine whether the size of the corticosterone responses over the first 60 min was related to the rate of corticosterone concentration decline after removal of a stressor. The results show that the higher the corticosterone concentrations at 60 min in little penguins, the faster the decline in corticosterone concentrations from 60 min to last samples (180 min, 240 min and 420 min). Individuals with a higher rank of corticosterone levels at 60 min also had a higher rank of corticosterone levels at 75, 90 or 120 min. Another significant finding in this study is that the total integrated corticosterone responses for the first 60 min were positively correlated with the integrated corticosterone responses from 60 min until the last sample. The integrated responses provide a measure of total corticosterone concentrations released over time which is related to the rates of secretion and clearance of corticosterone (Breuner *et al.*, 1999). Birds that reacted to a stressor with more pronounced corticosterone responses had greater rates of decline of corticosterone after the end of the stressor than birds with a less pronounced response. This relationship appears to have not been investigated using this method in other species. However, the results of the current study are in agreement with the findings of Liebl *et al.* (2013) that in house sparrows the increase in corticosterone concentrations in response to a stressor was correlated with the decrease in corticosterone concentrations in response to dexamethasone (a synthetic glucocorticoid which is injected into birds to determine feedback sensitivity to corticosterone). Individuals that released more corticosterone in response to stressors also reduced corticosterone secretion faster than the other individuals. The ability to

reduce corticosterone concentrations is one regulatory element of the stress response, like the ability to increase corticosterone concentrations. The strong correlation between the two regulatory elements might be a result of co-selection on the traits as they are functionally related (Liebl *et al.* (2013).

2.4.5 Corticosterone responses of pre-breeding and breeding birds

Seasonal variation has been investigated in many species but not in little penguins. Previous studies on the corticosterone responses of little penguins were performed during the non-breeding season in winter (Carroll *et al.,* 2016; Cockrem *et al.,* 2016). The current study compared the corticosterone responses between pre-breeding and breeding individuals, with wintering and pre-laying birds being considered as pre-breeding birds, and early-chick rearing birds being considered as breeding birds. Mean corticosterone responses of pre-breeding birds were greater than mean corticosterone responses of breeding birds (Fig. 2.8). There was no difference in mean corticosterone responses between wintering and pre-laying little penguins (Fig. 2.8). Similarly, corticosterone responses during pre-breeding were higher than breeding corticosterone responses in great tits (*Parus major*; Lattin *et al.*, 2016), red knots (*Calidris canutus islandica*; Reneerkens *et al.*, 2002), dusky flycatchers (*Empidona oberholseri*; Pereyra and Wingfield, 2003), snow buntings (*Plectrophenax nivalis*; Walker *et al.,* 2015b), Lapland longspurs (*Calcarius lapponicus*; Walker *et al.,* 2015b), tree swallows (*Tachycineta bicolor*; Lattin *et al.,* 2016) and Atlantic puffins (*Fratercula arctica*; Rector *et al.*, 2012) In contrast to these species and to little penguins, grey-faced petrels (*Pterodroma*

macroptera gouldi) showed higher responses during breeding compared to pre-breeding (Adams *et al.*, 2005).

Many hypotheses have been proposed to explain seasonal variation in corticosterone responses in birds, but most of them fail to explain the seasonal patterns of all species of birds. There is abundant opportunity to better understand the reasons behind seasonal variation in corticosterone responses. Additionally, future studies may investigate the corticosterone responses of little penguins during moulting to develop a fuller picture of the seasonal pattern of corticosterone responses of little penguins.

It is interesting that the current study found that the mean corticosterone responses did not differ between wintering (June and July 2012) and pre-breeding birds (later September 2016) even though they were sampled in different seasons and in different years. Cockrem *et al.* (2016) also reported that the mean corticosterone responses of little penguins sampled at the same time of the year in 2012 and in 2013 did not differ. This could be due to the little penguins living in a marine environment, which is relatively constant and stable (Cockrem *et al.*, 2016). It will be worthwhile to compare the full corticosterone responses across seasons and to determine if there are any seasonal changes in the ability to reduce corticosterone concentrations.

2.4.6 Sex differences in corticosterone responses

The current study found that the corticosterone responses of males were higher than females during the pre-breeding period (Fig. 2.9). This is consistent with the results reported by Cockrem *et al.* (2016), in which there was a significant difference in corticosterone responses between female and male little penguins in 2013. However, the same study also reported that there was no significant difference between the two sexes in 2012. Similarly, Carroll *et al.* (2016) reported that there are no sex differences in the corticosterone responses of little penguins. While these studies were performed during the non-breeding season, other studies also reported that there were no sex differences in the corticosterone responses of other penguin species during the breeding season, such as the Adelie penguins and Magellanic penguins (Fowler *et al.*, 1995; Vleck *et al.*, 2000).

It has been proposed that there will be little difference between males and females in corticosterone responses if both contribute equally to parental care during the breeding season (Carroll *et al.*, 2016), and bi-parental care is common in penguins. However, the current study found that pre-breeding females had lower corticosterone responses than males (Fig. 2.9). This may be due to unmated-males being mainly responsible for displaying and preparing nests to attract potential mates. Further work is required to examine sex differences in corticosterone response of little penguins during different times of year.

2.5 Conclusion

The results of this study demonstrate that the mean duration of corticosterone responses of little penguins after 60 min of handling and restraint is two hours, with corticosterone concentrations not significantly different from initial concentrations two hours after birds were returned to their nestboxes. Additionally, the rate of decrease in corticosterone concentrations after the end of a period of exposure to a stressor of stressors was significantly related to the rate of increase in corticosterone concentrations during exposure to the stressor. Future studies may look into the repeatability in the ability of little penguins to reduce corticosterone concentrations. It will also be worthwhile to investigate the relationships between the ability to reduce corticosterone concentrations and the fitness or health of little penguins.

Chapter 3 General discussion

3.1 Introduction

Almost all studies of corticosterone responses in birds report corticosterone concentrations whilst birds were experiencing stressor, but provide no information about corticosterone concentrations once birds are no longer experiencing stress the stressor. Free-living birds are caught and restrained, generally for up to 60 min, and then released. It would be difficult to recapture birds at defined times after release, so it is difficult to describe the time course of the corticosterone response and determine the time at which corticosterone concentrations have returned to initial values. The current study was conducted with free-living little penguins that were sampled in nestboxes. This provided an opportunity to sample birds at intervals after the stressor had ended, and thereby describe the full time course of corticosterone responses.

Mean corticosterone concentrations had not changed 15 and 30 min after birds were returned to the nestboxes. Concentrations declined significantly one hour after birds were returned to the nestboxes and after two hours the concentrations were not significantly different from initial concentrations. Mean corticosterone concentrations 30 min after birds were returned to their nestboxes were 94.2% of concentrations at the end of the 60 min period of restraint. Concentrations 60 min after birds were returned to their nestboxes had declined to 39.3% of concentrations at the end of the 60 min period of restraint. There is limited information on the metabolic clearance rate of corticosterone in birds. The metabolic clearance rate was 10.5 ml/min/kg in chicken and 44.6 ml/min/kg in duck (Carsia and Harvey, 2000). Metabolic clearance rate varies depending on species and changes according to physiological status and age (Carsia, 2015). The half-life (time required for hormone concentrations in blood to

reduce to half of original concentrations) of corticosterone in birds is considered to be less than 10 min (Carsia, 2015), as shown in studies on male quail (9.8 min; Kovács and Péczely, 1983)) and ducks (7.5 min; Holmes *et al.*, 1974). Corticosterone concentrations in penguins in the current study remained much higher than would have been predicted from the corticosterone half-life if secretion had stopped at the time when birds were returned to their nestboxes. Furthermore, mean corticosterone concentrations 15 min after birds were returned to their nestboxes (115.99±17.21 ng/ml) remained high and were not significantly different from mean concentrations at the end of one-hour sampling (109.02± 6.69 ng/ml).

The magnitude of corticosterone responses to a stressor is considered to be one of the physiological traits of coping styles (Cockrem, 2007; Koolhaas *et al.*, 2010). Birds that are considered to have reactive personalities have relatively high corticosterone responses and are relatively cautious and non-aggressive. In contrast, proactive individuals show low corticosterone responses, and are relatively bold and aggressive (Cockrem, 2007). Relatively high corticosterone responses to stressors are characteristics of animals that have relatively high sensitivity to stimuli from their immediate environment. It is animals with reactive personalities are thought to be better able to cope with changes in their environment than animals with proactive personalities and relatively low corticosterone responses (Cockrem, 2013a).

Discussions of corticosterone responses in birds and cortisol responses of mammals in relation to personality have considered the available data in which the glucocorticoids have been measured whilst animals were experiencing a stressor. The absence of data on the full time course of glucocorticoid responses means the ability of animals to reduce glucocorticoid secretion following removal of a stressor has not been considered. The rate at which an

animal can shut off its glucocorticoid response to a stressor may also indicate an individual's ability to cope with a stressor (Romero, 2004). As with the elevation of corticosterone concentrations in response to a stressor, the ability to reduce corticosterone concentrations varies among individuals. We have shown for little penguins that there is a relationship between the rates of increase and decrease in corticosterone concentrations, with individuals with more pronounced corticosterone responses being able to bring down corticosterone concentrations faster than those with less pronounced responses. There was also variation between individuals in the rate at which they reduced corticosterone concentrations and as yet we have no information about the repeatability of the shut off of the corticosterone response. Studies of full corticosterone responses will provide valuable information about the characteristics of a successful individual.

3.2 Major conclusions

The main aims of this study were to determine the total duration of the corticosterone responses to handling and restraint and to determine the relationship between the rates of increase and decrease of plasma corticosterone concentrations. The major conclusions from this study are:

1. The initial plasma corticosterone concentrations increase as the time taken to collect a blood sample increases. There were some extended sampling times in the current study, and the measured corticosterone concentrations in the initial samples were not considered to reflect corticosterone concentrations in undisturbed birds.

2. The total duration of the corticosterone responses of little penguins to handling and restraint was two hours after the birds were returned to their nestboxes at the end of a one-hour duration of a stressor.

3. The rate of decline in corticosterone concentrations after the removal of a stressor was strongly correlated to the magnitude of corticosterone concentrations in response to the stressors. Individuals with relatively high corticosterone responses to a stressor had relatively high rates of corticosterone decline.

4. There was marked individual variation in the magnitude of the corticosterone responses and in the rate of decline in corticosterone concentrations following removal of a stressor.

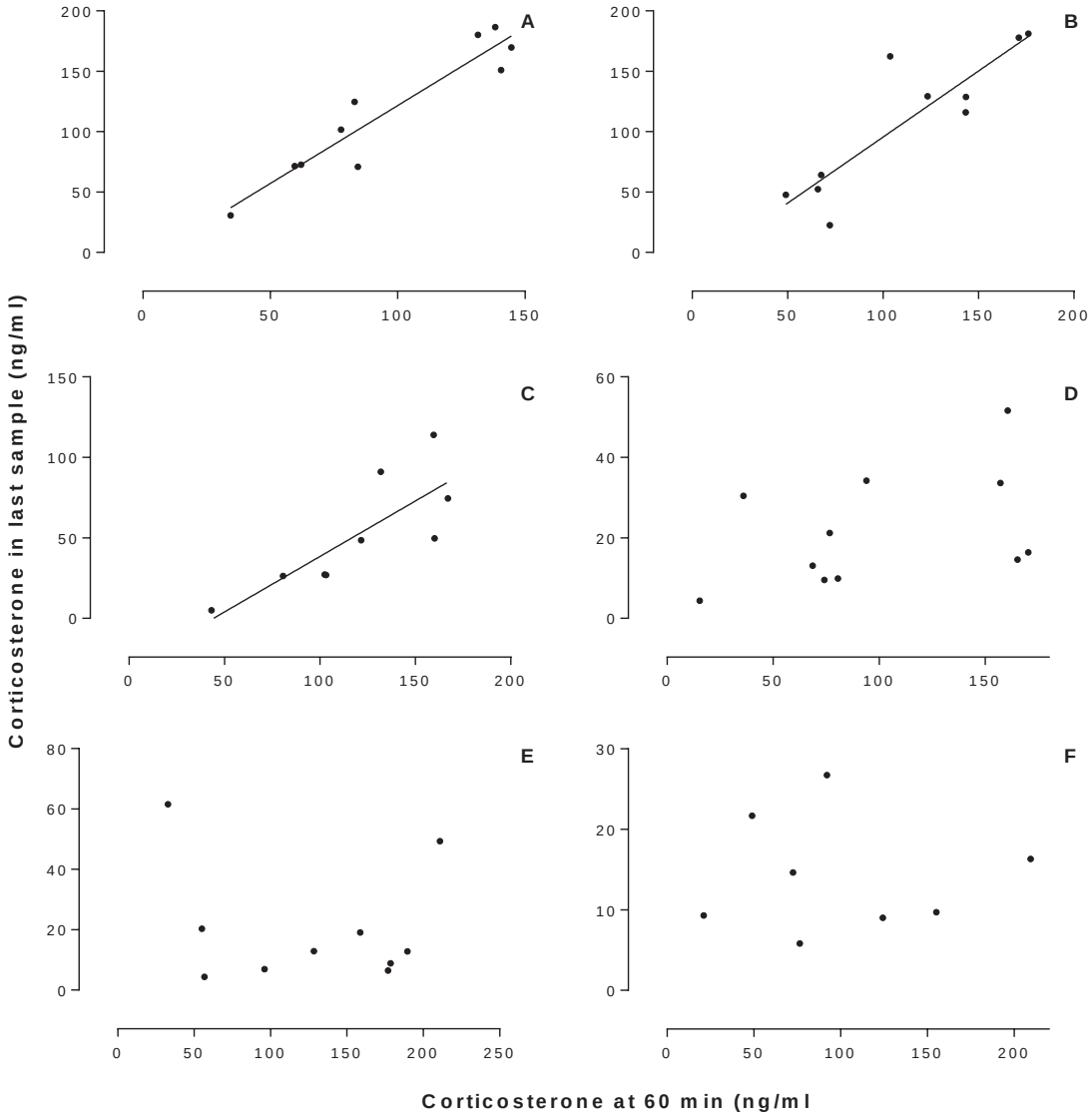
5. Corticosterone responses of little penguins did not differ between birds sampled in winter and birds sampled during the pre-laying period. Responses of birds sampled during early chick rearing were lower than responses of birds during winter or during the pre-laying period.

3.3 Future studies

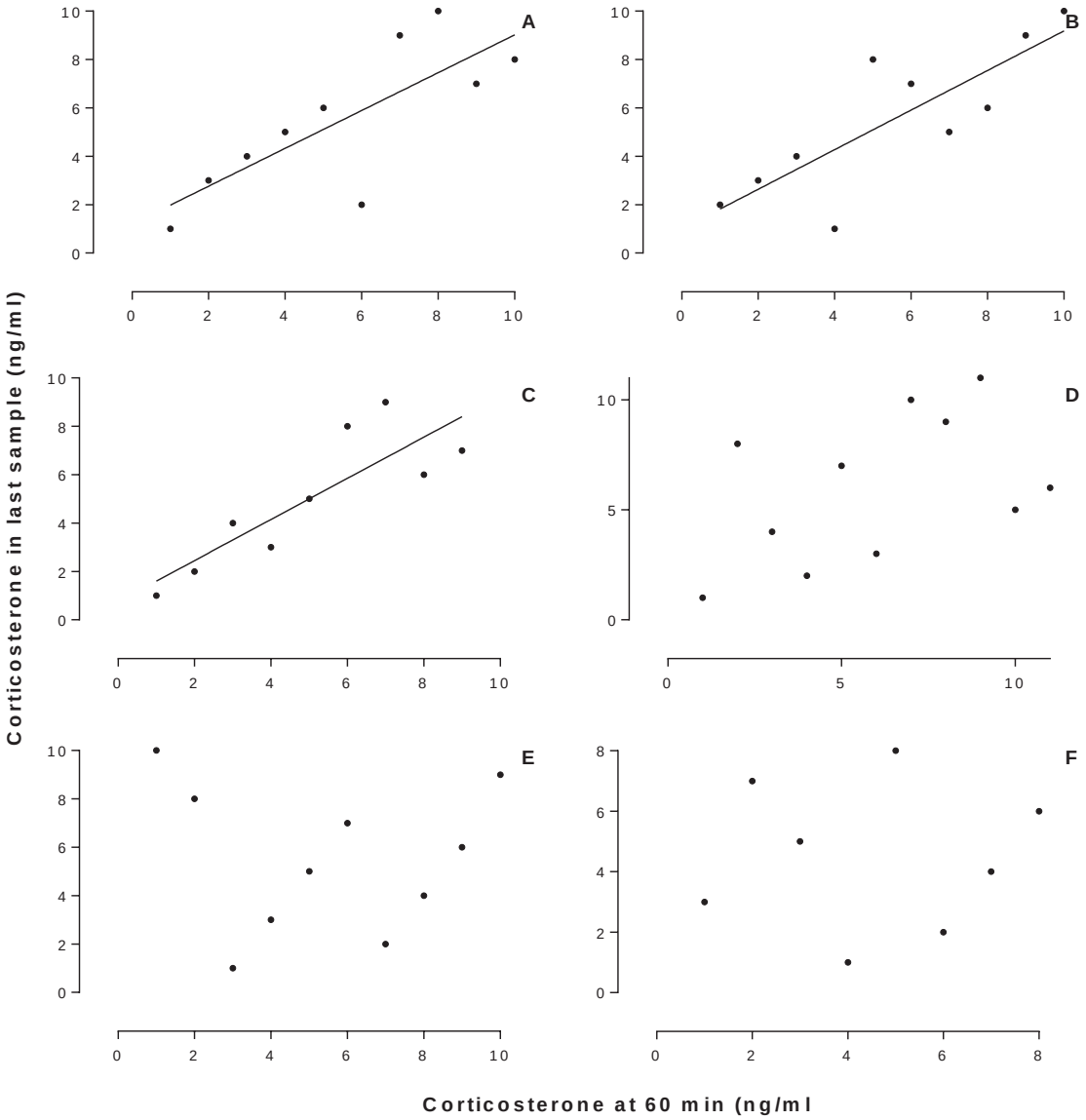
Many questions remained about the corticosterone responses of free-living animals. Many studies have proposed that delays in reducing corticosterone concentrations following removal of a stressor would adversely affect the health and fitness of animals. Further studies are required to determine the correlation between the rate of decline in corticosterone concentrations and the body mass or the age of animals. It would also be worthwhile to examine fitness in individuals that are able to reduce corticosterone concentrations quickly and individuals that are not able to do so. Previous studies have revealed that corticosterone responses of individuals to a stressor are repeatable and relatively constant across years

(Cockrem *et al.*, 2016). However, these studies only measured the repeatability in the increase of corticosterone concentrations in response to a stressor. Future work is required to determine the repeatability of the complete corticosterone response. It will be worthwhile to measure corticosterone responses of little penguins during moulting and in early winter to develop a full picture of seasonal variation in the corticosterone responses of little penguins. Corticosterone responses of male and female little penguins can also be compared at different stages of the annual cycle. Measurement of seasonal variation in the complete corticosterone responses of individuals would make it possible to determine whether the ability to reduce corticosterone concentrations changes across seasons.

Appendix



Appendix Fig. 1. Relationships between corticosterone concentrations in the last sample and corticosterone concentrations at 60 min. Last samples were collected 15 (A), 30 (B), 60 (C), 120 (D), 240 (E) or 360 (F) min after birds were returned to their nestbox.



Appendix Fig. 2. Relationships between the ranks of corticosterone concentrations in the last sample and the ranks of corticosterone concentrations at 60 min. Last samples were collected 15 (A), 30 (B), 60 (C), 120 (D), 240 (E) or 360 (F) min after birds were returned to their nestbox.

Appendix Table 1. Summary of studies that examined full corticosterone responses of birds.

Species	**Methods**	**Results**	**Notes**	**References**
Japanese quail *Coturnix coturnix japonica*	Blood sample of undisturbed birds were taken to measure basal corticosterone levels. Quail were held in a "crush cage" for 10 min. Birds were immediately bled then or returned to home cages for 10, 30, 50, or 110 min then bled.	Corticosterone levels increased significantly following exposure to stressor. After the birds were returned to home cages, corticosterone levels returned to basal within 10- 30 min.	The experiment tested corticosterone responses of two genotypes: long tonic immobility (LTI) and short tonic immobility (STI). STI birds had stronger corticosterone responses.	Hazard *et al*. 2008
Northern brown kiwi *Apteryx mantelli*	Birds were picked up and bled at 0, 30 and 60 min. Birds were kept in a box between sampling occasions. After the 60 min sample birds were returned to their nest box or burrow (exits were blocked). Blood samples were taken 2, 4, 6 or 8 h later.	Nocturnal house kiwi: Corticosterone returned to basal levels 120 min after returned to nest box or burrow. Outdoor penned kiwi: Corticosterone levels decreased 360 min after birds were returned to nest box or burrow but remained higher than basal levels.	The experiment tested responses of birds from different habitats.	Adams, 2005

Species	**Methods**	**Results**	**Notes**	**References**
Great tit *Parus major*	Birds were exposed to a stimulus object in their aviary for 30 min. Blood samples were collected at either 0, 0.5, 2 or 5 h after the end of the period of exposure to the stimulus.	Birds responded strongly to the predator stimulus, with a significant increase in corticosterone 30 min after exposure to stimulus. Corticosterone levels returned to basal 2 h after the end of the exposure to the stimulus.	Corticosterone levels did not change after birds were exposed to a brambling (a bird that is not a predator of great tits) or to a moving box.	Cockrem & Silverin, 2002

Appendix Table 2. Mean (± SE) plasma corticosterone concentrations of penguins sampled 0, 15, 30 and 60 min after handling and restraint began, and then sampled once after return to their nestbox after the 60 min stressor exposure.

Time of last sample after beginning of 60 min stressor	*N*	0 min	15 min	30 min	60 min	Last sample
75 min	10	30.23 ± 2.96	60.54 ± 9.05	82.88 ± 12.37	95.63 ± 12.60	115.99±17.21
90 min	10	17.24 ± 2.96	65.79 ± 10.91	93.77 ± 11.98	111.54 ± 14.68	108.19±18.29
120 min	9	13.73 ± 3.04	63.39 ± 9.34	97.26 ± 15.70	118.84 ± 13.72	51.44±11.76
240 min	11	24.06 ± 3.80	69.73 ± 11.14	105.22 ± 13.23	99.84 ± 16.51	21.73±4.28
360 min	10	19.59 ± 5.64	62.95 ± 9.22	108.04 ± 16.90	128.39 ± 20.29	20.25±17.84
420 min	8	13.51 ± 4.14	61.99 ± 7.24	98.21 ± 13.25	100.01 ± 21.47	14.16±2.53

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