The development of the corticosterone stress response of kororā (little penguin, *Eudyptula minor*) chicks in response to frequent handling

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Abstract

In birds, when a stressor is encountered, the hypothalamo-pitiutary-adrenal (HPA) axis is activated to produce the corticosteroid, corticosterone. The production of this hormone incites a flight or fight response in order to best avoid long-term damage from the stressor. However, prolonged or repeated exposure to high levels of corticosterone can have damaging behavioural and physiological effects. For this reason, high levels of the hormone should be avoided where possible in developing chicks. Chicks of altricial species in particular, seldom hatch with a highly functioning HPA axis so as to avoid these detrimental effects while their parents are still protecting them from major stressors such as predators, adverse weather and low food availability. Previously, studies have mapped the development of the HPA axis in a range of species or studied the effects of stress from a young age on future behavioural or physiological responses. This current study aimed to not only describe any adverse effects observed as a result of increased encounters with stressors but also track the development of the HPA axis in kororā (little penguin, *Eudyptula minor*) chicks.

Kororā chicks from the Oamaru Blue Penguin Colony were selected from both available sites and grouped based on their age at the time of sampling, 2 weeks old, 4 weeks old and 6 weeks old and a further two groups were formed for chicks that were handled twice a week from 2 weeks of age until 4 weeks or 6 weeks at which point they were sampled. Blood sampling and handling and capture stress responses were done following a modified standard protocol of a blood sample at 0 mins, 15 mins and 30 mins at which point they were returned to their nest to avoid over or under heating depending on the age and weather. The chicks being exposed to frequent human interactions were weighed twice a week and also had their flipper length and beak width, length and depth measured to graph the growth rates and body condition indices which has never previously been done in penguin chicks. It was found that frequent interactions with people did not have a significant effect on the stress responses when comparing previously handled chicks with unhandled chicks of the same age and that the previously handled chicks developed their adult-like stress response at a similar age to those that had not been previously handled. With regards to body condition indices and growth rates, this study showed that the parents leave the nest just after body condition indices drop to the low point in the chicks’ development.

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1. General introduction

1.1 Introduction

Avian chicks experience a range of stimuli from the environment as they develop from hatching to fledging. Some of these stimuli may be perceived as threats and lead to activation of the hypothalamo-pituitary-adrenal axis and the release of corticosterone. Corticosterone can have a range of effects in the short term and long term development of the chick (Cockrem, 2007; Busch and Hayward, 2009). Reactions of chicks of different ages to stressors have been studied in a range of species including northern mockingbirds (Sims and Holberton, 2000), grey-faced petrels (Adams *et al.,* 2008), Florida scrub-jays (Rensel and Schoech, 2011) and red-legged kittiwakes (Kitaysky *et al.*, 2001a) . There have been few studies on the corticosterone responses of Sphenisciforme, or penguin, chicks. The aim of this review is to summarise the available literature on corticosterone responses in developing chicks. There is a particular emphasis in this review on how corticosterone responses can change with developmental stages or can affect body condition.

1.2 Stress

1.2.1 Definition of stress

Stress in animals can be defined loosely as the body's response to any demand that could be physiologically threatening (Fink, 2010). This demand, or stressor, can be physical, interoceptive or psychological and can be either a short-term stressor (an acute stressor) or a long-term stressor (a chronic stressor). The body's response to a threatening stimulus can be both behavioural and physiological and often referred to as a "fight or flight" response (Boonstra, 2013).

1.2.2 Types of external stressors and how to measure stress

The stress response can be stimulated by variety of stressors. Stress responses and can be measured by taking blood or saliva samples to measure glucocorticoid concentrations. Cortisol is the main glucocorticoid in mammals other than rodents and in fish, and corticosterone is the main glucocorticoid in rodents, birds, reptiles and amphibians. Blood samples are spun to separate the plasma from the red blood cells. The plasma is then removed and glucocorticoid concentrations measured by radioimmunoassay. Plasma corticosterone concentrations in birds can vary greatly between species, populations and even individuals. Stress responses occur when animals respond to stressors. Examples of stressors include capture and handling (Adams *et al.*, 2008; Cockrem *et al.*, 2016), , food deprivation (Mora *et al.*, 1996; Kitaysky *et al.*, 2005; Walker *et al.*, 2005b), obligate siblicide (Tarlow *et al.*, 2001) or tourist encounters (Giese, 1996; Müllner *et al.*, 2004a; Kerbiriou *et al.*, 2009).

Note: corticosterone treatment or ACTH injections raise corticosterone concentrations but are not stressors as the complete HPA axis is not activated in response to internal or external stimuli in these situations.

1.2.3 Emergency life history stages

All organisms must adapt in order to survive and thrive in their ecosystem. Many changes to their ecosystem can be predicted and responded to appropriately; these predictable changes can also indicate the initiation of a different life history stage. Birds are able to transition between the breeding, non-breeding and moult stages based on predictable conditions such as photoperiod, rainfall and temperature (Wingfield and Kitaysky, 2002; Wingfield, 2013) (see Fig. 1). Transition into the emergency stage can occur at any time following a labile perturbation factor (LPF). When birds are in this stage energy mobilisation and diversion away from the current stage's priorities occurs and energy is used to maintain homeostasis in order to survive the unpredictable perturbation (Frigerio *et al.*, 2001; Wingfield and Kitaysky, 2002).

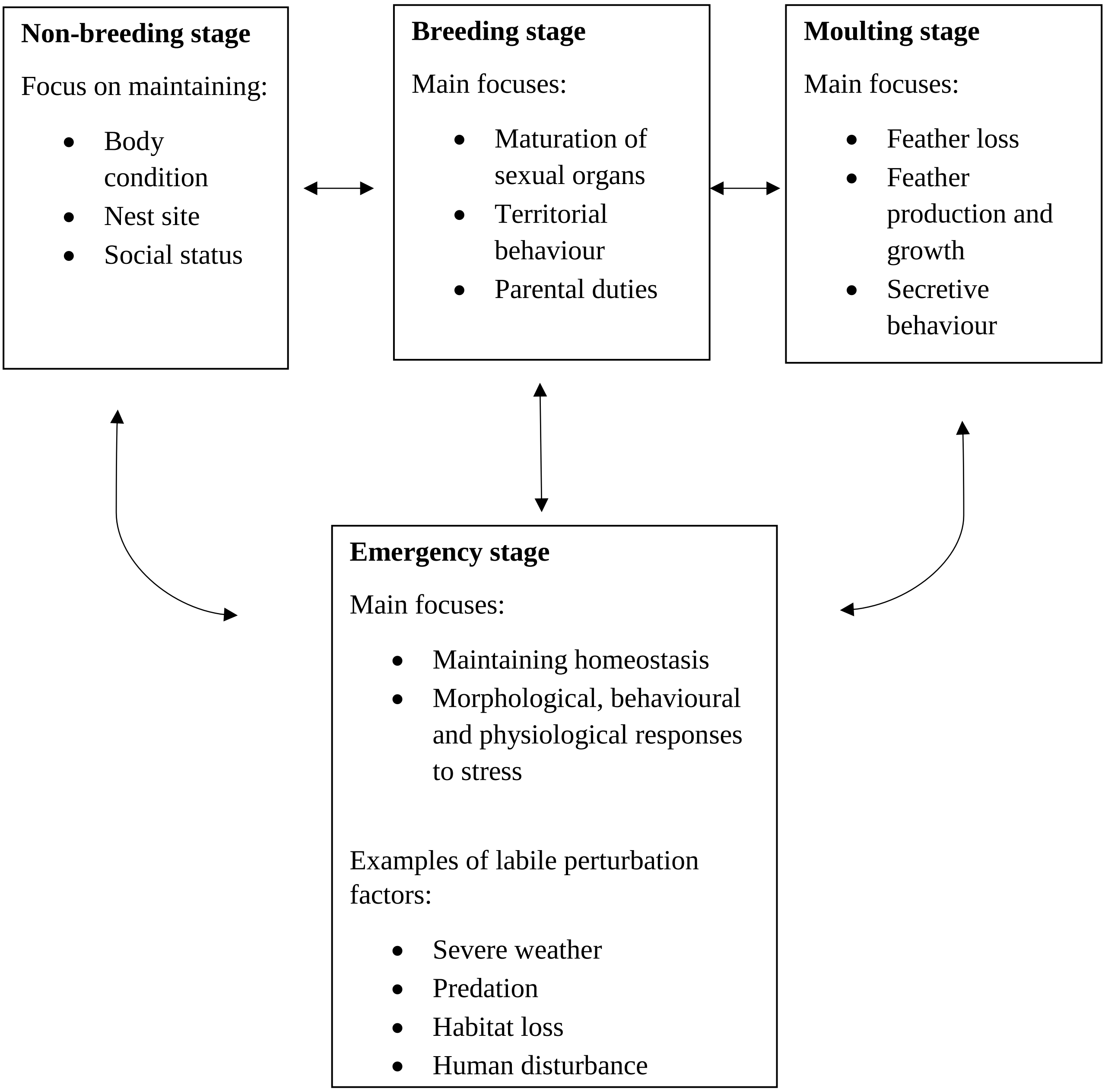

Fig. 1. Simplified series of life history stages in birds including the main focus during each stage and examples of unpredictable events or labile perturbation factors (LPFs) that can result in a transition from either the breeding or non-breeding stages to the emergency stage. Modified from Wingfield et. al (1998) and Wingfield & Kitaysky (2002).

Some birds live in “extreme” conditions such as deserts, alpine or arctic habitats and do not experience stress in conditions that would affect other birds (Martin and Wiebe, 2004), and the definition of extreme conditions requiring a switch to an emergency life history stage is different for each species (Wingfield *et al.*, 2011). Even the harshest of environments are not completely predictable or constant, neither is an environment completely unpredictable. This means that all organisms must adapt to suit their environment as best they can and once an LPF occurs, they must switch to the emergency stage and adapt their morphology, physiology and behaviour in order to survive (Wingfield and Kitaysky, 2002).

While many consider the response of an individual to an “emergency” is the flight-or-fight response, the response to transitioning into an emergency life history stage takes far longer than the seconds to minutes it takes an individual to enter into and return from the fight-or flight response (Wingfield *et al.*, 1998). The switch to the emergency life history stage in response to an LPF can take hours to develop and the resulting changes can take weeks from which to recover (Wingfield *et al.*, 1998). This is because the bird is responding to a stressor that threatens its survival meaning it must interrupt any other stage it is going through including abandoning nests and offspring in order to maintain its own long-term chance of survival and lifetime fitness (Wingfield *et al.*, 1995).

A bird can transition into the emergency life history stage in response to any LPF which may be an acute stressor or a chronic stressor. The duration of the stressor will determine how long the bird will be in the emergency life history stage and can affect the corticosterone concentrations. Increased concentrations of corticosterone can have lasting effects on the bird despite the bird transitioning back into another life history stage. If the life history stage was moulting for example, an elevated corticosterone concentration during an emergency life history stage could result in an inability to regrow feathers properly due to a breakdown of proteins (Romero *et al.*, 2005). The morphological, physiological and behavioural changes experienced during the emergency life history stage can be beneficial or detrimental to the bird and will be discussed in depth later in this chapter.

1.3 The hypothalamo-pituitary-adrenal (HPA) axis in birds

1.3.1 Components

The HPA axis (fig. 2) is a neuroendocrine system which has a number of different functions including regulating stress responses by releasing glucocorticoids (corticosterone in birds). The hypothalamus, the start of the system, is located in the brain and one of its most important functions as part of the HPA axis and the brain is to connect the nervous system to the endocrine system via the pituitary gland (hence the HPA axis is a neuroendocrine system). In response to a stressor, the hypothalamus releases corticotropin releasing hormone (CRH) from the hypothalamic paraventricular nucleus. When stimulated by CRH, the anterior pituitary gland releases adrenocorticotrophin hormone (ACTH) which stimulates the adrenal cortex to releases glucocorticoids (Boonstra, 2004). Glucocorticoids maintain homeostasis at multiple sites throughout the body to regulate processes such as immune function, metabolism, stress responses and reproductive system. To avoid over-exposure to glucocorticoids, which can have damaging effects (Walker *et al.*, 2005b), glucocorticoids on the hypothalamus and completes a negative feedback loop by shutting off the release of CRH.

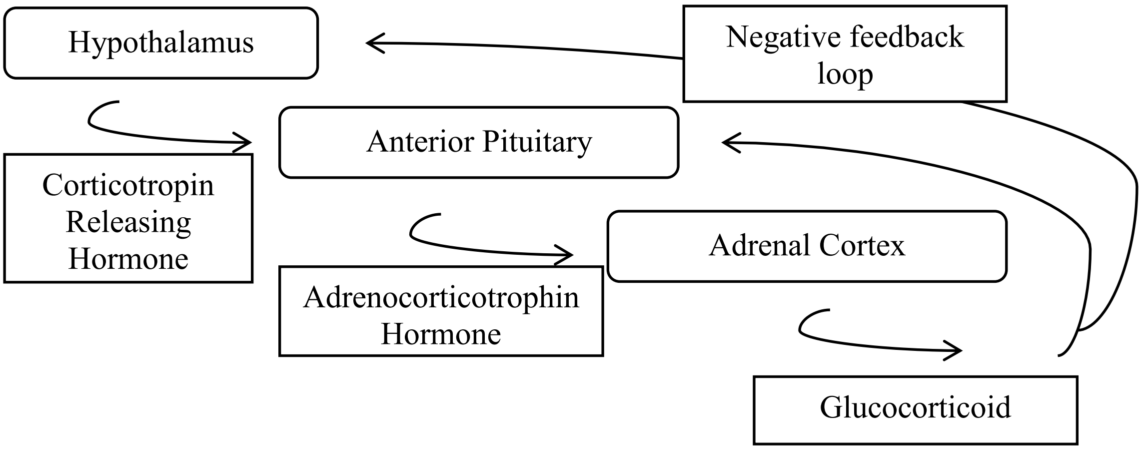

Fig.2. Schematic diagram of the HPA axis including organs and hormones involved and how they interact to create a negative feedback loop.

1.3.2 Corticosterone

Stress and fear are important primal emotions which, when experienced, start a physiological chain reaction to avoid any immediate danger. In birds, these primal emotions trigger the HPA axis (Cockrem, 2007) in order to release corticosterone which is the main glucocorticoid found in birds (Evans *et al.*, 2006). Corticosterone plays an important role in the functioning of a bird's HPA axis as it is the 'switch' that initiates the negative feedback loop that shuts off the hypothalamus and in turn, the axis if levels are sustained at a level which could be detrimental to the bird's wellbeing (Walker *et al.*, 2005b), these negative effects will be discussed in detail later in this review.

1.3.3 Function

It is believed that the stimulation of the HPA axis and the hormones it releases is used to change physiological and behavioural processes from current functions to perform life-saving responses such as the mobilisation of energy or increasing food intake (Sims and Holberton, 2000; Angelier *et al.*, 2008). As well as being beneficial in stressful situations, corticosterone also plays a part in some non-stressful processes necessary for development and survival including preparation for fledging and dispersal after hatching (Heath, 1997; Belthoff and Dufty, 1998). It is therefore crucial for offspring survival that the HPA axis is fully developed before fledging to not only prepare the body for fledging but to survive stressful situations the chick may encounter as an adult. In order to be complete by the time of fledging as well as being able to cope with stressful situations as a chick, the development of the HPA axis has to start before the chick has hatched, in the egg.

1.4 Development of the hypothalamo-pituitary-adrenal axis in chicks

1.4.1 Prenatal development

The development of the HPA axis of embryos inside eggs has been well studied due to the ease of access as the majority of the development occurs after the egg has been laid. The development of the axis does not happen in order (i.e. the hypothalamus, then the pituitary gland, then the adrenal cortex) and the three levels of the axis do not develop at the same rate or time (Table 1). The incubation period for chicken eggs is 22 days (Perry, 1988). By day five of embryonic development the adrenal glands are capable of secreting glucocorticoids when stimulated by ACTH (Pedernera, 1971). This indicates that the adrenal glands are functional at five days of development but they are not being stimulated by ACTH naturally. On day six, blood vessels connecting the tissues that give rise to the hypothalamus and the pituitary gland have been observed (Daikoku *et al.*, 1974). The connection of these tissues, which later make up the organs of the HPA axis, is the very beginning of developing the communication route used by these organs. By day seven of embryonic development, ACTH is naturally secreted (Jozsa *et al.*, 1979). The secretion of ACTH means that the anterior pituitary can start playing a role in the axis and ACTH can have an effect on the adrenal cortex. By day eight glucocorticoids can be naturally secreted by the adrenal cortex (Pedernera, 1971). At this point, the adrenal cortex is still capable of functioning independently from the anterior pituitary until between 12 and 14 days of development (Girouard and Hall, 1973). The beginning of the adrenal cortex's dependency on the rest of the axis is seen at around day 11 when supplementary cortisol injections result in negative feedback on ACTH (Kalliecharan and Buffett, 1982). Until day 14 of embryonic development, the adrenal cortex appears to be self-regulatory and can independently secrete corticosterone. At around day 14, the adrenal cortex loses this autonomy and is influenced by the anterior pituitary and by ACTH. Between days 14 and 16, the hypothalamic level of control becomes apparent and the HPA axis is morphologically completed and can self-regulate using the negative feedback loop (Wise and Frye, 1973). During development, each section of the HPA axis has a differing level of autonomy with the adrenal cortex having full autonomy as well as it being the last organ in the axis; the adrenal cortex can be controlled by the anterior pituitary, the next organ in the axis, which can then be controlled by the hypothalamus, the first organ in the axis.

Table 1. Timetable of prenatal development of HPA axis in chicken (*Gallus gallus domesticus*) chicks.

Approximate embryonic day	Developmental event
5	Adrenal cortex capable of secreting glucocorticoids when stimulated by ACTH
6	Blood vessels connect hypothalamus to pituitary gland
7	ACTH is naturally secreted
8	Glucocorticoids are naturally secreted
11	Negative feedback loop of glucocorticoids on ACTH occurs
14	HPA axis is naturally regulated

While the HPA axis is developing within the foetus, the foetus is developing within the yolk. The yolk is used by the developing foetus for sustenance and, before laying receives hormones such as corticosterone directly from the mother (Haussmann *et al.*, 2012). Despite being lipid soluble, corticosterone does not evenly distribute itself through the yolk (Hayward *et al.*, 2006). When freshly laid eggs were injected with corticosterone, it was found that corticosterone did not disperse evenly but rather concentrated at a small area at the top of the yolk (von Engelhardt *et al.*, 2009). It is thought this is in order to give the developing embryo the opportunity to synthesise extra corticosterone it receives corticosterone from the mother and potentially gain some control over the effects of elevated maternal stress (von Engelhardt *et al.*, 2009). This is plausible as the embryo develops at the top of the yolk in the same area as the corticosterone accumulates; this means that the embryo would be exposed directly to the corticosterone as opposed to corticosterone mixing through the yolk that is used by the embryo for sustenance throughout the incubation period. The developing embryo may have some control over the mother's corticosterone once it has been transferred to the egg. Maternal hormones are a necessary tool used to prepare and adjust the offspring for life outside of the egg in order to maximise fitness (Rettenbacher *et al.*, 2009).

1.4.2. Postnatal development

Life outside the egg can be testing on the HPA axis that was only fully developed approximately a week before hatching (Wise and Frye, 1973; Perry, 1988). Both altricial and precocial chicks go through developmental stages in the functioning of their HPA axis. These stages vary greatly along the altricial to precocial scale as well as between species (Table 2).

Altricial and semi-altricial chicks hatch at least somewhat dependent on their parents for protection from both the elements and predators as well as being dependent on food from their parents (Blas *et al.*, 2005). It has been suggested that because there is such a large environmental stress buffer from altricial chicks' parents that any response from the HPA axis early on in life would be detrimental to the chicks' survival when exposed to mild stressors in comparison to the stress they will experience later in life (Sims and Holberton, 2000). While there are many benefits to being exposed to corticosterone including reducing the physiological impact of the stressor and the mobilisation of energy to escape stressors (Walker *et al.*, 2005b), the costs of prolonged exposure can include decreased growth rates and compromised cognitive abilities later in life (Kitaysky *et al.*, 2003). It is thought that, because prolonged exposure can have such detrimental effects and the majority of environmental stressors are limited due to chicks being dependent on parents, altricial chicks have no adaptive advantage in having a responsive HPA axis early on in life (Sims and Holberton, 2000). Despite the apparent lack of need for an HPA response in altricial and semi-altricial chicks, it has been shown that an increase in corticosterone can increase aggressive and begging behaviours (Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003) both of which would be beneficial to the survival of the chick. The increase in begging behaviour especially would be a chick-specific response to an environmental stress indicating that there is a benefit to having an HPA stress response early on in life. This is likely due to the fact that, despite the parents' best efforts to mitigate any stressful factors, there will always be factors that are out of their control such as nest-mate competition, predators, parasites and variations in weather conditions and food availability (Blas *et al.*, 2005).

Many of the stressors encountered by altricial chicks also affect precocial and semi-precocial chicks. Precocial and semi-precocial chicks are largely capable of foraging, thermoregulating and surviving independent of their parents (Adams *et al.*, 2008). Precocial chicks still have the same costs and benefits of increased plasma corticosterone (Kitaysky *et al.*, 2003; Walker *et al.*, 2005b), do not require in extra begging behaviours, and yet may still benefit from aggressive behaviours (Kitaysky *et al.*, 2001b). Due to precocial chicks' ability to forage and feed themselves, they do not have to wait for their parents to get back with food but will still be at a disadvantage when food availability drops (Blas *et al.*, 2005). Precocial chicks are also susceptible to changes in weather, parasites and predators all of which would require a functioning HPA axis response to reduce the physiological impacts of these stressors (Blas *et al.*, 2005). It is, therefore, believed that because precocial birds hatch as much more independent birds, they have robust corticosterone responses from the time of hatching in order to avoid any environmental stressors including most of the ones that altricial chicks encounter as well as those that come with not being fed or protected by their parents.

Table 2. Postnatal development of corticosterone responses in chicks of different species.

Species	Age	Altricial or Precocial	Changes	Reference
Grey-faced petrel	2-4 days post-hatching until fledging (118 days)	Precocial	Mean corticosterone responses remained unchanged	(Adams *et al.*, 2008)
Northern Mockingbirds	5-7 days post-hatching until fledging (12 days)	Altricial	Stress response increased with age until a peak to adult levels just before fledging	(Sims and Holberton, 2000)
Greylag geese	Hatching until fledging (55 days)	Precocial	Response peaked at hatching and decreased thereafter	(Frigerio *et al.*, 2001)
White-crowned sparrows	1-3 days post-hatching until fledging (10 days)	Altricial	Hyporesponsive period at 1-3 days of age Response increases until a peak to adult levels just before fledging	(Wada *et al.*, 2007)

Species	Age	Altricial or Precocial	Changes	Reference
Domestic chickens	Embryos until hatching	Precocial	Hyporesponsive period at 1-7 days of age	(Wise and Frye, 1973)
American kestrels	10 days post-hatching until fledging (30 days)	Altricial	Stress response increases with age until they have adult levels a week before fledging Levels continue to increase and 28 day old nestlings had larger responses than 1 year old adults	(Love *et al.*, 2003)
Magellanic penguins	5-6 days post hatching until fledging (90-120 days)	Altricial	Baseline response higher as hatchling Response increases until fledging	(Walker *et al.*, 2005b)
Mallard ducks	From hatching until 180 days old (fledge at 50-65 days)	Precocial	Transient declines between days 4-14 Maximal increase in corticosterone was 1 day post-hatch and then decreased After 21 days post-hatch corticosterone increases significantly in response to stress	(Holmes *et al.*, 1989)

1.5 Costs and benefits of elevated corticosterone concentrations

1.5.1 Effects of acute elevation

Corticosterone concentrations are increased in response to a stressor; if a stressor poses a short-term, immediate threat, the elevation in corticosterone concentrations is considered acute. During acute elevation, corticosterone concentrations rise for the short period of time during which the stressor poses a threat and then drop to baseline concentrations shortly after (Boonstra, 2004). Short-term threats can include but are not limited to predators, capture and handling, and adverse weather conditions (e.g. storms).

During periods of acutely elevated corticosterone concentrations, the HPA axis negative feedback mechanisms operate efficiently so steroid concentrations quickly return to normal after a spike in concentrations seen after a stressor (Boonstra, 2004). This quick return to normal concentrations means that most effects on the body are short lived and many of them are positive in order to avoid any repercussions to the birds' survival as a result of the immediate danger or any injuries caused by the stressor.

Some of the first benefits in the elevation of corticosterone can be shown by the mobilisation of energy so that muscles can perform at high rates and fat stores can be used to avoid compromising the survival of the bird as a result of encountering an acute stressor such as territory protection or adverse weather conditions (Belthoff and Dufty, 1998; Frigerio *et al.*, 2001). Another benefit of acutely elevated corticosterone concentrations is an increase in aggression (Mora *et al.*, 1996). An increase in aggressive behaviour would be useful for fighting off predators or defending territory in older birds, or outcompeting a nest-mate for food in young birds which can also result in increased growth rate (Tarlow *et al.*, 2001). Birds exposed to corticosterone as a response to a stressor can also become more responsive to future stressors (Mora *et al.*, 1996). An increased responsiveness is possibly due to the bird being better able to identify stressors early on and react appropriately in order to increase their chance of survival. This trait is particularly important in birds living in harsh environments so they are able to identify the best time to start nesting or determine when a storm is bad enough to justify abandoning a nest they have already started brooding (Wingfield *et al.*, 1995).

Even after an acute stressor, such as a predator, is no longer present and the corticosterone concentrations are dropping, the effects of the elevation can still be beneficial and to some extent, long lasting. It has been shown that the immune response of an animal can increase after an acute rise in corticosterone concentrations (Dhabhar, 2009). It has also been shown that if stress is experienced at the time of developing an immune response, such as the stress from an injury along with the introduction of novel microbes, it can enhance innate and adaptive immune responses to better cope with disease or illnesses in the future (Dhabhar, 2009). A heightened immune system is very important after sustaining wounds so the bird can make a quick recovery. This would be particularly useful in birds as any sign of illness can lead to being ostracised by the flock or singled out by a predator. Another longer term benefit from acutely increased corticosterone concentrations is seen in older chicks just before fledging as an increase in corticosterone can facilitate behaviours needed for dispersal such as predator avoidance and searching for food (Sims and Holberton, 2000). These benefits are important immediately before fledging, especially for chicks that were not previously taught how or where to hunt or key skills for survival. By heightening their responsiveness and increasing dispersal behaviours, naïve fledglings will have an increased chance for survival compared with those that are less prepared.

These benefits ensure that the bird has the best chance of surviving any dangerous situation that may require a stress response (Belthoff and Dufty, 1998). They also ensure that any infections as a result of non-life threatening wounds can be dealt with as quickly and efficiently as possible by increasing the birds' immune functions (Dhabhar, 2009).
While there are many benefits to acutely increasing corticosterone concentrations, there are also drawbacks. Even though corticosterone concentrations quickly return to normal after an acute stressor (Boonstra, 2004), there can be immediate negative effects that are short-lasting and in some cases, delayed, longer lasting effects.

Despite only lasting in the system for a short amount of time, corticosterone can have detrimental effects on protein synthesis hindering both muscle and feather production (Romero *et al.*, 2005). By slowing or stopping the synthesis of proteins, in order to increase energy concentrations, muscle and feather production can be inhibited for a short period of time. In feathers, this results in fault bars (Bortolotti *et al.*, 2009). If muscle development is slowed or stopped then body mass can be stunted in younger birds going through early growth and developmental stages (Mora *et al.*, 1996).

Younger birds are particularly at risk from the detrimental effects that even briefly elevated corticosterone concentrations in response to an acute stressor can cause. This is likely due to the fact that at the time of the stressor, if the bird is still young, it is going through important developmental processes and if these processes are delayed, slowed or stopped the effects on the birds' behaviour, morphology and physiology can be long lasting. In Galápagos Nazca boobies and blue-footed boobies these behavioural changes are seen in the form of increased aggression (Mora *et al.*, 1996) as well as reduced social interactions in the Galápagos Nazca boobies (Tarlow *et al.*, 2001). These changes in behaviour could be highly detrimental when the bird is older and relies on social behaviours to attract a mate. Morphological changes have been seen in Northern mockingbirds (Sims and Holberton, 2000) when an increased concentration of corticosterone in response to an acute stressor has resulted in an inhibition of the production of growth hormone. Similar morphological changes have been seen in blue-footed boobies which were deprived of food and could subsequently not fully regain pre-stressor body mass even after they were no longer deprived of food (Mora *et al.*, 1996). These birds also exhibited lasting physiological effects of short term food deprivation in the form of increased baseline corticosterone concentrations and a heightened responsiveness to external stressors (Mora *et al.*, 1996).

While these changes in behaviour, morphology and physiology can be detrimental to young birds, overall the benefits of short-term elevation of corticosterone concentrations outweigh the costs as a stress response can lead to the survival of the bird from predation, disease, adverse weather or other stressors. This is particularly true for older birds as acute stress results in fewer long-term costs as they are better able to adapt to their environment rather than developing around it as young birds do. This adaptability means that older birds are able to better cope with stressors such as adverse weather by abandoning a clutch and starting a new one (Wingfield *et al.*, 1995) or enhancing their immune system after being injured by a predator (Dhabhar, 2009). These adaptations may be useful advantages to older birds when faced with acute stressors with minimal associated costs; the advantages and disadvantages become vastly different when the stressor and associated elevated corticosterone concentrations become chronic.

1.5.2 Effects of chronic elevation

While the effects of acute stressors can be long-lasting, the majority of the costs and benefits are generally short term to benefit the immediate survival of the animal. When the stressor is chronic, the effects of the raised concentrations of corticosterone can do more harm than good. Chronically elevated corticosterone concentrations occur when the stressor responsible for raising the concentrations becomes a long-term threat. These long-term threats can have deleterious effects on the survival and fitness of the individual (Sockman and Schwabl, 2001; Kitaysky *et al.*, 2003) so it is beneficial to only chronically elevate when stressors such as low food availability, constant food competition or constant predation pose an ongoing threat to the individuals' survival.

Despite it being widely accepted that chronic stress is detrimental to any individual, there are some benefits to having a constantly high concentration of corticosterone in response to a chronic stressor. While some of these benefits may be short-lived, such as an increased metabolism (Spencer and Verhulst, 2008), in order to maintain homeostasis throughout the stressor, other effects are beneficial to the individual in the long-term, such as improved spatial memory (Pravosudov, 2003).

Chronic stress can begin as early as *in ovo* as a result of the mother being exposed to stressors at the time of egg production and laying. Benefits to fledged chicks of an increased corticosterone concentration before hatching have been reported. Fledgling European starlings injected with corticosterone *in ovo* performed better in flight trials than control fledglings (Chin *et al.*, 2009). This was due to having more functionally mature flight muscles as well as a lowered wing loading. This benefit was not just to maintain homeostasis and survive exposure to stressors (Spencer and Verhulst, 2008). Rather, this was a long-term benefit that might likely increase the individuals' survival as a result of exposure to a stressor earlier on in life.

There can also be benefits from chronic stress when chicks have post-natal elevation of corticosterone. In black-legged kittiwakes, a chronic increase in corticosterone results in greater begging of chicks for food (Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003). This was similar to mountain chickadee adults which increased their food consumption when exposed to chronically high concentrations of corticosterone (Pravosudov, 2003). However, because black-legged kittiwakes are semi-altricial, they are unable to forage for themselves and so chronic corticosterone elevation in these chicks results in their parents being away from the nest for longer in order to keep up with the increased dietary needs and begging of their chicks (Kitaysky *et al.*, 2001b).

Corticosterone concentrations are elevated in response to a chronic stressor. The rise in glucocorticoid concentrations initiates behaviours such as food caching, increased foraging, fat deposition or nest abandonment in adult birds (Gray *et al.*, 1990; Breuner and Hahn, 2003; Pravosudov, 2003). Arctic and Antarctic breeding birds are constantly exposed to a chronic stressor: harsh weather. While living in these areas, there is always the risk that breeding may fail or the birds' survival might be at risk if the weather was to become too severe, so it can be beneficial to maintain a stress response that is sustained long-term so as to always be prepared to migrate or abandon a nest in the interest of long-term survival (Pravosudov *et al.*, 2001; Breuner and Hahn, 2003). While there are some benefits to having chronically elevated corticosterone concentrations as a response to long-term stressors, in some cases, down-regulation of corticosterone may occur so as to avoid negative effects of chronic elevation (Romero *et al.*, 2005; Walker *et al.*, 2005b). The benefits of chronic stress are not well documented with the majority of studies only noting minimal or no benefits of chronic stress. Amongst the studies that do show benefits, there is a small number of species including black-legged kittiwakes (Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003), white-crowned sparrows (Breuner and Hahn, 2003) and mountain chickadees (Pravosudov *et al.*, 2001; Pravosudov, 2003). This small range of species may indicate that the benefits outlined above do not occur in all species or ages of birds, with many not experiencing any of these benefits and others experiencing some but not all.

Once a stressor becomes chronic, the feedback signals in the HPA axis weaken (Boonstra, 2004) and while, in some cases, this can lead to down-regulation of corticosterone (Romero *et al.*, 2005; Walker *et al.*, 2005b), in most cases, the entire stress response system remains activated for long periods of time to maintain an appropriate response to chronic stressors which often results in long-term, detrimental effects on the physiology, morphology and behaviour of the bird.

It has been shown that chronic stress from an early age can lead to life-long detrimental effects. This has been shown in studies of sibling competition (Walker *et al.*, 2005b), supplementary oral corticosterone (Spencer and Verhulst, 2008) and dietary restrictions (Kitaysky *et al.*, 2001a) among others (Nowicki *et al.*, 1998; Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003; Cyr and Romero, 2007). This variation in chronic stressors has led to a wide variety of effects over a large range of species, with many of the effects being detrimental and supporting the idea that chronic stress is inherently bad for most individuals. These effects can last into adulthood with some studies showing that nutritional stress in chicks can be detrimental to brain development resulting in compromised cognitive abilities affecting song learning (Nowicki *et al.*, 1998) and spatial task solving (Kitaysky *et al.*, 2003). This could affect the long-term survival and fitness of the individual as it may be harder for them to find mates and reproduce without an appropriate repertoire of vocalisations as well as having trouble foraging because of difficulties in solving spatial tasks. Many studies show that chicks exposed to chronically high concentrations of corticosterone have lower body mass as well as lowered growth efficiency when compared to chicks who have not been chronically stressed (Kitaysky *et al.*, 2003; Walker *et al.*, 2005b; Spencer and Verhulst, 2008). A lower body mass and a lowered growth efficiency can lead to reduced survival rates along with other problems while the chick is still young right up until they fledge and even into adulthood (Tarlow *et al.*, 2001; Walker *et al.*, 2005b).

Chicks are not the only age group susceptible to the adverse effects of chronic stress and long-term elevation of corticosterone concentrations. Adult birds from a range of species have also been exposed to chronic stressors in order to identify the resulting detrimental effects. The experimental increase in corticosterone concentrations in adults has been achieved using slightly different methods from those used on chicks, including corticosterone implants (Gray *et al.*, 1990), increased predation and decreased food availability (Clinchy *et al.*, 2004), moulting, loud noises and novel objects (Romero *et al.*, 2005; Cyr and Romero, 2007) and migration (Jenni *et al.*, 2000), amongst others (Nowicki *et al.*, 1998; Pravosudov *et al.*, 2001). Unlike the chicks which suffer from lowered growth rates and lowered body mass as a result of chronic stress, the adult birds are no longer growing and tend to only fluctuate in mass. This difference means that many adult birds experience changes in fat deposition as opposed to changes in growth rates (Gray *et al.*, 1990; Jenni *et al.*, 2000; Pravosudov, 2003; Clinchy *et al.*, 2004) in response to a chronic stressor. In some birds this is an increase in fat deposition such as dark-eyed juncos (Gray *et al.*, 1990) and mountain chickadees (Pravosudov *et al.*, 2001) but in others this comes as an increase in free fatty acids from the breakdown of triglycerides resulting in much lower fat stores (Jenni *et al.*, 2000; Clinchy *et al.*, 2004). Chronic stress in adults can also lead to lowered brood sizes and reproductive success (Clinchy *et al.*, 2004; Cyr and Romero, 2007), increased protein breakdown including inhibition of feather regrowth (Jenni *et al.*, 2000; Romero *et al.*, 2005), changes to future stress responses including baseline corticosterone and response to acute stressors and emergency situations (Jenni *et al.*, 2000; Clinchy *et al.*, 2004; Cyr and Romero, 2007) as well as haematological and immunological changes including increased anaemia resulting in polychromasia and increased basophils (Clinchy *et al.*, 2004).

While all of these detrimental effects could significantly reduce the bird's chances of long-term survival as well as reducing its lifetime fitness, these costs are the result of the putting the bird's survival of the stressor above all else. Sometimes these chronic stressors are necessary in order to survive long-term such as moulting and migration (Jenni *et al.*, 2000; Romero *et al.*, 2005) and often they arise from the bird or chick's environment such as adverse weather or sibling competition for limited food resources (Nowicki *et al.*, 1998; Wingfield and Hunt, 2002; Walker *et al.*, 2005b). The necessity to go through and outlast the stressor means that despite these often highly detrimental effects of chronic stress, the benefits more often than not, outweigh the costs, provided the individual is able to survive the stressor.

1.6 Corticosterone responses of chicks

Results from studies of responses in chicks to a variety of stimuli are summarised in Table 3.

Table 3. Responses of chicks of different species to a variety of stimuli.

Species	Stimuli	Response	Age	Reference
Northern Mockingbirds	Handling	Breakdown of skeletal muscle Inhibition of growth hormones Facilitate behaviours needed for dispersal	5-7 days post-hatch, 8-9 day old nestlings, >10 days old and still in nest, 5 days post-fledging, 18-28 days post-fledging (still in natal territory), independent juveniles, Adults	(Sims and Holberton, 2000)
Black-legged kittiwakes	Exogenous corticosterone implants	Low growth efficiency Compromised cognitive abilities Parents spent less time brooding/guarding Increased food intake Increased begging behaviour Increased aggression	Approximately 15 days post-hatching	(Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003)

Species	Stimuli	Response	Age	Reference
Magellanic penguins	Capture and handling; ACTH injections	Reduced growth rate Muscles breakdown Reduced thyroid function Facilitation of energy Increased food intake Outcompete nest-mate (smaller chick often dies)	Shortly after hatching – shortly before fledging	(Walker *et al.*, 2005b)
Galápagos Nazca boobies	Obligate siblicide within first week post-hatching	Facilitates siblicide Reduce social behaviours Induce submissive behaviours Increase mass before second chick hatches Increases aggressive behaviour	0-1 week old	(Tarlow *et al.*, 2001)
Blue-footed boobies	Short-term food deprivation (48hrs)	Increase in aggressive behaviour Unable to fully regain body mass Increased baseline corticosterone Increased responsiveness to external stimuli	15-20 days old	(Mora *et al.*, 1996)

Species	Stimuli	Response	Age	Reference
Tufted puffins	Short-term food deprivation (24hrs), prolonged restrictive feeding regime	Decreased baseline concentration of corticosterone Decreased thyroid hormones	4-18 days old	(Kitaysky *et al.*, 2005)
Zebra finches	Oral corticosterone	Increased metabolic rate Lowered body mass	11-13 days old and 55-65 days old	(Spencer and Verhulst, 2008)
Adélie penguins	Scientific nest checking and recreational visits	Chick survival rate lowered Hatching success lowered	Throughout time spent in nest	(Giese, 1996)
Choughs	Eco-tourists in the area	Juvenile survival lowest in peak tourist month Juvenile survival negatively correlated with number of visitors	From juveniles to adults	(Kerbiriou *et al.*, 2009)

Species	Stimuli	Response	Age	Reference
Hoatzins	Eco-tourists approaching by boat	Juvenile survival at fledging stage lower Juveniles have lower body weights at tourist-disturbed nests	Juveniles and nestlings	(Müllner *et al.*, 2004a)
Yellow-eyed penguins	Unregulated ecotourism and visitor access	Fledging weight reduced Juvenile survival rate lowered	Fledglings	(McClung *et al.*, 2004)
Grey-faced petrels	Capture/handling	Responded at adult concentrations from hatching	Hatchlings to fledglings	Adams *et al.*, 2008)

1.6.1 Stimuli

A range of different stimuli have been used to initiate corticosterone responses in chicks. A standard protocol that has been widely used is the capture and handling of free-living birds. A bird is caught and the first blood sample is taken as quickly as possible. This is called the initial sample or, erroneously, the baseline sample. The bird is then put in a box or bag and subsequent blood samples are collected at various times up to 30 or 60 minutes post-capture before returning the bird to its nest or releasing it. This standardised protocol (Wingfield *et al.*, 1992), developed so that corticosterone responses could be compared across species, has been widely used with some variation in the timing of each blood sample and the amount of handling of birds (Sims and Holberton, 2000; Sockman and Schwabl, 2001; Adams *et al.*, 2008; Cockrem *et al.*, 2016). Other artificial stimuli have included restrictive feeding regimes (Kitaysky *et al.*, 2005), and corticosterone has been artificially elevated by exogenous corticosterone implants (Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003), ACTH injections (Walker *et al.*, 2005b), and oral corticosterone (Spencer and Verhulst, 2008). Corticosterone responses have also been measured to other stimuli including food deprivation (Mora *et al.*, 1996; Kitaysky *et al.*, 2005; Walker *et al.*, 2005b), obligate siblicide (Tarlow *et al.*, 2001) and tourist encounters (Giese, 1996; Müllner *et al.*, 2004a; Kerbiriou *et al.*, 2009).

1.6.2 Corticosterone responses

Initial corticosterone and corticosterone responses of chicks exposed to different stimuli vary between species. The initial concentration of corticosterone varies greatly not just across species or ages but between individuals as well. Initial corticosterone concentrations decreased in tufted puffins that experienced short-term food deprivation (Kitaysky *et al.*, 2005). However, blue-footed boobies had the opposite response with , increased initial corticosterone concentrations (Mora *et al.*, 1996). In response to an acute, as well as chronic, food shortage, the tufted puffin chicks also show lowered concentrations of thyroid hormones when compared to chicks fed *ad libitum* (Kitaysky *et al.,* 2005). These results show that it is difficult to compare responses of chicks of different species to the same stimuli. It is also important to note that results from a single study cannot be generalised to say that the response of chicks of one species to a stimulus will always be the response seen in just one study.

In some species of birds, although two chicks hatch, only one chick will survive due to nest-mate competition (Tarlow *et al.*, 2001; Walker *et al.*, 2005b). These species may produce "insurance" eggs to ensure a successful breeding season (Boersma, 1991; Clifford and Anderson, 2001; Rafferty *et al.*, 2005). In Galápagos Nazca booby chicks obligate siblicide occurs within a week of hatching (Tarlow *et al.,* 2001). However, in Magellanic penguin chicks nest-mate competition is prolonged as competition for food provided by the parents continues until one of the chicks dies from malnourishment (Walker *et al.*, 2005b). Development of corticosterone responses to stressors with age and changes in baseline concentrations in response to extreme nest-mate competition were examined in both of these studies. In the Nazca boobies, asynchrony is observed in hatch dates so one chick always starts as the 'larger chick'. These larger chicks are often the dominant chick that ends up surviving the siblicide. Tarlow *et al.* (2001) measured testosterone, progesterone and corticosterone concentrations of both the larger and smaller chicks as well as singular chicks which hatched with no siblings. It was found that testosterone did not significantly differ between larger, smaller or singular chicks. Progesterone concentrations were significantly lower in singular chicks than in the smaller and larger chicks but in the singular chicks, progesterone concentrations increased with age whereas progesterone decreased with age in the chicks that were subjected to siblicide at a young age (Tarlow *et al.*, 2001). Development of the reproductive system may be affected by corticosterone in chicks that experience stress from a young age. Corticosterone concentrations were significantly higher in smaller chicks than larger chicks, and after siblicide had occurred corticosterone decreased with age in the larger chicks but did not, however, change in singular chicks (Tarlow *et al.*, 2001). It is possible that this indicates that larger chicks are less able to respond to stressors post-fledging than singular chicks that did not commit siblicide.

In the Magellanic penguin the chicks develop their corticosterone stress response over time, with little to no response shortly after hatching, a slightly stronger response at 45 days and a fully robust response near fledging (Walker *et al.*, 2005b). In this example, like the previous one, there is a developmental difference between the sibling chicks with one, usually the first hatched, being larger than the other. At hatching, there was no difference in either the baseline or the integrated corticosterone concentration between the chicks. However, in middle-aged chicks baseline corticosterone concentrations were significantly higher in smaller than larger chicks but there was no significant difference between integrated corticosterone concentrations. Once the chicks had reached fledging age, baseline concentrations were similar in small and large chicks but the integrated corticosterone concentrations were significantly lower in smaller chicks (Walker *et al.*, 2005b). The results from the Nazca boobies and from the Magellanic penguins are insufficient to allow generalisations about corticosterone in chicks that experience challenges during development, and more studies are needed to determine whether exposure of chicks to stressors affects them as adults.

1.6.3 Behavioural and other responses

When chicks respond to a stimulus with activation of the HPA axis and increased secretion of corticosterone the chicks may also have behavioural responses and there may be changes in body condition. For example, responses of blue-footed booby chicks to an acute food shortage included an increase in aggressive behaviour, an inability to regain lost body mass, and an increased responsiveness to external stimuli (Mora *et al.*, 1996). Tufted puffin chicks that experienced prolonged food shortage showed obvious signs of nutritional deficit and had significant weight loss as well as lowered endogenous lipid reserves (Kitaysky *et al.*, 2005). These responses were in addition to corticosterone responses mentioned earlier.

Another situation in which corticosterone secretion in chicks may be increased is when ecotourists and scientists approach nest sites. A common response of birds to human approach to nest sites approached is a lowered survival rate in the young birds (Giese, 1996; McClung *et al.*, 2004; Müllner *et al.*, 2004a; Kerbiriou *et al.*, 2009). It is possible that this reduction in survival rate could be due to increased corticosterone concentrations in chicks having a negative effect on their development or that the parents flee the nest when approached possibly reducing food availability for the chicks or exposing them to predators or harsh weather. In smaller colonies of Adélie penguins, chick survival rate and hatching success was lowered when visited by eco-tourists or scientists (Giese, 1996). For choughs, juvenile survival rate was lowest in the peak tourist month for the ski field in which they lived and juvenile survival rate was negatively correlated with the number of tourists present (Kerbiriou *et al.*, 2009). Hoatzin chicks are a very popular sight for ecotourists due to their unusual appearance and ability to climb with their wings. However, tourist visits can have detrimental effects with lower body weights and reduced likelihood of fledging in chicks in highly visited areas (Müllner *et al.*, 2004a). Unfortunately, these tourist encounters often bring money to the region and allow for the protection of the species involved and their environment and in some situations, it is more beneficial for the ecosystem as a whole to have reduced fledgling survival in the focal species than to have no protection for any of the flora or fauna present.

1.6.4 Treatment with corticosterone or with ACTH

Corticosterone can be artificially elevated to examine the effects on birds of elevated corticosterone concentrations. Plasma corticosterone can be increased using exogenous corticosterone implants (Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003), ACTH injections (Walker *et al.*, 2005b), or oral corticosterone (Spencer and Verhulst, 2008). These treatments produce concentrations and patterns of corticosterone that are not physiological and it is difficult to draw conclusions about the relevance of results from these studies to physiological roles of corticosterone. However, these experiments do give great insight into development of the HPA axis and whether the adrenal gland of chicks of different ages is capable of secreting corticosterone at the same rate as adrenal glands of adult birds. For example, it was shown that Magellanic penguin chicks secreted mature concentrations of corticosterone in response to ACTH injections but did not generate adult corticosterone responses when subjected to handling at a young age (Walker *et al.*, 2005b).

Experiments in which birds are treated with corticosterone have induced a range of responses. For example, in black-legged kittiwake chicks with exogenous corticosterone implants chicks with higher corticosterone concentrations had lowered growth efficiency despite and increased food intake due to increased begging behaviours which resulted in parents spending less time in the nest (Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003). It was also found that the chicks with artificially elevated corticosterone showed impaired cognitive abilities later in life (Kitaysky *et al.*, 2003). These impairments were an inability to recognise visual cues and a reduced ability to solve spatial tasks to acquire food. It is possible that these cognitively impaired chicks would be less able to acquire food post-fledging as they could struggle with recognising visual cues of food such as changes in the sea's surface. There might be a reduced survival rate in fledglings that had prolonged, elevated corticosterone earlier in life (Kitaysky *et al.*, 2003).

Another way to induce high concentrations of corticosterone in chicks is to feed them oral corticosterone. Zebra finch chicks fed daily with 0.0125 mg of corticosterone dissolved in peanut oil daily had an increased metabolic rate resulting in a lowered body mass when compared to control chicks (Spencer and Verhulst, 2008). A lowered body mass at fledging could be detrimental to these chicks as it could affect their survival rate, especially if they had similar cognitive deficits to the black-legged kittiwake chicks (Kitaysky *et al.*, 2003) and had an impaired ability to solve tasks so as to successfully forage for food. These results indicate that elevated concentrations at a young age can have detrimental effects on the developmental, growth and cognitive abilities of chicks.

1.7. Conclusion

The literature review of corticosterone in chicks has shown that there are gaps in the knowledge we currently have about stress in chicks, how their corticosterone responses develop and if this development is altered by human interaction. Furthermore, little is known about corticosterone in penguin chicks although penguins are a group of birds that is quite frequently the subject of ecotourism (Giese, 1996; Fraser and Patterson, 1997; Fowler, 1999; McClung *et al.*, 2004; Cockrem *et al.*, 2016).

1.8 Outline of thesis

The study described in the following chapter addressed questions about corticosterone and development in kororā (little penguin) chicks. The study considered how the magnitude of corticosterone responses changes with age in kororā chicks and how frequent handling can affect these responses. Corticosterone responses and growth rates were compared between chicks at two study sites, and whether mass at fledging can be affected by the number of chicks in the nest was investigated. The last chapter in the thesis discusses the wider implications of the results and the possibility of further research in this field. These implications include but are not limited to, whether or not acclimatisation of wild bird populations to human contact is detrimental, how handling from a young age can affect survival rates after fledging, and if survival after fledging is related to mass and if this is affected by the number of chicks in the nest. The study and the discussion of the results and their implications aimed to increase the available information on kororā development as well as contribute to the conservation of this declining species.

2. Results

2.1. Introduction

Birds experience throughout their lives situations in which they respond to stimuli from the environment. When the stimuli are perceived as threats the hypothalamo-pituitary-adrenal (HPA) axis is activated and the corticosteroid hormone corticosterone is secreted (Cockrem, 2007). Stimuli that initiate corticosterone responses are known as stressors. The HPA axis can also be activated in response to metabolic stimuli such as low blood glucose concentrations, and corticosterone secretion can increase during fasting. Whilst corticosterone is thought to hep birds adjust to situations in which they are responding to a stressor, corticosterone may also affect the fitness and survival of birds.

The secretion of corticosterone in response to a stressor is known as a stress response (Cockrem, 2007). The development of corticosterone responses in birds from the time of hatching until fledging has been studied across a range of species and ages of chicks (Heath, 1997; Frigerio *et al.*, 2001; Kitaysky *et al.*, 2001b; Kitaysky *et al.*, 2003; Love *et al.*, 2003; Rubolini *et al.*, 2005; Walker *et al.*, 2005b). A range of corticosterone responses to different stressors has been reported. Although there can be marked differences between precocial (Holmes *et al.*, 1989) and altricial (Wada *et al.*, 2007) chicks in the pattern of development of corticosterone responses, it is difficult to make generalisations as there is a range of patterns of development within each of the broad classifications.

Corticosterone responses to capture and restraint have been measured in adult Adelie (Cockrem *et al.*, 2006), Magellanic (*Spheniscus magellanicus;* Hood *et al.*, 1998; Walker *et al.*, 2004; Walker *et al.*, 2006), king (*Aptenodytes patagonicus*; Holberton *et al.*, 1996), gentoo (*Pygoscelis papua*; Holberton *et al.*, 1996), yellow-eyed (*Megadyptes antipodes*;

Ellenberg *et al.*, 2007)) and little (*Eudyptula minor*; Cockrem *et al.*, 2017) penguins. The development of the corticosterone response with age has been described in the Magellanic penguin chicks (Walker *et al.*, 2005b) but not in other species of penguins.

Kororā (little penguins) will breed in nestboxes and can readily be studied at the Oamaru Blue Penguin Colony. The aim of the current study was to describe the development of corticosterone responses to handling in kororā chicks at Oamaru and to investigate relationships between corticosterone responses and factors including the frequency of handling and body condition. It was expected that the younger chicks would have an immature HPA axis (Adams *et al.*, 2008) and an inability to recognise potential threats (Sims and Holberton, 2000), and that corticosterone responses would increase with age from small responses in young chicks to adult-like responses in older chicks. It was also expected that regular handling of chicks would result in conditioning of the chicks so they perceived the opening of their nestbox and removal from the box for handling to be a weaker stressor than would chicks that were not regularly handled. The relationship between body condition and corticosterone responses was also examined. It was expected that there would be an inverse relationship and that this relationship would become weaker in chicks that experienced long durations of low body condition (Walker *et al.*, 2005b). Results from the study of corticosterone and development in kororā chicks will add to knowledge of corticosterone in avian chicks and provide information to assist the conservation of a native species that is at risk and declining.

2.2. Materials and methods

2.2.1. Study site and sample collection

This study was conducted at two colonies of kororā in Oamaru (45˚ 07’ S, 170˚ 58’ E) on the Northern Otago coast of New Zealand. The first of these sites was at the Oamaru Blue Penguin Colony (OBPC) and is known as the ‘Quarry colony’ (site 1). This site has 250 nestboxes and is managed as an ecotourist facility. Site 2 is approximately 1km away on a small, fenced reserve with approximately 200 nestboxes. Site 2 is closed to the public and is monitored by the OBPC on a weekly basis. Site 2 is also used as a control site by the resident marine biologist to ensure tourist visitation does not impact the wellbeing or growth of the kororā. Chicks from both colonies are either banded with a metal flipper band on their right flipper or have a PIT transponder tag inserted at the base of the back of their neck once they reach six weeks of age. Adults are sexed using a combination of their bill dimensions as well as their breeding behaviour which can be determined at weekly checking of nestboxes (Agnew *et al.*, 2014). Kororā chicks were sampled over $1^{1}/_{2}$ months from early October until mid-November 2016.

Chicks were randomly assigned to one of five groups (Table 2.1) that varied in the age at sampling and whether or not the chicks would be handled from two weeks of age until the time of sampling. Group 1 chicks were sampled at two weeks old. Group 2 chicks were handled twice weekly from two weeks until sampled at four weeks of age, group 3 chicks were sampled at four weeks of age, group 4 chicks were handled twice weekly from two weeks until sampled at six weeks of age, and group 5 chicks were sampled at six weeks of age.

Table 2.1. Groups of birds used in this study.

Group number	Number of individuals	Age sampled	Handling from 2 weeks of age
1	20	2 weeks	Not handled
2	16	4 weeks	Handled twice weekly
3	22	4 weeks	Not handled
4	16	6 weeks	Handled twice weekly
5	15	6 weeks	Not handled
6	14	Adults	Not handled

Blood samples for the measurement of plasma corticosterone in response to handling were collected from kororā chicks of varying ages in nestboxes. Chicks younger than 2 weeks of age were not handled to avoid possible impacts on the chick development. Chicks were removed from their boxes by hand and blood was collected through a 25 g needle into a 1ml heparinised syringe or into a heparinised capillary tube after puncturing a vein with a 25 g needle. Birds were weighed in a cloth bag to determine body mass and beak width, beak depth, beak length and flipper length were then measured. The chicks were then held in the hand until 15 min had elapsed from when the birds had been picked up from their nestbox. A second blood sample was taken at 15 min and the chicks were then placed in an opaque plastic box (29 x 39 x 29 cm) with a lid containing holes for ventilation. Chicks were removed from the box for the collection of a final blood sample at 30 min before being returned to their nestbox. The collected blood was transferred immediately after collection into numbered, heparinised 1.5 mL Eppendorf tubes which were kept cool until the end of the day before being centrifuged and plasma withdrawn at -20^{o}C. The total time from the removal of the chicks from the nestbox or the plastic box until the completion of blood sampling was recorded in min and sec and was then converted to total sec. Adult corticosterone was collected for a separate study using the same methods but were used in this study as an example of a different age group of kororā at the same time of year in the same geographical location.

The primary interest was in the magnitude of corticosterone responses to capture and handling in the different age groups and in whether repeated handling had an effect on the development of corticosterone responses. The plasma corticosterone concentrations in the first blood sample from each chick are referred to as the initial corticosterone concentrations. These concentrations are not considered to be baseline as corticosterone concentrations when sample collections were completed are likely to have begun to rise from concentrations in resting or undisturbed chicks before capture. This study was conducted under permits from the Massey University Animal Ethics Committee and the Department of Conservation.

2.2.2 Radioimmunoassay of corticosterone

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%.

2.2.3. Statistics

Data analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc.). Relationships between the time taken to collect blood samples and corticosterone concentrations were determined using linear regression. Corticosterone concentrations were log transformed before statistical analyses to compare concentrations between two or more groups of birds. Two-way repeated measures ANOVAs were performed to determine the relationships between age and corticosterone responses (post hoc comparisons made with Tukey’s multiple comparisons tests) and between handling experience and corticosterone responses (post hoc comparisons made with Sidaks multiple comparisons tests). 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). One-way ANOVA with post hoc comparisons made with Sidak’s multiple comparisons tests was used to compare mean integrated corticosterone responses between chicks of different ages and between chicks that had or had not been regularly handled. One-way ANOVA with post hoc comparisons made with Sidak’s multiple comparisons tests was used to compare mean body mass between chicks of different ages and between chicks that had or had not been regularly handled. Relationships between body mass and initial corticosterone concentrations and between fledging weight and integrated corticosterone responses were determined using linear regression. A *t*-test was used to compare mean fledging weights between chicks handled for two and for four weeks. Two-way repeated measures ANOVAs with post hoc comparisons made with Sidak's multiple comparisons tests were performed to determine relationships between colony location and corticosterone responses. *t*-tests were used to compare mean integrated corticosterone responses and body masses at fledging between colony locations.

Beak width data were not used because of frequent occurrences when the measured width decreased between successive measurements, indicating discrepancies in the placement of the measurement calipers on the beak. A body condition index was calculated as body mass/beak length x 100 000. Beak length was used as a measure of body size as Lubbe *et al.*, 2014 reported that head length was a better measure of body size than flipper length. One-way repeated measures ANOVAs with post hoc comparisons made with Sidak's multiple comparisons tests was used to compare mean body mass, beak length, beak depth, flipper length and body condition index between ages.

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 minutes and seconds then converted to a decimal value. This time ranged from 0.7 to 8.5 min. The mean times taken to collect initial samples were 1.9 ± 0.3, 2.8 ± 0.4, 2.2 ± 0.3, 2.6 ± 0.4 and 2.2 ± 0.4 min for groups of chicks (2 weeks old, 4 weeks old not handled and handled and 6 weeks old not handled and handled) and was 4.9 ± 0.8 min for adults. There were significant linear regression relationships between corticosterone concentrations in initial blood samples and the time taken to collect the samples for two of the five groups of chicks (Fig. 2.1; see Table 2.2 for statistics). Corticosterone concentrations in the first samples are called initial concentrations and are not considered to represent corticosterone concentrations in undisturbed penguins.

Table 2.2. Results of linear regressions of relationships between corticosterone concentrations in initial blood samples and the time taken to collect the samples.

Group	r^2	F	**Degrees of freedom**	p
2 weeks old not handled	0.067	1.22	1,17	0.285
4 weeks old handled	0.066	0.98	1,14	0.338
4 weeks old not handled	0.296	8.39	1,20	0.009
6 weeks old handled	0.112	1.76	1,14	0.206
6 weeks old not handled	0.536	15	1,13	0.002
Adults	0.218	3.35	1,12	0.092

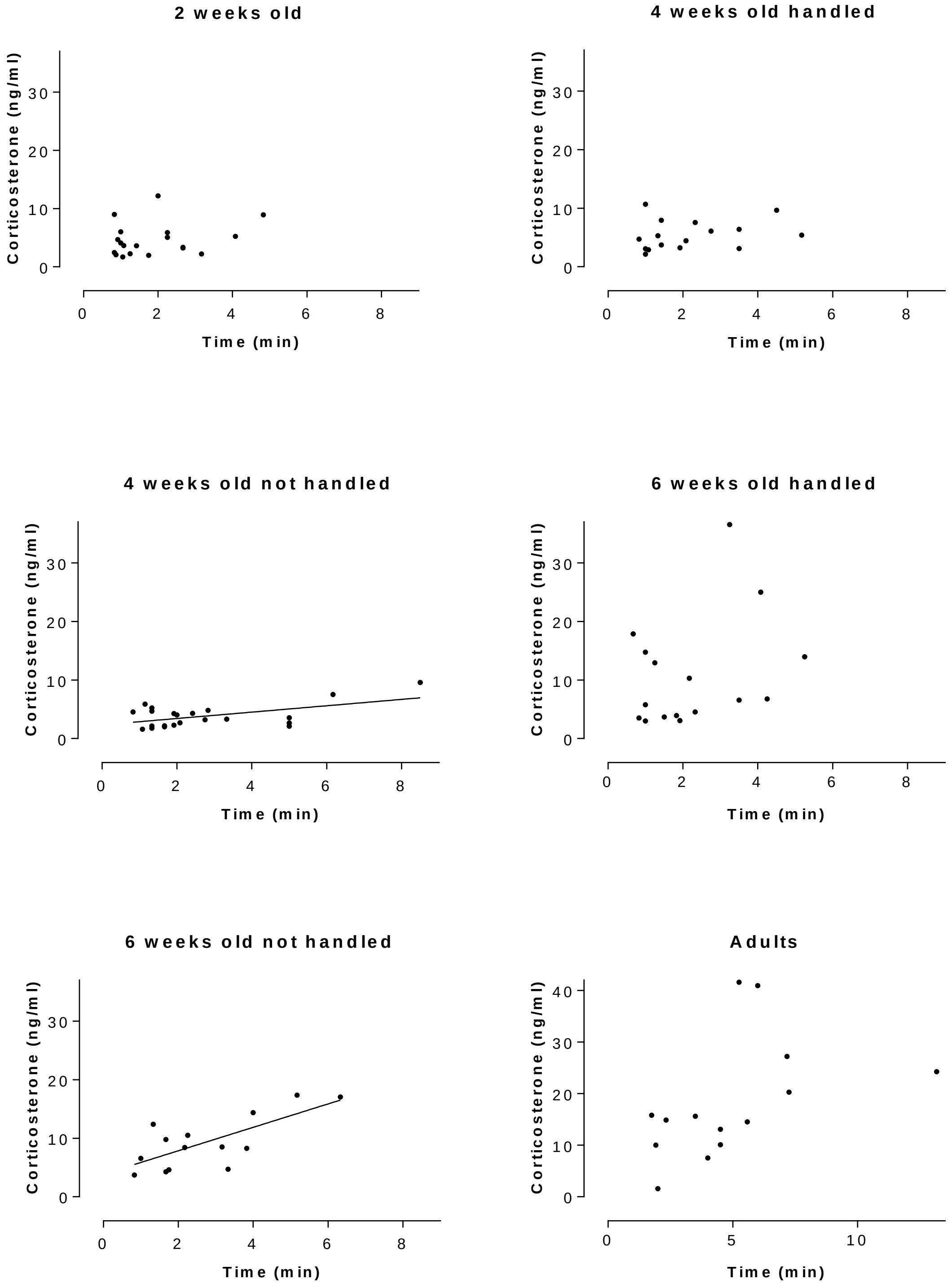

Fig. 2.1. Corticosterone concentrations in the initial samples versus time taken to collect the samples. Sample sizes were 19, 18, 20, 16, 15 and 14 respectively.

2.3.2 Development of corticosterone responses in relation to age

Corticosterone responses varied between individual birds at different ages and between individual adults (Fig. 2.2). Mean responses of chicks of different ages and of adults are shown in Fig. 2.3. A two-way repeated measures ANOVA showed that there were significant overall effects of time and age on plasma corticosterone concentrations, and a significant interaction ($F_{2,\,128} = 642.8$, $p < 0.001$; $F_{3,\,64} = 15.99$, $p < 0.001$; $\mathrm{F}_{6,128} = 12.21$, $p < 0.001$ respectively. Mean corticosterone concentrations did not differ between chicks of 2 and 4 weeks of age, were greater in chicks 6 weeks of age than chicks 4 weeks of age ($p = 0.0004$), and did not differ between chicks 6 weeks of age and adult penguins.

Mean corticosterone concentrations in initial samples (0 min) did not differ between chicks of 2 and 4 weeks of age, were greater in chicks 6 weeks of age than chicks 4 weeks of age ($p < 0.0001$) and were lower in chicks 6 weeks of age than in adult penguins age ($p = 0.0021$). Mean corticosterone concentrations at 15 min and at 30 min did not differ between chicks of 2 and 4 weeks of age, between chicks of 4 and 6 weeks of age, or between chicks of 6 weeks of age and adult penguins.

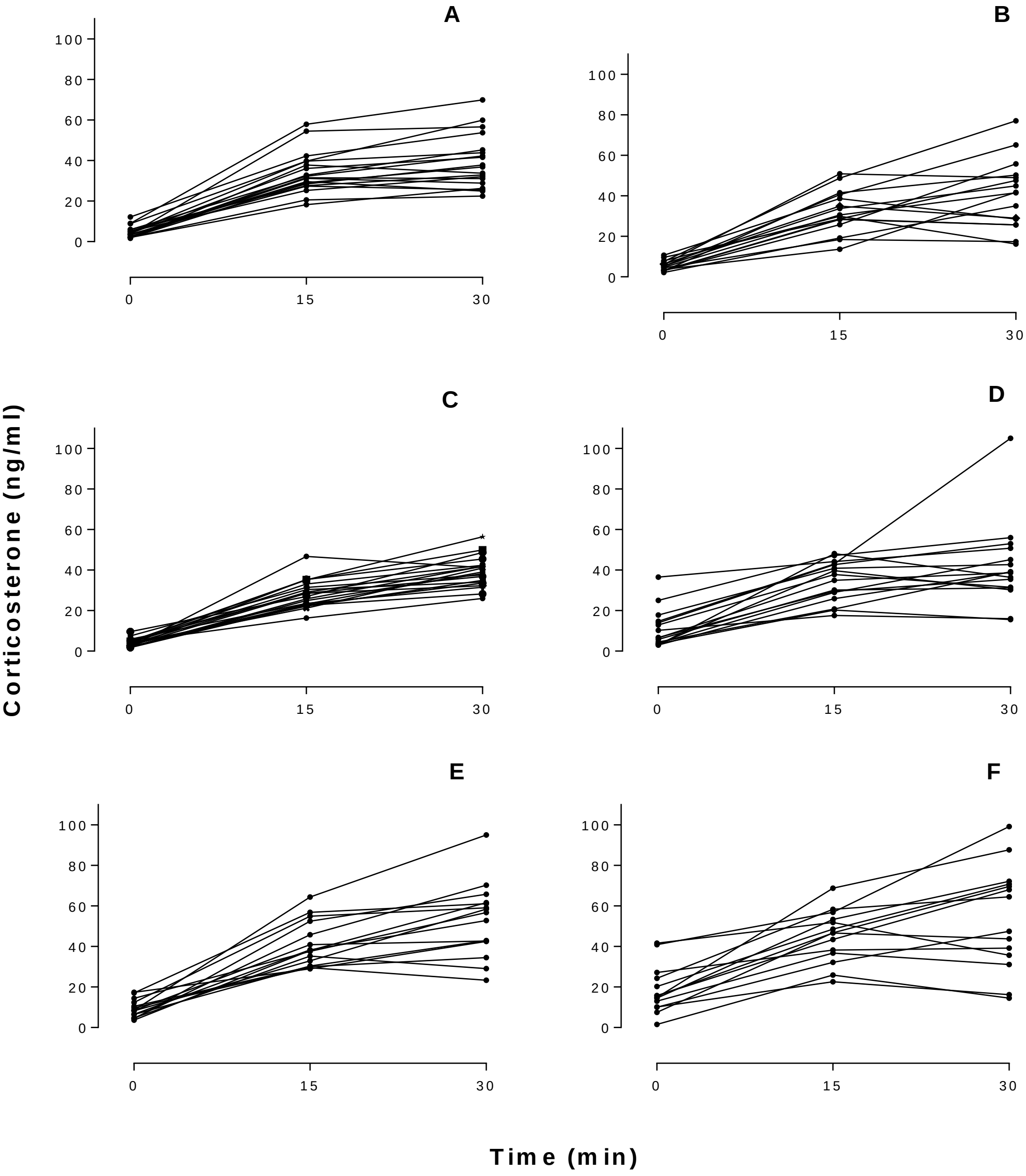

Fig. 2.2. Individual plasma corticosterone responses of little penguins. The birds were 2-weeks-old (A), 4-weeks-old handled (B), 4-weeks-old unhandled (C), 6-weeks-old handled (D), 6-weeks-old unhandled (E) and adult birds that had not been previously handled (F). Sample sizes were 19, 18, 20, 16, 15 and 14 respectively.

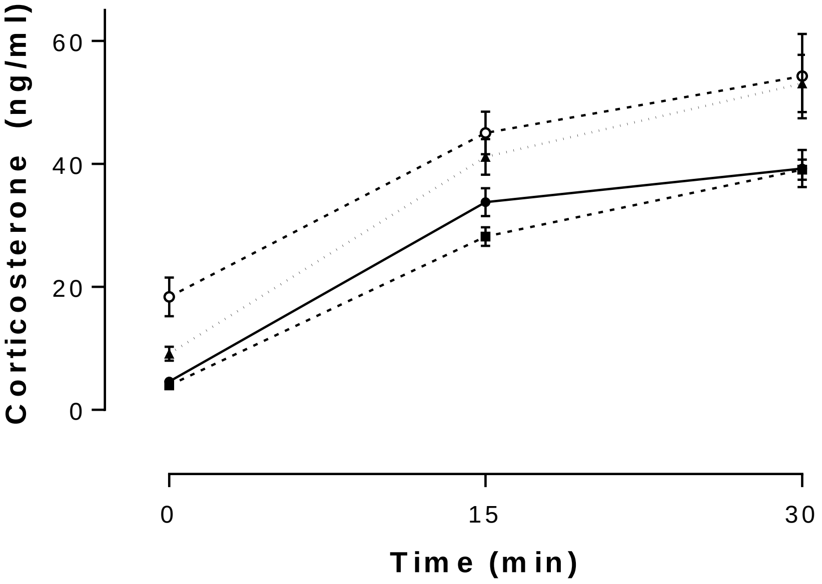

Fig. 2.3. Mean plasma corticosterone responses of little penguin chicks 2 (— ● —), 4 (--- ■ ---) and 6 (··· ▲ ···) weeks of age, and responses of adult penguins (--- ○---). Sample sizes were 19, 20, 15 and 14 respectively.

2.3.3 Corticosterone responses in relation to regular handling

Corticosterone responses of chicks handled twice weekly from 2 to 4 weeks of age and from 2 to 6 weeks of age were compared with responses of chicks that were not handled (see Fig. 2.4). Separate two-way repeated measures ANOVA showed that for chicks sampled at 4 weeks of age and for chicks sampled at 6 weeks of age there were significant overall effects of time but not regular handling on plasma corticosterone concentrations, and no significant interactions ($F_{2,72} = 473.2$, $p < 0.001$ and $F_{2,58} = 164$, $p < 0.001$ for effects of time).

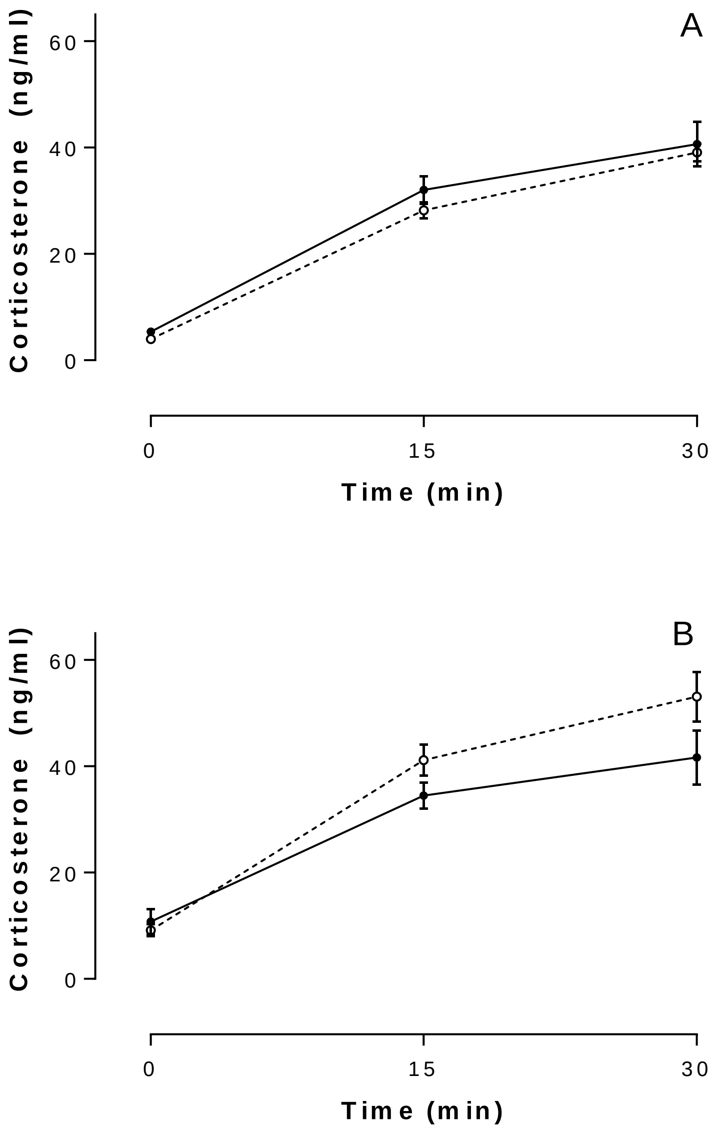

Fig. 2.4. Mean plasma corticosterone responses of little penguin chicks 4 weeks of age (A) or 6 weeks of age (B) that had been handled from 2 weeks of age (— ● —) or had not been handled before the time of sampling (--- ○ ---). Sample sizes were 16, 20, 16 and 15 respectively for chicks of 4 and 6 weeks of age.

2.3.4 Integrated corticosterone responses in relation to age and previous handling experience

Integrated corticosterone responses varied between individual birds (see Fig. 2.5 for individual and mean integrated responses). A one-way ANOVA showed that integrated responses differed between groups ($F_{5, 94} = 6.534$, $p < 0.0001$). Mean integrated responses did not differ between chicks of 2 and 4 weeks of age, were greater in chicks 6 weeks of age than chicks 4 weeks of age ($p = 0.0031$), did not differ between chicks 6 weeks of age and adult penguins, and were lower in chicks handled twice weekly from 2 to 6 weeks of age than in adults ($p = 0.0187$). Mean integrated responses did not differ between chicks handled twice weekly and chicks not handled twice weekly for chicks sampled at 4 and 6 weeks of age.

2.3.5 Relationships between body mass, handling and corticosterone responses

Mean body mass in chicks sampled at 4 weeks of age was 0.82 ± 0.02 kg in chicks that had been handled twice weekly from 2 weeks of age and 0.81 ± 0.02 kg in chicks not handled twice weekly. Mean body mass at 6 weeks of age for chicks that had been regularly handled was 1.09 ± 0.04 kg and was 1.03 ± 0.04 kg in chicks that had not been handled. Mean body masses did not differ between chicks handled twice weekly and chicks not handled twice weekly for chicks sampled at 4 and 6 weeks of age. There were no significant relationships between body mass and integrated corticosterone responses (see Table 2.3).

Table 2.3. Results of linear regressions of relationships between body mass and integrated corticosterone responses.

Group	r^2	F	**Degrees of freedom**	p
2 weeks old not handled	<0.001	<0.001	1,17	0.997
4 weeks old not handled	0.135	2.817	1,18	0.111
6 weeks old not handled	0.229	3.86	1,13	0.071
Adults	0.002	0.027	1,12	0.873

Mean body mass at fledging (1.17 ± 0.02 and 1.10 ± 0.04 kg) did not differ between chicks handled twice weekly from 2 weeks of age and blood sampled at 4 or 6 weeks of age. There were no significant relationships between the fledging weights of these chicks and their integrated corticosterone responses (see Table 2.4).

Table 2.4. Results of linear regressions of relationships between body mass at fledging and integrated corticosterone responses.

Group	R^2	F	**Degrees of freedom**	p
4 weeks old not handled	0.0054	0.076	1,14	0.786
6 weeks old not handled	0.0087	0.123	1,14	0.731

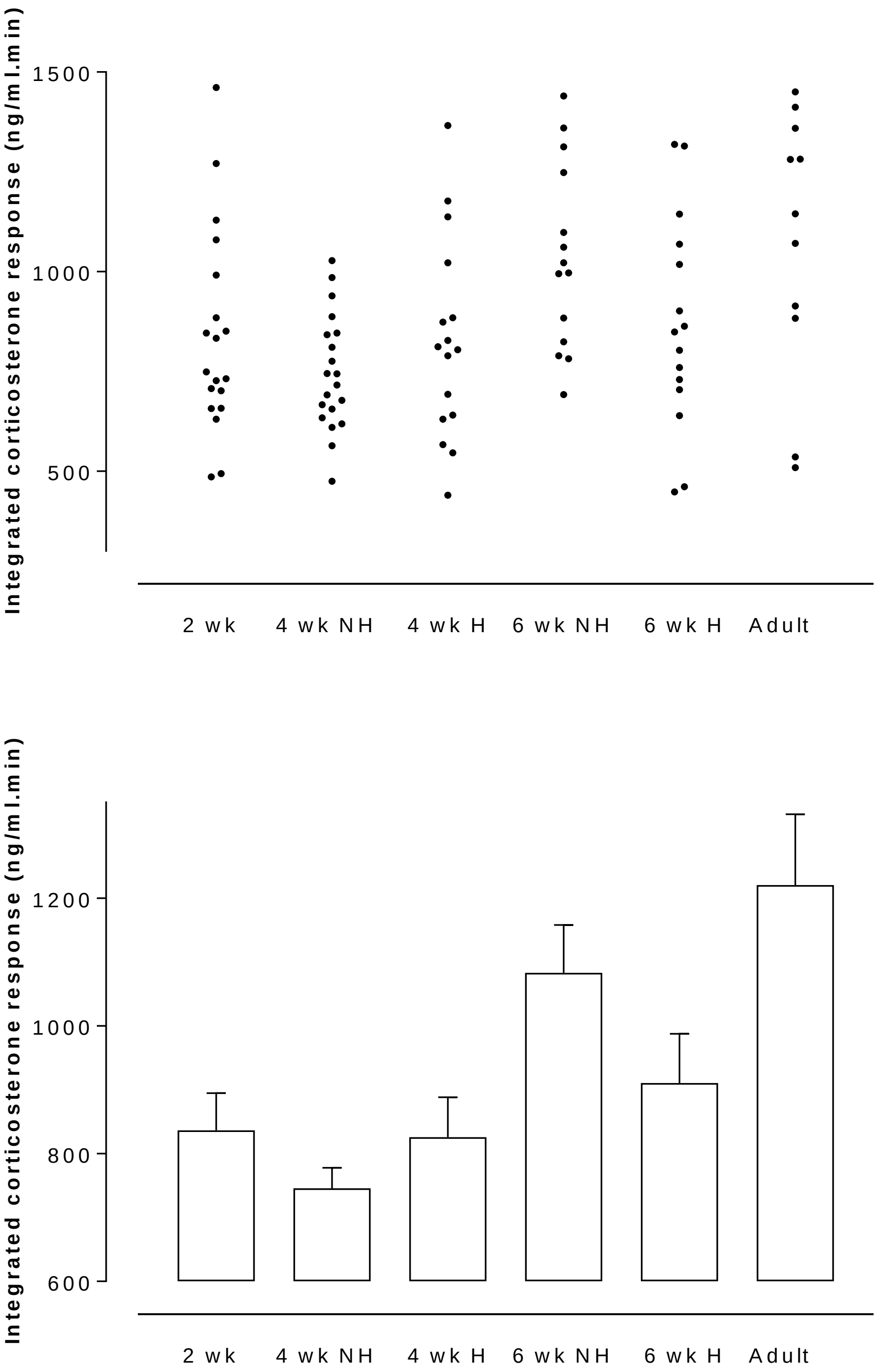

Fig. 2.5. Individual (top) and mean (bottom) integrated corticosterone responses of chicks that were not handled (NH), chicks that were handled twice weekly from 2 weeks of age (H) and adult penguins. Sample sizes were 19, 20, 18, 15, 16 and 14 respectively.

2.3.6 Corticosterone responses and fledging body masses at two kororā colonies at Oamaru

Individual and mean plasma corticosterone responses of chicks at the creek and quarry colonies are shown in Figs. 2.6 and 2.7. Two-way repeated measures ANOVAs showed that there were significant overall effects of location on plasma corticosterone concentrations in chicks 2 weeks of age ($F_{1, 19} = 6.165$, $p = 0.023$), and no significant effects in chicks of 4 or 6 weeks of age. Mean corticosterone concentrations in initial samples at 0 min were lower in chicks 2 weeks of age at the creek (3.53 ± 0.45 ng/mL) than at the quarry (5.80 ± 1.18 ng/mL; $p = 0.027$). Corticosterone concentrations at 15 and 30 min in chicks 2 weeks of age did not differ between the creek and the quarry. Mean integrated corticosterone responses (Fig. 2.8) were lower at the creek than at the quarry ($t_{13.85} = 2.993$, $p = 0.010$) in chicks 2 weeks of age and did not differ between locations in chicks of 4 or 6 weeks of age. Body masses at fledging (Fig. 2.9) did not differ between chicks at the creek and the quarry for chicks handled twice weekly from 2 weeks of age and blood sampled at 4 or 6 weeks of age.

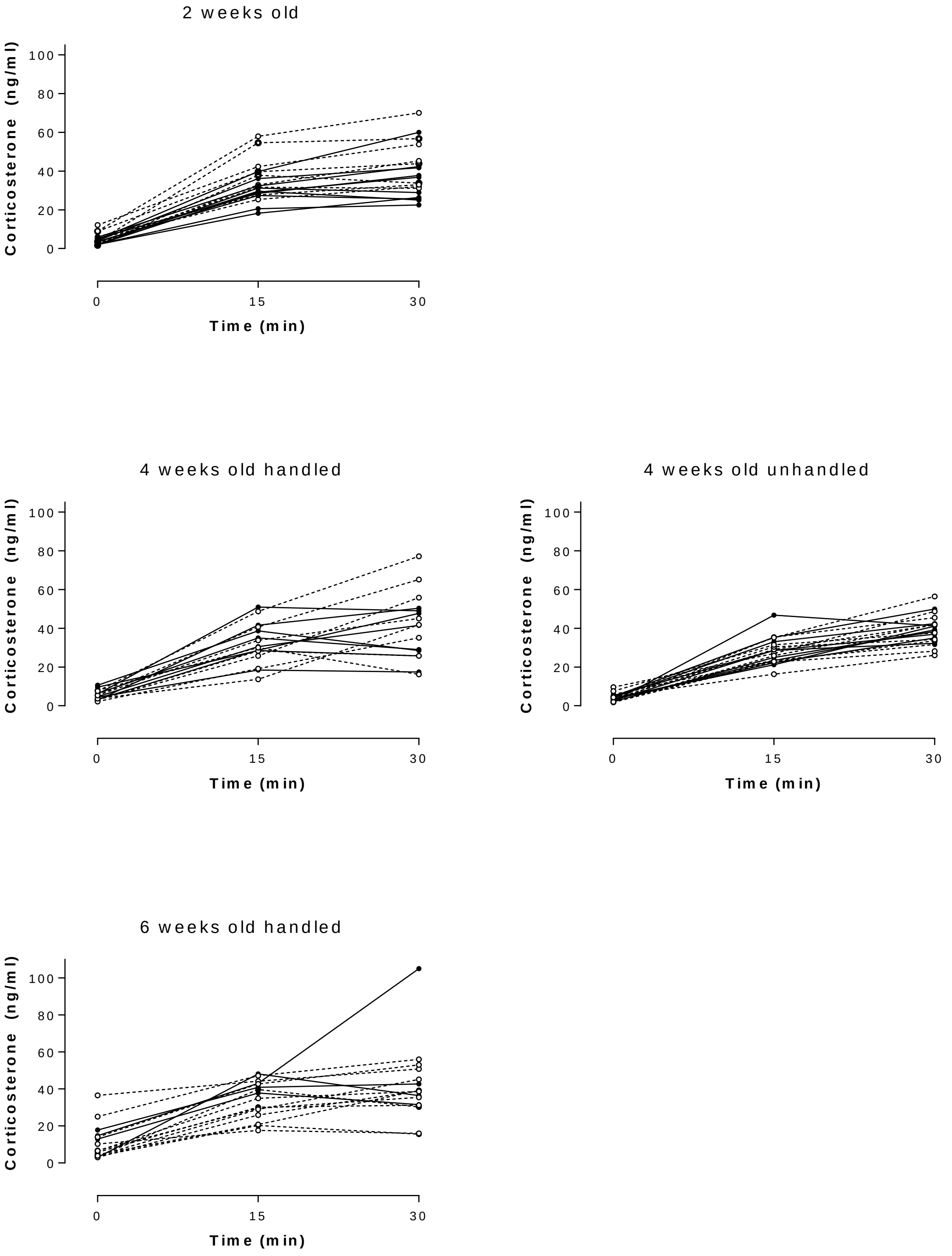

Fig. 2.6 Individual plasma corticosterone responses of kororā chicks at the creek (— ● —) and quarry (--- ○ ---) colonies at Oamaru. Sample sizes were 8 - 12 except for chicks 6 weeks of age at the creek colony for which the sample sizes were 4 chicks.

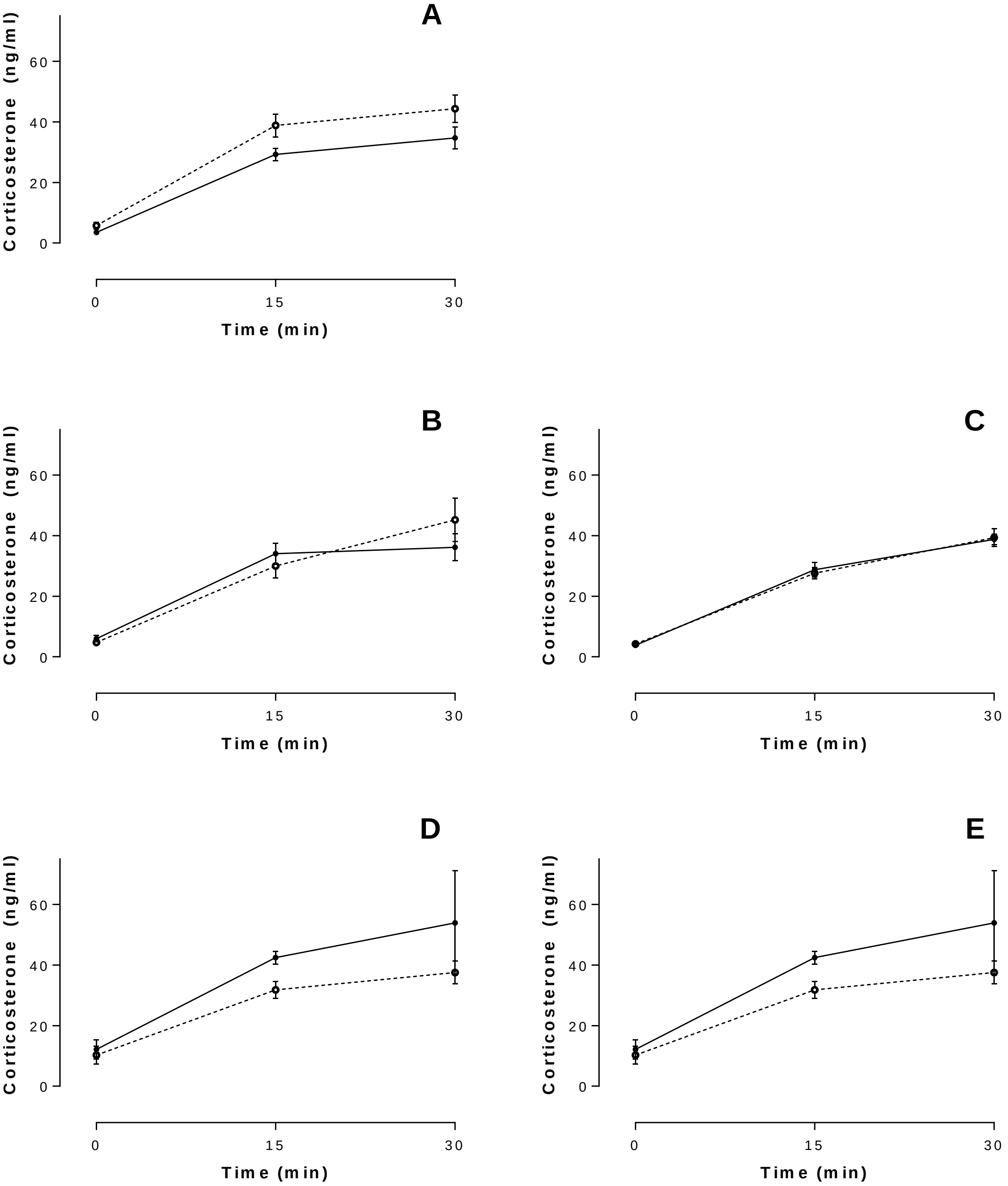

Fig. 2.7. Mean plasma corticosterone responses of kororā chicks at the creek (— ● —) and quarry (--- ○ ---) colonies at Oamaru. The birds were 2-weeks-old (A), 4-weeks-old handled (B), 4-weeks-old unhandled (C), 6-weeks-old handled (D) and 6-weeks-old unhandled (E). Sample sizes were 8 - 12 except for chicks 6 weeks of age at the creek colony for which the sample sizes were 4 chicks.

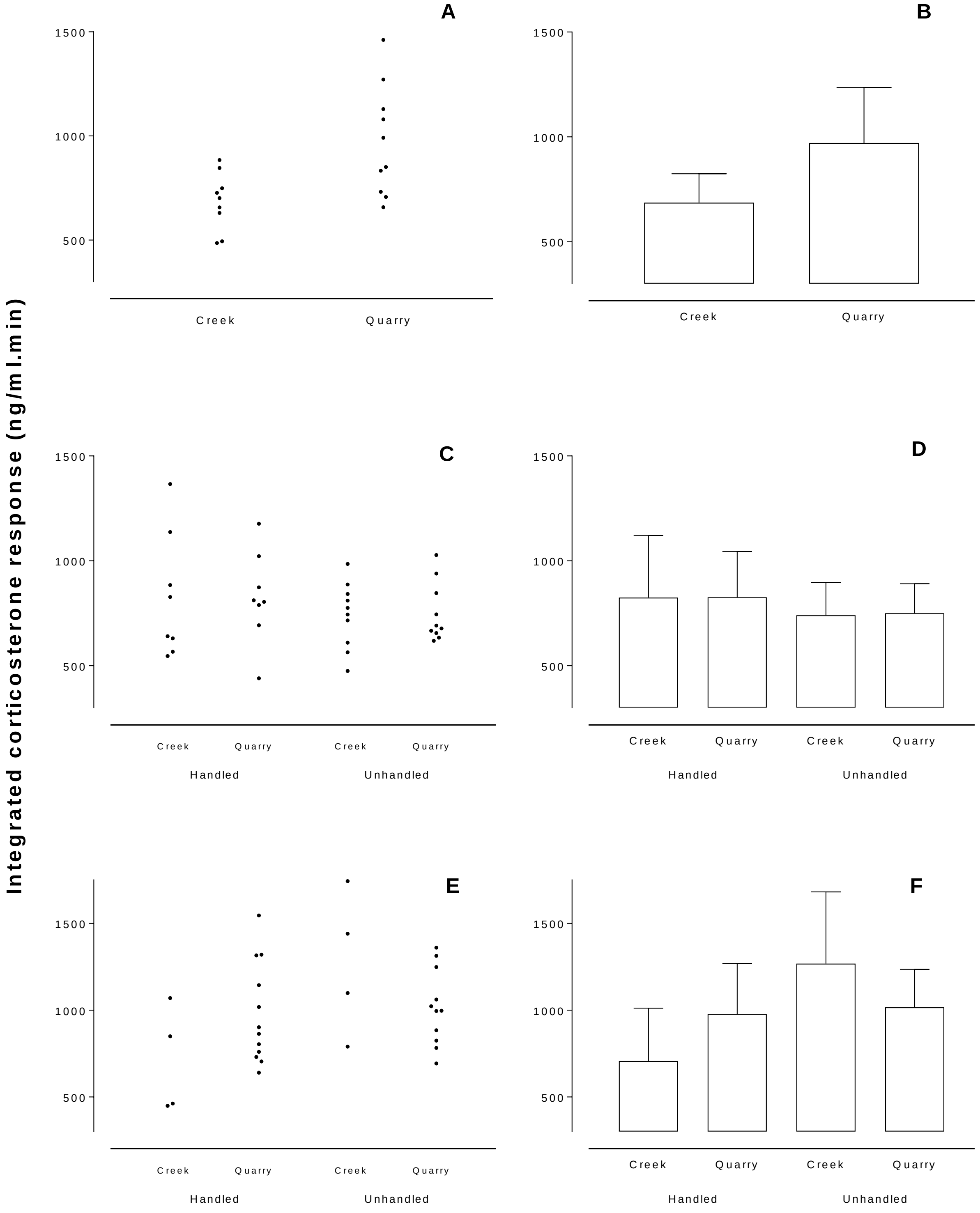

Fig. 2.8. Individual and mean integrated corticosterone responses of chicks at the creek and quarry colonies. Chicks were 2 (A and B), 4 (C and D) and 6 (E and F) weeks of age.

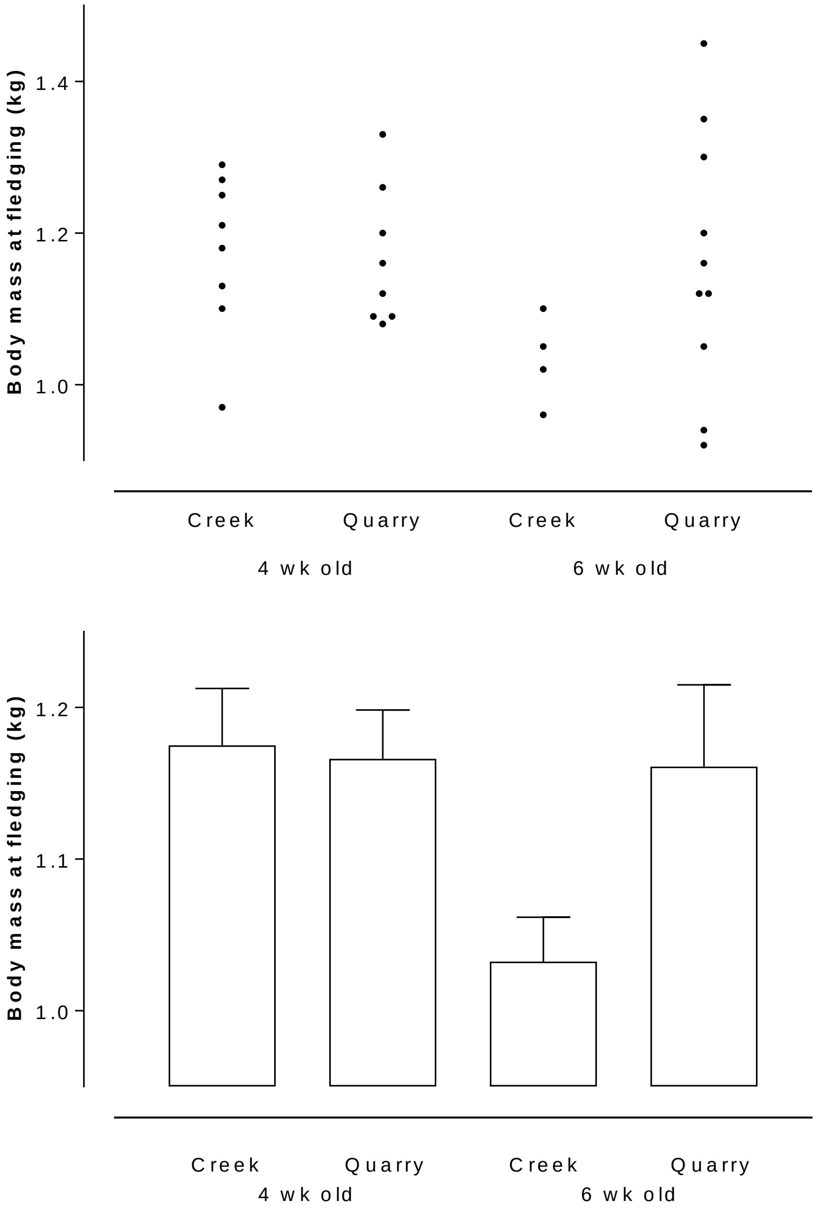

Fig. 2.9. Individual and mean fledging body masses of chicks at the creek and quarry colonies. Chicks were handled twice weekly from 2 weeks of age and blood sampled at 4 (4 wk old) or 6 (6 wk old) weeks of age.

2.3.7 Body mass, body dimensions, and body condition index

Mean body masses of kororā chicks weighed twice weekly from 2 weeks of age are shown in Fig. 2.10. Mean body mass increased steadily from 14 to 30 days of age. The rate of increase was slower from 30 to 50 days of age, then body mass did not increase further from around 50 days of age. A repeated measures one-way ANOVA showed that mean body mass changed with age ($F_{3.07,\ 49.13} = 353$, $P < 0.0001$). Comparisons of body masses at weekly intervals showed that body mass increased from 14 to 21 ($p < 0.0001$), 21 to 28 ($p < 0.0001$), 28 to 35 ($p < 0.0001$), 35 to 42 ($p = 0.0012$), and 42 to 49 ($p = 0.0002$) days of age, and did not change significantly from 49 to 56 days of age.

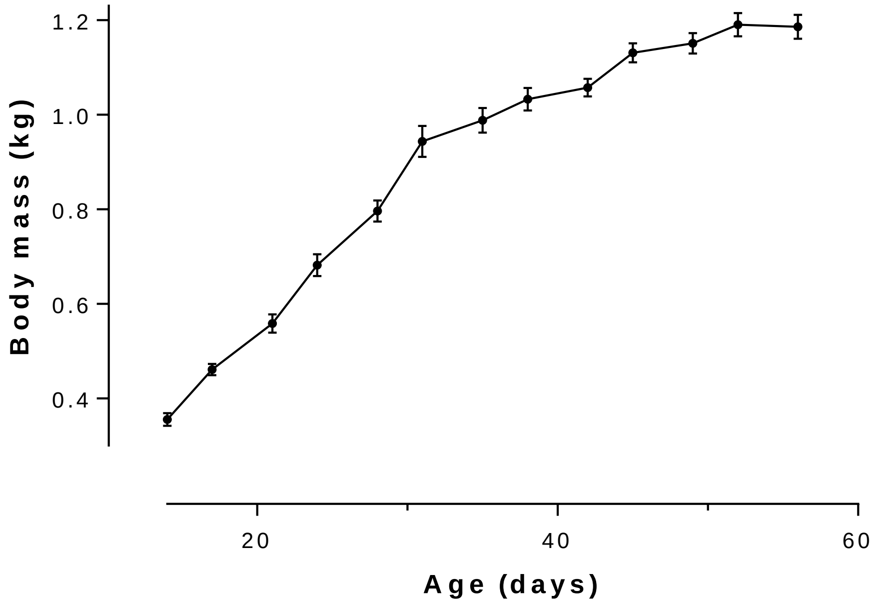

Fig. 2.10. Mean body masses of kororā chicks handled twice weekly from 2 weeks of age ($n = 32$).

Mean beak length and depth increased steadily from 17 to 38 days of age, whereas flipper length increased from 17 to 31 days of age and then did not change significantly (Fig. 2.11; see Table 2.5 for statistics).

Table 2.5. Statistical analyses of beak length, beak depth and flipper length in kororā from 2.5 to 6 weeks of age.

	F	Degrees of freedom	*p*
Beak length	136.4	1.65, 18.11	<0.0001
Beak depth	32.72	1.74, 17.43	<0.0001
Flipper length	76.33	2.25, 29.29	<0.0001
Comparisons between ages.			
Beak length			
17 vs. 24 days		11	<0.0001
24 vs. 31 days		11	<0.0001
31 vs. 38 days		11	<0.0001
Beak depth			
17 vs. 24 days		10	0.0153
24 vs. 31 days		10	0.0153
31 vs. 38 days		10	0.0001
Flipper length			

	F	Degrees of freedom	*p*
17 vs. 24 days		13	<0.0001
24 vs. 31 days		13	0.0132
31 vs. 38 days		13	0.8803

Note: The first three rows show the results of one way repeated measures ANOVA for the effects of age on body dimensions. The remaining rows show the results of Sidak's comparisons of ages for each measurement.

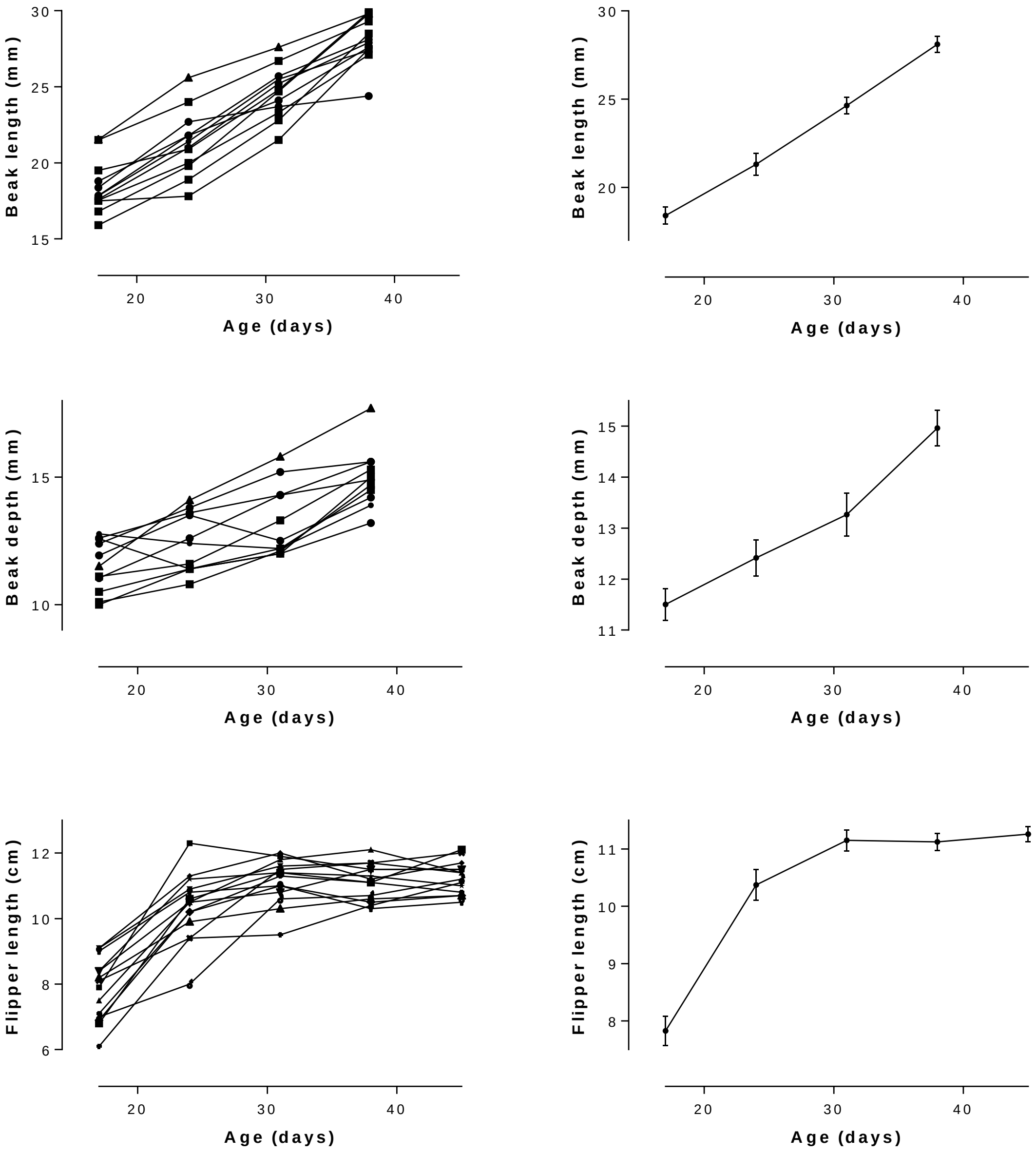

Fig. 2.11. Individual and mean beak and flipper dimensions of chicks handled twice weekly from 2 weeks of age and blood sampled at 4 or 6 weeks of age.

Mean body mass, beak length and flipper length are shown together on the same x-axis scale in Fig. 2.12 to illustrate how the pattern of increase in beak length was similar to the increase in body mass, whereas the pattern of increase in flipper length differed from the pattern of increase in body mass. A body condition index was calculated as body mass/beak length x 100 000. A repeated measures one-way ANOVA showed that mean body condition index changed with age ($F_{1.34,\,17.45} = 5.205$, $p = 0.0269$; see Fig. 2.13). Mean body condition indices did not differ between 17 and 24 days of age, decreased from 24 to 31 days of age ($p = 0.0125$), and decreased from 31 to 38 days of age ($p = 0.0004$).

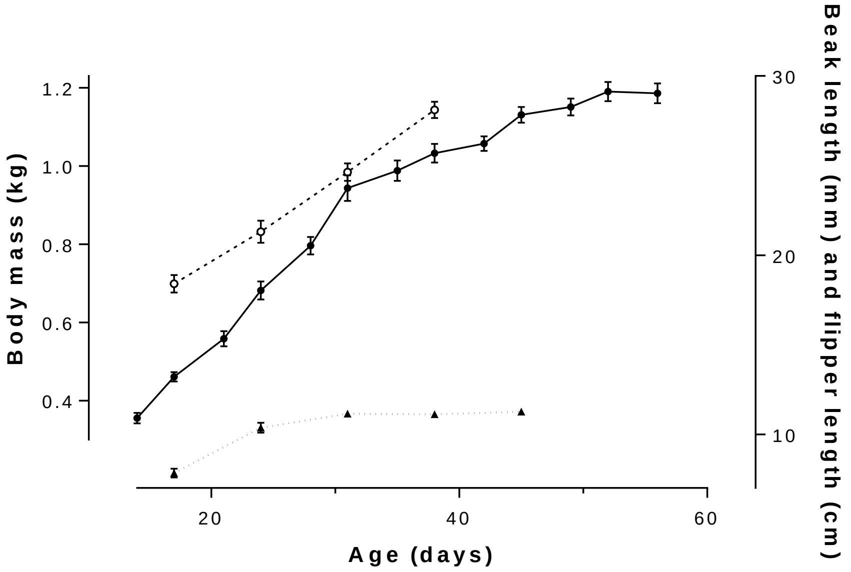

Fig. 2.12. Mean body mass (— ● —), beak length (--- ○ ---) and flipper length (···▲···) of chicks handled twice weekly from 2 weeks of age and blood sampled at 4 or 6 weeks of age.

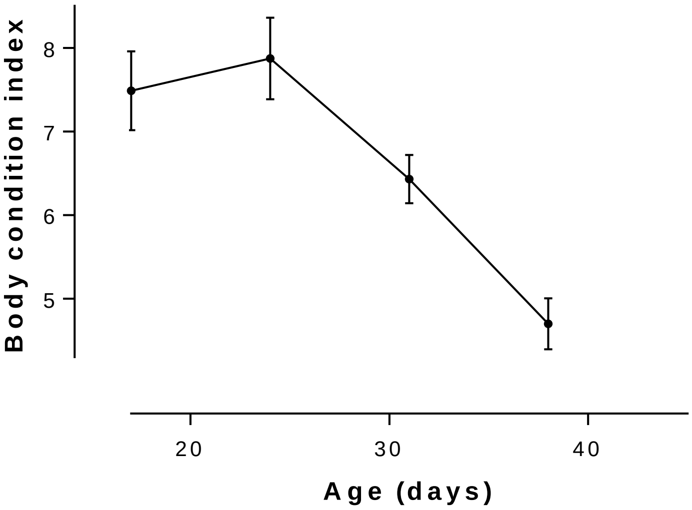

Fig. 2.13. Mean body condition index of chicks handled twice weekly from 2 weeks of age and blood sampled at 4 or 6 weeks of age.

2.4. Discussion

This was the first study of the development of corticosterone responses in kororā chicks, and the first study of effects of regular handling on corticosterone responses in penguin chicks. It was also the first to measure body condition indices will how body mass and fledging weight may be related to corticosterone responses of penguin chicks. This study also compared corticosterone responses and body mass at two sites at Oamaru. This study did not look at the responsiveness to natural stressors which means that comments on how previously handled chicks might respond to naturally encountered stressors are speculative. We also did not test the responsiveness of the HPA axis directly with ACTH or CRF thus we cannot make assumptions on how handling has affected the development of the HPA axis in kororā chicks.

Kororā chicks two weeks of age had clear corticosterone responses to handling. Mean responses did not change from two and four weeks of age, then were greater and similar to adult responses in chicks six weeks of age. There was no significant difference in corticosterone responses between chicks that were not handled and chicks that were handled twice weekly chicks from 2 to 4 weeks of age, or from 4 to 6 weeks of age. The mean integrated response of handled chicks was lower, however it was not significantly lower, than the integrated response of unhandled chicks 6 weeks of age. Fledging weight and body mass at 2, 4 or 6 weeks of age were not related to the magnitude of corticosterone responses of penguin chicks. Corticosterone responses and initial corticosterone concentrations differed between the creek and the quarry colonies in chicks two weeks of age, whilst there were no differences between the colonies in chicks for or six weeks of age. There was also no significant difference between the fledging weights between the two sites. Beak size (width, length and depth) grew at a linear rate as the chicks got older whereas flipper length increased markedly to 31 days and not thereafter. Mean body condition, calculated from body mass and beak length, decreased from 24 to 38 days of age.

2.4.1. Initial plasma corticosterone and sample collection times

When birds are captured and handled to measure a corticosterone response the corticosterone concentration in first sample is often called the baseline corticosterone concentration and is considered to reflect the plasma corticosterone concentration of a bird before it was captured or picked up (Kitaysky *et al.*, 2001b; Pravosudov *et al.*, 2001; Clinchy *et al.*, 2004; Kitaysky *et al.*, 2005; Walker *et al.*, 2005b; Angelier *et al.*, 2008). It has generally been considered that samples collected within three minutes of capture can be considered to be baseline samples, and that effects of capture and handling on corticosterone concentrations do not become apparent until after three minutes from the time of capture (Wingfield *et al.*, 1982; Kitaysky *et al.*, 1999; Scheuerlein *et al.*, 2001; Romero and Romero, 2002; Romero and Reed, 2005).

However, corticosterone concentrations in birds can be markedly elevated above concentrations in undisturbed birds within less than three minutes, and there have been recent reports challenging the "three minute rule" and the idea that samples collected within three minutes of capture reflect undisturbed corticosterone concentrations (Baugh *et al.*, 2013; Newman *et al.*, 2017; Small *et al.*, 2017). The time taken to collect the first samples from chicks in our study, and from adults, was often greater than three minutes and corticosterone concentrations measured in these first samples were often relatively high for some of the groups of chicks. There were significant relationships between time taken to collect the sample and plasma corticosterone concentrations. We therefore termed the corticosterone concentrations in the first sample to be initial concentrations rather than baseline concentrations.

2.4.2 Development of corticosterone responses in kororā chicks

Kororā chicks two weeks of age had clear corticosterone responses to handling. Chicks were not sampled when they were less than two weeks of age, so we do not have information about corticosterone responses of kororā chicks shortly after hatching. Corticosterone responses did not change significantly between two and four weeks of age, whereas responses were significantly higher in chicks six weeks of age that were approaching the time of fledging. These findings are similar to those found in other altricial or semi-altricial species such as Magellanic penguins (Walker *et al.*, 2005b), Northern mockingbirds (Sims and Holberton, 2000), American kestrels (Love *et al.*, 2003) and white-crowned sparrows (Wada *et al.*, 2007). In these species corticosterone responses increase with age in chicks, and responses of chicks before fledging similar to responses of adults. This is a common pattern seen amongst altricial and semi-altricial chicks (Adams *et al.*, 2008). It has been suggested that lower responses in younger chicks are related to the protection that young chicks receive from their parents and to the possibility that corticosterone responses of young chicks might be more detrimental than beneficial (Sims and Holberton, 2000; Kitaysky *et al.*, 2003).

Kororā chicks are covered with down when they hatch and require brooding by their parents until 2-3 weeks of age when the demand for food for the chicks mean that both parents must be away during the day to forage for food which they bring to the chicks in the evening. The parents will continue this until chicks are around 7-9 weeks of age when they have developed a waterproof layer of feathers and can fledge (Agnew *et al.*, 2014). It can be suggested that this semi-altricial progression from hatching to fledging and reliance on the parents means that the chicks do not require full adult corticosterone responses until just before fledging (Sims and Holberton, 2000; Kitaysky *et al.*, 2003). If chicks have lower magnitudes of corticosterone responses at a young age they could avoid possible detrimental effects of high concentrations of corticosterone such as the inhibition of growth hormones, catabolism of skeletal muscle and lowered growth efficiency, as well as a reduction in social behaviours and compromised cognitive abilities (Sims and Holberton, 2000; Kitaysky *et al.*, 2001b; Tarlow *et al.*, 2001; Kitaysky *et al.*, 2003; Walker *et al.*, 2005b). The postulated potential negative effects could compromise the survival rate of the chicks in their first year post-fledging, most of which is spent at sea, which is only 38% (Agnew and Houston, 2008; Agnew *et al.*, 2014).

2.4.3 Effect of twice weekly handling on corticosterone responses

Twice weekly handling did not have significant effects on corticosterone stress responses of kororā chicks 4 or 6 weeks of age. The results indicate that the HPA axis of the handled chicks developed a corticosterone stress response of adult-like magnitude between 4 weeks of age and fledging, at a similar time to the unhandled chicks.

It has been well documented that human interactions with wild animals, especially young or breeding animals can have a detrimental effect on their physiology, breeding success, survivability and behaviour (Hockey and Hallinan, 1981; Giese, 1996; Fowler, 1999; Blackmer *et al.*, 2004; Müllner *et al.*, 2004b). In hoatzin juveniles and Magellanic penguin chicks, these interactions have been recorded as having negative effects on their behavioural acclimatisation to humans as well as higher corticosterone and increased sensitivity of their HPA axes (Müllner *et al.*, 2004b; Walker *et al.*, 2005a). On the other hand, there are also studies showing that regular interactions with people did not have detrimental effects on birds. It was found that, in African penguin chicks, the frequent handling required to hand rear them, did not have damaging effects on the birds post-fledging (Barham *et al.*, 2008; Sherley *et al.*, 2014). When exposed to higher amounts of human interactions in the form of tourist visits, an African penguin colony had a higher breeding success than the colonies exposed to fewer tourist visits (Sherley *et al.*, 2012). Penguins in the more disturbed colony had a smaller response to humans approaching them than those in the other colonies (Pichegru *et al.*, 2016) which indicates that African penguins at these disturbed sites become conditioned to not view human disturbance as such an imposing stressor. The kororā chicks, their parents and other birds in the colonies tested in this current study often choose to breed in nest boxes near the path or under walkways and steps and are therefore frequently exposed to human sounds and interactions (P. Agnew, pers. comm.) indicating that kororā also acclimatise to human interactions so as to not perceive being approached as a life-threatening stressor.

The results from the current study showing that handling of kororā chicks twice weekly did not have significant effects on HPA axis responsiveness to handling do not exclude the possibilities of other detrimental behavioural and physiological effects on the chicks. Due to the novelty of this study, we do not have data on the long-term effects seen in adult kororā that were exposed to handling stress as chicks nor do we have comparative information on the responsiveness of kororā chicks to natural stressors. It is therefore likely that the previously handled chicks perceive human interactions to be less of a stressor when they are tested, however, this does not mean that the responsiveness of the chicks has been altered when confronted by a natural stressor.

2.4.4 Integrated corticosterone responses

The integrated corticosterone responses of the kororā chicks in this study showed the same patterns as the mean plasma corticosterone responses, with mean integrated responses not differing between chicks of two and four weeks of age, with responses of six week old chicks not significantly different from those of adults. There was also no significant difference in the integrated corticosterone response of the handled and unhandled chicks at four or six weeks of age. In white-crowned sparrows, there is a similar pattern with a hyporesponsive period in the early stages after hatching, and then the corticosterone response increases and is similar to that of adults and chicks at the time of fledging (Wada *et al.*, 2007). It has also been found that in Magellanic penguin chicks which have been frequently disturbed by humans from hatching, show a significant difference in integrated corticosterone levels at a young age (Walker *et al.*, 2005a), this current study was unable to compare younger chicks as we had been asked not to remove the chicks from their nest boxes until 2 weeks of age. However, mottled petrel chicks that were hand reared, had a significantly lower corticosterone response than those of parent reared chicks (Adams *et al.*, 2005). This pattern was also observed in eastern bluebirds with their corticosterone responses developing at a similar rate before the frequently handled chicks' responses significantly decreasing (Lynn *et al.*, 2013). It is possible that the chicks in this current study were not disturbed or handled frequently enough to show significant effects on the integrated corticosterone responses of the chicks at any age tested but with more frequent interactions, there may be a significant effect on the responses.

2.4.5 Body mass and fledging weight in relation to corticosterone responses

Body mass did not differ between chicks handled twice weekly from 2 weeks of age and chicks that were not handled when chicks were weighed at 4 or 6 weeks of age. Whilst we were not able to obtain data on fledging weights of chicks handled twice weekly from 2 to 4 weeks of age or twice weekly from 2 to 6 weeks of age, the absence of a handling effect on body mass at 4 or 6 weeks of age indicates that it was likely that there was no effect of twice weekly handling on fledging weights of kororā chicks.

These results are contrary to previous studies which have shown a decreased body mass of chicks raised in frequently disturbed colonies when compared to their less disturbed counterparts (McClung *et al.*, 2004; Müllner *et al.*, 2004b; Schroeder *et al.*, 2012). However, these studies cannot be directly compared with the chicks in the current study as they compare fledging masses after take one sample and having that sample be the first time the chicks were handled. Further research is need to determine if the kororā chicks' fledging weights to follow similar patterns to those of yellow-eyed penguins and house sparrows (McClung *et al.*, 2004; Schroeder *et al.*, 2012)

It was also found that chick body mass at fledging was not related to integrated corticosterone responses. These results added to the plethora of studies looking into the relationship between body mass and corticosterone responses all of which have varied results. In red-legged kittiwakes (*Rissa brevirostris*), it was found that lowered body mass resulted in an increase in baseline and stress-induced corticosterone levels (Kitaysky *et al.*, 2001a). However, in tufted puffin chicks (*Fratercula cirrhata*), a lowered body mass resulted in the suppression of the HPA axis and a decrease in both baseline and maximum stress-induced plasma corticosterone levels (Kitaysky *et al.*, 2005). It was also found in redpolls (*Carduelis flammea*), that an increase in fat stores resulted in a more robust corticosterone response at one breeding site but a decrease in plasma corticosterone levels at another (Romero *et al.*, 1998). Finally, as with the kororā chicks, Atlantic canary (*Serinus canaria*) chicks showed no relationship between body mass and corticosterone responses.

2.4.6 Chick mass and corticosterone responses at two Oamaru kororā colonies

The quarry and creek kororā colonies at Oamaru are 1 km apart and birds from the colonies are likely to forage in the same areas of ocean. Breeding success and survival rates are similar for the two colonies (Agnew, 2007; Agnew and Houston, 2008; Cockrem *et al.*, 2016). Body mass of chicks at fledging did not differ between the colonies in the current study. Mean corticosterone responses were lower at 2 weeks of age in chicks at the creek compared with the quarry colony and did not differ between colonies in chicks at 4 and 6 weeks of age. This is a positive outcome for the penguins at the quarry colony which is the Oamaru Blue Penguin Colony tourist site, as it indicates that the restrictions on tourist viewing are sufficient to avoid detrimental effects on the chicks' corticosterone responses or growth rates.

2.4.7 Growth in kororā chicks

The chicks' body condition index and flipper length growth rates were not linear with age. The pattern of growth that the body condition indices follow is likely due to the parents' behaviour. The low point is at around the time both parents need to leave the nest during the day to provide food for the chicks and then begins to climb to the point of having the best chance of survival post-fledging and when the parents stop feeding them (Chiaradia and Nisbet, 2006; Agnew *et al.*, 2014). The body mass of the chicks increased steadily with age before slowing, but still increasing, at around 45 days of age. This pattern varies slightly from that of kororā chicks in Phillip Island, Australia who grew until a peak at 6-7 weeks of age before declining in mass by 10-20% (Chiaradia and Nisbet, 2006). It was also found that peak mass and fledging masses were highest and obtained at a later age in good breeding years in the Phillip Island colony (Chiaradia and Nisbet, 2006) so it is possible that because we did not study these chicks over multiple years, we may not have observed the same decline if 2016 was considered to be a good year. The flipper length of the kororā chicks followed a similar pattern to the flipper growth in African penguin chicks wherein the flippers grow relatively quickly before plateauing at a length similar to adults at a young age (Lubbe *et al.*, 2014).

Both the beak width and depth grew at linear rates. These results are similar to those recorded in a colony of kororā chicks on Penguin Island, Australia (Wienecke *et al.*, 2000). It has been suggested that this less rapid growth rate of the beak dimensions when compared to the flipper length is most likely due to thermoregulatory demands (Gales, 1987) and a hastened growth rate is also seen in foot development (Wienecke *et al.*, 2000) which were not measured in this study.

2.5. Conclusions

This was the first study of the development of corticosterone responses in kororā chicks, and the first study in penguins of effects of regular handling on corticosterone responses of chicks. It was also the first study of relationships between corticosterone responses and body mass in growing kororā chicks and body mass at fledging. This study also compared corticosterone responses and body mass at two sites at Oamaru. Mean corticosterone responses were similar in chicks 2 and 4 weeks of age, whereas responses were higher and similar to adult responses in chicks 6 week of age. Twice weekly handling from 2 weeks of age did not have significant effects on corticosterone stress responses of kororā chicks 4 or 6 weeks of age. The results indicate that corticosterone responses of handled chicks increased to be similar to adult responses between 4 weeks of age and fledging, at a similar time to the unhandled chicks. A body condition index decreased as chicks became older, reflecting different patterns of growth of body mass and body size. This research has paved the way for future research that could look at survival rates and breeding success of birds that had been handled twice weekly as chick and that return to the colony to breed when two or three years old. It would also be possible to measure compare adult corticosterone responses with response of birds when they were chicks to determine if the magnitude of corticosterone stress responses of chicks as chicks is predictive of the responses of the birds as adults. In addition, relationships between corticosterone responses of chicks and survival of birds to breeding age could be investigated to determine whether chicks with relatively low or high corticosterone responses (and hence relatively proactive or reactive personalities) have different survival probabilities.

3. General Discussion

3.1. Introduction

Whilst there have been many studies of the development of the HPA axis in avian chicks, from pre-hatching to post-fledging, few studies have considered and the effects of human interactions on this development. In most studies, corticosterone responses of free-living chicks are measured at different ages using the standard capture and restraint protocol (Wingfield *et al.*, 1992). Each chick is handled once, with mean corticosterone responses at different ages measured in order to determine the magnitude of the corticosterone response in relation to the age of the chicks. There have also been previous studies of behavioural responses of chicks to human interactions. There have been few studies of physiological responses of chicks to human interactions. The current study used the standard capture and restraint protocol, with the duration of the protocol limited to 30 minutes to avoid potential detrimental effects on the chicks that could have arisen from the chicks being out of the nest for longer. Some of the chicks were sampled once whereas other chicks were handled twice weekly from two weeks of age for body condition measurements. Corticosterone responses of chicks that were handled twice weekly were compared with responses of chicks that had not been handled to determine how handling might affect corticosterone responses, and hence to determine whether the chicks became accustomed to (habituated) to handling by humans.

Kororā chicks two weeks of age had well-developed corticosterone responses to handling. The magnitude of the responses did not change between two and four weeks of age, whereas responses of six week old chicks were greater and were not significantly different from responses of adults. There were no significant differences in corticosterone responses between chicks handled from 2 to 4 weeks of age and chicks that were not handled, or between chicks handled from 2 to 6 weeks of age, and chicks that were not handled. There are limited data available on effects of handling on corticosterone responses of penguin chicks. Magellanic penguin chicks, while exhibiting behavioural habituation to humans, also showed no difference in corticosterone responses when compared to undisturbed chicks (Walker *et al.*, 2005a). It was expected that the kororā chicks would follow the same response pattern. There is no information available on the effects of twice weekly handling of chicks on post-fledging survival in kororā. The only information on survival of penguins that had been handled whilst chicks comes from studies of African penguin chicks. Survival of African penguin chicks that were hand reared did not differ from the survival of chicks that were not handled (Barham *et al.*, 2008; Sherley *et al.*, 2014). It is possible that if kororā chicks were handled more frequently than twice weekly there could be effects of handling that were not seen in the present study. For example, in hoatzins the nestlings did not show strong physiological impacts of human interactions but disturbed juveniles had a stronger corticosterone stress response and a lowered survival rate (Müllner *et al.*, 2004a).

Body mass, fledging weight and body condition measurements in the form of beak width and length and flipper length were also considered in this study. It was found that twice weekly handling did not affect the body mass of chicks at four and six weeks of age, so it is unlikely that twice weekly handling had an effect on fledging weights. It has been previously recorded that increased disturbance can decrease the body mass of avian chicks (McClung *et al.*, 2004; Müllner *et al.*, 2004b; Schroeder *et al.*, 2012) and it was hypothesised that the kororā chicks would exhibit the same body mass reduction. It is possible that twice weekly handling affected body mass at fledging, but measurements were not made of fledging body mass in chicks that had not been handled so this could not be tested in the current study. Linear growth rates were observed in both beak measurements but flipper length grew rapidly until around 24 days of age before slowing to a gradual increase. A body condition index derived from body mass and beak length gradually declined as chicks became older. Due to penguins' unique morphologies and a lack of previous studies in the area, we were unable to draw comparisons between kororā chicks and those of other, similar species.

Corticosterone responses of chicks of different ages have been described in a range of species. The number of species of birds that has been studied is limited, especially in penguin species, with many studies of chicks being focussed on behaviour. There are few studies of corticosterone responses of chicks that experience different levels of human interaction, and no information on corticosterone responses of adult birds that have been handled as chicks. Possible consequences of the effects of interactions of chicks with humans on their breeding success and survival are largely unknown. The current study of corticosterone responses and growth rates in relation to handling in chicks of different ages is novel just for kororā but for penguins generally.

3.2. Major conclusions

The goal of this study was to describe the development of corticosterone responses in kororā chicks and to determine whether twice weekly handling had an effect on this development. The major conclusions from this study are:

1. There were positive linear relationships between sampling time and initial plasma corticosterone concentrations in chicks four and six weeks of age that had not previously been handled. Corticosterone concentrations in initial samples collected from chicks were not considered to represent concentrations in undisturbed birds.

2. Kororā chicks two weeks of age had clear corticosterone responses to handling. Mean responses did not change from two and four weeks of age, then were greater and similar to adult responses in chicks six weeks of age.

3. There was no significant difference in corticosterone responses between chicks that were not handled and chicks that were handled twice weekly chicks from 2 to 4 weeks of age, or from 4 to 6 weeks of age.

4. The integrated corticosterone responses of the kororā chicks study showed the same patterns as the mean plasma corticosterone responses, with mean integrated responses not differing between chicks of two and four weeks of age, and responses of six week old chicks not significantly different from those of adults. Integrated corticosterone responses of chicks six weeks of age that had been handled twice weekly from two weeks of age were lower than integrated responses of adults

5. Fledging weight and body mass did not differ between handled and unhandled chicks. There were no significant relationships between fledging weight or body mass and plasma corticosterone responses or integrated corticosterone stress responses.

6. Corticosterone responses of two week old chicks were lower at the Creek colony and at the quarry colony at Oamaru. There were no differences between the colonies and responses of chicks for six weeks of age.

7. Body mass of chicks increased from 2 to 7 weeks old then did not change significantly. Beak length increased in a similar way to body mass up to 38 days whereas flipper length increased to 31 days old and then did not change significantly. Body dimensions were not measured in birds older than 38 days. A body condition index calculated using beak length decreased from 24 to 38 days of age.

3.3. Future studies

While this study addressed some novel questions about kororā and added to the available knowledge on avian chicks, many questions regarding the development of corticosterone stress responses, growth rates and the long-term effects of human interactions at an early age still remain. Studies of the chicks sampled in the study when they are adults would be useful to determine the long-term of handling effects on stress personalities, survival rates and behavioural responses to human interaction. These studies would have to be done one or more years after the chicks fledged when they began to return to the colony for the first time. Further studies could also be done on the different responses of siblings, late season chicks or chicks hatched as the result of a second (not replacement) clutch in the same season. Very little is known about the repeatability of stress responses in chicks and it is unknown at what age kororā develop their proactive or reactive personalities (Cockrem, 2007) and the extent to which these are inherited. Further studies in these areas would be worthwhile. Finally, it would be useful to extend this current study with mass and flipper length measurements at fledging at 7-9 weeks of age (Agnew *et al.*, 2014) for both handled and unhandled chicks to compare the effects of handling and human interaction on both the mass at fledging and body condition indices.

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