Corticosterone responses to stressors and the regulation of the hypothalamic-pituitary-adrenal axis in Japanese quail

A thesis presented
in partial fulfilment of the requirements
for the degree of
Doctor of Philosophy
in Physiology

at Massey University, Palmerston North
New Zealand

Wei-Hang Chua

2003

Abstract

Corticosterone is the major adrenal glucocorticoid in birds. It is secreted in response to stressors, with plasma concentrations typically rising to a peak within 10 to 15 minutes and then declining over 30 to 60 minutes once the stressor is removed. The increase in corticosterone is thought to help the animal to adjust to the stressor, although corticosterone may also inhibit the reproductive axis. The corticosterone response to a stressor varies between individuals, although the level of the hypothalamic-pituitary-adrenal axis at which this variation arises in birds has not been identified.

The goals of this research were to determine in Japanese quail (*Coturnix coturnix japonica*): (1) the effects of corticosterone and of various stressors on sex steroid secretion; (2) corticosterone responses to 5, 10 or 15 min exposure to manual restraint and 15 min exposure to different stimuli; (3) quantifying individual variation in the corticosterone response; and (4) whether individual variation in the corticosterone response to stimuli used in goal 2 is regulated by differences in pituitary or adrenal sensitivity to corticotropin releasing factor (CRF) and adrenocorticotrophic hormone (ACTH) respectively.

A prolonged elevation in plasma corticosterone concentrations can inhibit reproduction. The effects of a short elevation in corticosterone are less understood, so the relationship between corticosterone and sex steroid secretion in quail was characterised by administering 1.2 mg corticosterone injections. Corticosterone treatment stimulated an increase in plasma corticosterone, while testosterone declined in both corticosterone-treated and control birds. Plasma LH was unaffected by challenge with corticosterone. Collectively, these data suggest that repeated handling associated with frequent blood sampling inhibited testosterone secretion directly at the testis.

Corticosterone responses to a variety of novel stimuli were characterised. Manual restraint for 10 or 15 min caused a significant response whereas restraint for 5 min or less did not lead to an increase in corticosterone 15 minutes from the start of the stressor. Corticosterone responses to 15 min of mechanical restraint varied between birds whereas 15 min of manual restraint elicited a maximal plasma corticosterone response in all birds. Manual and mechanical restraint caused declines in plasma testosterone of a similar magnitude. There was more variation within than between birds in their corticosterone response to mechanical restraint. Despite this, the general pattern of the corticosterone response was repeatable for individuals.

Quail with low or high plasma corticosterone responses to 15 minutes of mechanical restraint were injected intrajugularly with a dose of ACTH that stimulated a sub-maximal corticosterone response. The plasma corticosterone response to ACTH did not differ between birds with low or high corticosterone responses to mechanical restraint, indicating that variation in corticosterone responses to restraint did not arise at the level of the adrenal gland. A preliminary study showed that injections of ovine CRF stimulated corticosterone secretion. However, CRF did not consistently stimulate an increase in plasma corticosterone in these birds and hence it was not possible to determine if pituitary responsiveness to CRF differed between birds with low or high corticosterone responses to restraint. These results indicate that variation in the corticosterone response between individual Japanese quail arises above the level of the adrenal gland in the HPA-axis, and may occur at the pituitary gland or due to differences in activation of neural pathways in the brain.

Massey University

Institute of Veterinary, Animal and Biomedical Sciences

Institute of Veterinary, Animal and Biomedical Sciences
Private Bag 11 222,
Palmerston North,
New Zealand
T: 64 6 350 4525
F: 64 6 350 5714

30th October 2004

CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled:

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Candidate's Name: Wei-Hang Chua

Signature:

Date: 14/10/2004

Supervisor's Name: John F. Cockrem

Signature:

Date: 14/10/2004

Acknowledgements

I would like to thank my supervisor, Dr. John Cockrem, for his support, guidance, encouragement, and the opportunities to travel to Japan, India, Scotland and Antarctica during my studies. Thanks also go to Prof. Tim Parkinson for his constructive criticism and insight during my research.

This research was supported by funding from the Institute of Veterinary, Animal and Biomedical Sciences (IVABS) Postgraduate Research Fund. Personal financial support was provided by the Massey University Scholarship Committee. A special thanks to Prof. Hugh Blair and Allain Scott of the IVABS Postgraduate Research Office.

Many thanks go to past and present members of the Conservation Endocrinology Research Group; Janis Bridges, Jane Candy, Janie Girling, Guy Hessell, and Raewyn Wheeler for their help and ideas over the years. Thanks also go to numerous students and staff in the Institute of Veterinary, Animal and Biomedical Sciences for their support.

I am grateful to Professor Susumu Ishii (Department of Education, Waseda University, Tokyo, Japan) for his kindness and hospitality during my visit to his laboratory in Tokyo. I would also like to thank Dr. Motoshi Kikuchi (Waseda University) who assayed blood samples for gonadotrophins and thyroid hormones, and Dr Richard Talbot (Roslin Institute, Midlothian, United Kingdom) who assayed plasma prolactin. I would like to acknowledge the advice, comments, and hospitality of Dr. Tim Boswell, Dr. Ian Dunn, and Prof. Peter Sharp during my stay at the Roslin Institute. Thanks go in particular to Tim for helping to get the quail c-*fos* mRNA assay setup when all looked lost.

I am indebted to my fellow students, Ellen Bennett, Emma Hawke, Sharon Henare, David and Sarah Simcock, and Mhairi Sutherland. Thank you for all the help with my experimental work, but also for the years of encouragement through the thick and thin, the great laughs we've all had, and especially the friendships. Good luck to all you guys!

Finally, I thank my parents and my brother, whom I will always be indebted to for their support and encouragement. Last but not least, appreciative pats and hugs go to my dogs Marley and Yuki for the stress relief and reminding me who gave them their mischievous streaks.

Table of contents

List of figures

List of tables

Chapter 1

General introduction

Birds respond to stressors with an increase in corticosterone secretion. The precise role of corticosterone in the stress response in not well understood in birds. It is thought to inhibit reproduction although this effect can vary between individuals.

The effect of corticosterone on sex steroid secretion was investigated in Japanese quail. Handling was found to have a profound effect on testosterone and the effect of handling and restraint on corticosterone and testosterone were investigated. Differences in the corticosterone response between individuals to the same stressor were quantified and the reasons for how they arise were investigated.

This is the first physiological study to use the New Zealand strain of Japanese quail and it was necessary to conduct pilot studies to identify the photoperiodic conditions for maintaining quail in the laboratory. These preliminary experiments are not included in this thesis, but the review of photoperiodic literature is included to provide relevant background information. The rest of the literature review provides an overview of the role of corticosterone in avian reproduction and variation of the corticosterone response.

1.1 Photoperiodic control of reproduction in birds

1.1.1 Photoperiodic induced gonadal growth and regression

It is well established that most birds are seasonal breeders, with reproduction timed to coincide with periods of ideal environmental temperature and food availability (Follett, 1984). Typically, exposure to a daylength of 12 hours or greater (long days; LD) stimulate the growth of the gonads, with onset of reproductive activity also influenced by other "ultimate" factors such as availability of food, competition, nesting conditions, predation pressure, and climate (Follett, 1984). Breeding is timed to occur when environmental conditions are such that the chances of successfully raising young are greatest. Near the end of the breeding season, most photoperiodic species undergo spontaneous gonadal regression while daylengths are the same or longer than those which stimulated vernal gonadal growth. This phenomenon is known as photorefractoriness (Deviche and Small, 2001). It occurs at a time of long daylengths, but prevents further breeding when any attempt to reproduce would be disadvantageous to the adult and potential young (Murton and Westwood, 1977). The photorefractive mechanism causes the rapid and spontaneous regression of the gonads. Photorefractoriness appears to be "programmed" into the hypothalamic-pituitary-gonadal axis (HPG-axis) during the first few days of photostimulation as reported in the American tree sparrow (*Spizella arborea*) (Reinert and Wilson, 1996a).

All seasonally breeding birds possess a unique critical daylength threshold, which once exceeded triggers the gonadal growth. Photic cues affect the hypothalamic-pituitary-gonadal axis via photoreceptive structures and centres within the avian brain involved with photoperiodic time measurement such as the mediobasal hypothalamus, the suprachiasmatic nucleus and pineal gland (Yasua *et al.*, 2003). Extra-ocular and extra-pineal receptors located close to or in the lateral septum and tuberal hypothalamic regions have been identified as the primary encephalic photoreceptors in the avian brain (reviewed by Deviche and Small, 2001). Wilson (1989) and Wilson (1990a) described the function of extraocular centres outside of the ocular photoreceptors in American tree sparrows (*Spizella arborea*). However, the photoreceptive mechanism may be mediated differently in different

species. Siopes and Wilson (1980) demonstrated that the photorefractive mechanism was mediated through retinal photoreceptors in quail, as blinded birds did not respond to a reduction in daylength. Photostimulation of the neural pathways associated with the photoreceptors is thought to directly influence the maintenance and numbers of gonadotrophin-releasing hormone (GnRH) neurons and the pulsatile secretion of GnRH from hypothalamic centres, which ultimately affect the secretion of gonadotropins from the anterior pituitary gland. Exposure to long days has been shown to stimulate an increase in GnRH secretion in Japanese quail (Follett and Maung, 1978) and gonadotrophin secretion increases in quail after just one day of exposure to long days (Follett *et al.*, 1977).

1.1.2 The hypothalamic-pituitary-gonadal axis

Exogenous cues such daylength, temperature and food availability are integrated in the brain and affect the hypothalamic-pituitary-gonadal axis through changes in GnRH secretion from the hypothalamus.

1.1.2.1 Gonadotrophin-releasing hormone

The secretion of luteinising hormone (LH) is under the control of GnRH. The effect of GnRH on follicle-stimulating hormone (FSH) secretion in birds is as yet unclear as previous *in vivo* GnRH treatment studies have proved inconclusive in chickens and Japanese quail (Hattori *et al.*, 1986). Gonadotropin-releasing hormone has been isolated from the chicken hypothalamus in two distinct forms, cGnRH-I and cGnRH-II (King and Millar, 1982; Miyamoto *et al.*, 1984). GnRH-II is the more potent stimulator of LH secretion *in vitro* (Millar *et al.*, 1986), although GnRH-I is likely to be the more important *in vivo* as it is secreted directly from the median eminence.

GnRH-I (from this point GnRH will be taken to mean GnRH-I) is secreted from the GnRH neurons which terminate in the median eminence. A fine network of blood vessels, also known as the hypothalamo-hypothyseal portal circulation, carries GnRH and other neuropeptides and neurotransmitters from the hypothalamus to the pituitary gland. GnRH stimulates gonadotrophin secretion from the anterior

pituitary gland or *pars distalis*. Photostimulation results in an increase in GnRH synthesis and secretion in the median eminence, as seen in Japanese quail in which GnRH immunoreactivity is higher in the median eminence in birds on long days than in birds held on short days (Foster *et al.*, 1988).

1.1.2.2 Gonadotrophins

The avian gonadotrophins LH and FSH are each composed of two glycoprotein subunits, a common α-subunit and a β-subunit specific to each hormone, with both units necessary for biological activity (Burke and Papkoff, 1979). Gonadotropin secretion is pulsatile and the frequency of pulses differs during hours of light and darkness. LH and FSH have specific roles within males and females in maintaining and controlling testicular and ovarian function.

LH induces ovulation of the largest ovarian follicle as well as stimulating differing modes of steroidogenesis in the largest ovarian follicles and small follicles. In contrast, FSH maintains and stimulates steroidogenesis in the smaller yolky follicles and the production of progesterone, androstenedione, and oestradiol from thecal cells of small follicles (reviewed in Scanes, 2000). LH stimulates Leydig cells to differentiate and produce testosterone in males, whilst FSH stimulates Sertoli cell differentiation and spermatogenesis (reviewed in Scanes, 2000).

1.1.2.3 Gonadal steroids

Gonadal steroids are synthesised and secreted in response to an increase in gonadotrophin secretion. In the female, a variety of gonadal steroids are secreted from the follicles and stromal tissue. Progesterone, oestradiol-17β and oestrone secretion are at their highest 4-6 hours prior to ovulation at the time of the preovulatory surge in LH (reviewed by Johnson, 2000). The gonadal steroids play essential roles in maintaining the passage of the ovulated follicle through the oviduct and in shell formation. In males, gonadotrophins stimulate the secretion of testosterone and androstenedione by Leydig cells, and the steroids influence spermatogenesis and the maintenance of testicular ducts and secondary sexual characteristics (reviewed by Kirby and Froman, 2000).

In addition to physiological effects, elevated gonadal steroids are associated with specific breeding behaviour. Oestrogens are involved in stimulating courtship and copulatory behaviour in females, with elevated progesterone and prolactin during the latter stages of follicular development inducing nest-building and incubation behaviour. Testosterone in males mediates copulatory behaviour indirectly through aromatization to oestrogens in the preoptic nuclei of the hypothalamus. Other courting behaviours such as strutting and crowing are stimulated through reduction of testosterone to 5α- and 5β-dihydrotestosterone (reviewed by Phillips *et al.*, 1985).

1.1.2.4 Prolactin

Prolactin is secreted from the pituitary gland under the influence of vasoactive intestinal polypeptide (VIP) from the hypothalamus (reviewed by Sharp *et al.*, 1998). Prolactin concentrations increase during photostimulation and prolactin is associated with brooding and incubation behaviour. Maximal prolactin concentrations during breeding are associated with and contribute to the suppression of LH secretion in incubating poultry and are also associated with the development of photorefractoriness (reviewed by Sharp *et al.*, 1998). The site of action of prolactin in the HPG-axis is unknown, although evidence shows that it is likely to act directly on GnRH and gonadotrophin secretion at the hypothalamus and anterior pituitary respectively. In turkeys, administration of prolactin results in a decline in hypothalamic GnRH and plasma LH (El Halawani *et al.*, 1991; Rozenboim *et al.*, 1993). However, the onset of photorefractoriness is likely to be independent of increasing prolactin concentrations, with prolactin being more involved with gonadal regression than with the initiation and maintenance of photorefractoriness (Juss, 1993). Alexander and Wolfson (1970) showed that twice daily injections of prolactin for 25 days did not affect ovarian or testicular weights but interrupted egg lay in quail, although Camper and Burke (1977) showed that ovarian and oviductal regression occurred following ovine prolactin treatment.

1.1.2.5 Regulation of gonadotrophin secretion

Gonadotrophin secretion is influenced by sex steroid feedback on GnRH secretion (reviewed by Scanes, 2000). The effect is predominantly inhibitory with LH

secretion being inhibited by oestrogens and androgens in male Japanese quail (Davies and Follett, 1980). While elevated oestradiol inhibits LH secretion in females, progesterone can have either a positive or negative feedback influence on LH secretion depending on the stage of the ovulatory cycle. The preovulatory LH surge in hens is stimulated by progesterone (Etches, 1990), although the stimulatory action of progesterone on GnRH and LH secretion has been suggested to only occur while oestrogen concentrations are elevated (Phillips *et al.*, 1985).

Inhibitory gonadal steroid feedback on the hypothalamus could influence gonadal state during the photorefractory period. It has been suggested that hypothalamic sensitivity to gonadal steroid inhibitory feedback increases in response to prolonged exposure to long days (Nicholls *et al.*, 1988). However, removal of the main sources of gonadal steroids by castration does not abolish the onset of photorefractoriness, with photorefractoriness manifesting itself in castrated birds at the same time as intact birds (Nicholls *et al.*, 1988). Wilson (1985) demonstrated in passerines that hypothalamic sensitivity to inhibitory gonadal steroid feedback decreased rather than increased after exposure to long daylength. A reduced hypothalamic sensitivity to negative feedback from steroids allows gonadal steroid secretion to increase resulting in the development of reproductive behaviour and development of accessory glands required during breeding (Nicholls *et al.*, 1988).

Hypothalamic sensitivity to gonadal steroids is restored upon exposure to short days. As a result, a low gonadal steroid concentration inhibits any increase in gonadotropin secretion from the anterior pituitary until daylength increases (Nicholls *et al.*, 1988). Exposure of a photosensitive bird to long days results in a decrease in hypothalamic sensitivity to negative feedback inhibition from gonadal steroids, allowing an increase in GnRH and gonadotropin secretion, gonadal development and increased steroid secretion (Nicholls *et al.*, 1988).

Hypothalamic sensitivity to gonadal steroids varies between species (Cockrem, 1995). Foster *et al.* (1987) and Goldsmith *et al.* (1989) reported a proliferation of GnRH perikarya in the median eminence of the hypothalamus in starlings once they were transferred from the photorefractory state to short days (8L:16D).

However, the increase in GnRH perikarya and GnRH immunoreactive regions of the hypothalamus which occur as the bird regains photosensitivity are not reflected by a corresponding increase in gonadotropin secretion when kept under short days (Wilson, 1990b). This indicates that photostimulation and the resulting increase in gonadotrophin secretion will not occur in the photosensitive bird without an increase in daylength. LH secretion did not change in photosensitive American tree sparrows in response to increasing daylength while photoperiod remained below the photostimulation threshold (Wilson, 1990c), and LH remained suppressed even after photosensitivity has been fully restored. LH only increased when daylength increased beyond the critical daylength threshold (Wilson, 1990b). However, in the non-passerine species that have been studied, photorefractoriness does not appear to be associated with a change in the sensitivity of the hypothalamus to negative feedback inhibition from gonadal steroids (Nicholls *et al.*, 1988).

1.1.3 Photorefractoriness

During the breeding season whilst daylength may be still increasing, gonadal regression can occur as birds lose their responsiveness to long days (Cockrem, 1995). The loss of photosensitivity generally occurs whilst photoperiod is still above the critical daylength threshold for photostimulation (Murton and Westwood, 1977). Photorefractiveness is a "reversible state of unresponsiveness to daylengths of gonadostimulatory duration" (Wilson, 1989), where the same daylength that stimulates the reproductive system (photostimulation) also induces the shutdown of reproduction (photorefractoriness). The photorefractory process encompasses a complex myriad of neuroendocrine mechanisms entrained to a variety of environmental cues or proximate factors but occurs to ensure that breeding terminates before environmental factors have the potential to impede reproductive success.

Photorefractoriness is initiated at or above the level of the hypothalamus (Cockrem, 1995). Associated with photorefractoriness is a decrease in GnRH and gonadotrophin secretion. It is unlikely that the pituitary gland or gonads become unresponsive to GnRH or gonadotrophins as gonadal development can be readily

induced by administration of synthetic GnRH and gonadotropin respectively (reviewed by Phillips *et al.*, 1985). Elevated gonadal steroid inhibitory feedback after photostimulation by long days is unlikely to be the primary cause of photorefractoriness. Wilson (1986) reported that the decrease in photosensitivity associated with photorefractoriness is independent of elevated plasma testosterone concentrations in American tree sparrows (*Spizella arborea*). However, it is important to note that the strength of the photorefractory response (degree of unresponsiveness to long days) varies between species (Cockrem, 1995) and species can be absolutely or relatively photorefractory.

1.1.3.1 Absolute photorefractoriness

Birds that undergo photorefractory gonadal regression and become completely unresponsive to daylengths that would otherwise be photostimulatory are known as absolutely photorefractory. In the starling (*Sturnus vulgaris*), a classic photorefractory model species, there is a reduction in areas of GnRH immunoreactive staining in the hypothalamic GnRH perikarya and median eminence as gonadal regression is nearly complete associated with the onset of photorefractoriness (Deviche and Small, 2001). Species such as the starling, house finch (*Carpodacus mexicanus*), and American tree sparrow (*Spizella arborea*) remain photorefractory as long as they are exposed to long days, and photosensitivity is only restored upon exposure to short days (reviewed by Deviche and Small, 2001).

1.1.3.2 Relative photorefractoriness

Species that are relative photorefractory retain gonadal photosensitivity to extremely long daylengths and do not undergo gonadal regression until there is a decrease in daylength. The Japanese quail is one such species that is not completely unresponsive to increasing daylength during the photorefractory process. Vernal gonadal development is stimulated in photosensitive quail at a photoperiod of 11.5 hours in quail at 52°N, whereas photorefractoriness is subsequently stimulated when the daylength falls below 14.5 hours (reviewed by Cockrem, 1995). However, rather than being completely unresponsive to long days during the photorefractory period, large gonads can be maintained indefinitely by chronic exposure of

photorefractory quail to unnaturally long day lengths, i.e. 20 hours (reviewed by Cockrem, 1995).

1.1.3.3 Thyroid hormones

Thyroid hormones are imperative for the regulation and control of whole body metabolism of birds. The effects of environmental factors such as diet, temperature and daylength on the release of thyroid hormones may explain how environmental cues influence reproductive development (reviewed by Phillips *et al.*, 1985). In many species, photorefractoriness is associated with the post-nuptial moult, and the cessation of mating behaviours and egg laying (reviewed by Phillips *et al.*, 1985). Thyroid hormones have been implicated as the primary humoral factor initiating and maintaining the photorefractory state (Wilson and Reinert, 1993), as well as having a primary role in the moult process. Thyroidectomy abolishes the main source of thyroid hormones, and blocks the onset of photorefractoriness (Wilson and Reinert, 1993). The secretion of T_4 and its metabolites increase after photostimulation, an effect which is required for gonadal growth (Reinert and Wilson, 1996b). However, thyroid secretion increases rapidly before the onset of photorefractoriness to concentrations that inhibit gonadotropin secretion at the same time as an increase in prolactin which also inhibits gonadotropin synthesis (reviewed by Sharp *et al.*, 1998; Sharp and Blache, 2003). Thyroid hormones are known to stimulate the growth of new feathers, which eject the old feathers. In starlings, thyroidectomy a few days after photostimulation abolishes the onset of photorefractoriness and there is no gonadal regression (Dawson *et al.*, 1986).

1.1.4 Gonadal growth and regression under artificial light cycles in Japanese quail

Japanese quail have been used extensively to study photoperiodic responses. Along with the starling and the domestic chicken, the photoperiodic response of the Japanese quail is one of the most extensively studied of any bird species (reviewed by Robinson and Follett, 1982; Wingfield, 1983; Follett and Nicholls, 1984; and Follett and Pearce-Kelly, 1990). The relative ease with which the timing of reproduction can be manipulated has made the Japanese quail an ideal species for photoperiodic studies. Japanese quail have also been selected for specific

photoperiodic responses due to the relative ease of housing quail and short time to sexual maturity.

Male and female Japanese quail (Figure 1.1) raised on long days from hatch are sexually mature by 5 and 6 weeks respectively (Siopes and Wilson, 1974). At sexual maturity, males and females of the wild-type randombred variety are sexually dimorphic by plumage. The increase in gonad size and androgen secretion results in the expression of a range of behaviours and secondary sexual characters such as crowing and development of a large cloacal gland (Oishi and Konishi, 1983). The cloacal gland produces a thick foam used during copulation (Adkins-Regan, 1999).

Females are on average heavier than males and begin laying eggs between 6-8 weeks of age at a regular daily interval. Wilson and Huang (1962) showed that females laid 75% of their eggs in the late afternoon between 1500 – 1800 when raised on a 12L:12D photoperiod (lights on: 0600 – 1800). Robinson and Follett (1982) reported that quail became photorefractory and underwent gonadal regression at 15 weeks of age when raised on natural daylengths of 13.5 – 14.5 hours of daylight. However, being relative photorefractory, gonadal regression can be prevented by maintaining birds on daylight hours greater than 15 hours (Robinson and Follett, 1982).

Conditions for maintaining Japanese quail with large gonads indefinitely are well established (Robinson and Follett, 1982), but maintaining birds with small gonads is more problematic due to differences in responsiveness to short days between strains of quail (Oishi and Konishi, 1983). Previous reports show that there are significant differences in the photoperiodic response between different populations or "strains" of Japanese quail. Some studies report a high degree of success inducing gonadal regression after a decrease in daylength (Robinson and Follett, 1982; Follett and Nicholls, 1984), while others have taken a different approach by preventing gonadal growth by raising chicks on short days (Follett and Farner, 1966; Wakabayashi *et al.*, 1992). The response of the different strains of quail to short days varies greatly and in some instances, additional stimuli such as

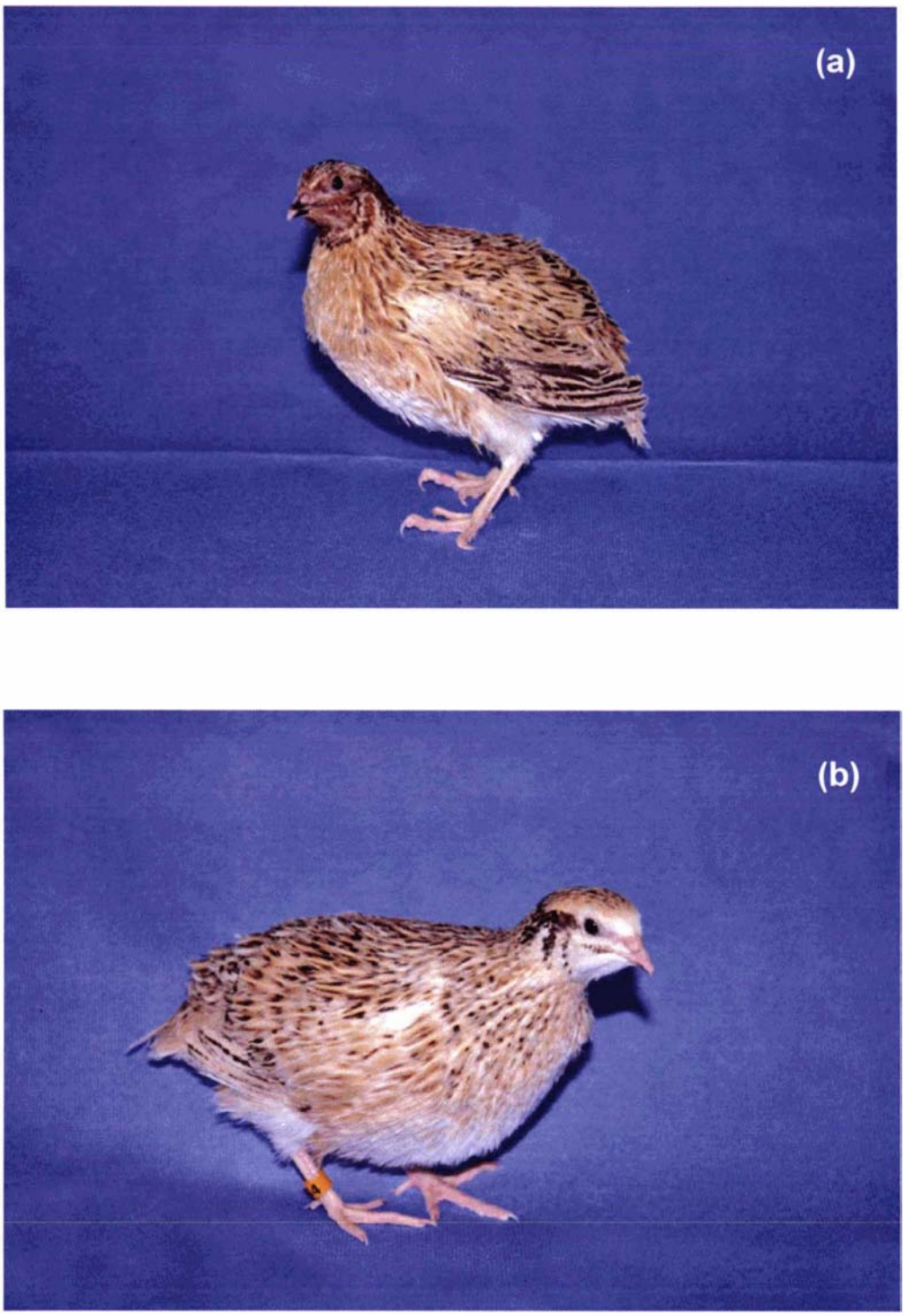


Figure 1.1 Eight week old male (a) and female (b) wild-type Japanese quail.

environmental temperature have been used in addition to a decrease in daylength to induce gonadal regression (Wada *et al.*, 1990).

The Japanese quail used in previous studies vary in origin from commercial "randombred" stock produced for egg or meat production, to strains genetically selected for particular photoperiodic responses. Japanese quail in New Zealand have been bred for both egg and meat production and nothing has been published on their photoperiodic response. Their photoperiodic response therefore had to be characterised in the present study as a prerequisite to studies of the effects of corticosterone on the control of the reproductive system.

1.1.4.1 Using changes in daylength to induce gonadal regression in sexually mature Japanese quail

Transferring quail with large gonads from long to short days can decrease reproductive function (Wilson *et al.*, 1965; Arrington *et al.*, 1969; Stein and Bacon, 1976; Robinson and Follett, 1982; Wada, 1993). Wilson *et al.* (1965) showed that egg production ceased 3-5 days after a reduction in daylength from 14L:10D to 8L:16D in 26-34 week old quail. Arrington *et al.* (1969) also showed that egg production had completely ceased 13 days after 12 week old birds were transferred from 14L:10D to a shorter daylength of 4L:20D. A similar pattern was repeated by Stein and Bacon (1976) where transfer from 14L:10D to 6L:18D caused a cessation in lay after 6 or 15 days in 89 or 140 day old females, respectively.

Regression of the gonads occurs as a result of a decrease in gonadotrophin secretion, as LH injections maintain lay in quail transferred from long to short days (Wilson *et al.*, 1965). There is also involvement of thyroid hormones as pharmacological doses of T_4 result in increases in FSH, LH and prolactin in quail transferred to short days (Follett *et al.*, 1988). Thyroid hormone concentrations also play a role in maintaining small gonads and are inherently higher in quail held on short days versus those on long days (Almeida, 1982).

Japanese quail with small gonads held on short days are extremely sensitive to subtle changes in daylength, and the utmost care must be taken in preventing an

increase in daylight hours or inadvertent exposure to any light outside the normal light regime. Hatanaka and Wada (1988) showed that 15 minutes exposure to light 12.5 – 14.5 hours after dawn during a 8L:16D photoperiod was enough to elevate LH. Plasma LH concentrations have been reported to increase within one day of exposure to increasing in daylength (Follett *et al.*, 1977; Wada, 1979).

There is a significant decrease in size of the androgen-dependent cloacal gland in some males when transferred from long to short days (Sachs, 1967; Siopes and Wilson, 1974; Siopes and Wilson, 1980; Follett and Pearce-Kelly, 1990). Cloacal gland area had been shown to be highly correlated with the endocrine function of the testis (Siopes and Wilson, 1975). Follett and Pearce-Kelly (1990) showed that the cloacal gland started to reduce in size 1-2 weeks after a transfer from long to short days. In some studies (Siopes and Wilson, 1974; Siopes and Wilson, 1980), testicular regression was inferred based on a reduction in cloacal gland area. Delville *et al.* (1985) showed that plasma testosterone concentrations dropped by up to 50% within a week of being transferred to short days and were lowest after 28-35 days of short days. However, without any increase in daylength, testosterone concentrations and cloacal gland area spontaneously increased after 40 days of short days in this strain of quail. A decrease in daylength has also been shown to suppress LH secretion in quail (Gibson *et al.*, 1975; Urbanski and Follett, 1982a; Urbanski and Follett, 1982b; Robinson and Follett, 1982; Follett and Nicholls, 1984).

Decreasing daylength can cause complete gonadal regression in some strains of quail, but these have been quail selected for gonadal regression in response to a reduction in daylength (Robinson and Follett, 1982; Follett and Nicholls, 1984). The response is much more varied in randombred quail sourced commercially, and in many of these strains some birds do not respond to decreases in daylength (Oishi and Konishi, 1983; Wada *et al.*, 1990). Oishi and Konishi (1983) showed that there was a correlation between the photoperiodic response and feather plumage in the Japanese commercial strain of quail, which underlines the genetic basis of this variation.

1.1.4.2 The use of changes in daylength to prevent gonadal growth in sexually immature Japanese quail

Another approach used to maintain birds with small inactive gonads is to raise immature birds on non-photostimulatory photoperiods and prevent gonadal maturation. Testicular growth is inhibited by raising male quail on less than 12L:12D of light (Tanaka *et al.*, 1965; Chaturvedi *et al.*, 1992). Follett and Farner (1966) showed that testicular growth was delayed for 170 days in males raised on 6L:18D, whereas males raised on long days reach sexual maturity by 35 days. Raising male quail on 8L:16D delayed the onset of testicular growth to 70 days of age (Konishi *et al.*, 1965; Siopes and Wilson, 1974), and this increased to 102 days where the photoperiod was 6L:18D (Siopes and Wilson, 1974). Testicular growth is delayed due to low gonadotrophin concentrations, as quail raised on 12L:12D respond to injections of ovine LH (McFarland *et al.*, 1964).

Ovarian growth and the onset of lay is also delayed by raising female quail on less than 12L:12D of light (Tanaka *et al.*, 1965; Siopes and Wilson, 1980; Delville *et al.*, 1985; Brain *et al.*, 1988), while the onset of lay is normal in birds raised on 14 hours or more of light (Wilson and Huang, 1962).

Raising quail on short days delays gonadal growth with the duration of the delay varying with daylength and the strain of quail. Despite the delay, spontaneous gonadal growth inevitably occurs in some if not all birds under non-photostimulatory photoperiods.

1.1.4.3 The effect of environmental temperature on gonadal regression

Responses to short days in different strains of quail range from complete gonadal regression (Wilson *et al.*, 1965; Arrington *et al.*, 1969; Stein and Bacon, 1976; Follett *et al.*, 1977; Robinson and Follett, 1982) to varying degrees of gonadal regression (Oishi and Konishi, 1983), or no regression (Wada *et al.*, 1992).

A range of environmental factors influence seasonal breeding in birds, and one of primary importance is temperature. As mentioned previously, testicular growth is delayed until 70 days of age in quail raised on 8L:16D (Konishi *et al.*, 1965).

However, Kato and Konishi (1968) showed that by lowering the environmental temperature from 25°C to 7-12°C, testicular regression occurred within a week in quail that had undergone testicular growth on 8L:16D, while no regression occurred in birds exposed to the same decrease in temperature but held on a continuous light regime. It has subsequently been shown that low temperatures along with short days are required to induce gonadal regression or prevent gonadal growth in the quail strain used in Japan (Tsuyoshi and Wada, 1992; Wada, 1993). Oishi and Konishi (1978) showed that both T_4 and T_3 concentrations are elevated in birds on short days, and that plasma T_3 concentrations increased once short day quail were moved from 23 to 9°C, while LH decreased rapidly leading to regression of the gonads (Wada, 1993). Thyroid hormones have some influence in short day and cold temperature induced gonadal regression, although the mechanism by which this occurs is unknown (Wingfield *et al.*, 1997a).

Clearly genetically selecting Japanese quail that undergo gonadal regression when exposed to short days results in a quail strain that is highly responsive to a decrease in daylength as used by Robinson and Follett (1982). Quail of a commercial origin such as those in New Zealand and Japan differ markedly in that the gonadal response to changes in daylength varies between individuals since no selection for photoperiodic response has occurred. Therefore the photoperiodic response of the New Zealand quail must be characterised before further studies on the effects of corticosterone on the control of the reproductive system.

1.2 Stressors, stress and the stress response

Individuals are exposed to challenges from the internal and external environment (stressors) on a daily basis. These challenges, sometimes called stress, can range from subtle environmental changes to an event that may threaten the survival of the individual. How the individual perceives the threat governs the magnitude of the stress response which includes behavioural and physiological responses that allow the animal to maintain or restore homeostasis. The individual's life history and experiences also contribute to how a threat is perceived and responded to;

therefore a neural component is just as much a part of the response as the physiological. An inability of an individual to respond to threats to homeostasis results in compromises to a multitude of body functions (e.g. muscular-skeletal, reproduction), leading to debilitation through injury and disease and may result in death.

1.2.1 Stress

Stress is a term that is often used to broadly describe the causes and effects of challenges that an animal may encounter. There is no correct definition of stress and the term can often be misleading. Silverin (1998) defines stress as "an animal is stressed when it is exposed to adverse conditions which produce physiological responses in the individual that adapt the animal to the stressor and return it to a homeostatic condition". In this study, "stress" refers to the state when an animal experiences a stress response.

1.2.1.1 Stressors

Stimuli that challenge homeostasis are commonly called "stressors" (Tilbrook *et al.*, 2000) and in this study, the term will define any stimuli which activate the hypothalamic-pituitary-adrenal (HPA) axis. Stressors in birds can originate from a variety of sources such as the environment (e.g. extremes in temperature (Beuving and Vonder, 1978), transport or housing (Carlisle *et al.*, 1998), physiological (injury or disease), psychological (fear or anxiety due to conspecifics or predators), or nutritional (starvation or food deprivation, Tanabe *et al.*, 1981)).

1.2.2 The physiological response to stressors

Exposure to a stressor activates the HPA-axis. Stimuli from the internal and external environment are integrated into neural information within the central-nervous system (CNS), where they may be consciously and/or subconsciously perceived as stressful or benign (Harvey *et al.*, 1984). The way in which the stimulus is perceived will determine if the sympathoadrenal and HPA-axis are activated. In mammals as well as birds, stimulation of the HPA-axis (see Figure 1.2) is characterised by activation of corticotrophin-releasing factor (CRF) neurons in the

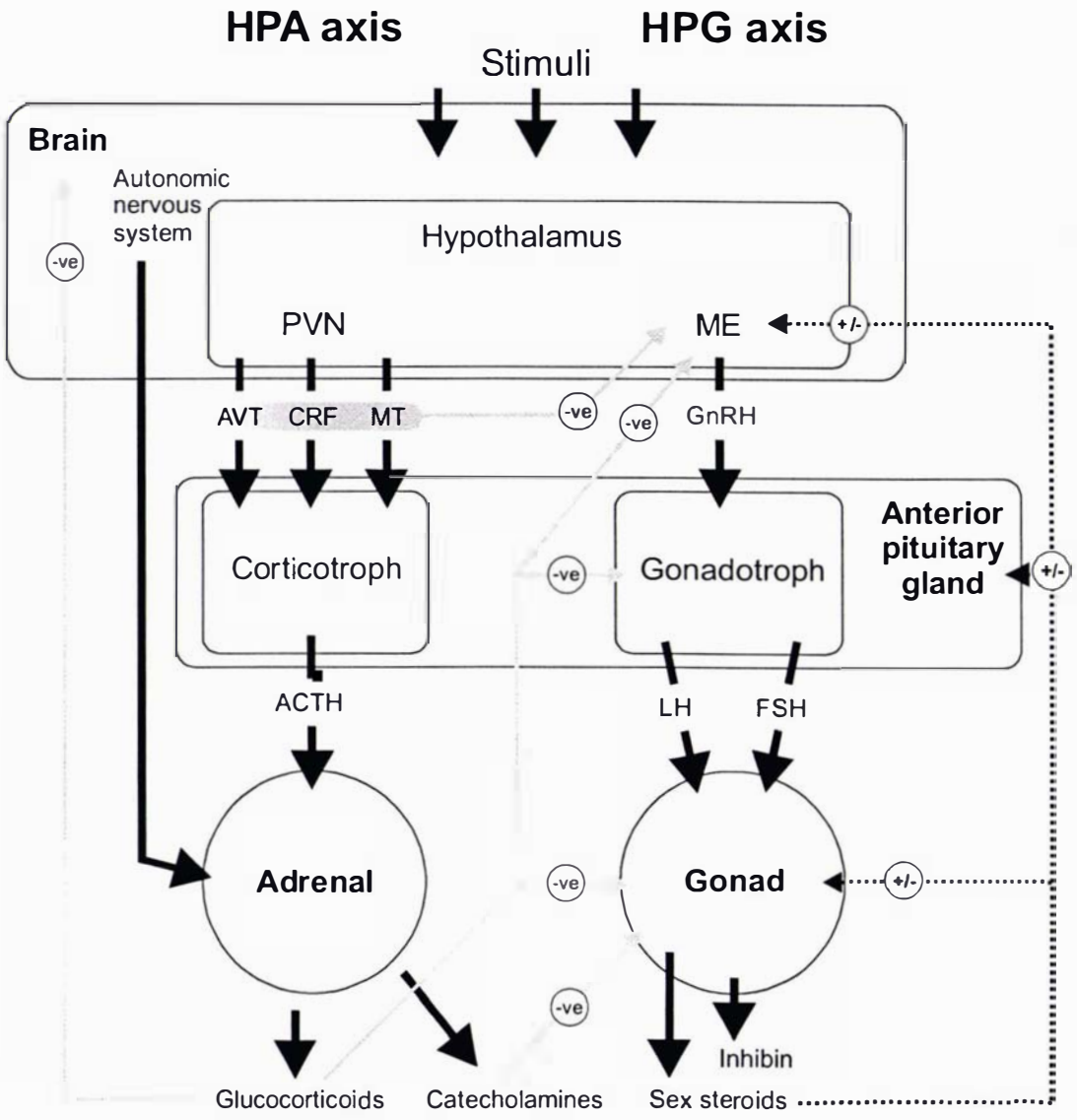


Figure 1.2 Schematic representation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes in birds, along with potential feedback (-ve and +/- indicate negative and positive/or negative feedback respectively) pathways which may regulate the gonadal axis. On the HPA-axis, the hypothalamo-hypophysial factors corticotrophin-releasing factor (CRF), arginine vasotocin (AVT) and mesotocin (MT) are released from neurons in the paraventricular nucleus (PVN) into the portal circulation stimulating adrenocorticotrophin hormone (ACTH) secretion from the adrenal gland. Glucocorticoids are secreted from the adrenal gland in response to ACTH. On the HPG-axis, gonadotrophin-releasing hormone (GnRH) is secreted from neurons terminating in the median eminence (ME) of the hypothalamus, and is released into the portal circulation stimulating secretion of the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from gonadotrophs in the anterior pituitary gland. The gonadotrophins stimulate steroidogenesis in the gonads resulting in an increase in sex steroid secretion.

paraventricular nucleus (PVN) and secretion of hypothalamo-hypophysiotrophic factors into the hypophysial portal system. CRF stimulates the production of adrenocorticotrophic hormone (ACTH), β-endorphin and α-melanocyte-stimulating hormone from corticotrophs in the pituitary gland, which are released during the stress response (Tilbrook *et al.*, 2000). ACTH stimulates the synthesis of glucocorticoids from the adrenal gland and their secretion into the systemic circulation. An increase in plasma corticosterone concentration occurs during the avian stress response and directs physiological and behaviour modifications that allow the bird to maintain or restore homeostasis. In this study, a stress response has not occurred if there is no increase in plasma corticosterone concentrations. The sympathoadrenal system is also activated when an individual is exposed to a stressor and is composed of the sympathetic nervous system and adrenal chromaffin cells. Activation of the sympathoadrenal system coupled with the preganglionic innervation within parts of the adrenal gland results in an increase in catecholamine secretion into the systemic circulation, principally adrenaline (Tilbrook *et al.*, 2000).Catecholamine secretion from avian adrenal chromaffin cells has been shown to be stimulated by ACTH (reviewed by Carsia and Harvey, 2000) and in many cases the glucocorticoids and catecholamines act synergistically at target tissues. The effects of the catecholamines are not included in this review which will concentrate on the HPA-axis.

1.2.3 The hypothalamo-pituitary-adrenal axis

1.2.3.1 Hypothalamo-hypophysiotrophic factors

1.2.3.1.1 Corticotrophin-releasing factor

The hypophysiotrophic role of CRF in mammals has been well established (reviewed by Rivier and Plotsky, 1986). CRF is a 41-amino acid polypeptide synthesised in the PVN and released into the median eminence (ME) and hypophysial portal system resulting in activation of the pituitary corticotrophs (Lovejoy and Balment, 1999). Expression of genes encoding CRF are also influenced by changes in serotonin, acetylcholine, cytokines and interleukins 1 and 6 (Chadwick *et al.*, 1993). In mammals, CRF is acknowledged as the predominant

hypophysiotrophic factor that stimulates ACTH secretion from the anterior pituitary during the stress response (Rivier and Plotsky, 1986).

The actions of CRF during the stress response are partly mediated through the two identified CRF receptor (CRF-R) subtypes; CRF-R type 1 is the main subtype and is present in the pituitary and brain, while CRF-R type 2 is present in the periphery and some areas of the brain (reviewed by Aguilera, 1998). The responsiveness of the HPA-axis during the stress response is controlled in part by changes in expression of CRF in the PVN as well as regulation of pituitary CRF receptors by CRF and glucocorticoid concentrations, and the possible role of a CRF binding protein (Behan *et al.*, 1995; Aguilera, 1998).

Despite the well established function of CRF in mammals, an avian CRF is yet to be characterised (Speiss *et al.*, 1998; Carsia and Harvey, 2000). However, there is measurable ovine CRF-immunoreactivity in the median eminence of the hypothalamus in Japanese quail (Mikami and Yamada, 1984) and chickens (Józsa *et al.*, 1984), while the avian pituitary corticotrophs are responsive to mammalian CRF. Ovine CRF treatment of avian pituitary cells *in vitro* increases ACTH secretion (Castro *et al.*, 1986; Carsia *et al.*, 1986), while Japanese quail respond to *in vivo* treatment with human CRF (Launay *et al.*, 1993). In addition, one of the two mammalian CRF receptor subtypes, CRF-R type 1, has been cloned in domestic fowl (Yu *et al.*, 1996).

1.2.3.1.2 Other hypothalamo-hypophysiotrophic factors

In mammals, arginine vasopressin (AVP) is another hypothalamo-hypophysiotrophic factor that controls ACTH concentrations (Konakchieva *et al.*, 1997; Ma *et al.*, 1997; Chrousos, 1998). AVP has a limited effect in birds (Gilles *et al.*, 1982; Westerhof *et al.*, 1992). Arginine vasotocin (AVT) is the natural hypothalamo-hypophysiotrophic factor in birds (Westerhof *et al.*, 1992), while mesotocin (MT) is also known to control ACTH secretion. Nerve fibres in the median eminence of Japanese quail show immunoreactive staining to both AVT and MT (Mikami and Yamada, 1984). *In vitro* studies using duck and chicken pituitary cell cultures have shown AVT and MT to be ACTH secretagogues (Castro *et al.*, 1986), although

treatment with AVT or MT have produced inconsistent results. It is possible that AVT and CRF act synergistically, with AVT potentiating the actions of CRF, as reported by Westerhof *et al.* (1992) and Romero and Wingfield (1998), where treatment with both factors stimulated a larger corticosterone response. The secretion of catecholamines immediately after exposure to a stressor and before activation of the HPA-axis is also thought to potentiate the responsiveness of pituitary to CRF, and thus enhance ACTH secretion (Harvey *et al.*, 1984).

In addition to changes in the synthesis and secretion of these hypothalamo-hypophysiotrophic factors, the actions of CRF, AVT and MT in the stress response are also mediated by changes in responsiveness of the pituitary corticotrophs. The plasticity of pituitary responsiveness to CRF, AVT and MT has been extensively reported in wild, free living species (Romero and Wingfield, 1998). Seasonal differences in the stress response, as measured by the corticosterone response, are associated with changes in pituitary and adrenal responsiveness to hypothalamo-hypophysiotrophic factors and ACTH respectively (Romero *et al.*, 1997; Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero and Wingfield, 1998). These results illustrate how the stress response is controlled at several different levels of the HPA-axis.

As well acting on the HPA-axis, AVT is also known to act on the HPG-axis. Male sexual behaviour is activated predominantly through the aromatization of testosterone to oestradiol. In Japanese quail, aromatase cell bodies and AVT fibres in the medial preoptic nucleus have been shown to be closely associated (Panzica and Viglietti-Panzica, 1999). AVT has been shown to have an inhibitory effect on reproduction in male Japanese quail, intracerebroventricular or systemic treatment with AVT has a profound inhibitory effect sexual behaviour in male Japanese quail (Panzica and Viglietti-Panzica, 1999; Panzica *et al.*, 2002).

1.2.3.2 ACTH

In both mammals and birds, the synthesis and secretion of corticosteroids from adrenocortical cells is primarily under the control of adrenocorticotrophic hormone (ACTH) which is secreted from the anterior pituitary (reviewed by Mikami and

Yamada, 1984; Mikami, 1986; Chrousos, 1998; Carsia and Harvey, 2000). As discussed previously, CRF and other hypothalamo-hypophysiotrophic factors, catecholamines and indoleamines stimulate ACTH secretion from the anterior pituitary (Harvey *et al.*, 1984).

ACTH is a 39-amino acid peptide synthesised from proopiomelanocortin, a large precursor protein complex (Hayashi *et al.*, 1991). ACTH is synthesised in the corticotrophic cells in the cephalic lobe of the anterior pituitary gland (Hayashi *et al.*, 1991). It is secreted under the control of hypothalamic-hypophysiotrophic factors (Carsia *et al.*, 1986; Castro *et al.*, 1986) and is also inhibited by glucocorticoid feedback (Carsia *et al.*, 1986; Herold *et al.*, 1992). ACTH is released into the systemic circulation and acts on adrenal cortical cells in the adrenal glands to stimulate the synthesis and secretion of corticosteroids and catecholamines.

In birds, ACTH stimulates the secretion of the glucorticoid corticosterone (Freeman and Manning, 1979; Davison *et al.*, 1980; Davison *et al.*, 1985; Beuving and Vonder, 1986), the mineralocorticoid aldosterone (Radke *et al.*, 1984; Radke *et al.*, 1985), and catecholamines (Zachariasen and Newcomer, 1971). Adrenal corticosterone release in the Japanese quail is under the control of pituitary ACTH (Mikami and Yamada, 1984; Mikami, 1986) and Carsia *et al.* (1988) showed that corticosterone secretion increased in quail adrenocortical cells incubated with ACTH. Corticosterone concentrations have been shown to increase within 5 minutes of treatment with ACTH in a variety of birds (reviewed by Carsia and Harvey, 2000). Plasma ACTH concentrations have been shown to peak 5-10 minutes after initiation of a stress response in geese (Kovács and Péczely, 1991) followed by a peak in corticosterone secretion several minutes later.

1.2.3.3 Corticosterone

Corticosterone is the primary avian glucocorticoid (Siegel, 1971; Harvey *et al.*, 1984; Carsia and Harvey, 2000). ACTH stimulates an increase in adrenocortical cell secretion of corticosterone and this increase is indicative of activation of the HPA-axis and a "non-specific stress response" (Harvey *et al.*, 1984). Corticosterone is carried in the systemic circulation in free and bound forms, the latter being bound

to corticosteroid binding globulins (CBG) (Wingfield *et al.*, 1984) which reduce the bioactivity and clearance of corticosterone (Carsia and Harvey, 2000).

1.2.3.3.1 Circadian rhythm of corticosterone secretion

There is a distinct daily rhythm of corticosterone secretion as shown in Japanese quail (Boissin and Assenmacher, 1970; Kovács and Péczely, 1983) and a variety of other birds (reviewed by Carsia and Harvey, 2000). Changes in basal corticosterone concentrations controlled by the HPA-axis appear to be mediated via the suprachiasmatic nucleus (King and Follett, 1997). Plasma corticosterone concentrations typically increase and peak during hours of darkness (Boissin and Assenmacher, 1970; Beuving and Vonder, 1977). Corticosterone concentrations remain low during hours of light, increase at the onset of darkness and remain elevated before decreasing at the onset of light (Boissin and Assenmacher, 1970). The timing of the peak varies and is influenced by photoperiod (Boissin and Assenmacher, 1970; Kovács *et al.*, 1983). In other Japanese quail studies, corticosterone has also been shown to peak in late morning and then decline (Kovács and Péczely, 1983; Kovács *et al.*, 1983). However, despite the change in concentrations during the day, overall basal corticosterone concentrations are low relative to the increase associated with a stress response.

1.2.3.3.2 Effects of increased corticosterone secretion on body and behaviour

The increase in corticosterone secretion in response to a stressor is thought to help the bird make physiological and behavioural modifications that allow it to maintain homeostasis. During the stress response, the main actions of glucocorticoids are on intermediary metabolism, immune function and behaviour (reviewed by Siegel, 1995; Carsia and Harvey, 2000). The effects on intermediary metabolism vary between species, but the most immediate change is in glucose metabolism, associated with gluconeogenesis via fat and protein catabolism (reviewed by Harvey *et al.*, 1984; Wingfield *et al.*, 1998; Carsia and Harvey, 2000). An increase in glucocorticoid secretion also alters immune function, although prolonged glucocorticoid concentrations can result in involution of the thymus, spleen and bursa of Fabricius (Carsia and Harvey, 2000).

There is clear evidence that corticosterone influences behaviours in a mechanism that allows an individual to alter behaviour to maximise short term and long term survival. Elevated plasma corticosterone is known to have a profound effect on behaviour in birds, including decreases in aggression, parental and reproductive behaviours (Wingfield and Silverin, 1986) and increases in locomotor activity (Breuner *et al.*, 1998) and foraging behaviour (Wingfield *et al.*, 1998). Breuner *et al.* (1998) avoided the disturbance caused by manually administrating corticosterone by infusing it directly into food, and clearly demonstrated an increase in locomotory activity in Gambel's White-Crowned Sparrows (*Zonotrichia leucophrys gambelii*). Fearfulness also appears to be related to plasma corticosterone (reviewed by Jones, 1996; Carsia and Harvey, 2000). Corticosterone treatment of laying domestic hens increased tonic immobility, an indicator of fearfulness (Jones *et al.*, 1988). In Japanese quail, selection for low or high adrenocortical responses to mechanical restraint also caused a divergence in behaviour. Quail with a high corticosterone response to mechanical restraint showed greater fearfulness than that of random-bred quail (Jones *et al.*, 1992a; Jones *et al.*, 1992b; Jones *et al.*, 1994a). Prolonged exposure to a stressor can result in suppression of the reproductive axis in birds as well as other vertebrates (reviewed by Deviche, 1983; Harvey *et al.*, 1984; Carsia and Harvey, 2000). Inhibition of the HPG-axis has been shown to occur at the hypothalamus, pituitary and at the gonads in mammals (Rivier and Rivest, 1991; Tilbrook *et al.*, 2000; Tilbrook *et al.*, 2002) and is thought to occur at the same levels in birds (see Figure 1.2) (reviewed by Carsia and Harvey, 2000).

1.2.3.3.3 Control of glucocorticoid secretion

Corticosterone secretion is regulated by feedback loops which are well established in mammals. Glucocorticoids exert a negative feedback affect on the hypothalamus and pituitary, through downregulation of brain corticosteroid receptors, and suppression of CRF, AVT and ACTH (Harvey *et al.*, 1984; Plotsky *et al.*, 1989). Glucocorticoids have been shown to inhibit hypothalamic CRF mRNA expression (Ma *et al.*, 1997) and release from the ME (Spinedi *et al.*, 1991). Glucocorticoids also inhibit POMC transcription resulting in a decrease in ACTH secretion by the corticotrophs (Levin and Roberts, 1991), as well as reducing adrenal sensitivity to ACTH (Harvey *et al.*, 1984). Negative feedback regulation limits glucocorticoid

secretion, although the response of specific levels of the HPA-axis to feedback may vary between species and be specific to the type of stressor the bird has experienced (Chadwick *et al.*, 1993). In rats the pattern of HPA-axis activation differs according to the type of stressor. Desensitization of ACTH responses to repeated stimuli occur for stressors such as cold exposure or repeated immobilization, while the responses are preserved after repeated stimuli of other stressors (foot shock, insulin hypoglycaemia and intraperitoneal hypertonic saline injections) (Aguilera, 1998).

1.2.4 Measurement of the stress response

The exposure of an individual to a stressor and activation of the HPA-axis can be assessed by measuring the hormones released in an adrenocortical response. Plasma concentrations of ACTH, catecholamines, and glucocorticoids all increase during the response , and measuring the change in concentrations is an ideal means of defining the stress response. Measuring plasma corticosterone is the widely accepted measure for defining the stress response given corticosterone is the predominant glucocorticoid secreted during activation of the HPA-axis (Carsia and Harvey, 2000). Plasma corticosterone concentrations increase rapidly (1-3 minutes) in response to a stressor in a variety of birds (Beuving and Vonder, 1978; Harvey *et al.*, 1980; Satterlee and Johnson, 1988) and the magnitude and duration of the response reflect the severity of the stressor (Harvey *et al.*, 1984). In Japanese quail, immobilisation causes corticosterone concentrations to increase within one minute, reaching a peak after four minutes and remained elevated during 20 minutes of immobilisation (Satterlee and Johnson, 1988).

Basal corticosterone concentrations vary between species and are low in domesticated species such as chickens (Freeman and Flack, 1980), Japanese quail (Satterlee and Johnson, 1988) and ducks (Harvey *et al.*, 1980), while significantly higher in some free-living species (Astheimer *et al.*, 1994; Smith *et al.*, 1994; Silverin, 1998).

The corticosterone response is assessed experimentally by the application of a stressor to each bird. The method described by Wingfield (1994) termed the

"capture stress protocol" is commonly used and involves the application of a standardised stressor (capture and handling). In wild birds, blood samples are collected before and then during the stressor, whereas in domesticated species housed in cages, samples can be collected after the stressor. The measurement of corticosterone concentrations allow the magnitude and duration of the response to be characterised. The collection of blood samples is in itself invasive and can stimulate a corticosterone response, therefore minimising the duration of the sampling procedure is important. Littin and Cockrem (2001) reported no increase in corticosterone concentrations after 15 minutes when blood was collected within 3 minutes in chickens, while there was no increase in Japanese quail after 15 minutes when samples were collected within 2 minutes (Boyd, 2000). However, Satterlee and Johnson (1988) showed plasma corticosterone increased 1 minute after being restrained and in further studies, blood was collected within 45 seconds to minimise measuring an adrenocortical response (Jones *et al.*, 2000).

The magnitude of the corticosterone response has been shown to vary and can be influenced by factors such as the bird's age (Romero *et al.*, 1998c), sex (Jones *et al.*, 1994b; Astheimer *et al.*, 1995), or stage of the breeding season (Romero *et al.*, 1997; Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero and Wingfield, 1998; Romero and Wingfield, 1999; Romero and Remage-Healey, 2000). These are factors to be mindful of when measuring the stress response in birds, although they can be minimised in laboratory species such as quail where birds are kept under controlled conditions.

1.2.5 Effect of stressors on reproduction

Corticosteroids are an integral component of the avian reproductive system and have a range of effects both stimulatory and inhibitory (reviewed by Carsia and Harvey, 2000; Wingfield and Sapolsky, 2003). It is generally accepted that the prolonged exposure to a stressor suppresses reproduction in birds (reviewed by Deviche, 1983; Harvey *et al.*, 1984) and this review will concentrate on the inhibitory effects of stress. However, the inhibitory relationship between stress and

reproduction cannot be generalised for all individuals of a species, some individuals exhibit an inhibitory response while others do not (Moore and Jessop, 2003).

From an ecological perspective, suppression of the reproductive system during which a bird is undergoing a period of stress is advantageous. Reproduction is an energetically expensive part of the life cycle, and reproduction during periods such as inclement weather and poor food availability when reproduction would be futile or compromise the survival of the individual is not ideal. Plasma corticosterone secretion can increase under such conditions, and exposure to continual high circulating concentrations of corticosterone for many days or even weeks contribute to suppression of the reproductive system in birds (reviewed by Deviche, 1983; Silverin, 1998; Wingfield *et al.*, 1998; Carsia and Harvey, 2000). Treatment of male and female Japanese quail with corticosterone or ACTH decreased oviposition in females and suppressed LH (Martin *et al.*, 1984).

The inhibitory effects of stress on reproductive function have been extensively studied in mammals and in particular rodents (reviewed by Rivier and Rivest, 1991; Tilbrook *et al.*, 2000; Tilbrook *et al.*, 2002). In almost all cases, the main result of prolonged exposure to a stressor is a decrease in GnRH secretion, which in turn influences gonadotrophin secretion and the gonads. It is important to highlight that the effects on reproduction depend very much on the type and duration of stressor, and the effects differ between sexes. Studies in the mammalian literature show that the neural and endocrine pathways activated during the stress response vary depending on the type and duration of the stressor, and these differences alter how the reproductive system is affected (reviewed by Rivier and Rivest, 1991; Tilbrook *et al.*, 2000; Tilbrook *et al.*, 2002).

In this review, the effect of stressors on reproduction will concentrate on the avian literature; however, reference will be made to mammalian research where appropriate.

1.2.5.1 Different types of stressors

In mammals, a wide variety of physical, metabolic and psychological stressors have been shown to activate the HPA-axis, resulting in a decline in LH secretion (reviewed by Tilbrook *et al.*, 2002). Similarly, birds show an adrenal response to a wide range of stressors (reviewed by Harvey *et al.*, 1984). While an inhibitory effect of corticosteroids has been shown in birds, there are relatively few studies which report the effects of different stressors on the reproductive system in species other than poultry (reviewed by Deviche, 1983). Repeated handling associated with frequent blood sampling has been shown to cause a decline in gonadotrophin and sex steroid secretion in poultry (Wilson and Sharp, 1975; Eskeland and Blom, 1979; Wilson *et al.*, 1979; Johnson, 1981). The results of Heiblum *et al.* (2000) conflict with these earlier studies, since short term mechanical restraint stimulated an increase in plasma testosterone concentrations at the same time as the corticosterone response in cockerels.

The adrenocortical response to different stressors has also been extensively studied in the Japanese quail. Plasma corticosterone increases in response to a variety of stimuli such as capture and brief manual restraint by the experimenter, social challenges, cold, crating, and food and water deprivation (Jones *et al.*, 2000). The magnitude and duration of HPA-axis activation may vary between the types of stimuli indicating that different stress response pathways may be activated. Differences in adrenocortical responsiveness between low and high stress response lines of Japanese quail have been shown to result in different changes in cloacal gland physiology to mechanical restraint between the lines (Satterlee *et al.*, 2002).

Studies in rats (Hennessy and Levine, 1978; Hennessy *et al.*, 1979) and starlings (Nephew *et al.*, 2003) show that the size and duration of the corticosterone response reflect the strength of the stressor. Plasticity of the corticosterone response to varying intensities of stimulation is likely to be an adaptive mechanism to minimise the change in energy balance involved in the stress response. Activation of a response of a precise size adequate to deal with the stressor is advantageous, in contrast to a non-specific maximal response to any stressor irrespective of type or duration. The size of the elicited response will also reflect an animal's individual

experiences and its own perception of whether the stimuli was a threat or benign. Modulation of the corticosterone response in free-living birds also extends to seasonal variation (Romero *et al.*, 1997; Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero and Wingfield, 1998) and the corticosterone response and reproduction are altered by unseasonal climatic conditions (Wingfield *et al.*, 1999).

Different stressors activate different parts of the brain, and the location of neuronal activity has been detected by localisation of immediate early gene expression in rats (reviewed by Akana and Dallman, 1997; Bonaz and Rivest, 1998; Van de Kar and Blair, 1999). In rats, the paraventricular hypothalamus and central amygdala are two brain regions noted for activation of c-*fos* in response to exposure to stressful stimuli (Campbell *et al.*, 2003; Zelena, 2003). Detection of the c-*fos* immediate early gene protein is used in the avian brain to detect neuronal activity following photoperiodic or sexual stimuli (Meddle and Follett, 1995; Meddle *et al.*, 1999) and also distress in quail (Takeuchi *et al.*, 1996). Further work in this area will aid in the understanding of how different stressors activate a response in the bird and how this could explain the difference in the magnitude and duration of the stress response for a given stimuli.

1.2.5.2 How do stressors affect reproduction?

The stress-induced activation of the HPA-axis could influence the avian reproductive system at the hypothalamus, pituitary gland or gonads or any number of these levels as it does in mammals (see Figure 1.2). In mammals, the main effects of stressors are generally accepted to be on the secretion of GnRH from the hypothalamus and LH from the anterior pituitary gland (Brann and Mahesh, 1991; Rivier and Rivest, 1991; Tilbrook *et al.*, 2000; Tilbrook *et al.*, 2002).

The effects are mediated directly at the gonadotrophs that secrete the gonadotrophins and indirectly through interactive effects that influence gonadotroph function such as feedback from gonadal steroids or inhibin (reviewed by Rivier and Rivest, 1991; Tilbrook *et al.*, 2000). In part, gonadotrophin suppression is mediated by glucocorticoid suppression of gonadotrophin release by acting at the level of the pituitary gland (Calogero *et al.*, 1999). Corticosterone

treatment of quail pituitary gonadotrophs *in vitro* has been shown to inhibit LH secretion (Connolly and Callard, 1987), and continuous corticosterone infusion by osmotic pump *in vivo* causes a decline in plasma LH concentrations in hens (Etches *et al.*, 1984; Williams *et al.*, 1985; Petitte and Etches, 1989). Despite the decline in LH, Etches *et al.* (1984) showed that the pituitary was just as sensitive to GnRH, indicating that corticosterone did not influence gonadotroph sensitivity to GnRH or the synthesis of LH. In contrast, subjecting Japanese quail to 3 days of food deprivation (nutritional stress) results in a decline in plasma LH caused by a decrease in gonadotrophin subunit mRNA expression (Kobayashi and Ishii, 2002). Wingfield and Silverin (1986) showed that subcutaneous corticosterone implants depressed testosterone but not LH in male song sparrows (*Melospiza melodia*). The differences in gonadotrophin response to corticosteroids *in vivo* and *in vitro* can be attributed to differences in species, sex and experimental protocol (Connolly and Callard, 1987). Differences in the way the HPG-axis is affected by the HPA-axis can also be attributed to the origin of the stressor that elicits the stress response. In the mammal, the origin and type of stressor has been clearly demonstrated to affect the neural pathways activated and how the HPA-axis is stimulated (reviewed by Day *et al.*, 1999; Tilbrook *et al.*, 2002). Further to this, it is likely that there are also differences in the way an individual responds to a stressor of psychological or psychogenic origin depending on the unique life experiences of that individual.

Prolonged (chronic) exposure to noxious stimuli generally results in a decrease in gonadotrophin secretion and the decline in the reproductive system. However, the effects of acute or repeated acute exposure to stressful stimuli in mammals as well as birds are less understood as there are varying reports ranging from no effect, to stimulatory and inhibitory effects on reproduction (reviewed by Brann and Mahesh, 1991; Tilbrook *et al.*, 2000). Differences in adrenocortical and reproductive axis responses to acute or chronic stressors are likely to be influenced by the type, intensity and duration of the stimuli, and the individual's perception of the stimuli.

1.2.5.2.1 Glucocorticoids

The structures of the HPG-axis in mammals and birds are fundamentally similar, and the avian and mammalian reproductive systems are likely to be influenced by

stressors in similar ways. In mammals, chronically elevated glucocorticoids exert an inhibitory effect on reproductive function, and the effect is thought to be mediated in part by influencing gonadal sex steroid feedback and the modulation of peptides that regulate GnRH synthesis and secretion (Ahima and Harlan, 1992). Central sites within the avian brain are thought to be the predominant sites of corticosteroid action in birds, and are generally accepted to influence gonadotrophin secretion directly and indirectly by influencing GnRH neurons (Deviche, 1983). Glucocorticoid receptors (GR) have been localised in GnRH neurons in several phylogenetic groups (fish (Teitsma *et al.*, 1999), mammals (Ahima and Harlan, 1992), and in the Japanese quail brain (Kovacs *et al.*, 1989)). In the rat brain, expression of GnRH mRNA and GnRH secretion are inhibited by corticosterone (Calogero *et al.*, 1999; DeFranco *et al.*, 1994) and the synthetic glucocorticoid dexamethasone (Attardi *et al.*, 1997; Calogero *et al.*, 1999; Schiml-Webb *et al.*, 2001).

The mammalian GnRH neuron receives synaptic input from more than 18 known peptides and neurotransmitters, two of which are ACTH and CRF (reviewed by Tilbrook *et al.*, 2002). CRF and GnRH neurons are found in some of the same regions of the Japanese quail brain and many perikarya terminate in the same regions (Mikami and Yamada, 1984; Mikami *et al.*, 1988). However, no functional relationship between the two (CRF and GnRH neurons) has yet been demonstrated in birds. In the rat, direct synaptic connections between CRF and GnRH neurons have been found in the preoptic area, with CRF immunoreactive terminals impinging upon GnRH neurons (reviewed by Lovejoy and Balment, 1999).

The ability of glucocorticoids to directly and indirectly inhibit GnRH synthesis and secretion suggests that the hypothalamus is one site of action where glucocorticoids may act to suppress the reproductive system. While the inhibitory effects of corticosterone on the avian reproductive system have been long recognised (Deviche, 1983), there has been little work to determine if corticosterone acts on GnRH in birds. Intra-hypothalamic corticosterone implants decreased LH and induced gonadal regression in photostimulated tree sparrows (Wilson and Follett, 1975), and daily corticosterone injections (3 mg/day) caused testicular regression and plasma LH to decline in ducklings (Deviche *et al.*, 1979). The continuous

infusion of corticosterone via osmotic pumps resulted in ovarian regression and a decline in plasma LH and oestradiol concentrations (Etches *et al.*, 1984; Williams *et al.*, 1985; Petitte and Etches, 1991). The anterior pituitary remained responsive to GnRH, suggesting that the decline in gonadotrophin secretion was caused by glucocorticoids influencing GnRH secretion. However, quail pituitary gonadotrophs show a significant reduction in LH secretion when incubated with corticosterone (Connolly and Callard, 1987) indicating that corticosterone action is not limited to GnRH secretion.

The association between elevated corticosterone and a decline in LH secretion is not always so clear, especially where the stimulus is of short duration. In Japanese quail, a single injection of corticosterone (1.2 mg/bird) caused a significant increase in plasma corticosterone without affecting plasma LH (Boyd, 2000). Corticosterone delivered via subcutaneous implants in male tree sparrows did not affect LH or testosterone secretion (Astheimer *et al.*, 2000).

The direct actions of glucocorticoids on gonadal steroidogenesis are poorly known as the effects are often transitory and rapidly reversible, and are often masked by the overall suppression of GnRH and gonadotrophin secretion at higher centres. However, it is well accepted that short term exposure to stressors can have a direct effect on gonadal steroidogenesis, as shown in mammals. Restraint in rats caused an increase in plasma corticosterone, which was shown to inhibit testicular steroidogenesis resulting in a decrease testosterone (Bambino and Hsueh, 1981; Mann and Orr, 1990; Orr and Mann, 1992; Monder *et al.*, 1994).

Elevated corticosterone alters testicular steroidogenesis in avian testicular cells *in vitro* (reviewed by Deviche, 1983). Deviche *et al.* (1982) showed that corticosterone injections in Japanese quail reduced the production of androstenedione which led to a decrease in plasma testosterone concentration and also inhibited photoinduced cloacal gland growth. In light of the evidence in avian and mammalian *in vitro* studies, the acute stress response and transitory elevation in corticosterone are a likely cause of transitory reductions in sex steroid concentrations. A similar mechanism may also be acting in studies where a short

period of handling (Wilson and Sharp, 1975; Wilson *et al.*, 1979; Johnson, 1981) or an injection of corticosterone (Boyd, 2000) has resulted in a rapid decrease in testosterone but where LH has remained unchanged.

1.2.6 Individual variation in the corticosterone response

The corticosterone response also varies within and between birds as shown in quail, domestic fowl and great tits (Satterlee and Johnson, 1988; Littin and Cockrem, 2001; Cockrem and Silverin, 2002a). Individual differences in adrenocortical responsiveness have been used by Satterlee and Johnson (1988) to select birds for divergent adrenocortical responses over many generations. These quail are an ideal model for testing hypotheses on the relationships between behavioural, physiological and genetic traits and different aspects of stress and the adrenocortical response (Jones *et al.*, 1992a; Jones *et al.*, 1992b; Jones *et al.*, 1994a; Jones *et al.*, 2000; Satterlee *et al.*, 2002; Odeh *et al.*, 2003a; Odeh *et al.*, 2003b).

Variation between birds in their corticosterone responses have been attributed to differences in sex, age, reproductive status and body condition, as well as changes in environmental conditions such as rainfall, temperature and food availability (reviewed by Wingfield *et al.*, 1992; Moore and Jessop, 2003; Wingfield and Sapolsky, 2003). The adrenocortical response to a standardised stressor has also been shown to vary during the season in some wild passerines, and is often referred to as "adrenocortical modulation" (Wingfield and Romero, 2001). Modulation of the adrenocortical stress response appears to be regulated by mechanisms both upstream and downstream of the anterior pituitary (Wingfield and Romero, 2001).

There has been less attention to differences in corticosterone responsiveness to stressors within and between individual birds under constant conditions, where variation due to differences in body condition and the external environment can be minimised under laboratory conditions. Birds have been selected for divergent adrenocortical responses to stressors such as the cold (Brown and Nestor, 1973), social stress (Gross and Siegel, 1985), or immobilisation (Satterlee and Johnson,

1988), indicating a genetic basis to this variation (as in other vertebrates, reviewed by Morméde *et al.*, 2003).

It has been assumed that a single measurement of a corticosterone response is sufficient to define the response for an individual bird. Studies in our laboratory on the domestic fowl and great tits (Littin and Cockrem, 2001; Cockrem and Silverin, 2002a) are the first to quantify within bird variation of the corticosterone response. The results showed that despite some variation, the adrenocortical response to 15 minutes of handling is generally repeatable within the same bird. The magnitude of the adrenocortical response to the same stressor varies between individuals in Japanese quail as shown by Satterlee and Johnson (1988). Despite the well established difference in adrenocortical responsiveness to immobilisation between individual quail, nothing is known of the causes of this variation.

1.2.6.1 Where is individual variation of the adrenocortical response mediated?

Variation between birds in their responses to emotional stressors could be due to differences in their perception of the stressor, or differences in the functioning of the HPA-axis. Differences in corticosterone responses are likely to be regulated at the CNS level (i.e. the individual's perception of the perturbation) and HPA-axis.

1.2.6.1.1 Adrenal gland

Individual variation in the adrenocortical response could be due to differences in adrenal sensitivity to ACTH. Carsia *et al.* (1988) showed that maximal ACTH-induced corticosterone secretion from isolated adrenocortical cells was greater in a strain selected for high corticosterone responses to immobilisation over random-bred quail. Carsia *et al.* (1988) postulated that selection for a high corticosterone response altered the maximal adrenocortical response to ACTH without affecting cellular sensitivity to ACTH, as basal corticosterone secretion did not differ between high response and random-bred quail. McIlroy *et al.* (1999) showed that the ACTH-induced corticosterone response of adrenocortical cells was enhanced by protein restriction in domestic fowl, and suggested that this was caused by either changes in adrenal sensitivity to ACTH, or changes in trans-membrane and intracellular

pathways associated with ACTH binding. The results of these two studies show that the adrenocortical cells in birds can intrinsically differ between individuals in their sensitivity and responsiveness to ACTH.

Studies by Satterlee and Johnson (1988) and Carsia *et al.* (1988) show that the adrenocortical response and adrenosteroidogenic properties of adrenocortical cells can differ significantly between quail exposed to a standardised stressor. Jones *et al.* (1992b) showed that selecting quail for divergent corticosterone responses to immobilisation also selected for other traits including different behaviour responses to stressors. Differences in intrinsic cellular properties of adrenocortical cells are not the sole cause of individual variation in the corticosterone response. Low or high corticosterone responses to immobilisation are clearly heritable, and differences are likely to be mediated at different levels of the HPA-axis as well as the CNS.

In vitro treatment of adrenocortical cells from random-bred and high response quail showed differences between lines in maximal ACTH-induced corticosterone secretion but not basal secretion (Carsia *et al.*, 1988). There does not appear to be an *in vivo* study where quail with differing corticosterone responses to mechanical restraint have been treated with a sub-maximal dose of ACTH. Such a study would elucidate whether differences in adrenal sensitivity to ACTH exist *in vivo* in quail with differing corticosterone responses. ACTH challenges have been used to identify changes in adrenal responsiveness in a range of passerines (Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero and Wingfield, 1998).

Measuring changes in plasma corticosterone concentrations following an ACTH challenge has been shown to be an accurate test for adrenal sensitivity in birds, since corticosterone is stimulated directly by ACTH. Plasma corticosterone increases rapidly (< 30 minutes) in response to intra-muscular (i.m.), intra-venous (i.v.), and intra-peritoneal (i.p.) administration of mammalian ACTH in a variety of bird species (Beuving and Vonder, 1978; Harvey *et al.*, 1980; Etches and Croze, 1983; Noirault *et al.*, 1999; Wilson and Holberton, 2001; Faure *et al.*, 2003). The use of the synthetic ACTH Synacthen™ in chickens stimulated corticosterone secretion within 30 minutes (Beuving and Vonder, 1978; Harvey *et al.*, 1980).

1.2.6.1.2 Anterior pituitary gland

The anterior pituitary corticotrophs of domestic fowl are responsive to CRF peptides of mammalian origin (Carsia *et al.*, 1986). In addition, AVT and MT have ACTH releasing properties in the isolated duck pituitary (Castro *et al.*, 1986). However, inter-specific differences in pituitary sensitivity to the three avian hypothalamo-hypophysiotrophic factors have been reported (Castro *et al.*, 1986; Westerhof *et al.*, 1992). CRF is established as the primary ACTH secretagogue in mammals (Owens and Nemeroff, 1991).

Individual variation in adrenal responsiveness could be mediated by differences in pituitary sensitivity to these releasing factors resulting in different rates of ACTH secretion between individuals. The administration of ACTH releasing factors *in vivo* has been shown to increase plasma corticosterone concentrations via action on ACTH secretion in pigeons (*Columba livia domestica*) (Westerhof *et al.*, 1992), Japanese quail (Launay *et al.*, 1993), and a variety of free-living passerines (Romero *et al.*, 1997; Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero *et al.*, 1998d; Romero and Wingfield, 1999). Assessing the effectiveness of ACTH-releasing factors by measuring corticosterone secretion is not ideal since they do not stimulate corticosterone directly (Romero *et al.*, 1998b). However, plasma corticosterone should reflect an ACTH response to CRF, AVT or MT.

Pituitary responsiveness to mammalian CRF was reported by Westerhof *et al.* (1992) where the intravenous treatment of pigeons with ovine CRF (oCRF) with doses of 10 or 100 μg/kg BWT stimulated increases in plasma corticosterone at 15 and 60 minutes. Japanese quail also showed a small but significant increase in corticosterone after human CRF (15, 30, 45 μg/kg BWT) was administered sub-cutaneously (Launay *et al.*, 1993). Clearly the Japanese quail pituitary is sensitive to mammalian CRF *in vitro* and *in vivo* (Carsia *et al.*, 1986; Launay *et al.*, 1993).

The importance of a permissive action of the other two avian hypothalamo-hypophysiotrophic factors AVT and MT cannot be discounted. Romero and Wingfield (1999) noted that that CRF and AVT administered together stimulated a more robust corticosterone response in Gambel's white-crowned sparrow than did

CRF alone. The assessment of the corticosterone response to CRF, AVT or MT has been used successfully to measure differences in pituitary responsiveness in a variety of species.

1.2.6.1.3 Central nervous system

Variation in the corticosterone response between individual quail is also likely to be mediated at the CNS. As discussed previously, the adrenocortical response is only activated after the processing of the stimuli within the CNS. Stimuli are first detected by the bird visually, aurally, or through the somatosensory system; the information is then processed and integrated within the CNS and an HPA and sympathoadrenal response initiated where the stimuli pose a threat to homeostasis. Subtle changes in the internal or external environment or immediate threats such as the presence of a predator, stimulate different parts of the brain. In rats, the amygdala and paraventricular nucleus are well established as two brain regions involved in the processing of stressful or fearful stimuli (Campbell *et al.*, 2003). Similarly, using c-*fos* immunoreactivity as a marker of immediate early gene expression, Takeuchi *et al.* (1996) showed that specific regions of the brain were activated in response to a social isolation stressor.

Variation in the adrenocortical response between individuals is also influenced by the perception of the severity of the stressor by that individual based on its own previous experience and associative memory. These non-genetic differences in the brain associated with each individual's experiences will influence the responses of birds to stimuli. In mammals, exposure to acute stressful stimuli has been shown to alter an individual's subsequent response and enhance the later ability to acquire new memories about associations between stimuli (Beylin and Shors, 2003). In rats, the elevation in glucocorticoids following exposure to noxious stimuli enhance the recognition and association of the stressor as a source of stressful stimuli in future challenges (Beylin and Shors, 2003).

The importance of a bird's perception of different stimuli and resulting activation or absence of a corticosterone response is illustrated by the study of Cockrem and Silverin (2002b). In their study, a marked corticosterone response was stimulated

by exposing great tits to a moving stuffed owl (a recognisable predator), whereas birds shown a moving box of similar size and colouration did not have a corticosterone response. This clearly showed that an individual's perception of the stimuli within the CNS, whether innate or learned, is a primary regulatory component of the adrenocortical response. Few studies have addressed the variation in adrenocortical responsiveness between individuals by examining differences in neural activation within specific regions of the brain. Measuring c-*fos* protein immunoreactivity to indicate expression of the immediate early gene has been the main method used to detect neuronal activation in the brain in mammals and birds (Meddle and Follett, 1995; Takeuchi *et al.*, 1996; Meddle *et al.*, 1999; Briski and Gillen, 2001). However, such measurements in birds with differing corticosterone responses to a standardised stressor have not been made.

Underlying the psychophysiological level of HPA-axis control is a genetic component to the adrenocortical response which has been reported in a wide range of vertebrates (reviewed by Morméde *et al.*, 2003). The Japanese quail is one such species which has been genetically selected over many generations for divergent adrenocortical responses to brief mechanical restraint (Satterlee and Johnson, 1988; Jones *et al.*, 1992a; Jones *et al.*, 1994a; Jones *et al.*, 1994b). The participation of genetic factors in variability of the stress response may occur at many levels of the HPA-axis, although the prevalence of molecular biology techniques and improved availability of avian gene sequences are making research in this area more accessible (Morméde *et al.*, 2003).

1.3 Outline of thesis

The overall aim of the research described in this dissertation was to determine the effects of different stressors on corticosterone and sex steroid secretion in Japanese quail, and to investigate the neuroendocrine basis of variation between birds in their responses to stressors.

The Japanese quail was used for this research as it is a convenient laboratory bird that had previously been studied in our laboratory. Boyd (2000) described

corticosterone responses of quail to 15 minutes of handling and found marked variation between birds in their responses. She also found that daily corticosterone injections for 6 days reduced the size of the cloacal gland, an androgen-dependent structure, but that testosterone concentrations 24 hours after corticosterone injections were not reduced. This discrepancy was resolved in a further study in which corticosterone was shown to be elevated and testosterone reduced 3 and 6 but not 12 hours after a corticosterone injection. These findings led us to investigate the detailed time course of changes in corticosterone and testosterone after a corticosterone injection, the dynamics of testosterone during physiological corticosterone responses to stressors compared with pharmacological responses to corticosterone injections, and the sites within the hypothalamic-pituitary-adrenal axis at which variation in corticosterone responses arises. The current study addressed the following questions:

1. What relationships are there between corticosterone, sex steroids and LH in the first 3 hours after a pharmacological elevation in plasma corticosterone?

2. What effect does the duration of a manual restraint stressor have on the magnitude and duration of the corticosterone response and testosterone secretion in male quail?

3. What effect do different stressful stimuli have on the magnitude and duration of the corticosterone response in male quail?

4. Are corticosterone responses to handling repeatable for individuals and what is the variation within and between individual quail?

5. Is variation in the corticosterone response between individual quail caused by differences in adrenal or pituitary sensitivity to ACTH or CRF respectively?

Chapters 2 and 3 describe a series of experiments which were designed to address questions one, two and three. Chapter 4 addresses question four, while Chapter 5

describes a series of experiments designed to address question five. Thesis results and future directions for research are addressed in Chapter 6 (general discussion).

Chapter 2

Effect of a single corticosterone injection on reproductive hormones in Japanese quail

2.1 Introduction

Stress in its various forms is well documented as having the potential to have a detrimental effect on the reproductive system in a wide variety of vertebrates (reviewed by Rivier and Rivest, 1991). Stressors which can be physical, psychological, nutritional, environmental, or social, trigger the release of adrenal steroids from the adrenal gland, which in turn stimulate a variety of physiological responses which allow the animal to respond or adapt to the stressor. Increased hypothalamic-pituitary-adrenal (HPA) axis activity can affect the function of the hypothalamic-pituitary-gonadal (HPG) axis in combination with other factors such as photoperiod and temperature, which were discussed in chapter 1. Exposure to a stressor results in an increase in plasma corticosterone concentrations in birds. Corticosterone is the major avian stress hormone released from the adrenal gland (Carsia and Harvey, 2000). The roles of corticosterone are diverse and it plays an important function in the maintenance and termination of breeding during the reproductive cycle in birds (reviewed by Deviche, 1983).

The inhibitory effects of corticosterone on the avian reproductive system have been long recognised (Deviche, 1983), and prolonged corticosterone treatment can suppress LH secretion and induce gonadal regression in a variety of species. Wilson and Follett (1975) showed that intra-hypothalamic implants of corticosterone caused gonadal regression and a decrease in LH secretion in

photostimulated tree sparrows (*Spizella arborea*). Likewise, a prolonged but subtle increase in plasma corticosterone concentrations caused gonadal regression and the cessation of lay in hens (Etches *et al.*, 1984; Williams *et al.*, 1985; Petitte and Etches, 1991).

While the effects of chronically elevated plasma corticosterone concentrations on reproduction have been studied extensively, the effect of an acute increase in corticosterone on sex steroid concentrations has not been well characterised. Boyd (2000) showed that plasma testosterone concentrations in Japanese quail decreased 3 hours after a single corticosterone injection that had raised plasma corticosterone above normal physiological concentrations. The current study was designed to provide more detailed information on the relationship between plasma corticosterone and testosterone in male Japanese quail, and to characterise how oestradiol, the major sex steroid in females, is affected by a single corticosterone injection. The nature of the ovulatory cycle with surges in sex steroid concentration at different stages of the cycle required that the timing of corticosterone injections in females was carefully planned. Van Tienhoven (1961) and Etches and Cunningham (1976) showed that exogenous corticosterone could induce ovulation in hens, while Wilson and Sharp (1976) and Etches and Croze (1983) showed that corticosterone could induce an increase in LH secretion.

The aims of the experiments were:

1. To determine the relationship between a dose of corticosterone and plasma concentrations of corticosterone, testosterone and luteinising hormone (LH) in male Japanese quail.

2. To determine the relationship between a dose of corticosterone and plasma concentrations of corticosterone and oestradiol in female Japanese quail given a single corticosterone injection.

2.2 Methods and materials

2.2.1 Animals and housing

2.2.1.1 Source and type of birds

Seven week-old wild-type male and female Japanese quail (*Coturnix coturnix japonica*) were purchased from our supplier (Rangitikei Game Birds Ltd., Bulls). The birds had been raised under a long-day photoperiod (15L:9D) at air temperatures of 20 to 25°C in mixed sex groups. The 7 week-old birds were sexually mature at time of purchase with all females laying eggs, while all males had developed cloacal glands and cloacal foam was present. Sexually mature males could be selected by choosing males with enlarged cloacal glands (relative to males with no cloacal gland) as this has been shown to be a positive indicator of spermatogenesis, an increase in testicular weight and testosterone secretion (Ottinger and Brinkley, 1979).

2.2.1.2 Housing conditions

Each bird was identified with a numbered leg band and housed in an individual cage measuring 20 cm (W) × 35 cm (D) × 24 cm (H) in animal rooms in the Veterinary Science building. Quail were held on a long day photoperiod (16L:8D; lights on from 0900 – 0100 h). An extractor fan provided ventilation for each room, and only one sex was held in a room during each experiment. A temperature control unit enabled air temperature to be maintained at 20°C. Light in each room was provided by two 75W incandescent light bulbs controlled by a 24 hour/7 day time switch (HPM Excel Light Switch and Timer, Cat XL770T). The light intensity in the quail rooms was 130 lux in cages at the top, and 15 lux in cages near the floor of the room (Minolta Illuminance meter).

2.2.1.3 Food and water

Quail were provided with fresh water and food (Harvey Farms quail layer mash) *ab libitum*. The food was the same as that used by Rangitikei Game Birds.

2.2.2 Experimental design

2.2.2.1 Experiment 1 – Acute response to a single subcutaneous corticosterone injection in 8 week old male Japanese quail

Twenty one 7 week old male Japanese quail were held with *ad libitum* access to food and water under long days (16L:8D) at 20°C for one week. The birds were then assigned into an untreated control group and two treated groups (n=7 each). These groups received either a single subcutaneous injection of a vehicle (sesame oil) or 1.2 mg corticosterone per bird (6 mg/kg) in sesame oil. A blood sample was taken at 0900 h (0 min, immediately before the injection) and then further samples were taken at 30 min, 1, 3, and 6 h after the injection. A terminal blood sample was obtained at 12 h when the birds were euthanased. Blood samples were collected from control birds at the same times. Body weight and the area of the cloacal gland were measured before the treatment and at 12 h. Plasma concentrations of corticosterone, testosterone and luteinising hormone (LH) were measured in all blood samples.

2.2.2.2 Experiment 2 – Acute response to a single subcutaneous corticosterone injection in 8 week old female Japanese quail

Twenty 7 week old female Japanese quail were obtained from our supplier and held with *ad libitum* access to food and water under long days (16L:8D) at 20°C for one week. The female experiment was conducted several months after the male study outlined above. The birds were then assigned into an oil- or corticosterone-treated group (n=10 each). These groups received either a single subcutaneous injection of a vehicle (sesame oil) or 1.2 mg corticosterone per bird (6 mg/kg) in sesame oil. Blood samples were collected at 0 min before the first injection, and then 30, 90 min, 3, 6, and 12 h after the injection. A terminal blood sample was obtained at 24 h when the birds were euthanased. The first blood sample was taken at 2000, in order to avoid the rise in corticosterone about the time of ovulation which occurs 15 – 30 min within oviposition (Woodard and Mather, 1964). The time of oviposition varied between 1400 - 1900 in these females. Body weight and the diameter of the cloacal opening were measured before the treatment and at 24 h. Plasma concentrations of corticosterone and oestradiol were measured in all blood samples.

The experiments were conducted under a protocol approved by the Massey University Animal Ethics Committee.

2.2.3 Data collection

2.2.3.1 Corticosterone administration

The corticosterone solution was prepared the day before the experiment and then kept on a magnetic stirrer (Chiltern Scientific) at 4°C. 250 mg of corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione, Sigma Chemical Co., St Louis, USA) was dissolved in 1 ml of benzyl alcohol (Sigma Chemical Co., St Louis, USA) then added to 9 ml of sesame oil (Sigma) producing a corticosterone 25 mg / ml stock solution. A solution was made from the stock producing a dose of 6 mg / kg of body weight in a 100 μl volume injection. 6 mg / kg was approximately 1.2 mg per bird, a dose used previously (in our laboratory) to stimulate a strong pharmacological corticosterone response and decrease in testosterone in male Japanese quail (Boyd, 2000). Subcutaneous injections were made under the skin on the abdomen of the quail using 1 ml insulin syringes and needles.

2.2.3.2 Tissue and blood samples

Blood samples were collected by venipuncture of the brachial vein in the wing with a 25 g needle, with 200 μl of blood collected into heparinised capillary tubes. All samples were collected within two minutes from the time the bird was removed from the cage. The final blood sample was collected when the birds were euthanased by stunning followed by decapitation. The blood was stored on ice until centrifugation at 2 000 g for 15 minutes at 4°C (Heraeus Christ 5000S refrigerated centrifuge). The plasma was removed and stored at -20°C until assayed. The width and height of the cloacal protuberance was measured (±0.1 mm) in males and body weight was measured (±0.1 g) in all birds on the day before the experimental treatment. The reproductive system was removed after euthanasia and the oviduct and ovary or testes were weighed (±0.001 g).

2.2.4 Hormone assays

Corticosterone, testosterone, oestradiol, and luteinising hormone (LH) concentrations in plasma were determined by radioimmunoassay.

2.2.4.1 Corticosterone

Corticosterone concentrations in extracted quail plasma were measured by radioimmunoassay, using a modification of the method described by (Wingfield *et al.*, 1997a). For extraction, 1 ml of plasma was thawed and spun in a 1.5 ml Eppendorf tube at 14 000 g for 5 min (IEC Micromax ventilated microcentrifuge OM3590) to separate clear plasma from lipid. A 20 μl aliquot of plasma was added to a screw top glass extraction tube (13 mm $\times$ 100 mm), and 1 ml of double-distilled dichloromethane (dichloromethane "AnalaR", BDH) was added using a Merck bottle-top dispenser. The plasma and dichloromethane were vortexed for 10 s, shaken together for 1 h on a Chiltern Scientific SS70 orbital shaker, and centrifuged at 4 500 g for 10 min in a Heraeus Christ 5000S refrigerated centrifuge to separate the organic and aqueous phases. An 800 μl aliquot of the organic phase was removed from each tube and dried under a stream of air in a heating block at 37°C. The dried extract was then reconstituted in 450 μl of phosphate-buffered saline with gelatine (PBSG; 0.1M, pH 7.0), vortexed, then shaken for 1 h at room temperature and then stored overnight at 4°C. The percentage recovery of corticosterone following the extraction process was determined by mixing tritiated corticosterone with 12 different plasma samples in triplicate. A 100 μl aliquot of the reconstituted extract containing label was taken for determination of the percentage recovery of the sample. This yielded a mean recovery for corticosterone of 99.7 $\pm$ 2.0% (n=12) for quail plasma, with this mean value used to calculate corticosterone concentrations in samples. All samples subsequently extracted were extracted without the addition of tritiated corticosterone.

Samples were assayed in duplicate and were randomly distributed throughout two assays. Reconstituted extracts were incubated with 100 μl of antibody (gift of Dr. R. J. Etches, University of Guelph, Ontario, Canada; 1:18 000 final dilution) and 100 μl of tritiated corticosterone (approximately 5 000 cpm; 1,2,6,7-^{3}H-corticosterone

TRK.406 Amersham, UK) at 4°C overnight. Bound and free components were separated by the addition of 500 μl dextran-coated charcoal (2.5 g/l charcoal, 0.25 g/l dextran (Dextran T70, Amersham Pharmacia) in PBSG) for 15 min at 4°C. Tubes were then centrifuged at 4 500 g for 15 minutes at 4°C and the supernatant poured off. 3 ml of scintillant (5 g/l PPO (2,5-diphenyl-oxazole, Sigma), 0.3 g/l dimethyl POPOP (1,4-bis-[4-methyl-5-phenyl-2-oxazoly]-benzene, Sigma) in toluene) was added, the samples were shaken for 1h, left for 1h at room temperature, and then each sample was counted for five minutes in a Wallac 1409-411 liquid scintillation counter.

The limit of sensitivity of the radioimmunoassay, defined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curve and expressed as corticosterone concentration in plasma, was 0.61 ng/ml. Serial dilutions of extracted quail plasma in assay buffer (PBSG) were parallel to the corticosterone standard curve (n=3). Recovery of corticosterone added to quail plasma was 93.9 ± 4.9%, 98.6 ± 7.3%, and 88.9 ± 5.5% for three samples. Solutions of corticosterone in PBSG were prepared at concentrations that gave approximately 20, 50, and 80% binding on the standard curve. These solutions were used as high, medium and low quality control samples in every assay. The intra-assay coefficients of variation for these samples were 13.2, 14.8, and 13.4% (n=20) and inter-assay coefficients of variation in all 12 assays were 4.9, 6.1, and 4.3%.

The cross-reactions of the antibody with other steroids tested by Etches (1976) were deoxycorticosterone (27.9%), cortisol (6.9%), progesterone (37.6%), 11β-hydroxyprogesterone (21.3%), oestradiol, testosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, aldosterone and pregnenalone (all < 5%).

2.2.4.2 Testosterone

Testosterone concentrations in extracted quail plasma were measured by radioimmunoassay, using a modification of the method described by (Wingfield *et al.*, 1997). For extraction, 1 ml of plasma was thawed and centrifuged in a 1.5 ml Eppendorf tube at 14 000 g for 5 min (IEC Micromax ventilated microcentrifuge

OM3590) to separate clear plasma from lipid within the plasma sample. A 20 μl aliquot of plasma was added to a screw top glass extraction tube (13 mm × 100 mm), and 1 ml of double-distilled dichloromethane (dichloromethane "AnalaR", BDH) was added using a Merck bottle-top dispenser. The plasma and dichloromethane were vortexed for 10 s, shaken together for 1 h on a Chiltern Scientific SS70 orbital shaker, and centrifuged at 4 500 g for 10 min in a Heraeus Christ 5000S refrigerated centrifuge to separate the organic and aqueous phases. An 800 μl aliquot of the organic phase was removed from each tube and dried under a stream of air in a heating block at 37°C. The dried extract was then reconstituted in 450 μl of phosphate-buffered saline with gelatine (PBSG; 0.1M, pH 7.0), vortexed, then shaken for 1 h at room temperature and then left overnight at 4°C. The percentage recovery of testosterone following the extraction process was determined by mixing tritiated testosterone with six different plasma samples in triplicate. A 100 μl aliquot of the reconstituted extract containing label was taken for determination of the percentage recovery of the sample. This yielded a mean recovery for testosterone of 96.6 ± 4.0% (n=18) for quail plasma, with this mean value used to calculate testosterone concentrations in samples. All samples subsequently extracted were extracted without the addition of tritiated testosterone.

Samples were assayed in duplicate and were randomly distributed throughout three assays. Reconstituted extracts were incubated with 100 μl of antibody (Endocrine Sciences CA, US; testosterone antiserum T3-125 raised in rabbit) and 100 μl of tritiated testosterone (approximately 5 000 cpm; ^{3}H-testosterone TRK.406 Amersham, UK) at 4°C overnight. Bound and free components were separated by the addition of 500 μl dextran-coated charcoal (2.5 g/l charcoal, 0.25 g/l dextran (Dextran T70, Amersham Pharmacia) in PBSG) for 15 min at 4°C. Tubes were then centrifuged at 4 500 g for 15 minutes at 4°C and the supernatant poured off. Three ml of scintillant (5 g/l PPO (2,5-diphenyl-oxazole, Sigma), 0.3 g/l dimethyl POPOP (1,4-bis-[4-methyl-5-phenyl-2-oxazoly]-benzene, Sigma) in toluene) was added, the samples were shaken for 1 h, left for 1 h at room temperature, and then each sample was counted for five min in a Wallac 1409-411 liquid scintillation counter.

The limit of sensitivity of the radioimmunoassay, defined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curve and expressed as testosterone concentration in plasma, was 0.36 ng/ml. Serial dilutions of extracted quail plasma in assay buffer (PBSG) were parallel to the testosterone standard curve (n=3). Recovery of testosterone added to quail plasma was 97.4 ± 1.7%, 99.1 ± 8.1%, and 102.0 ± 2.7% for three samples. Solutions of testosterone in PBSG were prepared at concentrations that gave approximately 20, 50, and 80% binding on the standard curve. These solutions were used as high, medium and low quality control samples in every assay. The intra-assay coefficients of variation for these three samples were 19.3, 7.8, and 5.5% (n=20). The inter-assay coefficients of variation for three assays were 9.5, 8.3, and 11.7% for high, medium and low quality controls respectively. The cross-reactions of the antibody with other steroids tested by Endocrine Sciences were dihydrotestosterone (20%), corticosterone (< 0.01%), oestradiol (0.14%), Δ-1-testosterone (52%), 4-androsten-3β-17β-diol (3%), 5α-androstan-3β-17β-diol (1.8%), Δ-4-androstenedione (0.5%), others (< 0.5%).

2.2.4.3 Oestradiol

Oestradiol concentrations in quail plasma were measured by radioimmunoassay using an ICN ImmunoChem™ Double Antibody 17β-estradiol ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA). Samples were assayed in duplicate and were run in one assay. For assay, plasma was thawed and centrifuged in a 1.5 ml Eppendorf tube at 14 000 g for 5 min (IEC Micromax ventilated microcentrifuge OM3590) to separate clear plasma from lipid within the plasma sample. A 10 μl aliquot of plasma was incubated with 100 μl of antibody (anti-17β-oestradiol Cat # 07-138113) and 100 μl of iodinated oestradiol (approximately 5 000 cpm; ^{125}I-oestradiol Cat # 07-138121) at 37°C for 90 minutes. After incubation, 100 μl of precipitant solution (Cat # 07-166624) was added to all tubes and mixed using a Chiltern Scientific SS70 orbital shaker for approximately 5 s. Tubes were then centrifuged at 4 500 g for 15 min at 4°C and the supernatant aspirated. The precipitates were then counted in a LKB Wallac 1261 Multigamma Gamma Counter.

The limit of sensitivity of the radioimmunoassay, defined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curve and expressed as oestradiol concentration in plasma, was 7.4 pg/ml. Serial dilutions of quail plasma in diluent buffer were parallel to the oestradiol standard curve (n=3). Recovery of oestradiol added to quail plasma was 97.4 ± 13.0%, 97.4 ± 12.3%, and 92.1 ± 11.1% for three samples. Solutions of oestradiol in PBSG were prepared at concentrations that gave approximately 20, 50, and 80% binding on the standard curve. These solutions were used as high, medium and low quality control samples in every assay. Intra-assay coefficients of variation have yet to be determined. No inter-assay coefficients of variation were calculated as all samples were analysed in one assay.

The cross-reactions of the antibody with steroids tested by ICN Biomedicals were oestradiol-17β (100.00%), oestrone (20.00%), oestriol (1.51%), oestradiol-17α (0.68%), ethinyl oestradiol, androstenedione, dehydroepiandrosterone (DHEA), 5α-dihydrotestosterone, 20α-dihydroprogesterone, deoxycorticosterone, progesterone, testosterone, pregnenolone, 17-hydroxypregnenolone, DHEA-sulphate, aldosterone, cortisol, 11-desoxycortisol, 17α-hydroxyprogesterone and cholesteronol (all < 0.01%).

2.2.4.4 Luteinising hormone (LH)

Luteinizing hormone (LH) concentrations in plasma were measured directly by Dr M. Kikuchi using a modification of the double- antibody radioimmunoassay for chicken LH described by (Hattori and Wakabayashi, 1979). Purified chicken LH (CANOMS12442B; Kikuchi and Ishii, 1989) was used for radioiodination, with a crude chicken LH fraction (AGC112B; Kikuchi and Ishii, 1989) for the reference standard (Silverin *et al.*, 1999), and an anti-avian LH antiserum (AL-MH#1) (Hattori and Wakabayashi, 1979).

Plasma was thawed and centrifuged in a 1.5 ml Eppendorf tube at 14 000 g for 5 min (IEC Micromax ventilated microcentrifuge OM3590) to separate clear plasma from lipid within the plasma sample. Samples were assayed in duplicate within a single assay.

Twenty five μl of plasma or standard was incubated overnight at 4°C with 400 μl of 0.1% gelatin in PBS (PBSG), 100 μl of diluted antiserum (1:100,000) in 1% normal rabbit serum (0.05M EDTA-PBS) (Hattori and Wakabayashi, 1979). One hundred μl of ^{131}I-LH in 0.1% PBSG was added the day after incubation and incubated overnight at 4°C. A goat anti-rabbit γ-globulin serum (H-4) was added to all tubes and then mixed before the supernatant was aspirated and the pellet counted (Hattori and Wakabayashi, 1979). The LH content in each sample was expressed in terms of the weight of CANOMS12442B.

The limit of sensitivity of the radioimmunoassay defined as the concentration of LH that corresponded to the percentage binding two standard deviations from the mean % bound of zero control tubes, was 0.06 ng/ml. The intra-assay coefficient of variation provided by Dr M. Kikuchi was 6.2%. Quail plasma produced dilution curves parallel to chicken LH standard curves.

2.2.5 Calculation of the area under the corticosterone curve

The area under the curve for plasma corticosterone concentrations versus time was calculated for each bird, using the method described by Cockrem and Silverin (2002a). The 'total integrated response' (Cockrem and Silverin, 2002a) is a measure of the amount of corticosterone secreted during the sampling period and is expressed as ng/ml·min. A second measure called the 'corrected integrated response' was also calculated by multiplying the corticosterone concentration at time 0 by the duration of experiment and subtracting this from the total integrated response area. The area of the curves for testosterone, oestradiol, and LH which had negative responses were also calculated for each bird. Areas under the curve were calculated using the trapezoid rule using GraphPad Prism 4.0 (GraphPad Software Inc.).

2.2.6 Statistical analyses

All results are presented as the mean ± SE (standard error) or individual data where appropriate. All the data were examined for normality using Shapiro-Wilks tests

and for equality of variance using Levene's homogeneity of variance test. Hormone data were $\log_{10}$ transformed to avoid heteroscedasticity.

If variances were homogenous then groups were compared with repeated measures two-way analyses of variance (ANOVA) with time and treatment as the grouping factors. Comparisons between times within each treatment and between treatments for each time were examined with post-hoc repeated measures contrasts. Data not complying with normality or variance criteria were compared using a Kruskal-Wallis non-parametric test followed by Mann-Whitney U independent group comparisons tests, or Friedman's test for repeat measures data. All comparisons of means were conducting using SPSS™ 11.0 (SPSS Inc., Illinois) or Systat™ 8.0 (SPSS Inc., Illinois).

2.3 Results

2.3.1 Experiment 1 - Acute response to a single subcutaneous corticosterone injection in 8 week old male Japanese quail

2.3.1.1 Gross morphology

The seven week old male quail weighed 187.9 – 248.1 g (mean 214.4 ± 4.0 g; Figure 2.1a) when collected, with no differences between the three randomly assigned treatment groups ($F_{2,18}$ = 0.385, p = 0.686). There was a slight but significant increase ($F_{1,18}$ = 41.485, p < 0.001) in body weight across all groups by the end of the experiment ranging from 200.0 – 249.1 g (mean 226.2 ± 3.3 g).

Cloacal gland areas were large in all groups, with no significant increases in area during the experiment and no effect of treatment ($F_{2,18}$ = 1.362, p = 0.281; Figure 2.1b). There were no differences in testis weight between groups at the end of the experiment ($F_{2,18}$ = 0.374, p = 0.694; Figure 2.1c). The testes were the same size as testes of adult birds held on long days.

2.3.1.2 Plasma corticosterone

Corticosterone declined after the first sample in all the untreated birds and then

remained relatively constant or increased, whereas there were no consistent changes in oil treated birds (Figure 2.2). Corticosterone rose to a peak (221.56 – 382.71 ng/ml) at 0.5 h in all birds injected with corticosterone, declined until 6 h after the injection and then remained constant. There was no relationship between body weight of the injected birds and peak corticosterone concentrations (r^2 = 0.059, p = 0.600).

Corticosterone varied between treatments and times, with a significant interaction between treatment and time (see Table 2.1 for all statistics for this analysis). Corticosterone concentrations were initially low in the three groups of birds (Figure 2.3a). Mean corticosterone concentrations in the untreated birds decreased 0.5 h after the initial sample, rose significantly from 1.5 to 3.0 h and remained constant and low thereafter, whereas there were no significant changes in corticosterone in the oil treated birds. Corticosterone in the birds injected with 1.2 mg corticosterone increased to high concentrations at 0.5 h (289.10 ± 20.56 ng/ml), declined to low concentrations at 6 h and remained low at 12 h. Corticosterone concentrations at 6 and 12 h did not differ from initial concentrations. There was a significant difference between groups in the total integrated corticosterone response ($F_{2,20}$ = 623.632, p < 0.001; Figure 2.5a), with the corticosterone treated group having a significantly greater response than the untreated and oil control groups (post-hoc Bonferroni test, p < 0.001 for both groups). It was not possible to calculate a corrected integrated corticosterone response for either control group as corticosterone concentrations dropped below 0 h values during the course of the experiment.

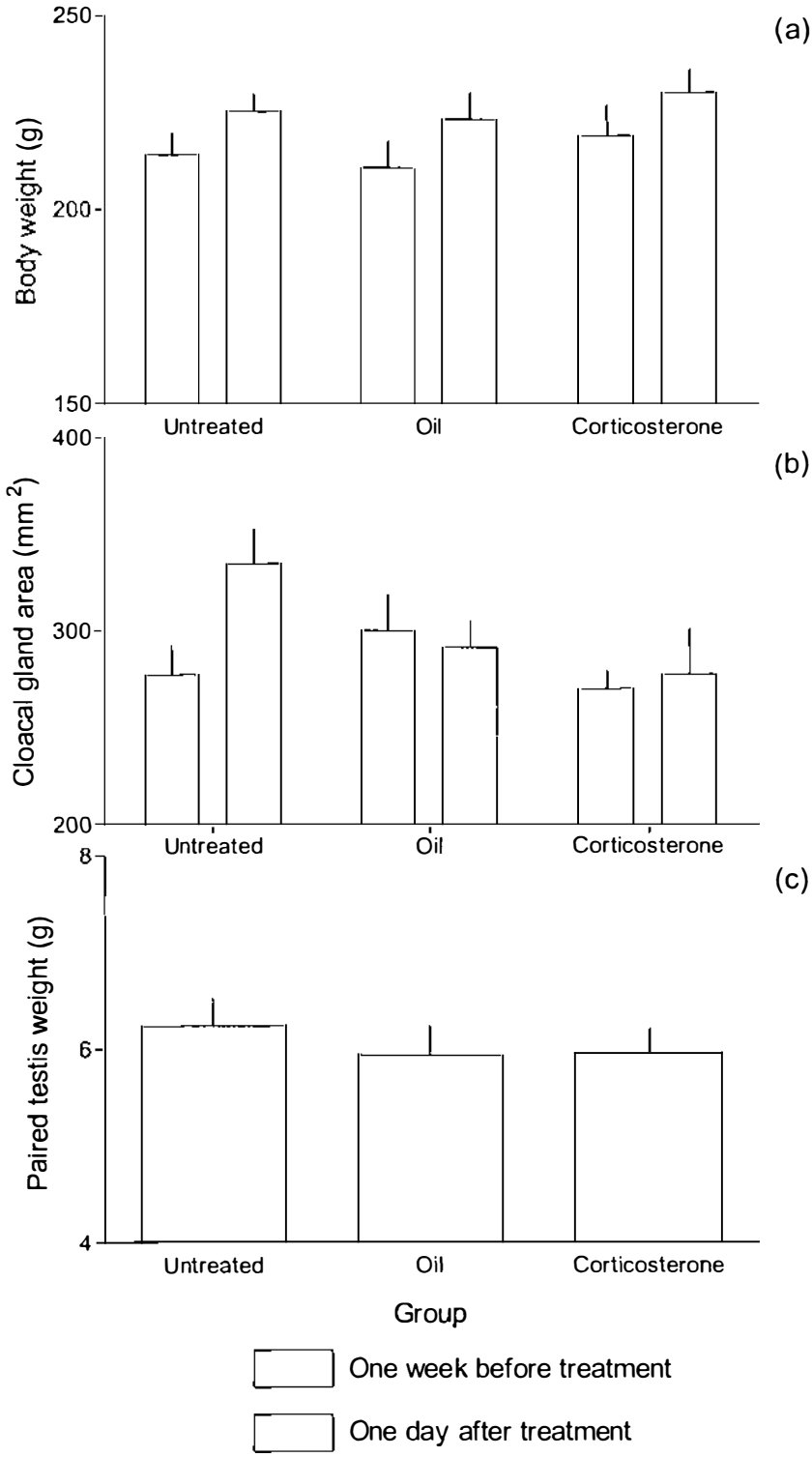


Figure 2.1 Body weight (a), cloacal gland area (b), and paired testis weights (c) in untreated male quail and in quail treated with oil or 1.2 mg corticosterone. Results are plotted as mean ± standard error.

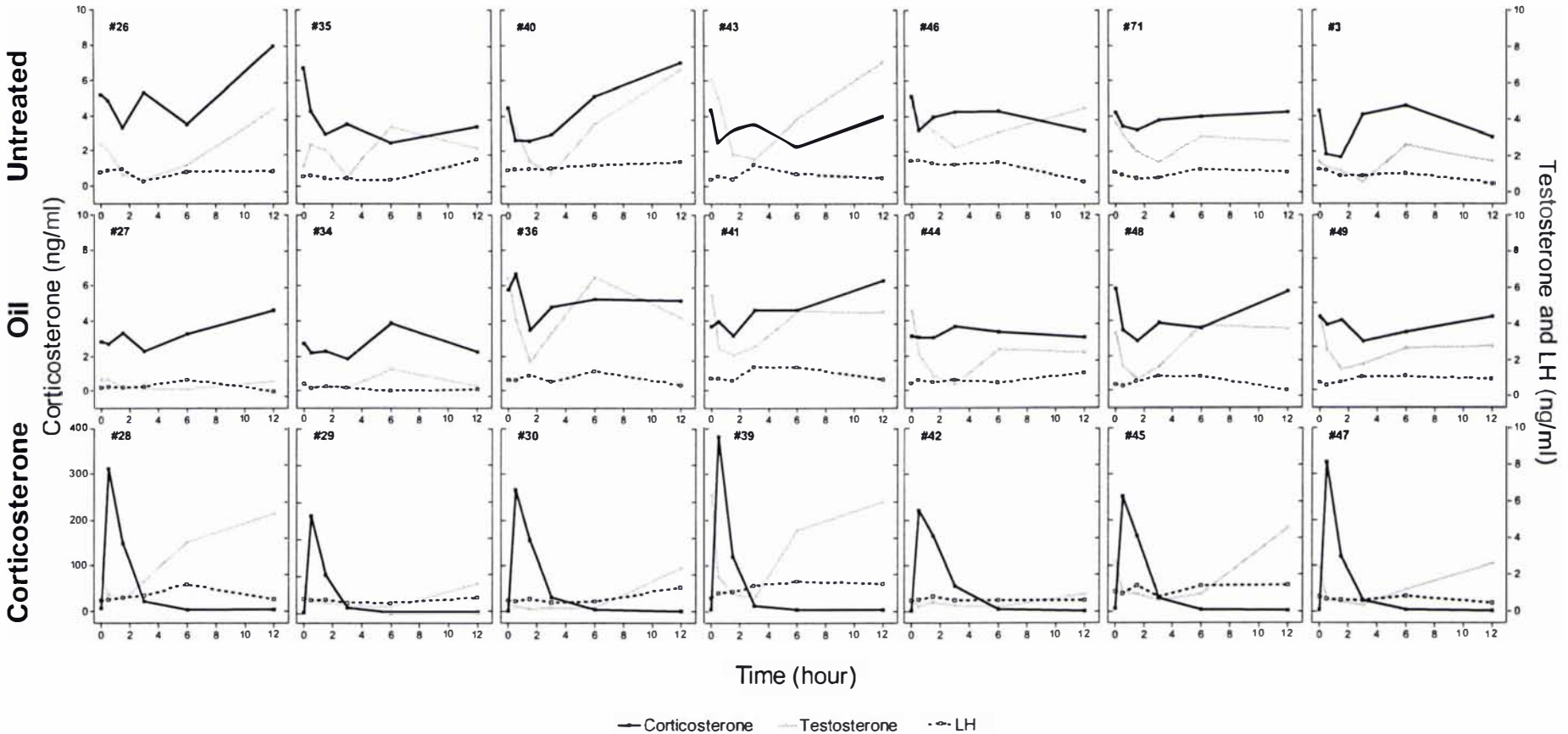


Figure 2.2 Plasma corticosterone, testosterone and LH in untreated male quail and in quail given a single injection of oil or 1.2 mg corticosterone. Note that the scale for the corticosterone axis differs between treatments. Results are plotted as raw data.

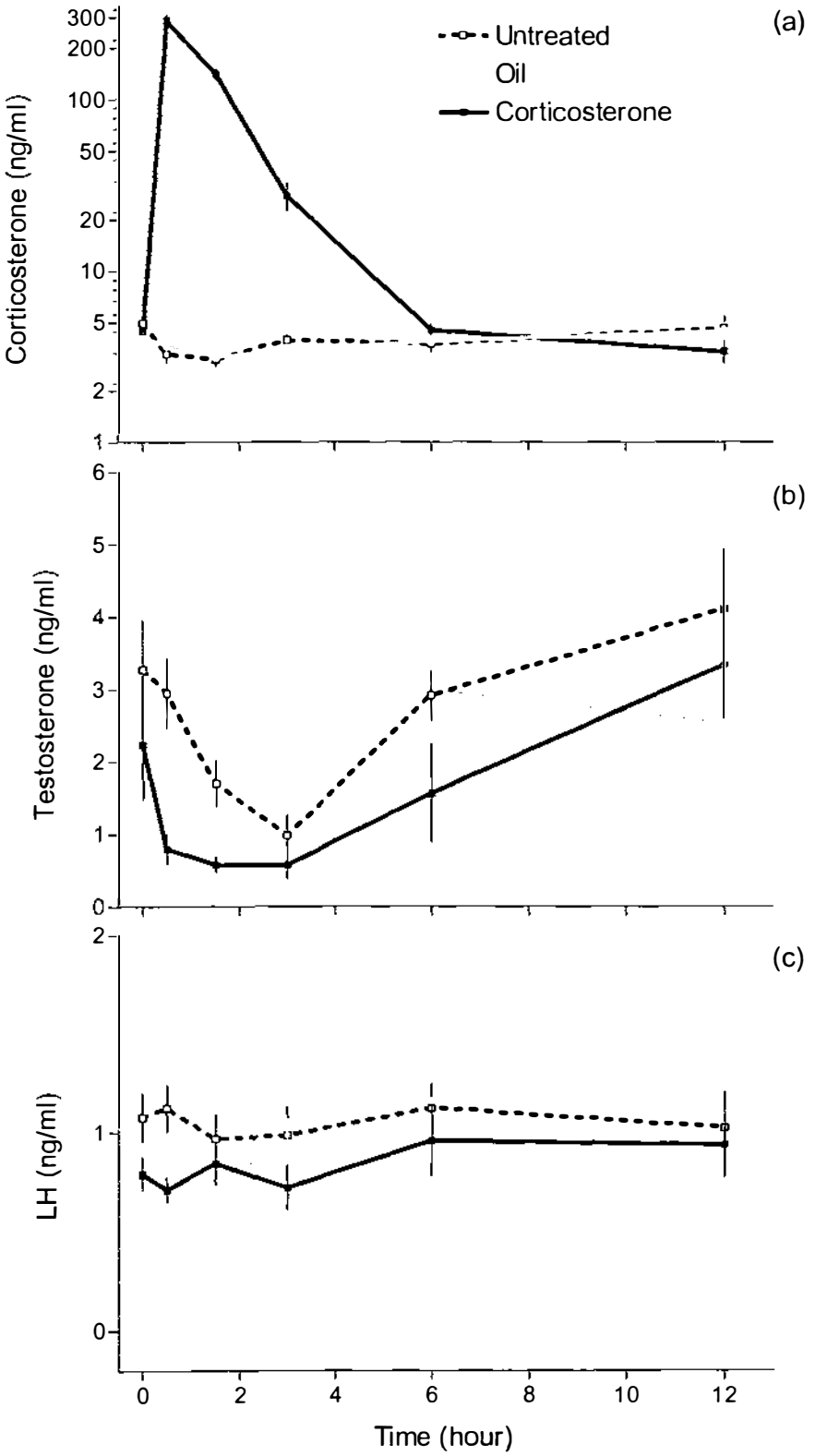


Figure 2.3 Plasma corticosterone (a), testosterone (b), and LH (c) in untreated male quail and in quail treated with oil or 1.2 mg corticosterone. Corticosterone concentration is plotted against a $\log_{10}$ vertical axis. Results are plotted as mean ± standard error.

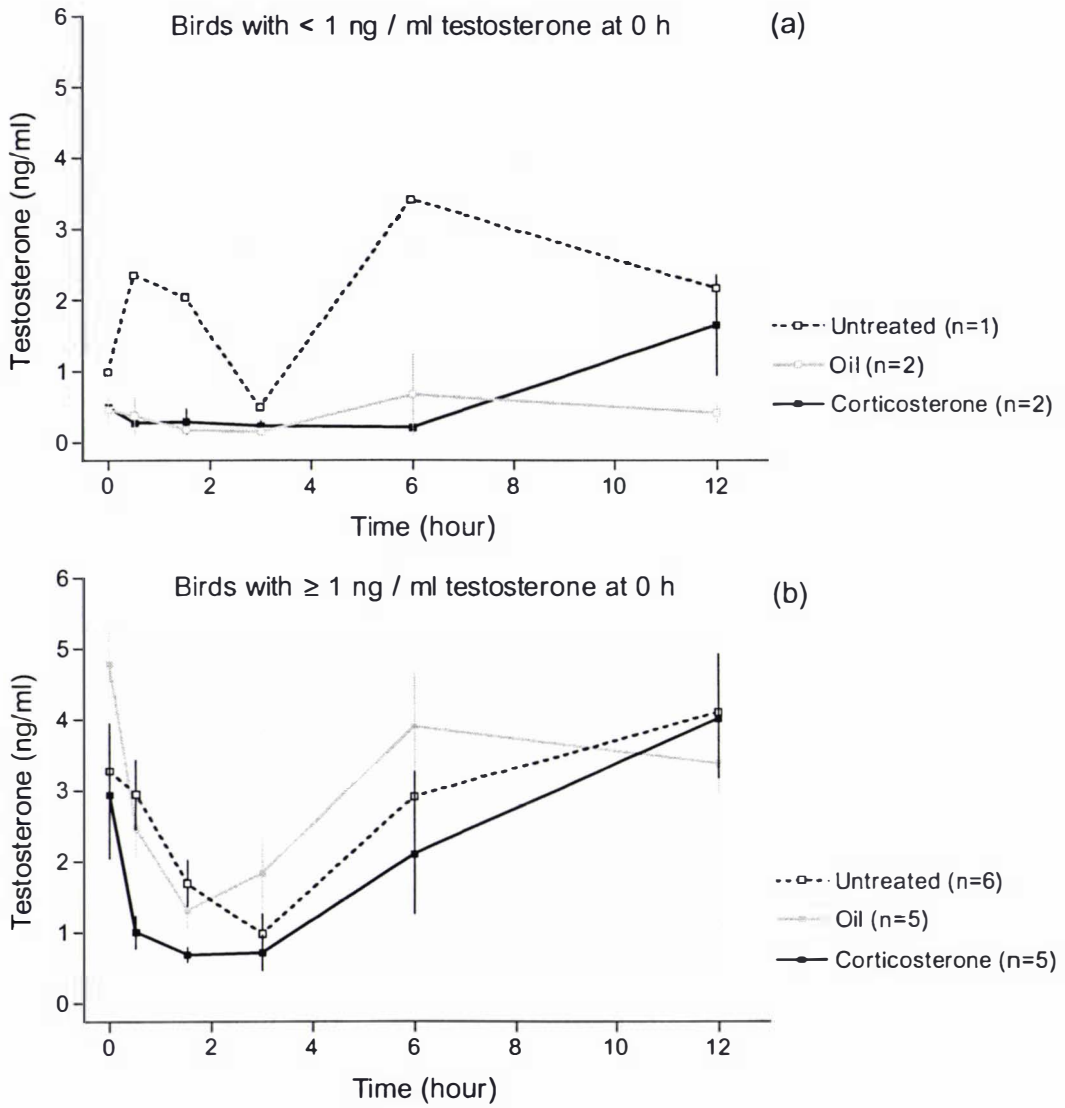


Figure 2.4 Plasma testosterone in male quail with initial concentrations < 1 ng/ml (a) or ≥ 1 ng/ml (b). Quail were untreated or given oil or corticosterone injections. Results are plotted as mean ± standard error.

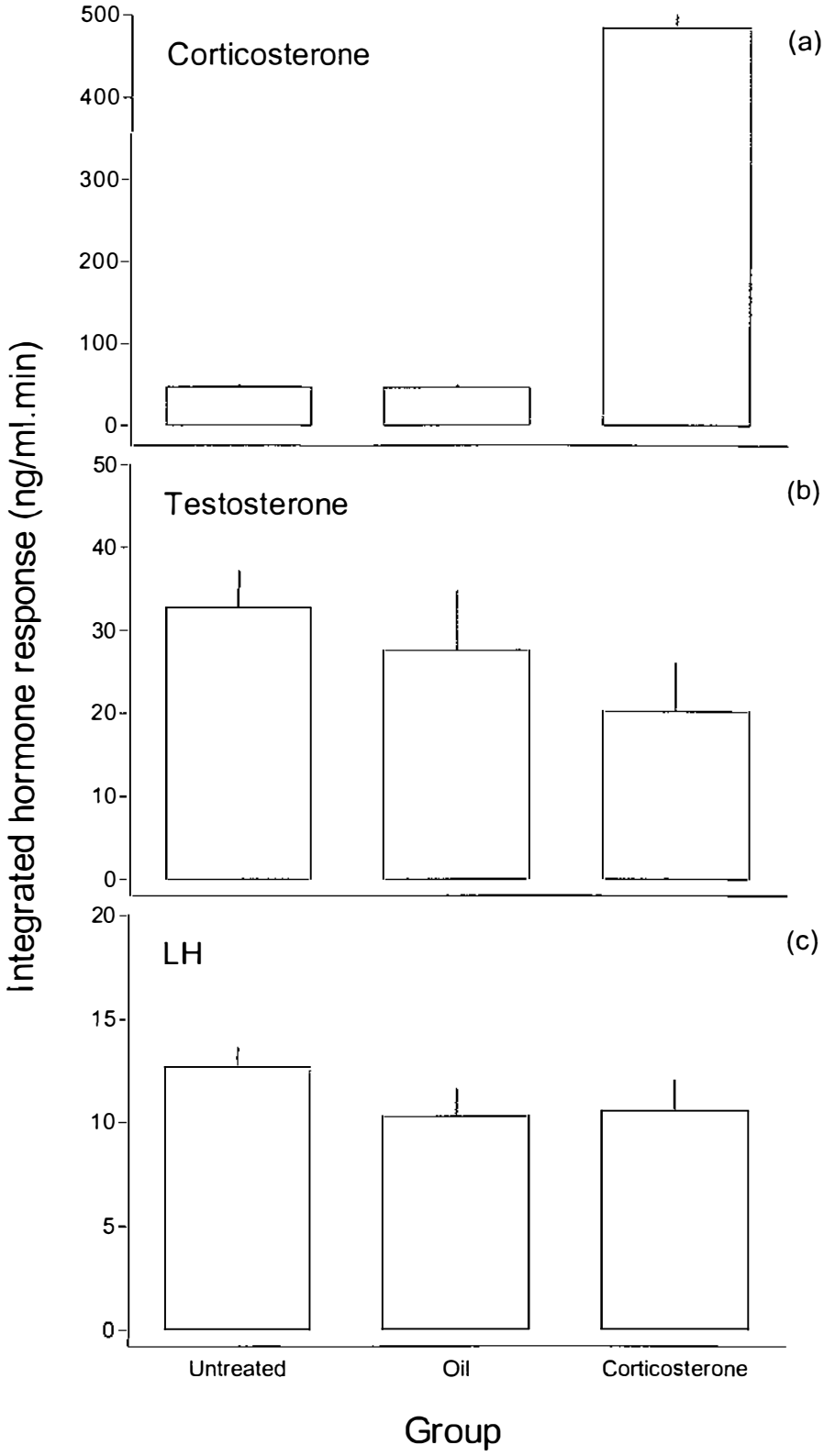


Figure 2.5 Total integrated responses for corticosterone (a), testosterone (b) and LH (c) in untreated male quail and in quail treated with oil or 1.2 mg corticosterone. Results are plotted as mean ± standard error.

Table 2.1 Two-way repeated measures ANOVA for corticosterone concentrations in untreated male quail and in quail treated with oil or 1.2 mg corticosterone.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Treatment		189.851	2,18	< 0.001	**
Time		81.796	5,90	< 0.001	**
Interaction of treatment and time		122.280	10,90	< 0.001	**
Comparisons within treatments for each treatment					
0 vs 0.5 h	Untreated	12.685	1,18	0.002	**
	Oil	0.702	1,18	0.413	
	Corticosterone	1150.135	1,18	< 0.001	**
0.5 vs 1.5 h	Untreated	0.346	1,18	0.564	
	Oil	1.175	1,18	0.293	
	Corticosterone	46.003	1,18	< 0.001	**
1.5 vs 3 h	Untreated	5.122	1,18	0.036	*
	Oil	0.102	1,18	0.753	
	Corticosterone	194.658	1,18	< 0.001	**
3 vs 6 h	Untreated	0.367	1,18	0.552	
	Oil	1.721	1,18	0.206	
	Corticosterone	172.807	1,18	< 0.001	**
6 vs 12 h	Untreated	1.084	1,18	0.311	
	Oil	0.300	1,18	0.590	
	Corticosterone	4.039	1,18	0.060	
0 vs 6 h	Untreated	3.295	1,18	0.086	
	Oil	0.003	1,18	0.954	
	Corticosterone	0.358	1,18	0.557	
0 vs 12 h	Untreated	0.823	1,18	0.376	
	Oil	0.438	1,18	0.516	
	Corticosterone	3.750	1,18	0.069	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 2.1 cont Two-way repeated measures ANOVA for corticosterone concentrations in untreated male quail and in quail treated with oil or 1.2 mg corticosterone.

		Plasma corticosterone		
Comparisons between treatments for each time		*F*	*df*	*p*
0 h	Untreated vs Oil	1.513	1,18	0.235
	Untreated vs Corticosterone	1.080	1,18	0.312
	Oil vs Corticosterone	0.036	1,18	0.851
0.5 h	Untreated vs Oil	0.468	1,18	0.502
	Untreated vs Corticosterone	808.564	1,18	< 0.001 **
	Oil vs Corticosterone	770.188	1,18	< 0.001 **
1.5 h	Untreated vs Oil	0.225	1,18	0.641
	Untreated vs Corticosterone	1069.055	1,18	< 0.001 **
	Oil vs Corticosterone	1038.240	1,18	< 0.001 **
3 h	Untreated vs Oil	0.920	1,18	0.350
	Untreated vs Corticosterone	92.683	1,18	< 0.001 **
	Oil vs Corticosterone	112.074	1,18	< 0.001 **
6 h	Untreated vs Oil	0.256	1,18	0.619
	Untreated vs Corticosterone	2.699	1,18	0.118
	Oil vs Corticosterone	1.294	1,18	0.270
12 h	Untreated vs Oil	0.011	1,18	0.918
	Untreated vs Corticosterone	2.322	1,18	0.145
	Oil vs Corticosterone	2.014	1,18	0.173

2.3.1.3 Plasma testosterone

Testosterone declined after the first sample in all but one of the birds when the initial plasma concentrations were greater than 1 ng/ml (Figure 2.2). Testosterone declined until 3 h, then increased until 6 h in all groups and then remained constant or continued to increase. Testosterone was less than 1 ng/ml in two birds in the oil group and two in the corticosterone treated group for the first three hours of the experiment and remained low (Figure 2.4a).

There was no effect of treatment on testosterone, but it did vary between time and there was a significant interaction between treatment and time (see Table 2.2 for all statistics for this analysis). Testosterone concentrations varied from 0.36 to 6.38 ng/ml in birds at 0 h, but there were no significant differences between the groups (Figure 2.3b). Mean testosterone concentrations in the oil and corticosterone treated birds decreased 0.5 h after the initial sample, and continued to decrease from 0.5 to 1.5 h in the oil group. Mean testosterone concentrations in untreated birds decreased from 0.5 to 3 h, and testosterone increased to initial concentrations in both control groups from 3 to 6 h. Testosterone in the birds injected with 1.2 mg corticosterone remained low between 0.5 and 3 h, then increased from 3 h and did not differ from initial concentrations at 12 h. There was no difference between the groups in the total integrated testosterone response ($F_{2,20}$ = 1.126, p = 0.346; Figure 2.5b), and no differences between birds treated with 1.2 mg corticosterone and the untreated and oil groups (post-hoc Bonferroni test, p = 0.458 and p = 1.000 respectively). It was not possible to calculate a corrected integrated testosterone response for any of the groups as testosterone concentrations dropped below 0 h values during the course of the experiment.

Table 2.2 Two-way repeated measures ANOVA for testosterone concentrations in untreated male quail and in quail treated with oil or 1.2 mg corticosterone.

Effect		Plasma testosterone			
		F	*df*	*p*	
Treatment		1.866	2,12	0.183	
Time		30.781	5,90	< 0.001	**
Interaction of treatment and time		2.995	10,90	0.006	**
Comparisons within treatments for each treatment					
0 vs 0.5 h	Untreated	0.181	1,18	0.675	
	Oil	24.474	1,18	< 0.001	**
	Corticosterone	51.572	1,18	< 0.001	**
0.5 vs 1.5 h	Untreated	9.728	1,18	0.006	**
	Oil	10.761	1,18	0.004	**
	Corticosterone	2.322	1,18	0.145	
1.5 vs 3 h	Untreated	12.753	1,18	0.002	**
	Oil	0.216	1,18	0.647	
	Corticosterone	0.232	1,18	0.636	
3 vs 6 h	Untreated	20.093	1,18	< 0.001	**
	Oil	9.528	1,18	0.006	**
	Corticosterone	3.678	1,18	0.071	
6 vs 12 h	Untreated	0.766	1,18	0.393	
	Oil	0.022	1,18	0.884	
	Corticosterone	16.733	1,18	0.001	**
0 vs 3 h	Untreated	28.188	1,18	< 0.001	**
	Oil	19.983	1,18	< 0.001	**
	Corticosterone	25.340	1,18	< 0.001	**
0 vs 6 h	Untreated	0.011	1,18	0.917	
	Oil	0.530	1,18	0.476	
	Corticosterone	0.143	1,18	0.036	*
0 vs 12 h	Untreated	1.820	1,18	0.194	
	Oil	2.353	1,18	0.142	
	Corticosterone	2.712	1,18	0.105	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 2.2 cont Two-way repeated measures ANOVA for testosterone concentrations in untreated male quail and in quail treated with oil or 1.2 mg corticosterone.

		Plasma testosterone			
Comparisons between treatments for each time		*F*	*df*	*p*	
0 h	Untreated vs Oil	0.124	1,18	0.729	
	Untreated vs Corticosterone	1.286	1,18	0.272	
	Oil vs Corticosterone	0.611	1,18	0.445	
0.5 h	Untreated vs Oil	2.680	1,18	0.119	
	Untreated vs Corticosterone	9.834	1,18	0.006	**
	Oil vs Corticosterone	1.246	1,18	0.151	
1.5 h	Untreated vs Oil	3.475	1,18	0.079	
	Untreated vs Corticosterone	7.422	1,18	0.014	*
	Oil vs Corticosterone	0.740	1,18	0.401	
3 h	Untreated vs Oil	0.003	1,18	0.956	
	Untreated vs Corticosterone	0.929	1,18	0.348	
	Oil vs Corticosterone	1.039	1,18	0.321	
6 h	Untreated vs Oil	0.377	1,18	0.547	
	Untreated vs Corticosterone	4.233	1,18	0.054	
	Oil vs Corticosterone	2.084	1,18	0.166	
12 h	Untreated vs Oil	2.537	1,18	0.129	
	Untreated vs Corticosterone	0.338	1,18	0.568	
	Oil vs Corticosterone	1.023	1,18	0.325	

2.3.1.4 Plasma LH

LH concentrations remained relatively constant for the duration of the experiment in all birds (Figure 2.2). LH did not vary between the treatments or between time, and there was no significant interaction between treatment and time (see Table 2.3 for all statistics for this analysis). Initial plasma LH concentrations were low and ranged from 0.49 – 1.67 ng/ml. LH concentrations in the untreated control group were higher than those of the other groups at 0 and 0.5 h, but not thereafter. There was no significant difference in the total integrated LH response between the groups ($F_{2,20}$ = 1.023, p = 0.379; Figure 2.5c). It was not possible to calculate a corrected integrated testosterone response for any of the groups as LH concentrations dropped below 0 h values during the course of the experiment.

Table 2.3 Two-way repeated measures ANOVA for LH concentrations in untreated male quail and in quail treated with oil or 1.2 mg corticosterone.

		Plasma LH			
Effect		*F*	*df*	*p*	
Treatment		2.297	2,18	0.129	
Time		1.001	5,90	0.406	
Interaction of treatment and time		1.091	10,90	0.380	
Comparisons within treatments for each treatment					
0 vs 0.5 h	Untreated	0.288	1,18	0.598	
	Oil	0.737	1,18	0.402	
	Corticosterone	1.004	1,18	0.330	
0.5 vs 1.5 h	Untreated	6.327	1,18	0.022	*
	Oil	2.278	1,18	0.149	
	Corticosterone	5.355	1,18	0.033	*
1.5 vs 3 h	Untreated	0.001	1,18	0.981	
	Oil	0.889	1,18	0.358	
	Corticosterone	1.742	1,18	0.203	
3 vs 6 h	Untreated	1.386	1,18	0.254	
	Oil	0.175	1,18	0.680	
	Corticosterone	3.451	1,18	0.080	
6 vs 12 h	Untreated	0.365	1,18	0.553	
	Oil	3.049	1,18	0.098	
	Corticosterone	0.001	1,18	0.979	
0 vs 6 h	Untreated	0.038	1,18	0.847	
	Oil	1.211	1,18	0.286	
	Corticosterone	0.343	1,18	0.565	
0 vs 12 h	Untreated	0.350	1,18	0.562	
	Oil	1.440	1,18	0.246	
	Corticosterone	0.350	1,18	0.562	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 2.3 cont Two-way repeated measures ANOVA for LH concentrations in untreated male quail and in quail treated with oil or 1.2 mg corticosterone.

Comparisons between treatments for each time		Plasma LH			
		F	*df*	*p*	
0 h	Untreated vs Oil	6.121	1,18	0.024	*
	Untreated vs Corticosterone	4.539	1,18	0.047	*
	Oil vs Corticosterone	0.118	1,18	0.735	
0.5 h	Untreated vs Oil	10.350	1,18	0.005	**
	Untreated vs Corticosterone	8.859	1,18	0.008	**
	Oil vs Corticosterone	0.058	1,18	0.812	
1.5 h	Untreated vs Oil	1.780	1,18	0.199	
	Untreated vs Corticosterone	0.634	1,18	0.436	
	Oil vs Corticosterone	0.289	1,18	0.597	
3 h	Untreated vs Oil	0.181	1,18	0.676	
	Untreated vs Corticosterone	2.170	1,18	0.158	
	Oil vs Corticosterone	1.098	1,18	0.309	
6 h	Untreated vs Oil	0.492	1,18	0.492	
	Untreated vs Corticosterone	0.763	1,18	0.394	
	Oil vs Corticosterone	0.030	1,18	0.865	
12 h	Untreated vs Oil	2.832	1,18	0.110	
	Untreated vs Corticosterone	0.072	1,18	0.791	
	Oil vs Corticosterone	2.000	1,18	0.174	

2.3.2 Experiment 2 - Acute response to a single subcutaneous corticosterone injection in 8 week old female Japanese quail

2.3.2.1 Gross morphology

The seven week old female quail weighed 204.7 – 277.6 g (mean 236.94 ± 4.72 g; Figure 2.6a) when collected, with no differences between the two randomly assigned treatment groups ($F_{1,19}$ = 0.012, p = 0.914) and no change ($F_{1,18}$ = 0.452, p = 0.510) in body weight in the two groups over the course of the experiment where body weights were 207.5 – 280.7 g (mean 238.3 ± 4.33 g).

All females had a palpable egg in the oviduct when they were collected from the supplier and continued to lay up to one egg a day until the end of the experiment. Ovary weight did not differ between the groups one day after the treatment (8.38 ± 2.65 in oil treated compared with 8.75 ± 2.77 g in corticosterone treated;

$F_{1,19}$ = 0.213, p = 0.650; Figure 2.6b). All ovaries had normal yellow follicular hierarchies and were the same size as those of adult birds held on long days.

2.3.2.2 Plasma corticosterone

Corticosterone declined after the first sample in 7 out of 10 birds treated with oil and then generally increased between 0.5 and 3 h (Figure 2.7). Corticosterone rose to a peak (75.84 – 229.91 ng/ml) at 0.5 h in all birds injected with corticosterone, declined until 12 h after the injection and then remained constant.

Corticosterone varied between treatment and between time, with a significant interaction between treatment and time (see Table 2.4 for all statistics for this analysis). Corticosterone concentrations were initially low in both groups of birds (Figure 2.8a). Mean corticosterone concentrations in the oil treated birds decreased 0.5 h after the initial sample, then increased and remained elevated from 1.5 to 6 h before decreasing and remaining constant and low after 12 h. Corticosterone in the birds injected with 1.2 mg corticosterone increased to high concentrations at 0.5 h (158.69 ± 9.63 ng/ml) and then declined to initial concentrations at 24 h. Corticosterone concentrations at 12 h were significantly higher than initial concentrations, but did not differ at 24 h. Birds treated with 1.2 mg corticosterone had a significantly greater total integrated corticosterone response than birds in the oil treated group ($F_{1,19}$ = 52.673, p < 0.001; Figure 2.9a). It was not possible to calculate a corrected integrated corticosterone response for the oil treated group as corticosterone concentrations dropped below 0 h values during the course of the experiment.

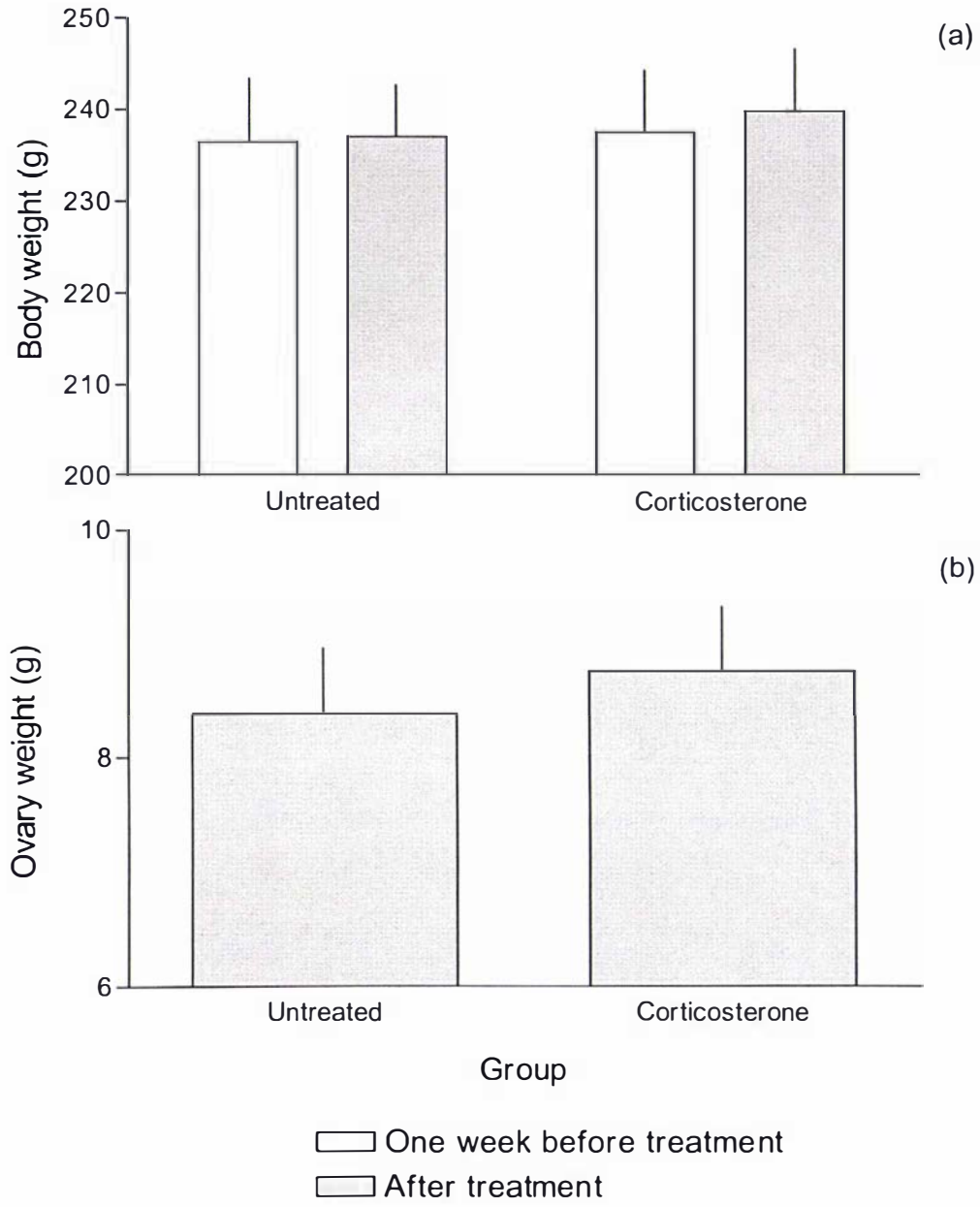


Figure 2.6 Body weight (a) and ovary weight (b) in untreated female quail and in quail treated with 1.2 mg corticosterone. Results are plotted as mean ± standard error.

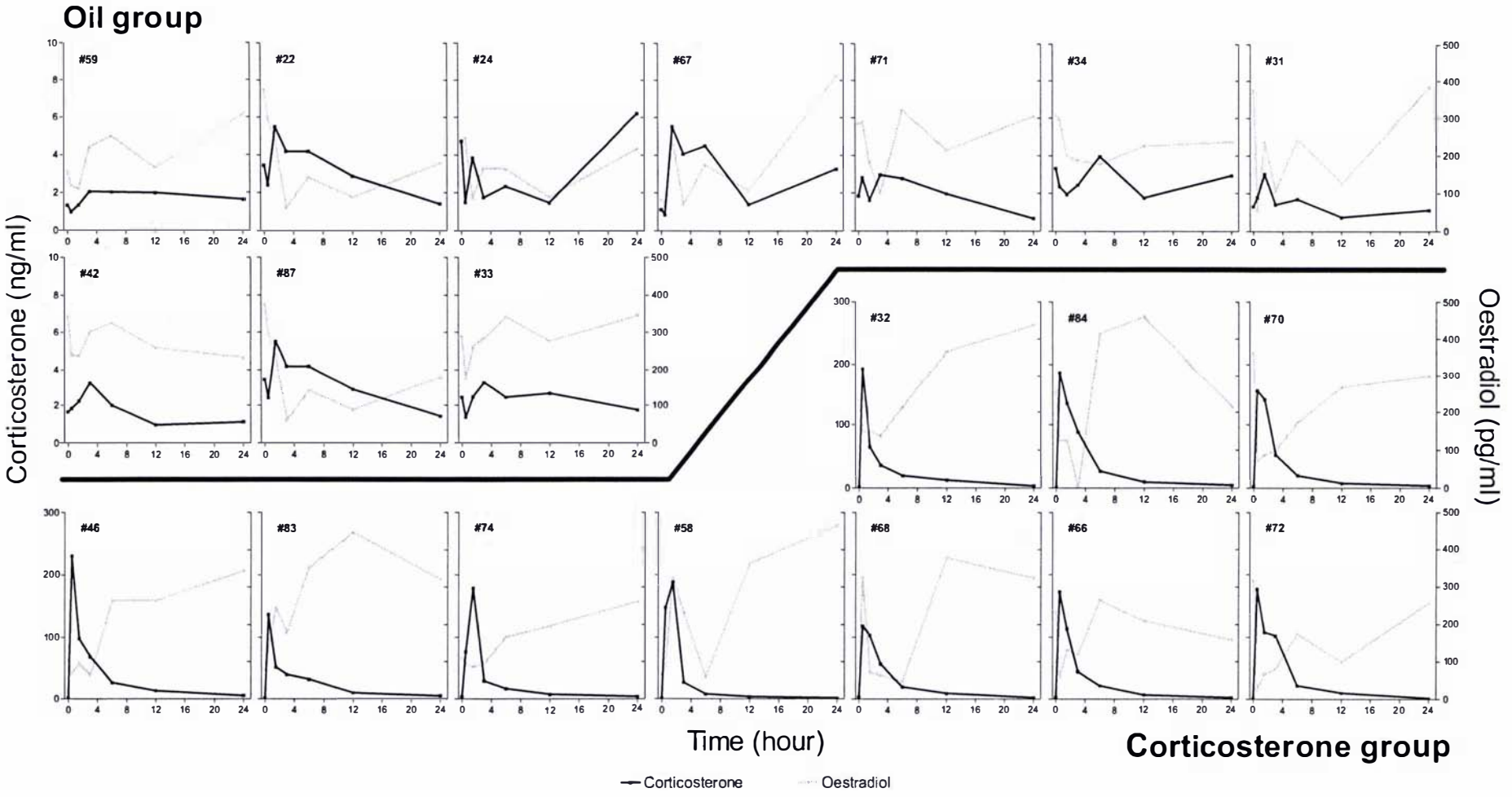


Figure 2.7 Plasma corticosterone and oestradiol responses in female quail treated with oil or 1.2 mg corticosterone. Note that the scale for the corticosterone axis differs between treatments. Results are plotted as raw data.

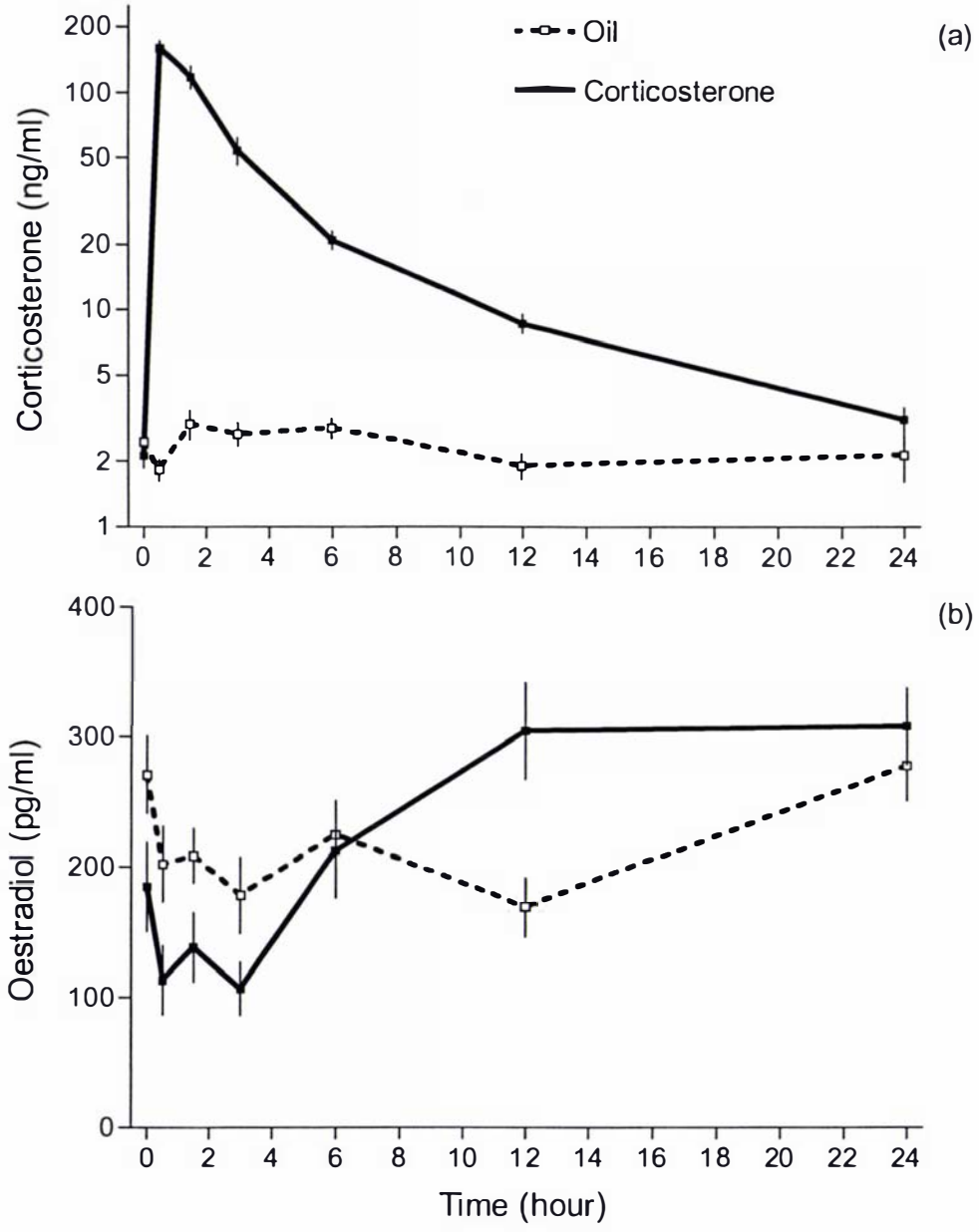


Figure 2.8 Plasma corticosterone (a), oestradiol (b) in female quail treated with oil or 1.2 mg corticosterone. Corticosterone concentration is plotted on a $\log_{10}$ axis. Results are plotted as mean ± standard error.

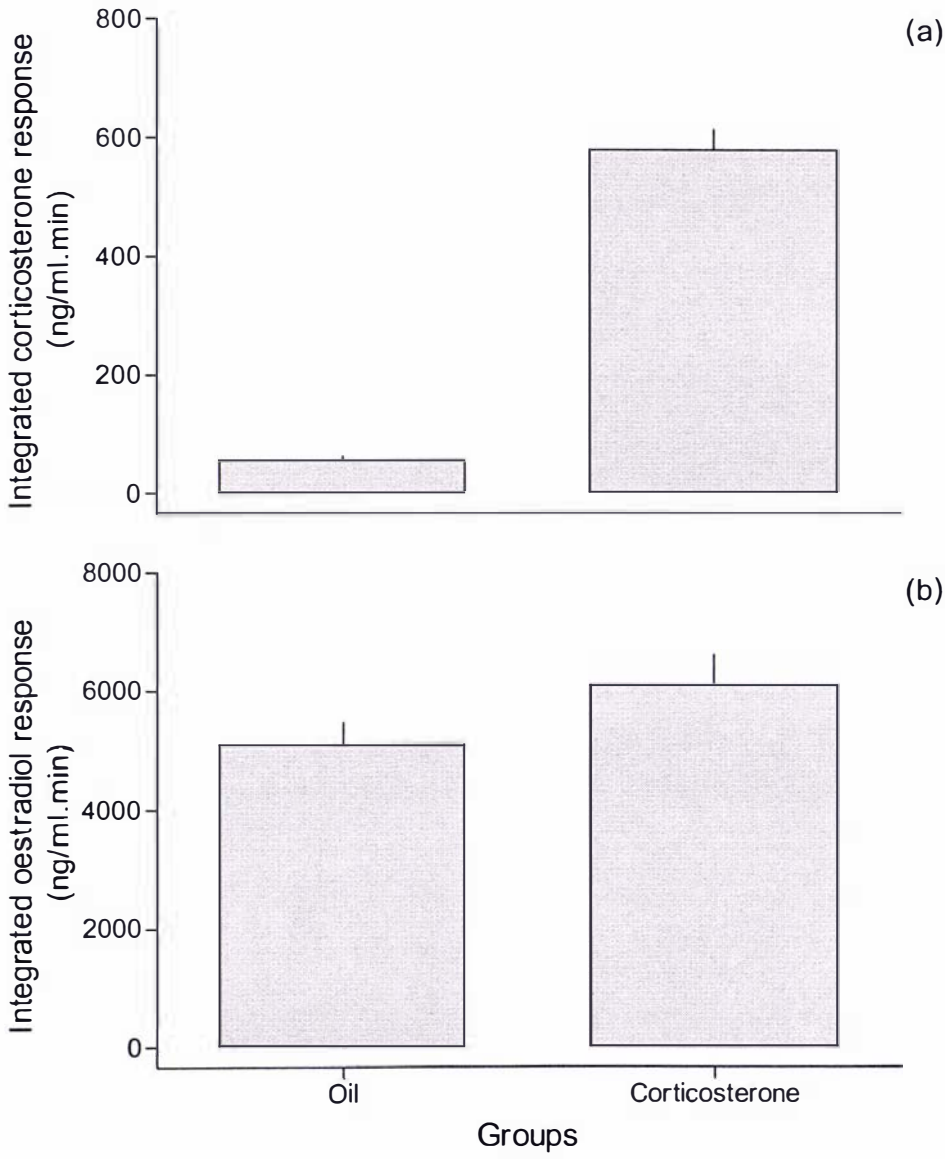


Figure 2.9 Total integrated responses for corticosterone (a) and oestradiol (b) in female quail treated with oil or 1.2 mg corticosterone. Results are plotted as mean ± standard error.

Table 2.4 Two-way repeated measures ANOVA for corticosterone concentrations in quail treated with oil or 1.2 mg corticosterone.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Treatment		483.720	1,18	< 0.001	**
Time		95.208	6,108	< 0.001	**
Interaction of treatment and time		83.501	6,108	< 0.001	**
Comparisons within treatments for each treatment					
0 vs 0.5 h	Oil	2.213	1,18	0.154	
	Corticosterone	641.039	1,18	< 0.001	**
0.5 vs 1.5 h	Oil	5.149	1,18	0.036	*
	Corticosterone	2.723	1,18	0.116	
1.5 vs 3 h	Oil	0.115	1,18	0.698	
	Corticosterone	19.348	1,18	< 0.001	**
3 vs 6 h	Oil	0.490	1,18	0.493	
	Corticosterone	66.776	1,18	< 0.001	**
6 vs 12 h	Oil	15.331	1,18	0.001	**
	Corticosterone	59.141	1,18	< 0.001	**
12 vs 24 h	Oil	0.002	1,18	0.965	
	Corticosterone	26.378	1,18	< 0.001	**
0 vs 12 h	Oil	2.292	1,18	0.147	
	Corticosterone	79.646	1,18	< 0.001	**
0 vs 24 h	Oil	1.363	1,18	0.258	
	Corticosterone	3.252	1,18	0.088	
Comparisons between treatments for each time		*F*	*df*	*p*	
Oil vs corticosterone					
	0 h	0.284	1,18	0.600	
	0.5 h	785.190	1,18	< 0.001	**
	1.5 h	349.392	1,18	< 0.001	**
	3 h	247.606	1,18	< 0.001	**
	6 h	153.785	1,18	< 0.001	**
	12 h	64.554	1,18	< 0.001	**
	24 h	3.602	1,18	0.074	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

2.3.2.3 Plasma oestradiol

Oestradiol declined after the first sample in 9 out of 10 birds treated with oil, and 7 out of 10 birds treated with 1.2 mg corticosterone (Figure 2.7). Oestradiol increased between 3 and 24 h in all corticosterone treated birds, while there was no consistent pattern in oestradiol concentrations after 0.5 h in the oil treated group.

Oestradiol varied in time but not between treatment, and there was a significant interaction between treatment and time (see Table 2.5 for all statistics for this analysis). Oestradiol concentrations were initially lower in the group treated with corticosterone (Figure 2.8b). Mean oestradiol concentrations in birds given an oil injection declined between 0 and 3 h, but did not differ from initial oestradiol concentrations after 6, 12 and 24 h. Mean oestradiol concentrations in birds treated with 1.2 mg corticosterone remained relatively constant between 0.5 and 3 h, rose significantly from 3 to 12 h and remained constant and high thereafter. Concentrations at 12 and 24 h were significantly higher than initial oestradiol concentrations. There was no significant difference in the total integrated oestradiol response between the groups ($F_{1,19} = 2.498$, $p = 0.131$; Figure 2.9b). It was not possible to calculate a corrected integrated oestradiol response for either group as corticosterone concentrations dropped below 0 h values during the course of the experiment.

Table 2.5 Two-way repeated measures ANOVA for oestradiol concentrations in quail treated with oil or 1.2 mg corticosterone.

		Plasma oestradiol			
Effect		*F*	*df*	*p*	
Treatment		3.570	1,18	0.075	
Time		6.688	6,108	< 0.001	**
Interaction of treatment and time		3.714	6,108	0.008	**
Comparisons within treatments for each treatment					
0 vs 0.5 h	Oil	1.620	1,18	0.219	
	Corticosterone	3.492	1,18	0.078	
0.5 vs 1.5 h	Oil	0.234	1,18	0.634	
	Corticosterone	1.226	1,18	0.283	
1.5 vs 3 h	Oil	2.083	1,18	0.166	
	Corticosterone	0.286	1,18	0.600	
3 vs 6 h	Oil	2.007	1,18	0.174	
	Corticosterone	4.472	1,18	0.049	*
6 vs 12 h	Oil	2.190	1,18	0.156	
	Corticosterone	5.131	1,18	0.036	*
12 vs 24 h	Oil	12.812	1,18	0.002	**
	Corticosterone	0.127	1,18	0.726	
0 vs 6 h	Oil	0.691	1,18	0.417	
	Corticosterone	0.396	1,18	0.537	
0 vs 12 h	Oil	3.794	1,18	0.067	
	Corticosterone	5.928	1,18	0.026	*
0 vs 24 h	Oil	0.067	1,18	0.799	
	Corticosterone	7.099	1,18	0.016	*
Comparisons between treatments for each time		*F*	*df*	*p*	
Oil vs corticosterone					
	0 h	3.607	1,18	0.074	
	0.5 h	4.774	1,18	0.042	*
	1.5 h	5.552	1,18	0.030	*
	3 h	2.021	1,18	0.172	
	6 h	0.472	1,18	0.501	
	12 h	8.496	1,18	0.009	**
	24 h	0.535	1,18	0.474	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

2.4 Discussion

The administration of a single 1.2 mg corticosterone injection to male quail caused a rapid increase in plasma corticosterone concentrations. Plasma corticosterone remained elevated 3 hours later and had returned to initial concentrations after 6 hours. Testosterone concentrations declined over 3 hours in both treated and control birds then returned to initial concentrations after 6 hours in control birds and after 12 hours in treated birds. LH did not change in any of the groups. Oestradiol declined after treatment then returned to initial concentrations after 6 hours in oil and corticosterone treated females. Oestradiol in both groups of treated females returned to initial concentrations whilst corticosterone remained elevated.

2.4.1 Male quail

2.4.1.1 Corticosterone

In the current experiment corticosterone concentrations remained elevated for at least 3 hours whereas Boyd (2000) reported that plasma corticosterone in males was elevated for at least 6 hours after a single 1.2 mg corticosterone injection. Plasma corticosterone concentrations 3 hours after treatment ranged between 70 and 220 ng/ml in the Boyd (2000) study, while they were 15 to 54 ng/ml in the current experiment. The corticosterone was delivered as a suspension in oil with stirring necessary to maintain the suspension's uniformity (Boyd, 2000). The different corticosterone profiles in the two studies could be due, at least in part, to differences in the actual amount of corticosterone delivered. Alternatively, the two groups of birds may have differed in their rates of clearance of corticosterone.

The first blood sample after the corticosterone injection was collected at 0.5 hours in the present study, compared with 3 hours by Boyd (2000). Beuving and Vonder (1978) and Davison *et al.* (1980) showed that plasma corticosterone increased 3.5 minutes after an injection of ACTH or corticosterone in chickens. Corticosterone in quail was elevated 0.5 hours after the injection, and it is likely that sampling closer to the injection time would reveal an earlier and higher corticosterone peak than observed in this study.

Despite all birds being similar in weight and receiving the same amount of corticosterone, there were a range of peak concentrations measured at 30 minutes. A number of factors are likely to have influenced and caused this variation between the birds such as, body weight, metabolic rate, the amount of corticosterone delivered, and the concentration of corticosterone binding globulin (CBG). All are factors that contribute to different rates of corticosterone clearance from the plasma.

Plasma corticosterone remained low in both control groups and there was no pattern of secretion during the experiment. Corticosterone secretion in quail follows a circadian rhythm, it remains low during hours of light, increases at the onset of darkness remaining elevated before decreasing at the onset of light (Boissin and Assenmacher, 1970). However, plasma corticosterone concentrations have also been reported to peak in late morning and then decline in other quail (Kovács and Péczely, 1983; Kovács *et al.*, 1983). In the current study, plasma corticosterone decreased in the few hours after the lights went on, and then remained relatively constant for the duration of the experiment. Corticosterone concentrations did not differ between the untreated and oil treated birds, showing the injection alone did not elicit a corticosterone response 0.5 hours later.

2.4.1.2 Testosterone

Mean testosterone concentrations in all groups decreased after 30 minutes and further decreased or remained low at 1.5 and 3 hours before rising at 6 hours. Testosterone concentrations in the corticosterone treatment group were lower between 0.5 and 3 hours, although this is attributed to this group having lower initial corticosterone concentrations. The results of the current study differ from Boyd (2000) who showed that testosterone concentrations decreased 3 hours after a 1.2 mg corticosterone injection, but did not change in oil treated quail. In contrast, Heiblum *et al.* (2000) showed testosterone concentrations increased within 30 minutes of an injection of ACTH in male chickens, which conflicts with previous studies showing declines in plasma testosterone during acute and chronic stressors (reviewed by Deviche, 1983). The decrease in testosterone concentration in the current experiment occurred over 1.5 – 3 hours and was unlikely to be circadian in nature. Ottinger and Brinkley (1979) showed that testosterone in quail is highest in

the early morning and slowly decreases over 9 hours to a minimum in the late afternoon before rapidly increasing in the evening.

Birds in the current study were bled at 0, 0.5 and 3 hours, whereas Boyd (2000) bled birds at 0 and 3 hours. The extra handling in the current study may have caused the decrease in plasma testosterone concentrations in all groups as seen in hens handled and blood sampled every 15 minutes (Wilson *et al.*, 1979). An effect of repeated handling and sampling by wing vein venipuncture on plasma testosterone has also been shown in other studies of chickens (Eskeland and Blom, 1979; Johnson, 1981). The authors of all three studies considered handling stress to be the cause of the decline in testosterone secretion without defining stress or considering a mechanism for stressors to inhibit testosterone secretion. A decrease in testosterone secretion following a handling stressor is said to be caused by an increase in corticosterone or cortisol with handling or restraint (Orr and Mann, 1990; Orr and Mann, 1992). Corticosterone in rats inhibits the conversion of 17α-OH progesterone to androstenedione, an androgen that is then converted into testosterone (Orr and Mann, 1990; Orr and Mann, 1992), and Deviche *et al.* (1982) showed that corticosterone injections in Japanese quail reduced the production of androstenedione which led to a decrease in testosterone. Satterlee *et al.* (2002) showed that genetically selecting quail for reduced corticosterone responses to mechanical restraint accelerated puberty. This was assessed by measuring the area and volume of the androgen-dependent cloacal gland, which was larger in birds with a low corticosterone response compared with quail with a high response.

The decline in testosterone in the control birds means that it is not possible to determine from this data whether corticosterone inhibited testosterone. However, a transient corticosterone increase due to handling might have contributed to the decline in testosterone in the control birds. The pattern of testosterone secretion varied considerably between birds. A few birds had low initial testosterone concentrations and testosterone remained low for the duration of the study. All of these birds had large testes, large cloacal glands and were producing cloacal foam.

2.4.1.3 LH

There was no change in plasma LH concentrations after birds were treated with corticosterone or in control birds, as found by Boyd (2000). Deviche *et al.* (1980) also showed that plasma LH and FSH remained unchanged whilst testosterone decreased in male ducks given a corticosterone injection. Handling did not affect LH in the present study, although Wilson and Sharp (1975) found that plasma LH decreased in hens blood sampling at 10 and 15 minute intervals.

The prolonged elevation of plasma corticosterone using osmotic pumps has been shown to cause ovarian regression and reduce plasma LH and oestradiol concentrations (Etches *et al.*, 1984; Williams *et al.*, 1985; Petitte and Etches, 1989). The decreases in plasma LH and oestradiol could be reversed by the administration of GnRH and PMSG (pregnant mare serum gonadotrophin) respectively (Etches *et al.*, 1984; Petitte and Etches, 1989). The responsiveness of the pituitary and gonad to exogenous GnRH and a gonadotrophin show that prolonged elevation of corticosterone acts in some part at the hypothalamus and pituitary gland to inhibit the reproductive axis in chickens. Corticosterone can inhibit LH secretion from quail pituitary cells in culture (Connolly and Callard, 1987). However, corticosterone has many influences in the avian reproductive system and injections can either cause a decrease or increase in plasma LH concentrations in laying hens depending on the timing of the injection within the ovulatory cycle (Etches and Croze, 1983).

Elevated corticosterone in the treated birds may have altered the pulsatile secretion of LH without a change in mean LH concentrations. LH secretion is pulsatile in the male Japanese quail with 6 – 10 pulses per day (Gledhill and Follett, 1976) that are 90 – 120 minutes in duration (Wilson and Sharp, 1975). Acute changes in cortisol and ACTH in mammals can decrease LH pulse frequency without affecting mean LH concentrations (Daley *et al.*, 1999; Van Lier *et al.*, 1999; Smith and Dobson, 2002), and this could also occur in birds.

The secretion of LH from the pituitary is in part controlled by negative feedback by testosterone (Gibson *et al.*, 1975; Davies *et al.*, 1976; Deviche *et al.*, 1979; Deviche

et al., 1980; Knight *et al.*, 1983). The permanent depression of testosterone following gonadectomy results in an increase in LH secretion in birds (Gibson *et al.*, 1975; Wilson and Sharp, 1975; Deviche *et al.*, 1980). The decline in testosterone concentration in both the current study and that of Boyd (2000) did not however lead to an increase in LH secretion.

2.4.2 Female quail

2.4.2.1 Corticosterone

Administration of a 1.2 mg corticosterone injection to female Japanese quail resulted in a significant increase in corticosterone concentrations after 30 minutes followed by a gradual decline, with corticosterone still elevated 12 hours after the injection. The period of elevated corticosterone was longer than in the male quail in the previous experiment and found by Boyd (2000). However, the current study was conducted after the male treatment study and can not be directly compared. Differences in peak corticosterone concentrations between the sexes are likely to be due to slight differences in treatment, and the fact that the corticosterone doses for either sex were identical can not be assumed.

2.4.2.2 Oestradiol

Plasma oestradiol concentrations gradually decreased in both oil and corticosterone-treatment birds after the injection, then increased after 3 hours. As was the case in the male study, it is likely that the handling associated with blood sampling caused this decreased, where oestradiol concentrations are lowest in both groups after 3 hours. A decrease in oestradiol following a pre-ovulatary surge may also be a possible cause of the decline in oestradiol concentrations during the first 1.5 hours after the injection. Oviposition occurred between 1400 – 1900 in all females while ovulation occurs within 15–30 minutes (Woodard and Mather, 1964). However, this cause is unlikely given that the time of oviposition varied greatly between females while the decrease in oestradiol after the injection was so synchronous within and between both groups.

The role of corticosterone in the ovulatory cycle in female birds makes studying the inhibitory effects of an acute increase in corticosterone difficult as the effect of corticosterone can be stimulatory or inhibitory to the reproductive axis depending on the stage of the ovulatory cycle that an injection is administered (Etches and Croze, 1983). In contrast, an increase in corticosterone concentrations by 1 – 2 ng/ml for 14 days in chickens implanted with osmotic pumps resulted in a decrease in plasma oestradiol and LH, and a cessation in lay (Etches *et al.*, 1984). The same dose of corticosterone administered for 14 days in immature hens prevented the increase in plasma LH and oestradiol that follow photostimulation (Petitte and Etches, 1988).

2.4.3 Summary

The aim of this study was to determine the effects of an acute increase in corticosterone on testosterone and LH in male and oestradiol in female quail. The steroids decreased in corticosterone-treated birds but also in birds that only received saline, whereas LH did not change. Gonadotrophin secretion (Etches *et al.*, 1984; Williams *et al.*, 1985; Petitte and Etches, 1989) has been previously shown to decrease in response to corticosterone treatment, although in all such cases corticosterone was delivered constantly over a period of days. Prolonged exposure to stressors over hours and days can have negative effect on breeding behaviours and the reproductive axis (Wingfield, 1994). Factors such as the species of bird, latitude, and type of stressor influence how the different levels of the reproductive axis respond to stressors, as reported in wild passerines (Astheimer *et al.*, 2000). Astheimer *et al.* (2000) proposed that the reproductive axis can become unresponsive to the inhibitory effects of stressors in Arctic birds so that breeding is not disrupted in species with a short period available for breeding. The natural breeding season of Japanese quail in their original range is not known, but the absence of an LH response to a stressor in quail could indicate the presence of a similar mechanism to that proposed for Arctic birds.

The aim of the study was not met because sex steroids fell in control as well as corticosterone-treated birds. This effect of a single handling event was unexpected, as Boyd (2000) showed no effect of handling on testosterone after 3 hours, and

testosterone in chickens declined only when blood sampling was repeated at < 5 minute intervals (Wilson *et al.*, 1979; Eskeland and Blom, 1979; Johnson, 1981).

An alternative method for the delivery of corticosterone to quail is the use of jugular cannulae. This method would allow hormone solution to be delivered and blood samples withdrawn remotely, thereby avoiding any effect of handling. However, cannulation is used only in larger birds such as chickens and turkeys and was not considered feasible for quail in our laboratory. Instead, the novel finding that a single handling event would reduce sex steroids was explored in detail in another study. The effects on corticosterone and testosterone of different periods of handling and of exposure to other stressors were quantified to determine relationships between corticosterone and testosterone responses.

Chapter 3

Adrenocortical and testosterone responses of Japanese quail to a range of novel stimuli

3.1 Introduction

Exposure to a stressor can have a negative effect on reproduction in vertebrates (reviewed by Rivier and Rivest, 1991; Tilbrook *et al.*, 2000; Tilbrook *et al.*, 2002; Wingfield and Sapolsky, 2003). In the previous chapter, repeated blood sampling in Japanese quail was shown to cause a decrease in plasma testosterone without affecting LH secretion. The decline in plasma testosterone following serial blood sampling has been well documented in chickens (Wilson *et al.*, 1979; Eskeland and Blom, 1979; Johnson, 1981). The duration of manual restraint or other stressors needed to initiate a corticosterone response and decline in testosterone secretion had not been reported in quail and was therefore determined in the study described in this chapter.

The corticosterone response to handling or mechanical restraint has been widely studied in a wide range of species such as quail (Boyd, 2000), chickens (Beuving and Vonder, 1978; Littin and Cockrem, 2001) and a variety of other free-living, wild species (Silverin, 1998). Handling can cause an increase in corticosterone concentrations within two minutes in chickens (Beuving and Vonder, 1978) and one minute in ducks (Harvey *et al.*, 1980), and corticosterone increases within one minute of restraint in quail (Satterlee and Johnson, 1988). Plasma corticosterone has been shown to increase 45 seconds after the start of a stressor and increase rapidly for the first 10 minutes in chickens restrained for 60 minutes (Beuving and Vonder,

1978). In Japanese quail, corticosterone increased to a peak four minutes after the start of restraint and remained relatively constant during 20 minutes of mechanical restraint (Satterlee and Johnson, 1988). However, the relationship between the length of the handling procedure and changes in plasma corticosterone have not been quantified in the quail. In the first part of the study the relationships between the duration of manual restraint, corticosterone and testosterone were determined. As testosterone was affected by the blood sampling procedure used in the previous chapter, separate groups of birds were sampled at each collection time, as described by Boyd (2000).

Another factor that can influence the size of the corticosterone response is the type of stressor. Handling (Boyd, 2000), mechanical restraint (Satterlee and Johnson, 1988), or social disruption (Satterlee *et al.*, 1983) can initiate corticosterone responses in Japanese quail whereas testosterone responses to different stressors in Japanese quail have not been described. In the second part of the present study corticosterone and testosterone responses to manual restraint, mechanical restraint, and social stimuli were characterised. In this study, as in those described by Satterlee and Johnson (1988), mechanical restraint covers a series of events in which quail were captured from their home cage, transported to the mechanical restraint area, blood sampled, then mechanically restrained before being returned to their cage.

3.2 Methods and materials

3.2.1 Animals and housing

3.2.1.1 Source and type of birds

Seven week-old male Japanese quail (*Coturnix coturnix japonica*) were purchased from our supplier (Rangitikei Game Birds Ltd., Bulls). The birds had been raised under a long-day photoperiod (15L:9D) at air temperatures of 20 to 25°C in mixed sex groups. The 7 week-old birds were sexually mature at time of purchase with all birds having large cloacal glands and cloacal foam present.

3.2.1.2 Housing conditions

Each bird was identified with a numbered leg band and housed in an individual cage measuring 20 cm (W) × 35 cm (D) × 24 cm (H) in animal rooms in the Veterinary Science building. Quail were held on a long day photoperiod (16L:8D; lights on from 0900 – 0100 h). An extractor fan provided ventilation for each room, and only one sex was held in a room during each experiment. A temperature control unit enabled air temperature to be maintained at 20°C. Light in each room was provided by two 75W incandescent light bulbs controlled by a 24 hour/7 day time switch (HPM Excel Light Switch and Timer, Cat XL770T). The light intensity in the quail rooms was 130 lux in cages at the top, and 15 lux in cages near the floor of the room (Minolta Illuminance meter).

3.2.1.3 Food and water

Quail were provided with fresh water and food (Harvey Farms quail layer mash) *ab libitum*. The food was the same as that used by Rangitikei Game Birds.

3.2.2 Experimental design

3.2.2.1 Experiment 1 – Quantification of the effect of manual restraint on plasma testosterone and corticosterone in the Japanese quail

Seventy 7 week old male Japanese quail were held with *ad libitum* access to food and water under long days (16L:8D) at 20°C for 10 days. The birds were assigned into 2 groups; birds in one group (n=35) were bled at time 0 and handled for approximately 2 minutes (called 'untreated group'), the time taken to collect a single blood sample. Birds in the second group (n=35) were bled at time 0 and manually restrained until 5 min total handling time had elapsed from when they were first removed from their cages, birds were returned to their cage after this time had past. Manual restraint consisted of a bird being held in one hand by the experimenter. Birds were removed from their cages and taken to another room where they were held firmly to prevent movement. Blood samples were collected from each group of birds (n=7) at 15, 30, 60, 120, or 240 min after the time 0 sample. Birds were returned to their cages between samples.

Individual sample groups for each sample time were required to provide a clear picture of what effect manual restraint has on plasma testosterone (as we had previously shown in Chapter 3) as repeated blood sampling in individual birds decreases plasma testosterone.

Both groups of birds were housed for another 10 days under the same conditions. After 10 days, the blood sampling procedure described above was repeated with manual restraint for 10 or 15 minutes in the place of 2 and 5 min. Birds were euthanased by stunning and decapitation at the final sample time (240 min) so that the testes could be weighed and a terminal blood sample collected. Body weight and the area of the cloacal protuberance were measured before the treatment. Plasma concentrations of corticosterone and testosterone were measured in all blood samples.

3.2.2.2 Experiment 2 – Adrenocortical and testosterone responses to manual restraint, mechanical restraint and social stimuli in Japanese quail

3.2.2.2.1 The effect of different stressors on the adrenocortical response

Fifty 7 week old male Japanese quail were obtained from our normal supplier (Rangitikei Game Birds) and held with *ad libitum* access to food and water under long days (16L:8D) at 20°C for 10 days. All quail were housed in individual cages.

After 10 days, the quail were assigned into five groups (n=10 per group). Birds in each group were given one of the following treatments, (a) 2 min of manual restraint by an experimenter (also called 'untreated group'), (b) 15 min of manual restraint by the same experimenter in the previous group, (c) 15 min of mechanical restraint that prevented movement, (d) 15 min in a cage with an unfamiliar quail, or (e) 15 min in a cage with an unfamiliar quail with birds separated by a wire mesh partition. Blood samples were collected from each bird at 0, 15, 30, 60, 120, and 240 minutes. Birds were returned to their cages between samples. Plasma corticosterone concentrations were subsequently measured by radioimmunoassay.

3.2.2.2.2 The effects of manual and mechanical restraint on testosterone and corticosterone

In the previous experiment (Section 3.2.2.2.1) 15 minutes of mechanical restraint caused a smaller and more variable corticosterone response than 15 minutes of manual restraint. The goal of the previous study was to identify a stressor that stimulated a similar but smaller response to that caused by manual restraint.

All 50 birds from the previous study were housed for another 10 days under the same conditions then assigned into five groups (n=10). All birds were blood sampled at time 0 prior to the treatment, and then each group was sampled once more at 15, 30, 60, 120 or 240 minutes. Half of the birds received 15 min of manual restraint (n=25) while the other half received 15 min of mechanical restraint. This protocol was repeated 10 days later to increase the sample size to 10 for each group. Thirty five birds were held for another 10 days, then assigned into five groups (n=7) and blood sampled at time 0 then each group was sampled once more at 15, 30, 60, 120 or 240 minutes without manual or mechanical restraint. These birds were untreated controls. Birds were euthanased at the end of the study, and terminal blood samples collected. Plasma concentrations of corticosterone and testosterone were measured in all blood samples.

The experiments were conducted under a protocol approved by the Massey University Animal Ethics Committee.

3.2.3 Data collection

3.2.3.1 Administration of stressors

3.2.3.1.1 Manual restraint

Birds were removed from their cage and the 0 min blood sample collected within 2 min. The 2 min manual restraint group were then returned to their cage. Birds in the other groups were then held by a handler for up to 5, 10 or 15 min of manual restraint then returned to their cages.

3.2.3.1.2 Mechanical restraint

Individual quail were placed into a firm well ventilated plastic bag that prevented the bird from flapping its wings but still allowed the bird free movement of its head outside the bag. The bird was then placed inside a metal cage 6 cm (W) × 15 cm (D) × 9 cm (H) that was larger than the quail. Quail were left in an upright dorso-ventral position for 15 min.

3.2.3.1.3 Exposure to an unfamiliar quail

Birds were removed from their cage and a blood sample collected. The bird was then returned to it's home cage which measured 20 cm (W) × 35 cm (D) × 24 cm (H). A quail that had not been housed nearby was then either (a) introduced into the adjacent cage with a wire mesh partition physically separating them or (b) introduced into the cage with the quail that had been blood sampled. In both treatments, birds were exposed to each other for up to 15 min after the initial blood sample and the stocking density remained 1 bird / 0.07 m^2. After the 15 min, the unfamiliar quail was removed and returned to it's home cage.

3.2.3.2 Tissue and blood samples

Blood samples were collected by venipuncture of the brachial vein in the wing with a 25 g needle, with 200 μl of blood collected into heparinised capillary tubes. All samples were collected within 2 minutes from the time the bird was removed from the cage. The final blood sample was collected when the birds were euthanased by stunning followed by decapitation. The blood was stored on ice until centrifuged at 2 000 g for 15 minutes at 4°C (Heraeus Christ 5000S refrigerated centrifuge). The plasma was removed and stored at -20°C until assayed. The width and height of the cloacal protuberance was measured (±0.1 mm) in males and body weight was measured (±0.1 g) in all birds on the day before the experimental treatment. The reproductive system was removed after euthanasia and the testes weighed (±0.001 g).

3.2.4 Hormone assays

Corticosterone and testosterone concentrations in plasma were determined by radioimmunoassay.

3.2.4.1 Corticosterone

Plasma corticosterone concentrations were determined by radioimmunoassay using the methodology outlined in Section 2.2.4.1. The inter-assay coefficients of variation for 9 assays were 9.7, 10.3, and 7.1% for high, medium and low quality controls respectively.

3.2.4.2 Testosterone

Plasma testosterone concentrations were determined by radioimmunoassay using the methodology outlined in Section 2.2.4.2. The inter-assay coefficients of variation for three assays were 9.1., 6.1, and 5.7% for high, medium and low quality controls respectively.

3.2.5 Calculation of the area under the corticosterone curve

The area under the curve for plasma corticosterone concentrations versus time was calculated for each bird that was repeat sampled, using the method described in Chapter 2.

3.2.6 Statistical analyses

All results are presented as the mean ± SE (standard error) or individual data where appropriate. All the data were examined for normality using Shapiro-Wilks tests and for equality of variance using Levene's homogeneity of variance test. Hormone data were $\log_{10}$ transformed to avoid heteroscedasticity.

If variances were homogenous then groups were compared with repeated measures two-way analyses of variance (ANOVA) with time and treatment as the grouping factors. Comparisons between times within each treatment and between treatments for each time were examined with post-hoc repeated measures contrasts. Data not complying with normality or variance criteria were compared

using a Kruskal-Wallis non-parametric test followed by Mann-Whitney U independent group comparisons tests, or Friedman's test for repeat measures data. All comparisons of means were conducting using SPSS™ 11.0 (SPSS Inc., Illinois) or Systat™ 8.0 (SPSS Inc., Illinois). Graphs were generated using GraphPad Prism 4.0 (GraphPad Software Inc.) and SigmaPlot 2001 (SPSS Inc., Illinois).

3.3 Results

3.3.1 Experiment 1 – Quantification of the effect of manual restraint on plasma testosterone and corticosterone in the Japanese quail

3.3.1.1 Corticosterone

Corticosterone varied between treatments and between times, with a significant interaction between treatment and time (see Table 3.1 for all statistics for this analysis).

Initial corticosterone concentrations were low in all four groups of birds, with differences between groups ($F_{3,136}$ = 5.752, p = 0.001; Figures 3.1a). Corticosterone remained low in untreated birds manually restrained for the minimum 2 min (Figures 3.1a). Plasma corticosterone in birds manually restrained for 5 min increased after 15 min, then decreased to similar concentrations to untreated birds by 30 min and thereafter. Corticosterone rose to a peak at 15 min in birds manually restrained for 10 min (6.84 – 18.13 ng/ml) or 15 min (9.23 – 17.78 ng/ml) and did not differ significantly from each other. Corticosterone decreased between 15 and 30 min in birds manually restrained for 10 min, and did not differ from initial concentrations after 30 min and was similar to untreated birds thereafter. Plasma corticosterone decreased between 15 and 30 min in birds manually restrained for 15 min and was still elevated at 30 min. Corticosterone continued to decrease and was similar to initial concentrations at 60 min, although it subsequently increased between 60 and 120 min before remaining constant. Overall, plasma corticosterone concentrations at 15 min appeared to be related to the duration of manual restraint.

3.3.1.2 Testosterone

Testosterone did not vary between treatments and there was no interaction between treatment and time, although there was an effect of time (see Table 3.2 for all statistics for this analysis). Testosterone concentrations were high and did not differ between the groups at the beginning of the experiment ($F_{3,136}$ = 0.521, p = 0.668; Figure 3.1b). Testosterone remained relatively constant in untreated birds. Plasma testosterone concentrations in birds manually restrained for 5 min decreased between 30 and 60 min, then returned to initial concentrations by 120 min. Testosterone remained unchanged between 0 and 30 min in birds manually restrained for 10 min then decreased after 30 min, was significantly lower than initial concentrations after 60 min then increased between 60 and 120 min and remained relatively constant. Testosterone initially (0-30 min) remained unchanged in birds manually restrained for 15 min, then dramatically decreased between 30 and 60 min and were significantly less than initial concentrations. Testosterone then increased back to pretreatment concentrations between 60 and 120 min and did not differ from untreated birds or initial testostosterone concentrations. Overall, the magnitude of the decrease of plasma testosterone concentrations by 60 min appeared to be related to the duration of manual restraint.

Mean plasma testosterone concentrations were significantly lower at 60 min in birds manually restrained for 15 min than in any other birds. Testosterone concentrations did not differ between treatments at any other time.

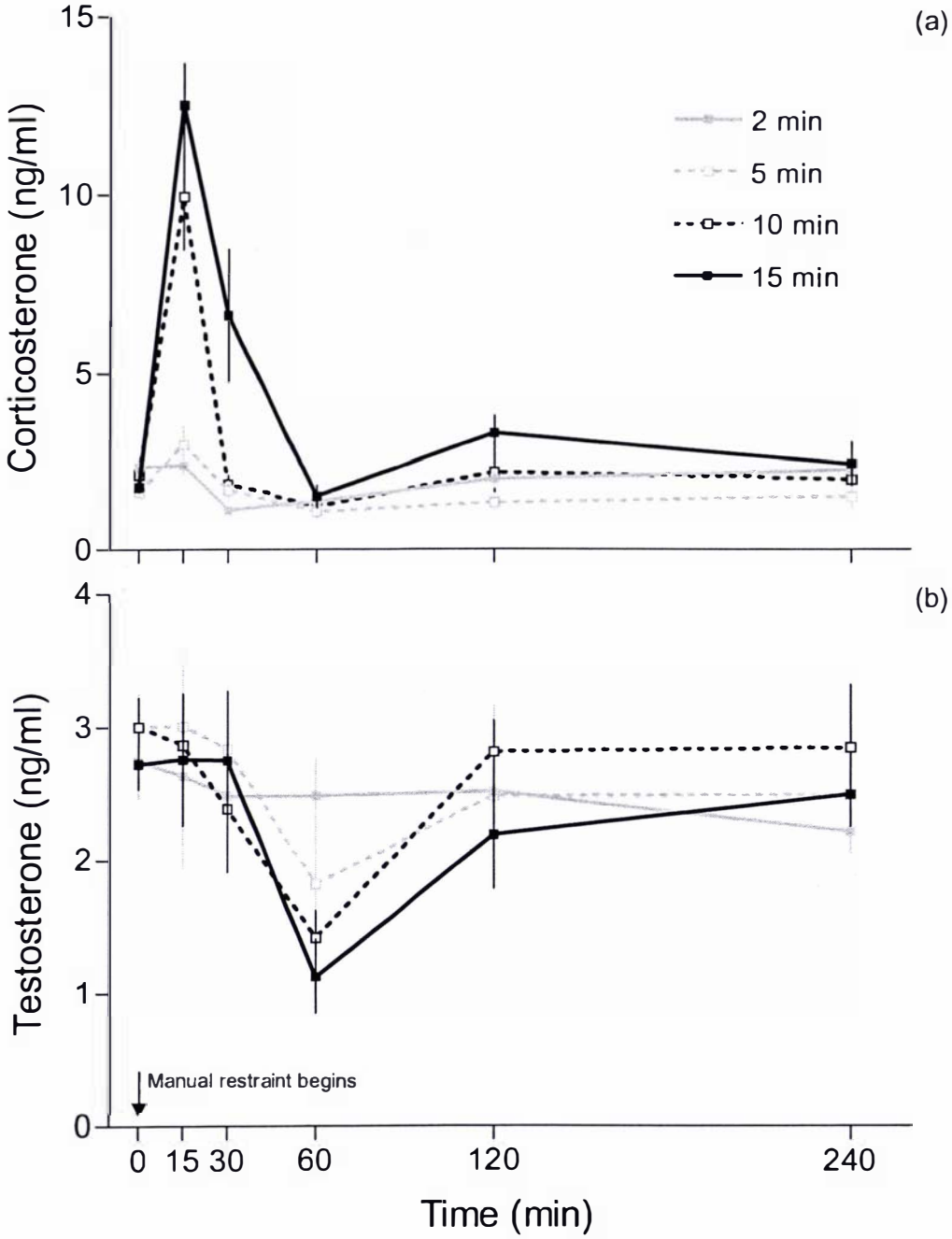


Figure 3.1 Plasma corticosterone (a) and testosterone (b) in male quail blood sampled at 0 min (2 min group) then manually restrained up to 5, 10 or 15 min after the initial blood sample, then blood sampled at 15, 30, 60, 120 or 240 min. Results are plotted as mean ± standard error.

Table 3.1 Two-way repeated measures ANOVA of corticosterone concentrations in male quail that were manually restrained for 2, 5, 10 or 15 minutes.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Treatment		18.298	3,256	< 0.001	**
Time		28.243	5,256	< 0.001	**
Interaction of treatment and time		6.282	15,256	< 0.001	**
Comparisons within treatments for each treatment					
0 vs 15 min	2 min manual restraint	0.064	1,256	0.801	
	5 min manual restraint	9.637	1,256	0.002	**
	10 min manual restraint	51.227	1,256	< 0.001	**
	15 min manual restraint	92.188	1,256	< 0.001	**
15 vs 30 min	2 min manual restraint	7.101	1,256	0.008	**
	5 min manual restraint	4.531	1,256	0.034	*
	10 min manual restraint	36.845	1,256	< 0.001	**
	15 min manual restraint	8.553	1,256	0.004	**
30 vs 60 min	2 min manual restraint	0.172	1,256	0.679	
	5 min manual restraint	3.247	1,256	0.073	
	10 min manual restraint	4.926	1,256	0.027	*
	15 min manual restraint	26.883	1,256	< 0.001	**
60 vs 120 min	2 min manual restraint	2.283	1,256	0.132	
	5 min manual restraint	0.493	1,256	0.483	
	10 min manual restraint	4.709	1,256	0.031	*
	15 min manual restraint	9.993	1,256	0.002	**
120 vs 240 min	2 min manual restraint	0.442	1,256	0.507	
	5 min manual restraint	0.433	1,256	0.511	
	10 min manual restraint	0.034	1,256	0.854	
	15 min manual restraint	2.342	1,256	0.127	
0 vs 30 min	2 min manual restraint	10.164	1,256	0.002	**
	5 min manual restraint	0.127	1,256	0.722	
	10 min manual restraint	0.461	1,256	0.498	
	15 min manual restraint	33.941	1,256	< 0.001	**
0 vs 60 min	2 min manual restraint	7.035	1,256	0.008	**
	5 min manual restraint	3.880	1,256	0.051	
	10 min manual restraint	12.563	1,256	< 0.001	**
	15 min manual restraint	0.753	1,256	0.386	
0 vs 120 min	2 min manual restraint	0.492	1,256	0.483	
	5 min manual restraint	1.131	1,256	0.289	
	10 min manual restraint	0.522	1,256	0.458	
	15 min manual restraint	10.325	1,256	0.001	**
0 vs 240 min	2 min manual restraint	0.024	1,256	0.876	
	5 min manual restraint	0.046	1,256	0.831	
	10 min manual restraint	0.255	1,256	0.614	
	15 min manual restraint	1.531	1,256	0.217	

Note. The first three rows show the results of the two-way ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 3.1 cont Two-way repeated measures ANOVA of corticosterone concentrations in male quail that were manually restrained for 2, 5, 10 or 15 minutes.

		Plasma corticosterone			
Comparisons between treatments for each time		*F*	*df*	*p*	
0 min	2 min vs 5 min	10.672	1,256	0.001	**
	2 min vs 10 min	0.317	1,256	0.574	
	2 min vs 15 min	7.356	1,256	0.007	**
	5 vs 10 min	7.309	1,256	0.007	**
	5 vs 15 min	0.308	1,256	0.580	
	10 vs 15 min	4.617	1,256	0.033	*
15 min	2 min vs 5 min	0.560	1,256	0.455	
	2 min vs 10 min	25.978	1,256	< 0.001	**
	2 min vs 15 min	36.350	1,256	< 0.001	**
	5 vs 10 min	18.909	1,256	< 0.001	**
	5 vs 15 min	27.886	1,256	< 0.001	**
	10 vs 15 min	0.869	1,256	0.352	
30 min	2 min vs 5 min	1.650	1,256	0.200	
	2 min vs 10 min	2.861	1,256	0.092	
	2 min vs 15 min	33.285	1,256	< 0.001	**
	5 vs 10 min	0.166	1,256	0.684	
	5 vs 15 min	20.113	1,256	< 0.001	**
	10 vs 15 min	16.628	1,256	< 0.001	**
60 min	2 min vs 5 min	0.869	1,256	0.352	
	2 min vs 10 min	0.889	1,256	0.347	
	2 min vs 15 min	0.029	1,256	0.866	
	5 vs 10 min	0.000	1,256	0.992	
	5 vs 15 min	1.214	1,256	0.272	
	10 vs 15 min	1.237	1,256	0.267	
120 min	2 min vs 5 min	3.031	1,256	0.083	
	2 min vs 10 min	0.081	1,256	0.777	
	2 min vs 15 min	3.311	1,256	0.070	
	5 vs 10 min	2.123	1,256	0.146	
	5 vs 15 min	12.679	1,256	< 0.001	**
	10 vs 15 min	4.425	1,256	0.036	*
240 min	2 min vs 5 min	3.054	1,256	0.082	
	2 min vs 10 min	0.584	1,256	0.445	
	2 min vs 15 min	0.141	1,256	0.708	
	5 vs 10 min	0.967	1,256	0.326	
	5 vs 15 min	1.883	1,256	0.171	
	10 vs 15 min	0.151	1,256	0.698	

Table 3.2 Two-way repeated measures ANOVA of testosterone concentrations in male quail that were manually restrained for 2, 5, 10 or 15 minutes.

		Plasma testosterone			
Effect		*F*	*df*	*p*	
Treatment		0.396	3,256	0.756	
Time		2.653	5,256	0.023	*
Interaction of treatment and time		0.841	15,256	0.631	
Comparisons within treatments for each treatment					
0 vs 15 min	2 min manual restraint	0.166	1,256	0.684	
	5 min manual restraint	0.022	1,256	0.882	
	10 min manual restraint	0.135	1,256	0.714	
	15 min manual restraint	0.007	1,256	0.935	
15 vs 30 min	2 min manual restraint	0.356	1,256	0.551	
	5 min manual restraint	0.000	1,256	0.997	
	10 min manual restraint	1.016	1,256	0.314	
	15 min manual restraint	0.057	1,256	0.811	
30 vs 60 min	2 min manual restraint	0.004	1,256	0.950	
	5 min manual restraint	1.985	1,256	0.160	
	10 min manual restraint	1.135	1,256	0.288	
	15 min manual restraint	6.750	1,256	0.010	*
60 vs 120 min	2 min manual restraint	0.001	1,256	0.973	
	5 min manual restraint	0.155	1,256	0.694	
	10 min manual restraint	4.109	1,256	0.044	*
	15 min manual restraint	4.346	1,256	0.038	*
120 vs 240 min	2 min manual restraint	0.076	1,256	0.782	
	5 min manual restraint	0.151	1,256	0.698	
	10 min manual restraint	0.086	1,256	0.770	
	15 min manual restraint	0.485	1,256	0.487	
0 vs 30 min	2 min manual restraint	0.132	1,256	0.717	
	5 min manual restraint	0.021	1,256	0.886	
	10 min manual restraint	0.873	1,256	0.351	
	15 min manual restraint	0.052	1,256	0.820	
0 vs 60 min	2 min manual restraint	0.197	1,256	0.657	
	5 min manual restraint	2.806	1,256	0.095	
	10 min manual restraint	5.333	1,256	0.022	*
	15 min manual restraint	12.829	1,256	< 0.001	**
0 vs 120 min	2 min manual restraint	0.238	1,256	0.626	
	5 min manual restraint	1.362	1,256	0.244	
	10 min manual restraint	0.095	1,256	0.759	
	15 min manual restraint	0.793	1,256	0.374	
0 vs 240 min	2 min manual restraint	0.017	1,256	0.896	
	5 min manual restraint	0.443	1,256	0.506	
	10 min manual restraint	0.005	1,256	0.944	
	15 min manual restraint	0.000	1,256	0.993	

Note. The first three rows show the results of the two-way ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 3.2 cont Two-way repeated measures ANOVA of testosterone concentrations in male quail that were manually restrained for 2, 5, 10 or 15 minutes.

Comparisons between treatments for each time		Plasma testosterone			
		F	*df*	*p*	
0 min	2 min vs 5 min	1.319	1,256	0.252	
	2 min vs 10 min	1.408	1,256	0.236	
	2 min vs 15 min	0.882	1,256	0.365	
	5 vs 10 min	0.001	1,256	0.969	
	5 vs 15 min	0.058	1,256	0.809	
	10 vs 15 min	0.078	1,256	0.780	
15 min	2 min vs 5 min	0.891	1,256	0.346	
	2 min vs 10 min	1.278	1,256	0.259	
	2 min vs 15 min	0.615	1,256	0.434	
	5 vs 10 min	0.035	1,256	0.852	
	5 vs 15 min	0.026	1,256	0.873	
	10 vs 15 min	0.120	1,256	0.729	
30 min	2 min vs 5 min	0.118	1,256	0.732	
	2 min vs 10 min	0.225	1,256	0.636	
	2 min vs 15 min	0.003	1,256	0.958	
	5 vs 10 min	0.669	1,256	0.414	
	5 vs 15 min	0.156	1,256	0.693	
	10 vs 15 min	0.178	1,256	0.673	
60 min	2 min vs 5 min	1.273	1,256	0.260	
	2 min vs 10 min	2.567	1,256	0.110	
	2 min vs 15 min	7.361	1,256	0.007	**
	5 vs 10 min	0.225	1,256	0.636	
	5 vs 15 min	2.512	1,256	0.114	
	10 vs 15 min	0.159	1,256	0.686	
120 min	2 min vs 5 min	0.590	1,256	0.443	
	2 min vs 10 min	0.153	1,256	0.696	
	2 min vs 15 min	0.438	1,256	0.509	
	5 vs 10 min	1.344	1,256	0.247	
	5 vs 15 min	0.011	1,256	0.915	
	10 vs 15 min	1.109	1,256	0.293	
240 min	2 min vs 5 min	0.011	1,256	0.918	
	2 min vs 10 min	0.140	1,256	0.708	
	2 min vs 15 min	0.097	1,256	0.756	
	5 vs 10 min	0.228	1,256	0.633	
	5 vs 15 min	0.171	1,256	0.679	
	10 vs 15 min	0.004	1,256	0.949	

3.3.2 Experiment 2 – Corticosterone and testosterone responses to manual restraint, mechanical restraint and social stimuli in Japanese quail

3.3.2.1 The effect of different stimuli on the corticosterone response

Corticosterone did not change significantly after the first sample in untreated birds (Figure 3.2). In birds that were exposed to an unfamiliar quail that was kept physically separated, corticosterone increased at 15 or 30 min in some birds while there was no response in others. The corticosterone concentrations of birds that were kept in the same cage as an unfamiliar quail did not differ from untreated birds, with one exception.

Corticosterone increased at 15 min following 15 min of mechanical restraint, then decreased between 15 and 60 min and remained relatively thereafter. The magnitude of the increase in corticosterone varied between birds and concentrations ranged from 1.24 to 6.06 ng/ml at 15 min. There was a strong corticosterone response in all birds manually restrained for 15 min with a peak in corticosterone at 15 min followed by a decrease to initial concentrations at 30 min.

Initial corticosterone concentrations differed between groups ($F_{4,45} = 2.671$, $p = 0.044$; Figure 3.3a). The changes in corticosterone from initial concentrations were therefore calculated for all birds (Figure 3.3b), with a value of 5 ng/ml added to each calculated value to enable them to be $\log_{10}$ transformed for statistical analysis. Overall, mean changes in corticosterone did not vary between treatment but did between time, and there was a significant interaction between treatment and time (see Table 3.3 for all statistics for this analysis).

Corticosterone concentrations remained low and relatively constant in birds that were untreated and those in contact with an unfamiliar quail. Corticosterone was elevated at 15 min in birds that were exposed to an unfamiliar quail but kept separate from them, then decreased and did not differ from untreated birds. Mean corticosterone concentrations in mechanically restrained birds increased 15 min after the initial sample and were higher than in birds kept separated from an unfamiliar quail. Corticosterone in the birds manually restrained for 15 min

increased to a peak at 15 min (6.11 ± 1.93 ng/ml), declined to low concentrations at 30 min and remained below initial concentrations at 60 and 120 min. Concentrations at 30, 60 and 120 min generally did not differ greatly between treatments.

There was a significant difference in the total integrated corticosterone response between the groups ($F_{4,45}$ = 7.305, $p < 0.001$; Figure 3.4a), although differences in baseline corticosterone between the groups confounded differences in the integrated response. Corrected corticosterone responses were calculated from 0 to 30 min. Corticosterone concentrations between 0 and 30 min varied between groups (Kruskal-Wallis t = 21.376, $p < 0.001$; Figure 3.4b). Untreated birds and those in direction contact with an unfamiliar bird showed little response over this time, whilst there was a significant response in birds that were mechanically restrained and those that were exposed to an unfamiliar bird but kept separated from it. These two responses did not differ (post-hoc Bonferroni test, $p = 0.356$). Birds that were manually restrained for 15 min showed the largest corrected corticosterone response between 0 and 30 min, and this was significantly greater than responses to either mechanical restraint or visual exposure to an unfamiliar quail (post-hoc Bonferroni test, $p = 0.043$ and $p = 0.022$ respectively).

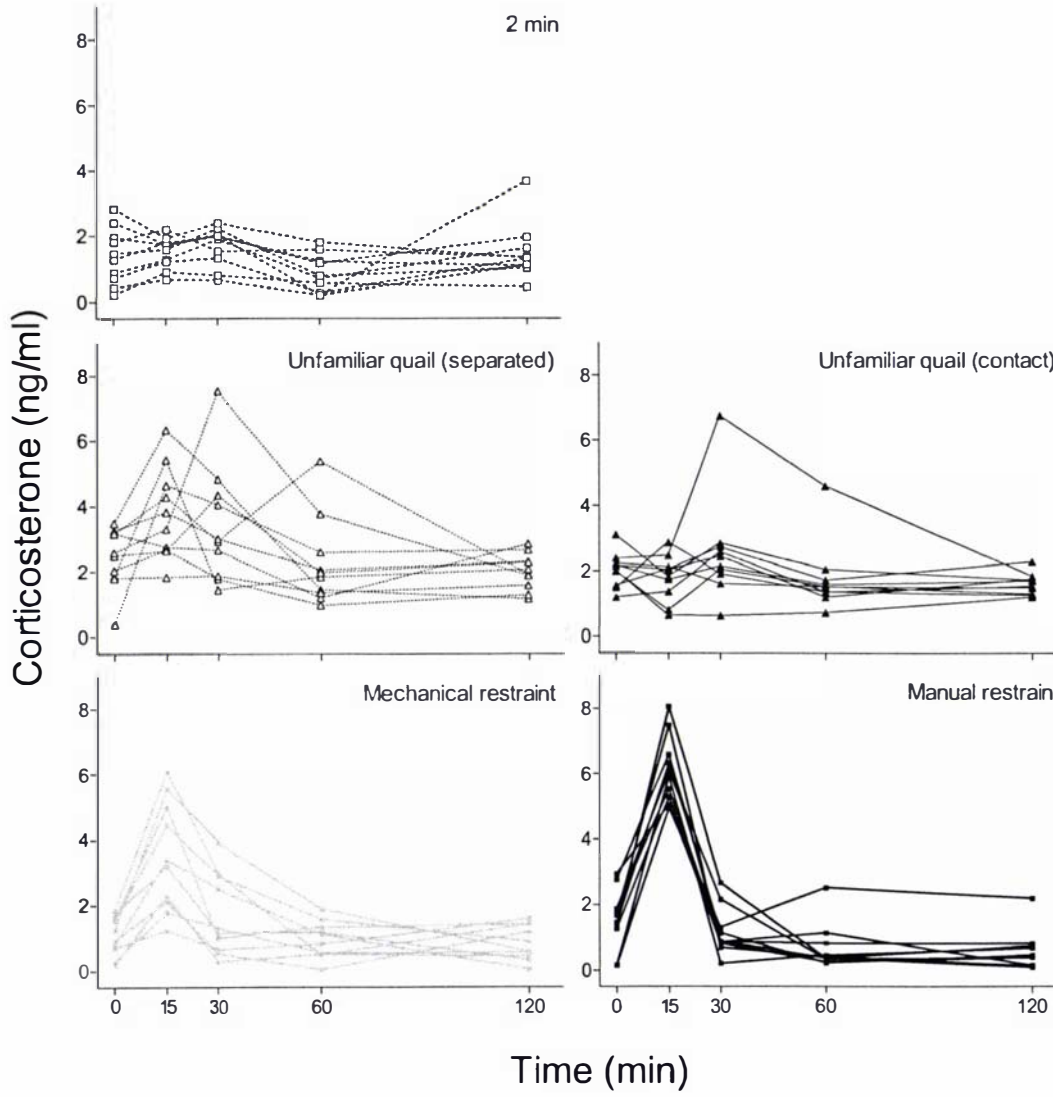


Figure 3.2 Plasma corticosterone in male quail that were blood sampled (2 min), or in addition were manually or mechanically restrained for 15 min, or exposed to an unfamiliar quail that was visible but separated by a wire mesh or in direct physical contact. Results are plotted as raw data.

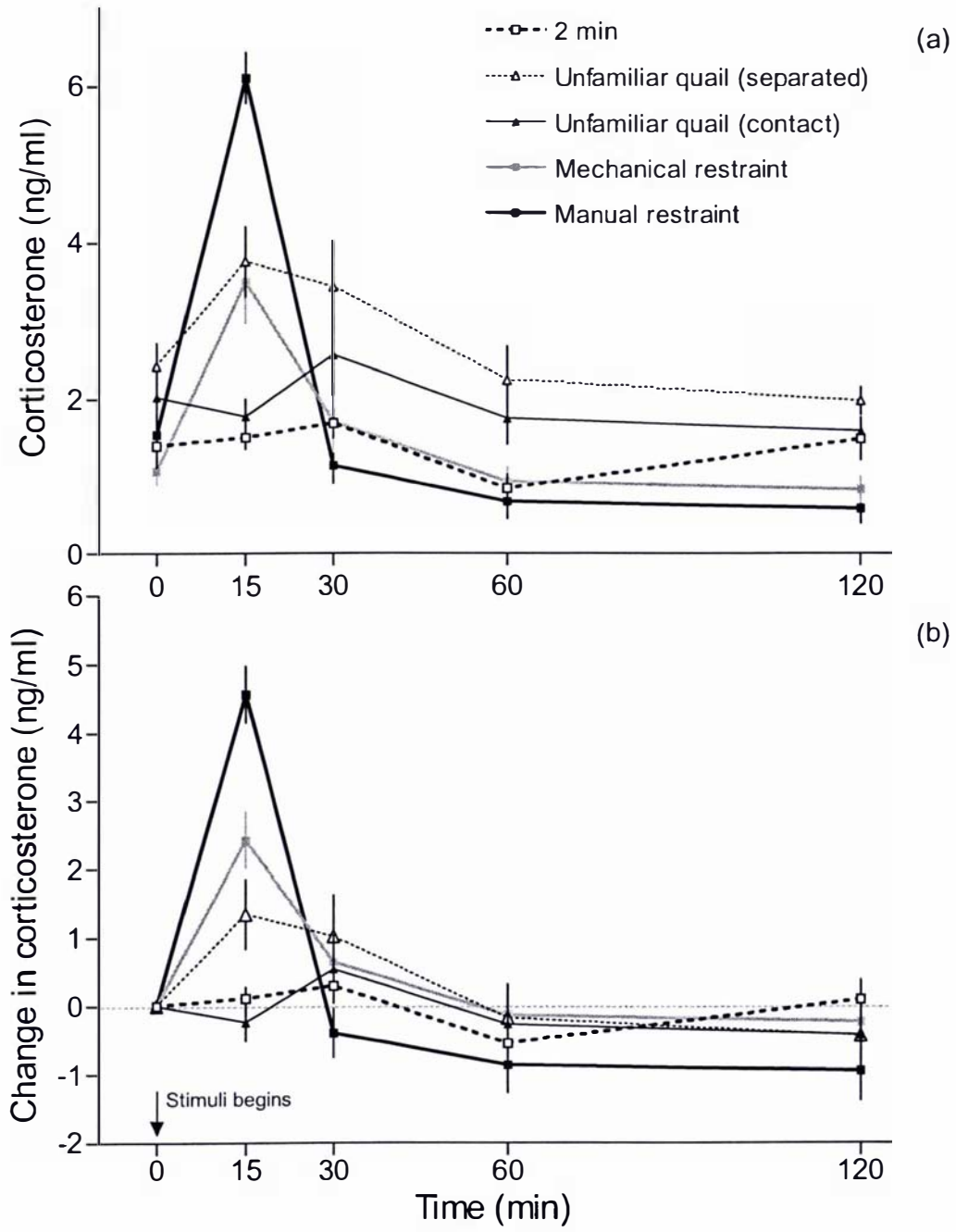


Figure 3.3 Plasma corticosterone (a) and change in plasma corticosterone relative to 0 min (b) in male quail that were blood sampled (2 min), or in addition were manually or mechanically restrained for 15 min, or exposed to an unfamiliar quail that was visible but separated by a wire mesh or in direct physical contact. Results are plotted as mean ± standard error.

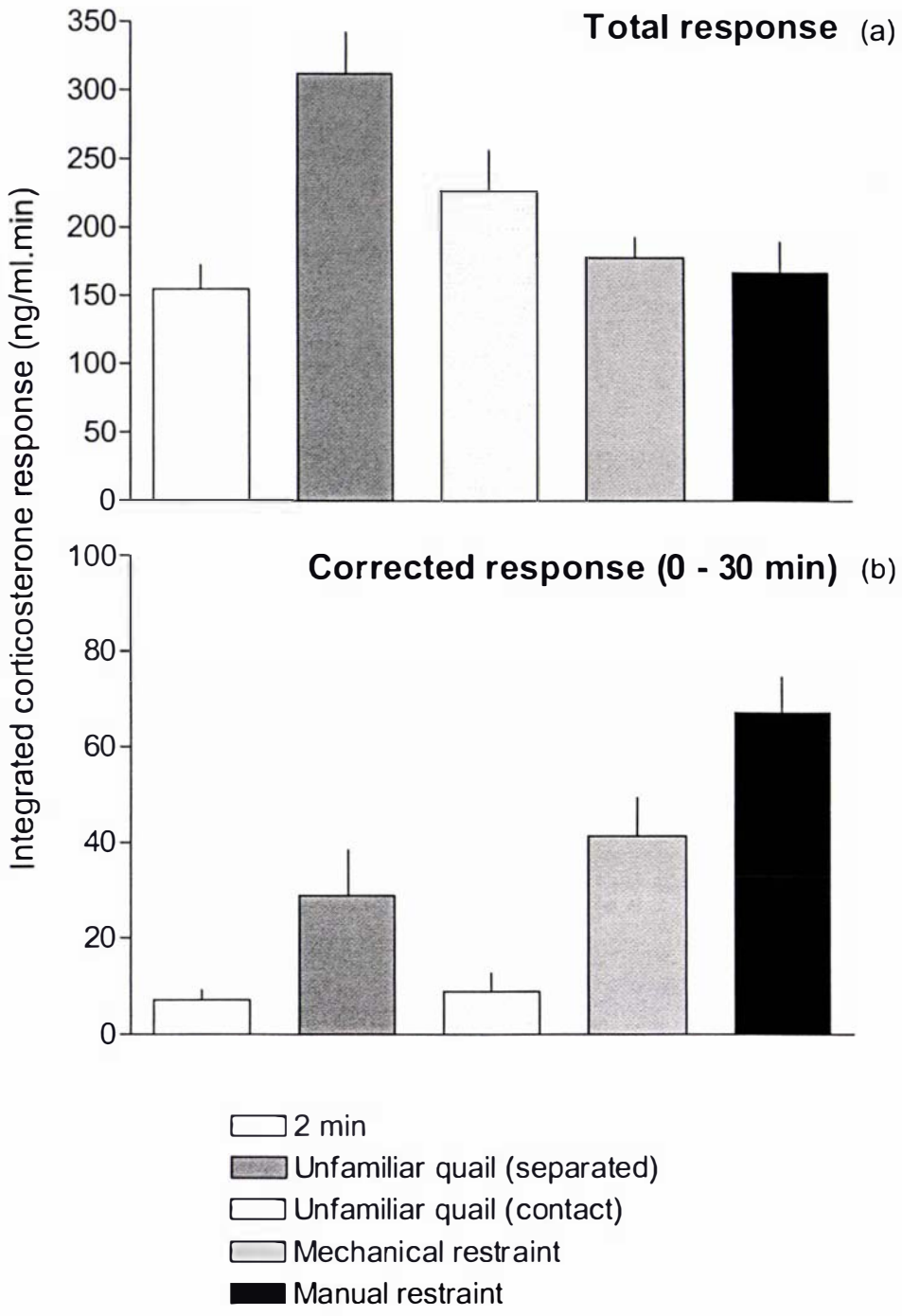


Figure 3.4 Total (a; all groups n=10) and corrected (b) integrated corticosterone responses (0-30 min) in male quail that were blood sampled (2 min, n=8), or in addition were mechanically restrained (n=10) or manually restrained for 15 min (n=10), or exposed to an unfamiliar quail that was visible but separated by a wire mesh (n=9) or in direct physical contact (n=6). Results are plotted as mean ± standard error.

Table 3.3 Two-way repeated measures ANOVA of corticosterone concentrations in male quail exposed to different stimuli.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Treatment		1.081	4,45	0.377	
Time		54.050	3,135	< 0.001	**
Interaction of treatment and time		12.632	12,135	< 0.001	**
Comparisons within treatments for each treatment					
15 vs 30 min	2 min	0.295	1,45	0.590	
	Separated	0.989	1,45	0.325	
	Contact	4.190	1,45	0.047	*
	Mechanical restraint	17.573	1,45	< 0.001	**
	Manual restraint	128.820	1,45	< 0.001	**
30 vs 60 min	2 min	7.539	1,45	0.009	**
	Separated	12.320	1,45	0.001	**
	Contact	4.956	1,45	0.031	*
	Mechanical restraint	4.613	1,45	0.037	*
	Manual restraint	3.040	1,45	0.088	
60 vs 120 min	2 min	3.677	1,45	0.062	
	Separated	0.328	1,45	0.570	
	Contact	0.127	1,45	0.723	
	Mechanical restraint	0.105	1,45	0.748	
	Manual restraint	0.262	1,45	0.611	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 3.3 cont Two-way repeated measures ANOVA of corticosterone concentrations in male quail exposed to different stimuli.

Comparisons between treatments for each time		Plasma corticosterone			
		F	*df*	*p*	
15 min	2 min vs Separated	6.177	1,45	0.017	*
	2 min vs Contact	1.064	1,45	0.308	
	2 min vs Mechanical restraint	21.783	1,45	< 0.001	**
	2 min vs Manual restraint	63.456	1,45	< 0.001	**
	Separated vs Contact	12.368	1,45	0.001	**
	Separated vs Mechanical restraint	4.760	1,45	0.034	*
	Separated vs Manual restraint	30.036	1,45	< 0.001	**
	Contact vs Mechanical restraint	32.474	1,45	< 0.001	**
	Contact vs Manual restraint	80.951	1,45	< 0.001	**
	Mechanical vs Manual restraint	10.881	1,45	0.002	**
30 min	2 min vs Separated	0.826	1,45	0.368	
	2 min vs Contact	0.034	1,45	0.855	
	2 min vs Mechanical restraint	0.260	1,45	0.613	
	2 min vs Manual restraint	2.557	1,45	0.117	
	Separated vs Contact	0.526	1,45	0.472	
	Separated vs Mechanical restraint	0.159	1,45	0.692	
	Separated vs Manual restraint	6.291	1,45	0.016	*
	Contact vs Mechanical restraint	0.106	1,45	0.746	
	Contact vs Manual restraint	3.179	1,45	0.081	
	Mechanical vs Manual restraint	4.448	1,45	0.041	*
60 min	2 min vs Separated	0.189	1,45	0.666	
	2 min vs Contact	0.276	1,45	0.602	
	2 min vs Mechanical restraint	0.829	1,45	0.367	
	2 min vs Manual restraint	0.973	1,45	0.329	
	Separated vs Contact	0.008	1,45	0.928	
	Separated vs Mechanical restraint	0.227	1,45	0.636	
	Separated vs Manual restraint	2.019	1,45	0.162	
	Contact vs Mechanical restraint	0.148	1,45	0.702	
	Contact vs Manual restraint	2.286	1,45	0.137	
	Mechanical vs Manual restraint	3.599	1,45	0.064	
120 min	2 min vs Separated	1.646	1,45	0.206	
	2 min vs Contact	1.082	1,45	0.304	
	2 min vs Mechanical restraint	0.387	1,45	0.537	
	2 min vs Manual restraint	7.279	1,45	0.010	*
	Separated vs Contact	0.059	1,45	0.809	
	Separated vs Mechanical restraint	0.437	1,45	0.512	
	Separated vs Manual restraint	2.002	1,45	0.164	
	Contact vs Mechanical restraint	0.175	1,45	0.678	
	Contact vs Manual restraint	2.749	1,45	0.104	
	Mechanical vs Manual restraint	4.309	1,45	0.044	*

3.3.2.2 The effect of mechanical and manual restraint on corticosterone and testosterone concentrations

3.3.2.2.1 Plasma corticosterone

Corticosterone varied between treatment and between time, with a significant interaction between treatment and time (see Table 3.4 for all statistics for this analysis). Half of each treatment group (n=10) were sampled on one day and the other half 10 days later. Corticosterone did not differ between the different sample days (see Table 3.6 for statistics for this analysis), thus the birds were combined for subsequent analyses.

Initial corticosterone concentrations were low (< 4 ng/ml) and remained constant in untreated birds (Figure 3.5a), and initial corticosterone concentrations did not differ between the three groups ($F_{2,132}$ = 0.631, p = 0.533). Mean corticosterone concentrations increased to a peak (5.34 ± 1.75 ng/ml) at 15 min in birds that were mechanically restrained, declined from this peak until 60 min and then remained constant. Concentrations at 60, 120 and 240 min did not differ from initial corticosterone concentrations or those of untreated birds. Manual restraint for 15 min elicited a larger increase in plasma corticosterone than mechanical restraint after 15 min (7.95 ± 2.51 ng/ml), with corticosterone then declining by 30 min, and were lower than initial values at 60 min and remaining relatively constant thereafter. Corticosterone at 60, 120 and 240 min did not differ from initial concentrations.

3.3.2.2.2 Plasma testosterone

Testosterone varied between treatment and between time, with a significant interaction between treatment and time (see Table 3.5 for all statistics for this analysis), while testosterone did not differ between the two sample days (Table 3.6).

Initial testosterone concentrations did not differ between the groups ($F_{2,132}$ = 0.218, p = 0.804; Figure 3.5b), and did not change with time in untreated birds. Testosterone declined between 0 and 30 min in birds that were mechanically restrained, was significantly lower than in untreated birds after 60 min and remained low until 120 min, before increasing to a concentration similar to

untreated birds at 240 min. 15 min manual restraint caused testosterone concentrations to decline until 60 min and remain low until 120 min before increasing between 120 and 240 min. The mean concentration at 240 min did not differ from the initial concentrations or from that of untreated birds at 240 min. Mean testosterone concentrations in mechanically restrained birds were most different from untreated birds at 60 min with manual restraint producing the most effect.

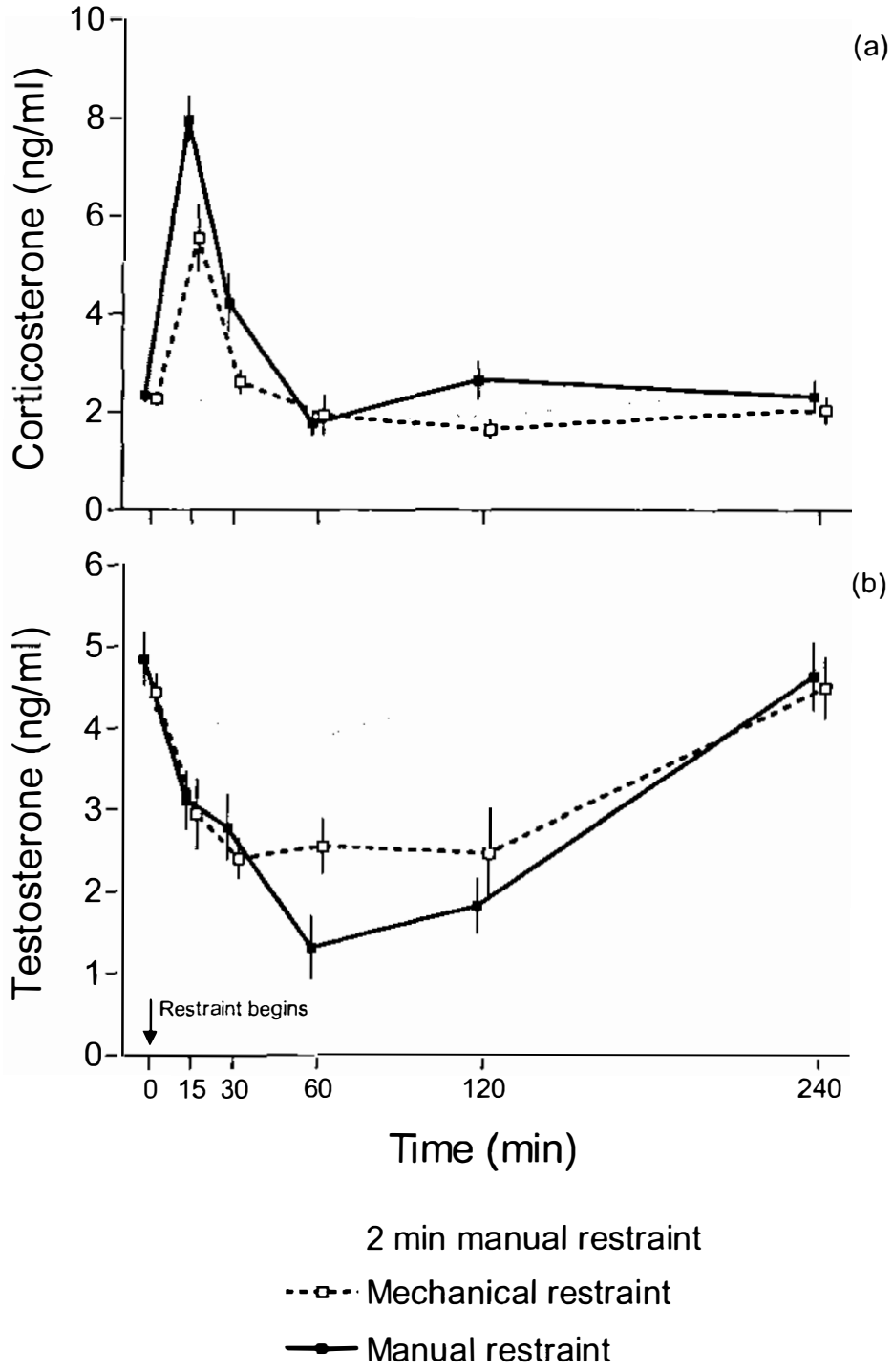


Figure 3.5 Plasma corticosterone (a) and testosterone (b) male quail blood sampled at 0 min (2 min manual restraint) then manually or mechanically restrained for 15 min, then blood sampled at 15, 30, 60, 120 or 240 min. Results are plotted as mean ± standard error.

Table 3.4 Two-way repeated measures ANOVA of corticosterone concentrations in male quail manually or mechanically restrained for 15 min.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Treatment		9.929	2,252	< 0.001	**
Time		17.434	5,252	< 0.001	**
Interaction of treatment and time		3.861	10,252	< 0.001	**
Comparisons within treatments for each treatment					
0 vs 15 min	2 min	0.234	1,252	0.629	
	Mechanical restraint	34.848	1,252	< 0.001	**
	Manual restraint	69.095	1,252	< 0.001	**
15 vs 30 min	2 min	0.107	1,252	0.744	
	Mechanical restraint	12.433	1,252	0.001	**
	Manual restraint	11.913	1,252	0.001	**
30 vs 60 min	2 min	0.402	1,252	0.527	
	Mechanical restraint	5.034	1,252	0.026	*
	Manual restraint	20.381	1,252	< 0.001	**
60 vs 120 min	2 min	0.115	1,252	0.735	
	Mechanical restraint	0.037	1,252	0.847	
	Manual restraint	3.278	1,252	0.071	
120 vs 240 min	2 min	1.517	1,252	0.219	
	Mechanical restraint	0.844	1,252	0.359	
	Manual restraint	0.265	1,252	0.607	
0 vs 30 min	2 min	0.004	1,252	0.950	
	Mechanical restraint	1.825	1,252	0.178	
	Manual restraint	14.872	1,252	< 0.001	**
0 vs 60 min	2 min	0.571	1,252	0.450	
	Mechanical restraint	2.389	1,252	0.123	
	Manual restraint	1.825	1,252	0.178	
0 vs 120 min	2 min	0.101	1,252	0.751	
	Mechanical restraint	3.222	1,252	0.074	
	Manual restraint	2.389	1,252	0.123	
0 vs 240 min	2 min	1.618	1,252	0.205	
	Mechanical restraint	0.371	1,252	0.543	
	Manual restraint	3.222	1,252	0.074	

Note. The first three rows show the results of the two-way ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 3.4 cont Two-way repeated measures ANOVA of corticosterone concentrations in male quail manually or mechanically restrained for 15 min.

Comparisons between treatments for each time		Plasma corticosterone			
		F	*df*	*p*	
0 min	2 min vs Mechanical restraint	0.196	1,252	0.658	
	2 min vs Manual restraint	1.240	1,252	0.267	
	Mechanical vs Manual restraint	0.546	1,252	0.461	
15 min	2 min vs Mechanical restraint	15.333	1,252	< 0.001	**
	2 min vs Manual restraint	35.218	1,252	< 0.001	**
	Mechanical vs Manual restraint	4.825	1,252	0.029	*
30 min	2 min vs Mechanical restraint	1.200	1,252	0.274	
	2 min vs Manual restraint	9.962	1,252	0.002	**
	Mechanical vs Manual restraint	5.128	1,252	0.024	*
60 min	2 min vs Mechanical restraint	0.064	1,252	0.801	
	2 min vs Manual restraint	0.000	1,252	1.000	
	Mechanical vs Manual restraint	0.000	1,252	1.000	
120 min	2 min vs Mechanical restraint	0.635	1,252	0.426	
	2 min vs Manual restraint	1.044	1,252	0.308	
	Mechanical vs Manual restraint	4.016	1,252	0.046	*
240 min	2 min vs Mechanical restraint	1.687	1,252	0.195	
	2 min vs Manual restraint	0.610	1,252	0.436	
	Mechanical vs Manual restraint	0.326	1,252	0.569	

Table 3.5 Two-way repeated measures ANOVA of testosterone concentrations in male quail manually or mechanically restrained for 15 min.

		Plasma testosterone			
Effect		*F*	*df*	*p*	
Treatment		14.219	2,252	< 0.001	**
Time		11.071	5,252	< 0.001	**
Interaction of treatment and time		4.207	10,252	< 0.001	**
Comparisons within treatments for each treatment					
0 vs 15 min	2 min	0.011	1,252	0.917	
	Mechanical restraint	3.899	1,252	0.049	*
	Manual restraint	4.304	1,252	0.039	*
15 vs 30 min	2 min	0.000	1,252	0.991	
	Mechanical restraint	0.149	1,252	0.700	
	Manual restraint	0.416	1,252	0.519	
30 vs 60 min	2 min	0.006	1,252	0.940	
	Mechanical restraint	0.000	1,252	0.984	
	Manual restraint	17.837	1,252	< 0.001	**
60 vs 120 min	2 min	0.161	1,252	0.689	
	Mechanical restraint	1.152	1,252	0.284	
	Manual restraint	2.825	1,252	0.094	
120 vs 240 min	2 min	0.136	1,252	0.713	
	Mechanical restraint	11.723	1,252	0.001	**
	Manual restraint	18.980	1,252	< 0.001	**
0 vs 30 min	2 min	0.014	1,252	0.905	
	Mechanical restraint	6.114	1,252	0.014	*
	Manual restraint	6.318	1,252	0.013	*
0 vs 60 min	2 min	0.000	1,252	0.982	
	Mechanical restraint	6.240	1,252	0.013	*
	Manual restraint	63.457	1,252	< 0.001	**
0 vs 120 min	2 min	0.292	1,252	0.590	
	Mechanical restraint	15.080	1,252	< 0.001	**
	Manual restraint	27.959	1,252	< 0.001	**
0 vs 240 min	2 min	1.031	1,252	0.311	
	Mechanical restraint	0.288	1,252	0.592	
	Manual restraint	0.113	1,252	0.737	

Note. The first three rows show the results of the two-way ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 3.5 cont Two-way repeated measures ANOVA of testosterone concentrations in male quail manually or mechanically restrained for 15 min.

Comparisons between treatments for each time		Plasma testosterone		
		F	*df*	*p*
0 min	2 min vs Mechanical restraint	0.005	1,252	0.941
	2 min vs Manual restraint	0.320	1,252	0.572
	Mechanical vs Manual restraint	0.295	1,252	0.588
15 min	2 min vs Mechanical restraint	2.081	1,252	0.150
	2 min vs Manual restraint	1.032	1,252	0.311
	Mechanical vs Manual restraint	0.221	1,252	0.639
30 min	2 min vs Mechanical restraint	3.259	1,252	0.072
	2 min vs Manual restraint	2.605	1,252	0.108
	Mechanical vs Manual restraint	0.044	1,252	0.833
60 min	2 min vs Mechanical restraint	3.033	1,252	0.083
	2 min vs Manual restraint	28.784	1,252	< 0.001 **
	Mechanical vs Manual restraint	15.943	1,252	< 0.001 **
120 min	2 min vs Mechanical restraint	9.926	1,252	0.002 **
	2 min vs Manual restraint	15.346	1,252	< 0.001 **
	Mechanical vs Manual restraint	0.714	1,252	0.399
240 min	2 min vs Mechanical restraint	0.196	1,252	0.658
	2 min vs Manual restraint	0.132	1,252	0.717
	Mechanical vs Manual restraint	0.008	1,252	0.930

Table 3.6 One-way ANOVA corticosterone and testosterone concentrations in quail sampled on Day 0 versus 10.

Effect		Day 0 versus 10		
		F	*df*	*p*
Corticosterone	Manual restraint	0.041	1,50	0.841
	Mechanical restraint	0.008	1,50	0.927
Testosterone	Manual restraint	2.526	1,50	0.120
	Mechanical restraint	0.062	1,50	0.804

3.4 Discussion

Manual restraint of male Japanese quail for 10 minutes or greater resulted in a significant rise in plasma corticosterone concentrations, with corticosterone remaining elevated for up to 30 minutes. The increase in corticosterone was

followed by a decline in testosterone. Testosterone was lowest after 60 minutes and remained low for up to two hours.

The adrenocortical response varied with the type of the stressor. Manual restraint consistently initiated a large corticosterone increase in every bird, while the response to mechanical restraint varied from a small increase to a strong response seen in handled birds. The introduction of an unfamiliar quail as a social stimulus resulted in no change in corticosterone when quail were placed in the same cage as the unfamiliar quail despite both birds displaying agonistic behaviour towards each other. In contrast, unfamiliar quail separated by a wire mesh showed a significant adrenocortical response, although there was no clear pattern to corticosterone secretion. Mechanical restraint causing immobilisation resulted in a significant decline in plasma testosterone for up to 2 hours, manual restraint was more effective and caused a larger decrease in testosterone at 60 minutes.

3.4.1 Quantification of the effect of manual restraint on plasma testosterone and corticosterone

3.4.1.1 Effect of manual restraint time on plasma corticosterone

This experiment showed that 5 or more minutes of immobilisation by manual restraint were necessary to significantly increase plasma corticosterone concentrations at 15 minutes in male Japanese quail. The response of quail to 15 min of manual restraint was similar to the response of handling reported previously in our laboratory (Boyd, 2000). All birds were blood sampled twice only, at 0 minutes and one further time to prevent the additive effect of serial blood sampling on corticosterone and testosterone as reported in previous studies (Wilson *et al.*, 1979; Eskeland and Blom, 1979; Johnson, 1981). All blood samples were collected within 2 min to ensure that measured corticosterone values did not reflect a response to the bleeding procedure. Corticosterone can increase after 2 minutes in quail used in our lab (Boyd, 2000).

Five minutes of manual restraint resulted in a small increase in corticosterone after 15 minutes. However, corticosterone may have increased during the 5 min period of restraint then decreased by the time of the 15 min sample. Plasma corticosterone

rises rapidly in response to manual restraint, increasing within two minutes in chickens (Beuving and Vonder, 1978), and one minute in ducks (Harvey *et al.*, 1980) and Japanese quail (Satterlee and Johnson, 1988). Plasma corticosterone concentrations in Japanese quail restrained for 20 min peak after four minutes and were no greater at 10 or 20 min (Satterlee and Johnson, 1988). Jones *et al.* (1994a) reported corticosterone increased significantly after 5.5 minutes of manual restraint, so the five minutes of manual restraint in the current study is likely to have caused a transient increase in corticosterone that was not detected after 15 minutes. The half-life of corticosterone in male Japanese quail is 10 minutes (Kovács and Péczely, 1983) thus an increase could have occurred and dissipated within 15 min. Therefore peak corticosterone concentrations after 5 min of manual restraint are likely to have been lower than after 10 and 15 minutes of manual restraint.

Ten minutes of manual restraint resulted in a five-fold increase in mean corticosterone concentrations after 15 minutes, followed by a decline to initial concentrations by 30 minutes. Ten minutes of manual restraint clearly initiated a strong adrenocortical response in our strain of Japanese quail, whereas halving the manual restraint time had substantially less effect on the corticosterone response. Peak corticosterone concentrations at 15 minutes in birds manually restrained for 10 minutes appeared less than birds manually restrained for 15 minutes but results were not statistically significant. There was a seven-fold increase in plasma corticosterone in birds manually restrained for 15 minutes, with corticosterone still elevated at 30 minutes. Boyd (2000) reported a three-fold increase at 15 minutes after handling, and a return to initial concentrations by 30 minutes. The differences in peak corticosterone concentration between the current study and that reported by Boyd (2000) may indicate different responses to continuous immobilisation by hand (current study) compared with repeatedly removing and returning a bird from a box. The higher peak and prolonged elevation in corticosterone concentrations in the current study indicate that continuous manual restraint is a more potent stressor than picking up and putting down.

3.4.1.2 Effect of duration of manual restraint on plasma testosterone

Manual restraint for 5 minutes or greater resulted in a significant decline in plasma testosterone after 60 minutes. Decreases in testosterone concentration following manual restraint and serial blood sampling have been found in quail (previous chapter) and chickens (Wilson *et al.*, 1979; Eskeland and Blom, 1979; Johnson, 1981). A different group of birds was blood sampled at each time to avoid measuring the decline in testosterone associated with serial blood collection from the same bird, This resulted in testosterone concentrations remaining unaffected for up to 30 minutes after the manual restraint in all groups. Collection of the first blood sample had no effect on subsequent testosterone concentrations in the untreated group. Mean testosterone in birds manually restrained for five minutes decreased, but not significantly, between 30 and 60 minutes. Manual restraint for 10 or 15 minutes resulted in plasma testosterone concentrations falling significantly below initial concentrations 60 minutes after the onset of manual restraint.

The delay in the decline of testosterone secretion until 30 minutes after the quail were first handled conflicts with what was found in the previous chapter. In Chapter 2, testosterone had decreased 30 minutes after the collection of the blood sample and injection of oil or corticosterone in oil. Blood sampling followed by the added injection procedure is the most likely cause of the initial decline in testosterone secretion by 30 minutes, as testosterone had not significantly decreased at 30 minutes in birds that were only blood sampled. In the current study, the first manipulation was restricted to a short period of blood collection taking less than 2 minutes, followed by manual restraint until 5, 10 or 15 minutes after birds were first removed from the cage. Testosterone did not decline for the 15 minutes after birds were first returned to their cages.

Heiblum *et al.* (2000) reported that testosterone increased in chickens 30 minutes after 10 minutes of immobilisation, returning to initial concentrations by 60 minutes. However, testosterone declined in response to mechanical restraint in one quarter of the chickens indicating individual variation in the testosterone response to mechanical restraint.

3.4.2 Adrenocortical and testosterone responses to different stressors

3.4.2.1 Adrenocortical responses to manual restraint, mechanical restraint and social stressors

The results of the experiment described in Section 3.3.1 clearly demonstrated that 10 or 15 minutes of immobilisation by manual restraint resulted in a decrease in testosterone by 60 minutes. The current experiment showed that the adrenocortical response varies depending on the type of stressor. As the aim of the study was to examine the adrenocortical response to different stressors, repeated sampling of the same bird was appropriate in this study.

Social disruption has previously been shown to initiate a corticosterone response in quail. Satterlee *et al.* (1983) reported that corticosterone was elevated at two hours in wild-type quail after the introduction of an albino quail. In the current study, corticosterone did not change when an unfamiliar male quail was introduced into the cage for 15 minutes. This was despite vigorous pecking and other agonistic behaviours being exhibited by both birds towards each other. Littin and Cockrem (2001) also reported no effect of mixing unfamiliar chickens. In contrast, the introduction of an unfamiliar quail that was physically separated by a wire partition resulted in a strong adrenocortical response after 15 minutes in 30% of quail. Both quail attempted pecking the other through the wire mesh during the 15 minutes, a behaviour that was infrequent between individuals that were normally housed next to each other.

Manual restraint for 15 minutes resulted in a significant increase in corticosterone in all birds. The magnitude of the response was similar for all birds indicating that the stressor elicited a maximum adrenocortical response. Corticosterone concentrations had declined to initial concentrations by 30 minutes, as reported for female quail handled for 15 minutes (Boyd, 2000). The adrenocortical responses varied between birds restrained and immobilised in a small metal cage for 15 minutes, with responses ranging from small increases in corticosterone to increases identical to those after 15 minutes of manual restraint. Mechanical restraint is a weaker stressor than manual restraint, so is preferable to manual restraint for investigating

differences between birds in their corticosterone responses. The response of quail to mechanical restraint in the current study is not unlike that reported by Satterlee and Johnson (1988), where mechanical restraint was used to discriminate between birds with low or high corticosterone responses to immobilisation.

3.4.3 Adrenocortical and testosterone response to manual restraint and mechanical restraint

3.4.3.1 Plasma corticosterone

Initial corticosterone concentrations in quail in the previous two experiments and in this experiment were about 2 ng/ml, higher than the 1 ng/ml reported in quail by Satterlee and Johnson (1988), but similar to concentrations reported for chickens (Beuving and Vonder, 1978; Littin and Cockrem, 2001). Fifteen minutes of manual restraint initiated a corticosterone response at 15 minutes, a result similar to that reported in Section 3.3.1, where corticosterone increased to a peak of 12.53 ± 4.74 ng/ml whilst it was 7.95 ± 2.51 ng/ml in the current study. The peaks in corticosterone are comparable with those reported previously for quail (4 to 18 ng/ml; Satterlee and Johnson, 1988) and chickens (4 to 16 ng/ml; Beuving and Vonder, 1978; Beuving and Vonder, 1986; Littin and Cockrem, 2001), whilst significantly lower than those reported in free-living, wild birds subjected to a similar stressor (Silverin, 1998). In the current study, manual restraint initiated a strong response of similar magnitude in all birds. Previous studies showed a rapid decrease in corticosterone after the end of the stressor, returning to basal after 30 minutes in quail (Boyd, 2000), and 40 minutes in chickens (Littin and Cockrem, 2001).

Mechanical restraint initiated a significant corticosterone response, although as discussed in the previous experiment (Section 3.3.2), the response varied between birds and the mean response was less than that of manually restrained birds at 5.54 ± 1.75 ng/ml at 15 minutes. This highlights the individual variation of the corticosterone response to mechanical restraint in the quail. Boyd (2000) also reported variation in the corticosterone response to handling after 15 minutes. Handling was a weaker stressor than manual restraint in the current experiment, whilst mechanical restraint in the current study initiated a varying adrenocortical

response resulting in varying responses as reported in the previous study and by Satterlee and Johnson (1988) and Boyd (2000).

3.4.3.2 Plasma testosterone

Manual and mechanical restraint resulted in a significant decrease in plasma testosterone concentrations in both groups after 15 minutes. Testosterone remained low for up to 120 minutes in the mechanical restraint group while it continued to decline until 60 minutes in the manual restraint group. In the previous chapter, the decrease in testosterone was more rapid following serial blood sampling of the same bird, while Heiblum *et al.* (2000) reported testosterone increased 10 minutes after mechanical restraint in 75% of chickens.

Testosterone declined more rapidly in this comparison of manual and mechanical restraint than that described in Section 3.4.1.2. In the current study, plasma testosterone concentrations started higher at 4.5 – 5 ng/ml compared with 2.7 – 3 ng/ml in the earlier study. It is possible that the inhibitory effect of manual and mechanical restraint on testosterone is greater for birds with higher basal testosterone. Testosterone concentrations were their lowest at 1 – 1.5 ng/ml, 60 minutes after birds were first taken from their cages and manually restrained for 15 minutes in both the current study and in Section 3.4.1.2.

15 minutes of manual and mechanical restraint clearly initiated a decline in testosterone concentrations. Testosterone was lowest at 30 and 60 minutes in mechanical and manual restraint groups respectively, while it was unchanged in untreated birds. In contrast, the results in the previous chapter showed that serial blood sampling resulted in a decline in testosterone concentrations in untreated as well as treatment birds. It is clear that blood sampling separate groups of birds at each sampling time is necessary when the effect of a single stressor on plasma testosterone is being examined.

Manual and mechanical restraint both initiated an increase in plasma corticosterone concentrations which was associated with a decline in plasma testosterone. However, it cannot be concluded from these results alone that elevated

corticosterone caused the decrease in testosterone secretion. A previous study showed that a single injection of corticosterone (1.2 mg/bird) causes a significant decline in plasma testosterone, still measurable after 3 hours (Boyd, 2000). Unlike the study in Chapter 3, the first blood sample after the injection was collected at 3 hours meaning repeated handling was unlikely to have caused the decline.

The decline in testosterone in response to mechanical restraint was of the same duration as that in manually restrained birds, but of a lesser magnitude which may reflect the smaller corticosterone response. However, as discussed earlier manual restraint initiated a similar corticosterone response in each bird, whilst mechanical restraint resulted in a range of responses. This difference could not be attributed to differences in gonad size as all birds had large testes, although Boyd (2000) reported a larger corticosterone response to manual restraint in female quail with larger gonads compared with birds with small gonads. This result indicates that the size of the corticosterone response may be related to the testosterone concentration of the individual. Previous studies have reported a positive correlation between high testosterone and a large corticosterone response (Boyd, 2000; Heiblum *et al.*, 2000).

3.4.4 Summary

The aims of this study were to determine the relationships between the duration of manual restraint and the magnitude of changes in plasma corticosterone and testosterone concentrations in quail, and to examine responses of quail to other stressors. Two minutes of manual restraint had no effect whereas 5, 10 or 15 minutes of restraint led to increased corticosterone and decreased testosterone.

Decreases in testosterone in quail and chickens have previously been shown only in birds that were handled on more than one occasion (Wilson *et al.*, 1979; Eskeland and Blom, 1979; Johnson, 1981; Chapter 2). The duration of reduced testosterone concentrations was greater than the duration of increased corticosterone, and the rate of testosterone decline was inversely related to initial testosterone concentrations.

The results are consistent with a transient inhibitory action of corticosterone on the control of testosterone secretion. If the testosterone response to restraint is indeed mediated by corticosterone then this may be directly at the gonads. A reduction in testosterone with no change in LH was shown in the previous chapter, and corticosterone has a direct inhibitory effect on testosterone synthesis in rat leydig cell *in vitro* (Mann and Orr, 1990) and on steroidogenesis in the quail cloacal gland (Deviche *et al.*, 1982).

Corticosterone responses to stressors in birds are already measured by restraining birds in bags (Wingfield *et al.*, 1992; Wingfield, 1994) or sometimes in a mechanical device (Satterlee and Johnson, 1988). The responses are therefore to artificial stressors, and little is known about the acute corticosterone responses of birds to natural stressors. Responses of quail to situations that might impose social stressors were therefore tested in the current study. Surprisingly, the introduction of an unfamiliar quail into a bird's home cage was not a stressor, whereas the introduction of a quail into an adjacent cage did induce corticosterone responses in some birds. Previous studies of quail have shown that the introduction of an unfamiliar albino quail into the cage did cause a corticosterone response (Satterlee *et al.*, 1983). The quail used by Satterlee are generally more responsive to stressors than the quail used in the current study (J. F. Cockrem *pers. comm.*), which may account for the different results. The absence of consistent corticosterone responses to social stimuli meant that they would not be used for more detailed studies of responses of quail to natural stressors.

Corticosterone responses to manual restraint in which birds were held by the experimenter for 15 minutes were consistently high, whereas responses to mechanical restraint varied markedly between birds. Manual restraint was therefore perceived by the birds to be a stronger emotional stressor than mechanical restraint. The range of responses was similar to that in chickens (Littin and Cockrem, 2001) and great tits (Cockrem and Silverin, 2002a). This variation in quail corticosterone responses was investigated in detail and quantified in a study reported in the next chapter.

Chapter 4

Variation within and between birds in corticosterone responses of Japanese quail

4.1 Introduction

Corticosterone is the major adrenal glucocorticoid hormone in birds (Carsia and Harvey, 2000). Exposure to a stressor initiates an increase in plasma corticosterone concentrations. In Japanese quail, immobilisation caused corticosterone concentrations to increase within one minute, reach a peak after four minutes and generally remain elevated during 20 minutes of mechanical restraint (Satterlee and Johnson, 1988). In the previous chapter it was shown that 15 minutes of handling or mechanical restraint resulted in a corticosterone peak at 15 minutes, followed by a return to basal concentrations between 15 and 60 minutes. The results of the previous experiment illustrated that significant variation in the corticosterone response to stressors exists between birds from the same population as reported previously in quail (Satterlee and Johnson, 1988) and willow tits (Silverin, 1997).

Differences in the adrenocortical response have been reported in a wide variety of free-living and domesticated birds. The majority of studies have concentrated on ecological factors such as differences between species (Wingfield *et al.*, 1992), geography (Silverin, 1997), or breeding or life stages (Wada *et al.*, 1999; Romero *et al.*, 1997; Romero and Remage-Healey, 2000). An area that has received little attention are the differences in hypothalamic-pituitary-adrenal (HPA) axis sensitivity to stressors within and between individual birds under constant conditions. Studies where birds are selected for divergent adrenocortical responses

to stressors such as low temperatures (Brown and Nestor, 1973), social stress (Gross and Siegel, 1985), or immobilisation (Satterlee and Johnson, 1988) are evidence of significant differences in the corticosterone response to stress between birds, irrespective of external influencing factors. There is likely to be a genetic basis to this variation in birds, as in other vertebrates (reviewed by Morméde *et al.*, 2003).

In almost all previous studies of corticosterone responses to handling, individual birds were bled on only one occasion. It has been presumed that the corticosterone response of a bird on a single occasion indicated its sensitivity to handling. However, recent studies in our laboratory on chickens (Littin and Cockrem, 2001) and great tits (Cockrem and Silverin, 2002a) were the first to show that individual responses are indeed repeatable and to quantify variation in responses. In addition, these two studies showed that the corticosterone response is generally repeatable within birds.

In the previous chapter, the results showed that the size of the corticosterone response varied between quail from the same population. The aim of the present study was to define variation in corticosterone responses within and between Japanese quail, and at the same time determine the repeatability of the response.

4.2 Methods and materials

4.2.1 Animals and housing

4.2.1.1 Source and type of birds

Eight week-old male Japanese quail (*Coturnix coturnix japonica*) were purchased from our supplier (Rangitikei Game Birds Ltd., Bulls). The birds had been raised under a long-day photoperiod (15L:9D) at air temperatures of 20 to 25°C in mixed sex groups. The birds were sexually mature at time of purchase with all birds having large cloacal glands and cloacal foam present.

4.2.1.2 Housing conditions

Each bird was identified with a numbered leg band and housed in an individual cage measuring 20 cm (W) × 35 cm (D) × 24 cm (H) in animal rooms in the Veterinary Science building. Quail were held on a long day photoperiod (16L:8D; lights on from 0900 – 0100 h). An extractor fan provided ventilation for each room during each experiment. A temperature control unit enabled the air temperature to be maintained at 20°C. Light in each room was provided by two 75W incandescent light bulbs controlled by a 24 hour/7 day time switch (HPM Excel Light Switch and Timer, Cat XL770T). The light intensity in the quail rooms was 130 lux in cages at the top, and 15 lux in cages near the floor of the room (Minolta Illuminance meter).

4.2.1.3 Food and water

Quail were provided with fresh water and food (Harvey Farms quail layer mash) *ab libitum*. The food was the same as that used by Rangitikei Game Birds.

4.2.2 Experimental design

Twenty 8 week old male Japanese quail were held with *ad libitum* access to food and water under long days (16L:8D) at 20°C for 10 days. The birds were assigned into two groups; birds in the untreated group (n=10) were blood sampled at 0, 15, 30, 60 and 120 min. Birds in the treated group (n=10) were blood sampled at 0 min and handled, handling comprised picking up and putting down the bird continuously for 15 min (Boyd, 2000). Blood samples in treated birds were then collected at 15, 30, 60, and 120 min. Birds were returned to their cage after time 0 in the untreated group, or 15 min in the handled group, and following all subsequent blood samples. Blood sampling and handling was repeated 10 and 20 days after the first instance. Birds were euthanased at the final sample time on day 20 and a terminal blood sample was collected.

4.2.3 Data collection

Blood samples were collected by venipuncture of the brachial vein in the wing with a 25 g needle and 200 μl of blood was collected into heparinised capillary tubes. All

samples were collected within 1.5 min from the time the bird was removed from the cage. The final blood sample was collected when the birds were euthanased by stunning followed by decapitation. The blood was stored on ice until centrifuged at 2 000 g for 15 minutes at 4°C (Heraeus Christ 5000S refrigerated centrifuge). The plasma was removed and stored at -20°C until assayed. The width and height of the cloacal protuberance was measured (±0.1 mm) and body weight was measured (±0.1 g) in all birds on the day before the experimental treatment. The reproductive system was removed after euthanasia and the testes weighed (±0.001 g).

The experiment was conducted under a protocol approved by the Massey University Animal Ethics Committee.

4.2.4 Hormone assays

Plasma corticosterone concentrations were determined by radioimmunoassay using the methodology outlined in Section 3.2.4.1. The inter-assay coefficients of variation for five assays were 7.4, 6.2, and 6.6% for high, medium and low quality controls respectively.

4.2.5 Calculation of integrated corticosterone responses

The area under the curve for plasma corticosterone concentrations versus time were calculated for each bleed for each bird, using the method described by Cockrem and Silverin (2002a). The 'total integrated response' (Cockrem and Silverin, 2002a) is a measure of the amount of corticosterone secreted during the sampling period and is expressed as ng/ml·min. The 'corrected integrated response' was also calculated by multiplying the corticosterone concentration at time 0 by the duration of experiment and subtracting this from the total integrated response area. Given that corticosterone peaks at 15 min and concentrations have largely returned to basal by 30 min, the corrected integrated response was calculated between 0 and 30 min and was not calculated for birds where subsequent corticosterone concentrations declined below the initial concentration. Areas under the curve were calculated using the trapezoid rule using GraphPad Prism 4.0 (GraphPad Software Inc.).

4.2.6 Statistical analyses

All results are presented as the mean ± SE (standard error) or individual data where appropriate. Plasma corticosterone concentrations were $\log_{10}$ transformed to minimise heteroscedasticity. All the data were examined for normality using Shapiro-Wilks tests and for equality of variance using Levene's homogeneity of variance test.

The changes in plasma corticosterone in birds sampled on three occasions were analysed using repeated measures two way ANOVA with time (0, 15, 30, 60 and 120 min) and bleed (first, second and third) as the grouping factors. Post-hoc comparisons were made between times for each bleed and between bleeds for each time using univariate F tests. Differences between bleeds in integrated corticosterone responses were examined by one way ANOVA followed by Bonferroni pairwise comparisons between pairs of bleeds. Differences between birds in corticosterone concentrations at each time and in integrated corticosterone responses were examined by one way ANOVA.

Data not complying with normality or variance criteria were compared using a Kruskal-Wallis non-parametric test followed by Mann-Whitney U independent group comparisons tests, or Friedman's test for repeat measures data. All comparisons of means were conducting using SPSS™ 11.0 (SPSS Inc., Illinois) or Systat™ 8.0 (SPSS Inc., Illinois).

4.3 Results

4.3.1 Corticosterone responses

The corticosterone response to handling was largely repeatable on three occasions within individual quail (Figure 4.1), with only two birds out of the 10 (#3 and 7) exhibiting marked variation between bleeds. Plasma corticosterone concentrations were low in the majority of birds when they were first sampled, although four birds had higher initial concentrations on the second sampling occasion. Plasma

corticosterone increased 15 min later in 90% of birds in each of the three blood sampling periods (Figure 4.1 and 4.2), then decreased between 15 and 30 min in all birds except for one (third bleed for bird 4; Figure 4.1). Corticosterone concentrations then remained relatively constant in all birds between 30 and 120 min (Figure 4.2). The corticosterone response to handling at 15 min was markedly larger for the second bleed in three birds (Nos. 2, 3, 8) and one bird for the third bleed (No. 2). Corticosterone was significantly higher at 15 min for the second bleed in two other birds (Nos. 5 and 7), but the size of the increase was no different from the first bleed. Initial corticosterone concentrations in bird no. 4 were high on all three occasions and declined after 15 min on the first and third bleed. On the second bleed, corticosterone remained high before declining by 30 min and remaining relatively constant.

Mean corticosterone concentrations in individual birds ranged from 1.01 ± 0.64 to 7.57 ± 0.28 ng/ml at 0 min, 4.57 ± 1.76 to 9.63 ± 1.67 ng/ml at 15 min, 1.85 ± 0.66 to 3.76 ± 1.84 ng/ml at 30 min, 1.09 ± 0.38 to 2.42 ± 0.68 ng/ml at 60 min, and from 0.91 ± 0.12 to 2.71 ± 1.25 ng/ml at 120 min (Figure 4.2). In all but bird no. 4, mean corticosterone concentrations increased after 15 min handling, then decreased to initial concentrations by 30 min and remained relatively constant.

Mean corticosterone concentrations remained low and relatively unchanged in birds that returned to their cages between samples (untreated; Figure 4.3a). In untreated birds corticosterone varied between bleeds and between times, but there was no significant interaction between bleed and time (Table 4.1). In handled birds, corticosterone varied between bleeds and between time, and there was a significant interaction between bleed and time (see Table 4.2 for all statistics for this analysis). Mean corticosterone concentrations at 0 min did not differ between bleeds in handled birds (Figure 4.3b) and increased after 15 min for all three bleeds. Corticosterone concentrations were higher at 15 min in the second bleed than in either of the other two bleeds. Plasma corticosterone declined after 15 min in all bleeds, and for the first bleed declined below initial concentrations and those of the other two bleeds at 30, 60 and 120 min. Elevated corticosterone concentrations at

15 min for all bleeds were significantly higher than in untreated birds for the same bleed, although corticosterone concentrations in the second bleed were significantly higher in handled birds versus untreated birds throughout the sampling period (Table 4.3).

The total integrated corticosterone responses varied between bleeds in handled birds ($F_{2,29} = 34.159$, $p < 0.001$; Figure 4.4b), with the response for the second bleed greater than the first and third bleeds (post-hoc Tukey tests, $p < 0.001$ and $p = 0.028$), and the third greater than than the first bleed (post-hoc Tukey tests, $p < 0.001$). Some of the birds had to be excluded from the calculation of the corrected integrated corticosterone response between 0 and 30 min due to corticosterone falling below initial concentrations. There were no differences in corrected corticosterone responses from 0 to 30 min between the three bleeds ($F_{2,13} = 1.029$, $p = 0.389$).

The total integrated corticosterone responses also varied in untreated birds ($F_{2,29} = 15.746$, $p < 0.001$; Figure 4.4a), with the response in birds for the third bleed greater than the responses for the first and second bleeds (post-hoc Tukey tests, $p = 0.001$ and $p < 0.001$). Corrected responses could be calculated for only a few birds, but did not appear to differ between bleeds.

4.3.2 Quantification of variation in corticosterone responses

Mean corticosterone concentrations for each time and the mean integrated corticosterone responses were calculated for each quail (Figure 4.5 and 4.6). There were close to significant differences between birds in corticosterone concentrations at the 0 and 15 min sampling times (see Table 4.4 for statistics for this analysis). The initial corticosterone concentrations in bird no. 4 was higher than most of the other birds. Using the method described by Cockrem and Silverin (2002a), the ratio of the largest to smallest value for corticosterone concentrations at each time and for integrated corticosterone responses were calculated (Table 4.5). The ratio was largest in the first sample, and was similar for all subsequent sampling times. The

ratio for the corrected integrated response was twice that for the total integrated response.

Using the method described by Cockrem and Silverin (2002a), variation within and between birds was quantified by calculating the coefficients of variation (CV) for corticosterone concentrations and integrated responses for each bird at each time (Table 4.6 and 4.7). Variation between birds, measured as the CVs of the mean of individual bird means, was greatest for corticosterone at 0 min (65.5%), and smaller at 15, 30, 60 and 120 min (24.5, 27.7, 24.4 and 24.5%). Variation within birds, measured as the means of the CVs of individual birds, was lower for corticosterone at 15 min (38.0%) than at other times. Variation between birds was lower for total integrated corticosterone responses (14.8%) than for corrected responses (36.0%), while variation within birds was higher for total integrated responses (45.0%) than for corrected responses (28.6%).

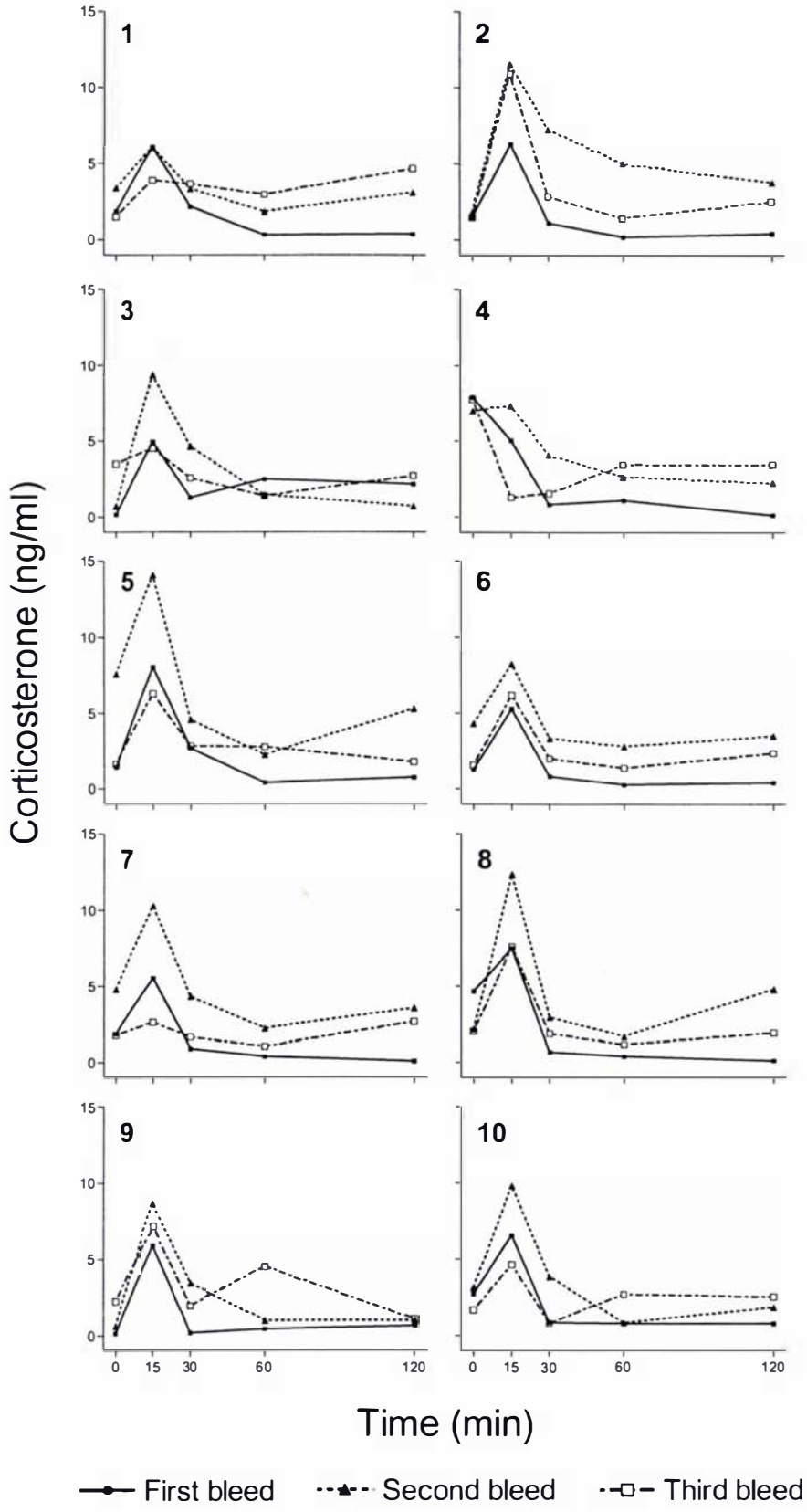


Figure 4.1 Plasma corticosterone in individual male quail handled for 15 min on three separate occasions at 10 day intervals. Results are plotted as raw data.

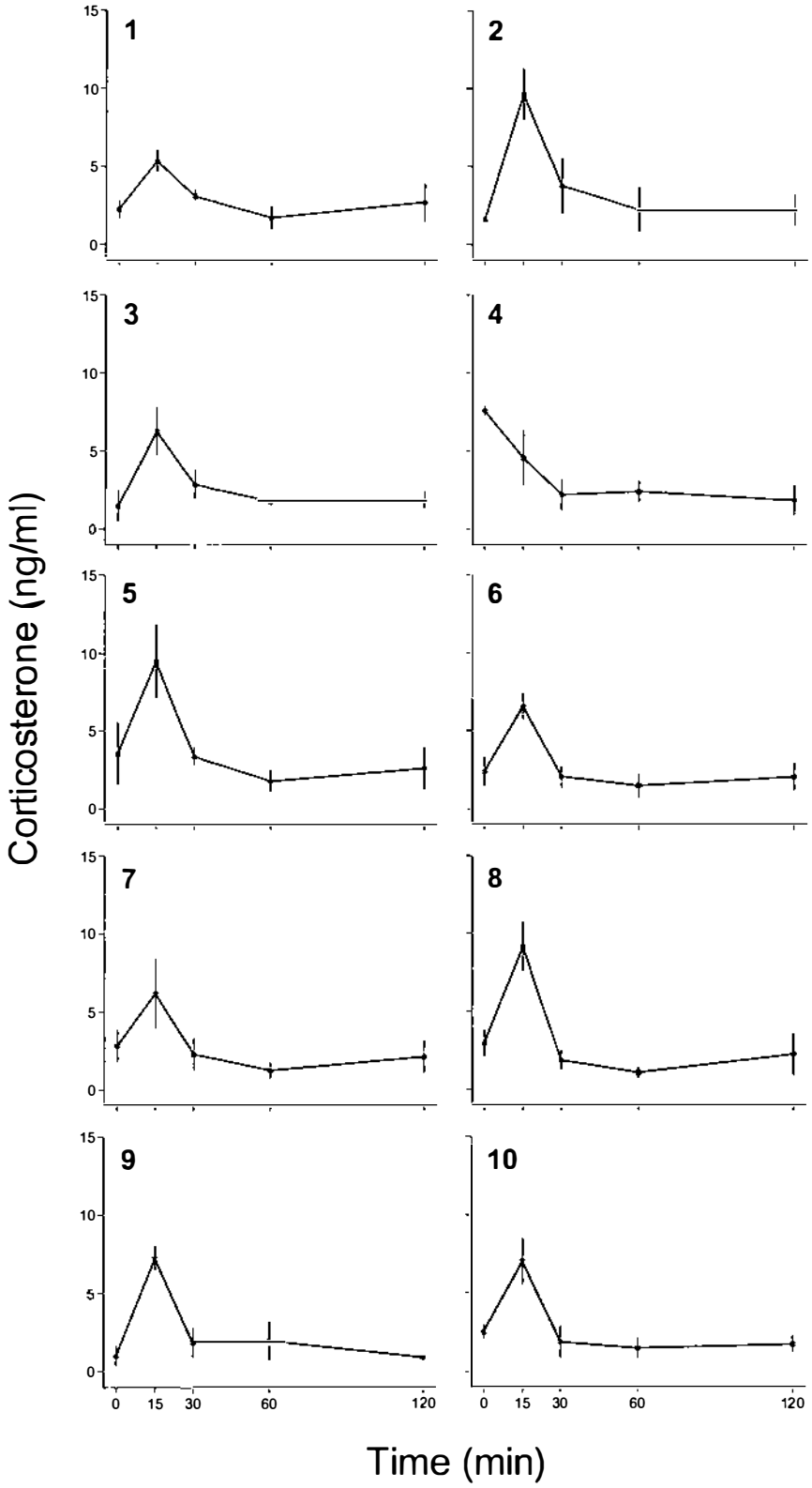


Figure 4.2 Mean plasma corticosterone concentrations in individual male quail handled for 15 min on three separate occasions at 10 day intervals. Results are plotted as means ± standard error.

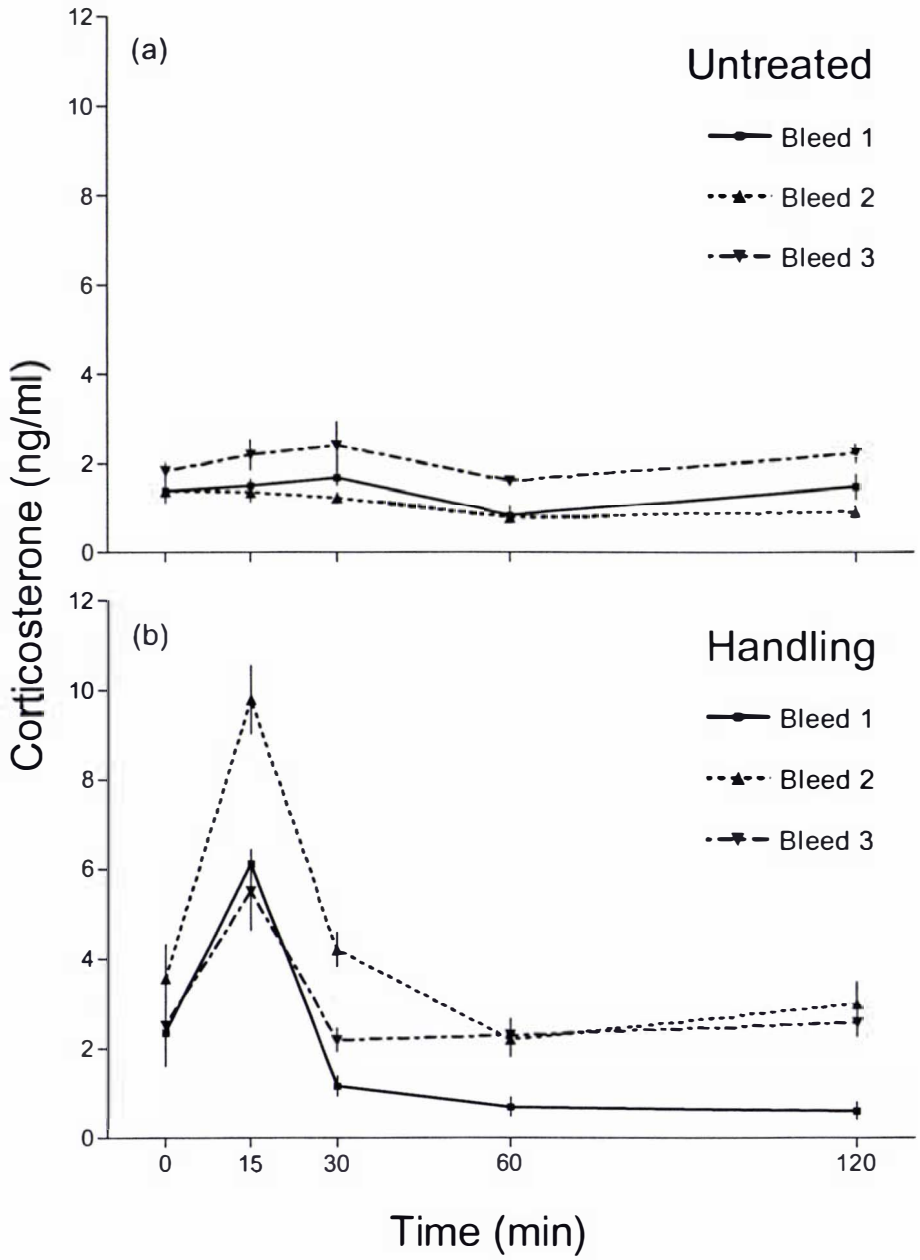


Figure 4.3 Mean plasma corticosterone concentrations in individual male quail that were returned to their cages between samples (untreated; a) or handled for 15 min (b) on three separate occasions at 10 day intervals. Results are plotted as means ± standard error.

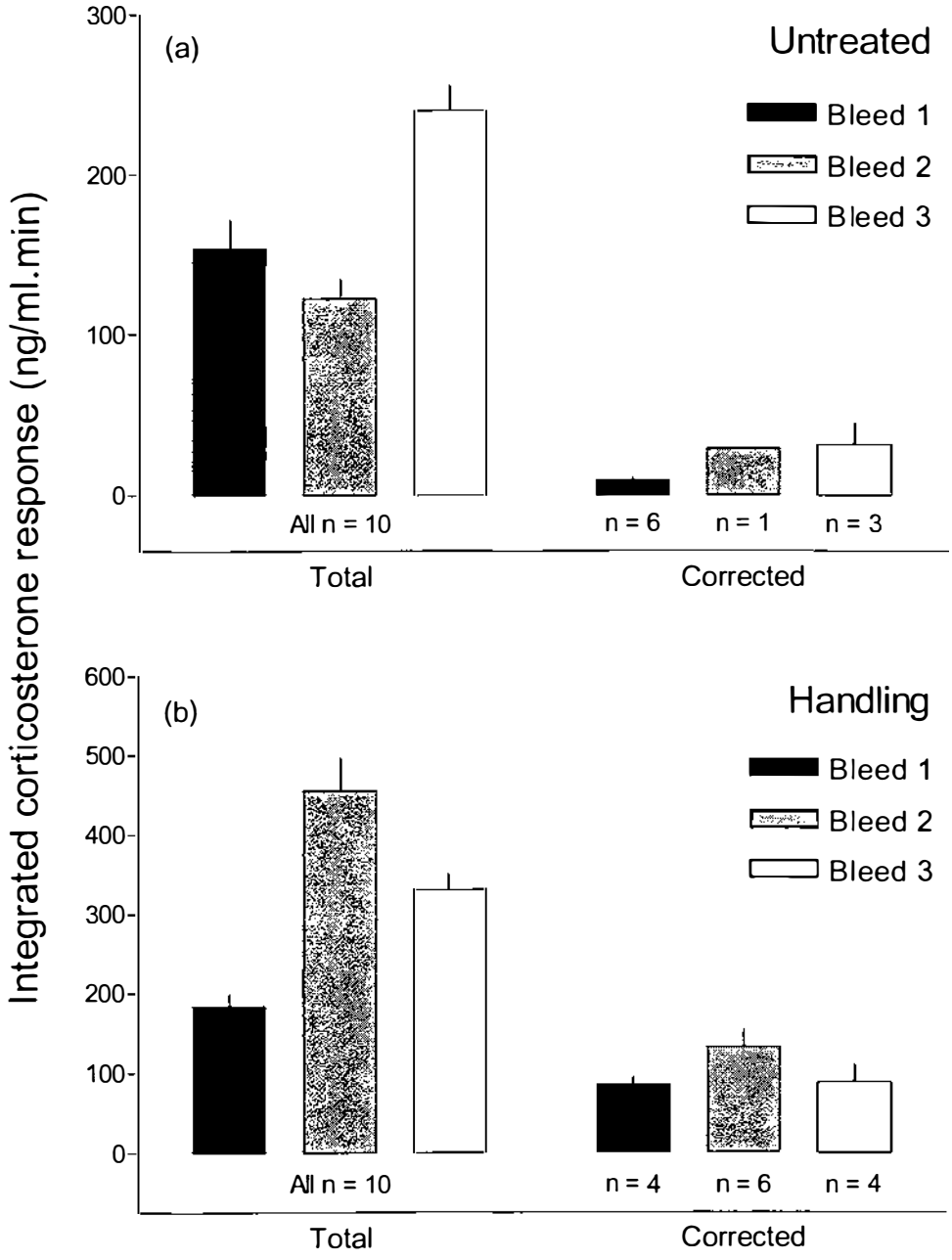


Figure 4.4 Total and corrected corticosterone responses of male quail that were (a) returned to their cages between samples (untreated); or (b) handled for 15 min on three separate occasions at 10 day intervals. Total integrated corticosterone responses were calculated from the areas under the corticosterone vs time curves for each bird. Corrected integrated corticosterone responses were calculated by subtracting the area under the corticosterone vs time (0–30 min) curve due to basal corticosterone concentrations from the total area. Results are plotted as means ± standard error for 10 birds.

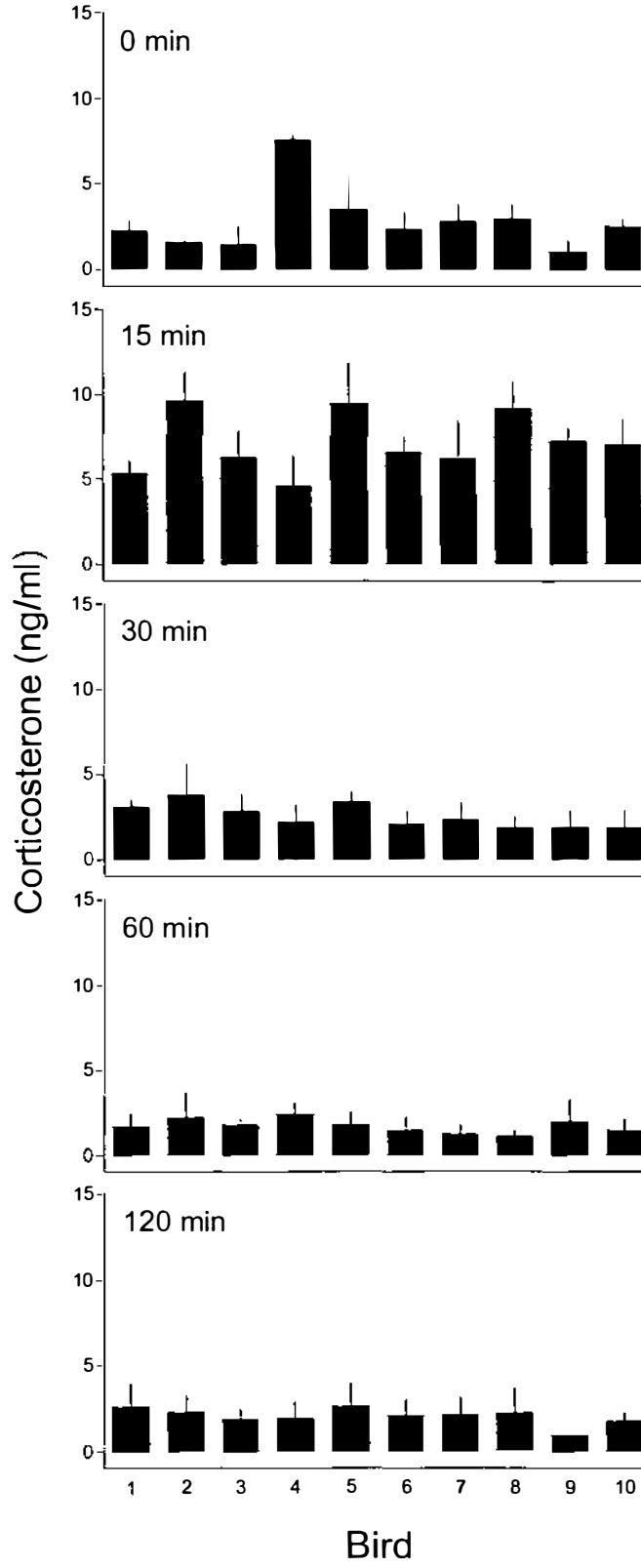


Figure 4.5 Mean corticosterone concentrations at 0, 15, 30, 60, and 120 min in individual male quail handled for 15 min on three separate occasions at 10 day intervals. Results are plotted as means ± standard error.

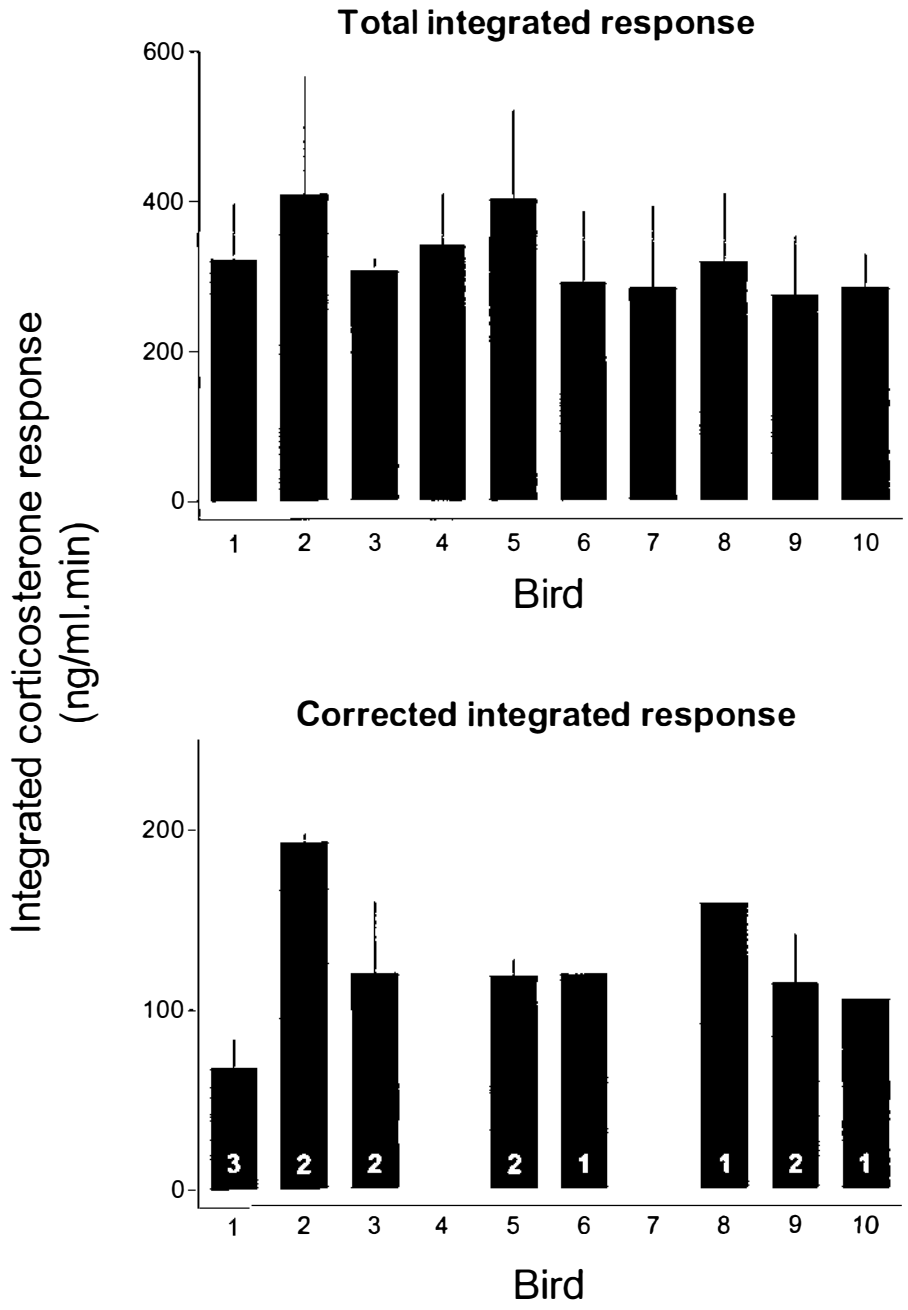


Figure 4.6 Mean total and corrected (0–30 min) integrated corticosterone responses in individual male quail handled for 15 min on three separate occasions at 10 day intervals. The number of corrected integrated responses per bird are shown inset in the columns. Results are plotted as means ± standard error.

Table 4.1 Statistical analysis for variation in the corticosterone response in birds that were returned to their cages between samples (untreated) on three separate occasions at 10 day intervals.

Effect		*F*	*df*	*p*	
Bleed		8.755	2,27	0.001	**
Time		7.333	4,108	< 0.001	**
Interaction of bleed and time		1.614	8,108	0.129	
Comparisons within treatments for each bleed					
0 vs 15 min	Bleed 1	1.481	1,27	0.234	
	Bleed 2	0.195	1,27	0.662	
	Bleed 3	0.335	1,27	0.567	
15 vs 30 min	Bleed 1	0.224	1,27	0.640	
	Bleed 2	0.053	1,27	0.819	
	Bleed 3	0.084	1,27	0.774	
30 vs 60 min	Bleed 1	22.515	1,27	< 0.001	**
	Bleed 2	6.797	1,27	0.015	*
	Bleed 3	2.399	1,27	0.133	
60 vs 120 min	Bleed 1	11.079	1,27	0.003	**
	Bleed 2	0.273	1,27	0.606	
	Bleed 3	2.092	1,27	0.160	
0 vs 30 min	Bleed 1	5.932	1,27	0.022	*
	Bleed 2	0.113	1,27	0.739	
	Bleed 3	1.555	1,27	0.223	
0 vs 60 min	Bleed 1	7.395	1,27	0.011	*
	Bleed 2	7.741	1,27	0.010	*
	Bleed 3	0.292	1,27	0.593	
0 vs 120 min	Bleed 1	1.238	1,27	0.276	
	Bleed 2	6.866	1,27	0.014	*
	Bleed 3	1.549	1,27	0.224	
Comparisons between bleeds for each time		*F*	*df*	*p*	
0 min	Bleed 1 vs Bleed 2	0.194	1,27	0.663	
	Bleed 1 vs Bleed 3	3.013	1,27	0.094	
	Bleed 2 vs Bleed 3	1.677	1,27	0.206	
15 min	Bleed 1 vs Bleed 2	0.898	1,27	0.352	
	Bleed 1 vs Bleed 3	1.812	1,27	0.189	
	Bleed 2 vs Bleed 3	5.262	1,27	0.030	*
30 min	Bleed 1 vs Bleed 2	2.280	1,27	0.143	
	Bleed 1 vs Bleed 3	2.527	1,27	0.124	
	Bleed 2 vs Bleed 3	9.608	1,27	0.004	**
60 min	Bleed 1 vs Bleed 2	0.195	1,27	0.662	
	Bleed 1 vs Bleed 3	13.092	1,27	0.001	**
	Bleed 2 vs Bleed 3	10.092	1,27	0.004	**
120 min	Bleed 1 vs Bleed 2	4.888	1,27	0.036	*
	Bleed 1 vs Bleed 3	5.251	1,27	0.030	*
	Bleed 2 vs Bleed 3	20.270	1,27	< 0.001	**

Note. The first three rows show the results of the two-way repeat measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each bleed and comparisons of bleed at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 4.2 Statistical analysis for variation in the corticosterone response in birds that were handled for 15 min on three separate occasions at 10 day intervals.

Effect		*F*	*df*	*p*	
Bleed		38.735	2,27	< 0.001	**
Time		30.958	4,108	< 0.001	**
Interaction of bleed and time		4.281	8,108	0.001	**
Comparisons within treatments for each bleed					
0 vs 15 min	Bleed 1	19.463	1,27	< 0.001	**
	Bleed 2	13.824	1,27	0.001	**
	Bleed 3	5.678	1,27	0.024	*
15 vs 30 min	Bleed 1	110.052	1,27	< 0.001	**
	Bleed 2	23.395	1,27	< 0.001	**
	Bleed 3	23.589	1,27	< 0.001	**
30 vs 60 min	Bleed 1	7.199	1,27	0.012	*
	Bleed 2	9.550	1,27	0.005	**
	Bleed 3	0.000	1,27	0.984	
60 vs 120 min	Bleed 1	1.002	1,27	0.326	
	Bleed 2	1.232	1,27	0.277	
	Bleed 3	0.505	1,27	0.483	
0 vs 30 min	Bleed 1	1.294	1,27	0.265	
	Bleed 2	1.664	1,27	0.208	
	Bleed 3	0.026	1,27	0.874	
0 vs 60 min	Bleed 1	8.056	1,27	0.009	**
	Bleed 2	0.896	1,27	0.352	
	Bleed 3	0.025	1,27	0.877	
0 vs 120 min	Bleed 1	9.013	1,27	0.006	**
	Bleed 2	0.026	1,27	0.874	
	Bleed 3	0.079	1,27	0.781	
Comparisons between bleeds for each time		*F*	*df*	*p*	
0 min	Bleed 1 vs Bleed 2	2.678	1,27	0.113	
	Bleed 1 vs Bleed 3	1.165	1,27	0.290	
	Bleed 2 vs Bleed 3	0.310	1,27	0.582	
15 min	Bleed 1 vs Bleed 2	6.825	1,27	0.015	*
	Bleed 1 vs Bleed 3	1.771	1,27	0.194	
	Bleed 2 vs Bleed 3	15.550	1,27	0.001	**
30 min	Bleed 1 vs Bleed 2	43.459	1,27	< 0.001	**
	Bleed 1 vs Bleed 3	11.968	1,27	0.002	**
	Bleed 2 vs Bleed 3	9.815	1,27	0.004	**
60 min	Bleed 1 vs Bleed 2	25.019	1,27	< 0.001	**
	Bleed 1 vs Bleed 3	26.636	1,27	< 0.001	**
	Bleed 2 vs Bleed 3	0.025	1,27	0.875	
120 min	Bleed 1 vs Bleed 2	33.523	1,27	< 0.001	**
	Bleed 1 vs Bleed 3	31.645	1,27	< 0.001	**
	Bleed 2 vs Bleed 3	0.027	1,27	0.871	

Note. The first three rows show the results of the two-way repeat measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each bleed and comparisons of bleed at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 4.3 One-way ANOVA analyses for comparison of untreated and handled corticosterone concentrations for each bleed at each time.

Comparisons between treatments for each bleed		*F*	*df*	*p*
Bleed 1	0 min	0.191	1,18	0.667
	15 min	126.210	1,18	< 0.001 **
	30 min	3.621	1,18	0.073
	60 min	0.597	1,18	0.450
	120 min	11.667	1,18	0.003 **
Bleed 2	0 min	5.641	1,18	0.029 *
	15 min	69.961	1,18	< 0.001 **
	30 min	84.544	1,18	< 0.001 **
	60 min	20.781	1,18	< 0.001 **
	120 min	16.582	1,18	0.001 **
Bleed 3	0 min	1.012	1,18	0.328
	15 min	13.482	1,18	0.002 **
	30 min	0.038	1,18	0.848
	60 min	1.964	1,18	0.178
	120 min	0.540	1,18	0.472

Note. One-way ANOVA for the main effects of treatment (untreated vs handled) for each bleed at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 4.4 One-way ANOVA analyses for corticosterone concentrations for each time and integrated corticosterone responses in birds that were handled for 15 min on three separate occasions at 10 day intervals.

Variable		*F*	*df*	*p*
Plasma corticosterone concentrations				
Time	0 min	2.386	9,29	0.051
	15 min	1.211	9,29	0.342
	30 min	0.555	9,29	0.817
	60 min	0.209	9,29	0.990
	120 min	0.260	9,29	0.979
Integrated corticosterone responses				
	Total	0.266	9,29	0.977
	Corrected	3.467	7,13	0.075

Table 4.5 Smallest and largest mean values of corticosterone concentrations and integrated corticosterone responses for individual quail.

	Corticosterone (ng/ml)					Integrated response (ng/ml·min)	
	0 min	15 min	30 min	60 min	120 min	Total	Corrected
Smallest value							
Bird number	9	4	8	9	9	9	7
Mean	1.01	4.57	1.85	1.09	0.91	274.66	52.55
Largest value							
Bird number	4	2	2	4	1	2	2
Mean	7.57	9.63	3.76	2.42	2.71	408.50	170.55
Ratio of smallest to largest value							
Mean	7.5	2.1	2.0	2.2	3.0	1.5	3.2

Table 4.6 Coefficients of variation of mean corticosterone concentrations and integrated corticosterone responses within and between quail.

	Corticosterone					Integrated response	
	0 min	15 min	30 min	60 min	120 min	Total	Corrected
All birds							
Mean	2.81	7.13	2.51	1.72	2.05	323.29	110.76
SE	0.41	0.52	0.29	0.23	0.27	25.79	12.32
CV	79.47	40.05	63.73	72.86	73.11	43.70	41.62
n	30	30	30	30	30	30	14
Variation between birds (means of the individual bird means)							
Mean	2.81	7.13	2.51	1.72	2.05	323.29	110.76
SE	0.58	0.55	0.22	0.13	0.16	15.15	14.09
CV	65.49	24.53	27.51	24.42	24.50	14.82	35.98
n	10	10	10	10	10	10	8
Variation within birds (means of the individual bird CVs)							
Mean	60.71	37.95	66.29	75.21	72.61	45.04	28.55
SE	12.57	5.17	7.25	7.77	7.38	5.60	5.49
n	10	10	10	10	10	10	5

Table 4.7 Coefficients of variation of mean corticosterone concentrations and integrated corticosterone responses within and between quail.

	Corticosterone at 0 min				Corticosterone at 15 min				Corticosterone at 30 min			
Bird	Mean (ng/ml)	SE	CV (%)	*n*	Mean (ng/ml)	SE	CV (%)	*n*	Mean (ng/ml)	SE	CV (%)	*n*
1	2.23	0.58	45.19	3	5.33	0.73	23.60	3	3.04	0.45	25.86	3
2	1.57	0.12	13.03	3	9.63	1.67	30.02	3	3.76	1.84	84.61	3
3	1.46	1.03	122.41	3	6.27	1.56	43.02	3	2.84	0.97	59.12	3
4	7.57	0.28	6.52	3	4.57	1.76	66.82	3	2.19	0.99	78.75	3
5	3.54	2.02	99.05	3	9.46	2.37	43.40	3	3.35	0.62	31.88	3
6	2.38	0.97	70.87	3	6.56	0.88	23.17	3	2.04	0.73	61.71	3
7	2.82	1.00	61.31	3	6.15	2.23	62.78	3	2.29	1.04	79.01	3
8	2.97	0.85	49.79	3	9.14	1.60	30.38	3	1.85	0.66	61.65	3
9	1.01	0.64	108.90	3	7.21	0.79	19.04	3	1.87	0.94	86.75	3
10	2.53	0.44	29.94	3	7.01	1.51	37.32	3	1.86	1.01	93.59	3

	Corticosterone at 60 min				Corticosterone at 120 min			
Bird	Mean (ng/ml)	SE	CV (%)	*n*	Mean (ng/ml)	SE	CV (%)	*n*
1	1.70	0.76	77.18	3	2.71	1.25	79.83	3
2	2.22	1.45	112.71	3	2.26	0.97	74.74	3
3	1.79	0.35	34.20	3	1.88	0.60	54.96	3
4	2.42	0.68	48.96	3	1.91	0.94	85.57	3
5	1.79	0.72	69.77	3	2.59	1.38	92.22	3
6	1.47	0.74	86.75	3	2.08	0.91	75.50	3
7	1.23	0.55	77.24	3	2.11	1.04	85.55	3
8	1.09	0.38	60.75	3	2.29	1.36	103.17	3
9	1.99	1.27	110.86	3	0.91	0.12	23.63	3
10	1.47	0.63	73.66	3	1.74	0.51	50.93	3

	Total integrated response				Corrected integrated response (0 – 30 min)			
Bird	Mean (ng/ml)	SE	CV (%)	*n*	Mean (ng/ml)	SE	CV (%)	*n*
1	323.00	73.73	39.54	3	52.55	7.45	24.55	3
2	408.50	155.91	66.10	3	170.55	17.45	14.47	2
3	306.03	16.92	9.58	3	119.65	39.55	46.75	2
4	340.73	68.83	34.99	3	-	-	-	-
5	402.36	119.02	51.23	3	93.69	15.31	23.11	2
6	290.80	95.91	57.12	3	72.23	-	-	1
7	283.56	108.88	66.50	3	-	-	-	-
8	318.83	90.01	48.90	3	158.30	-	-	1
9	274.66	77.76	49.04	3	113.92	27.29	33.87	2
10	284.40	44.94	27.37	3	105.20	-	-	1

4.4 Discussion

4.4.1 Variation in corticosterone responses

The results of this study contribute to our understanding of individual variation of the adrenocortical response from work previously conducted on chickens and great tits in our laboratory. Plasma corticosterone in quail increased after handling and the response was largely repeatable in individual quail, although the profile of the corticosterone response and basal corticosterone varied between birds. Variation in basal corticosterone concentrations between birds has been reported previously (Brown and Nestor, 1973; Schwabl, 1999). Earlier studies have concentrated on the influence of environmental factors such as season, geography or breeding state on the temporal modulations of the adrenocortical responses in birds (Wingfield *et al.*, 1992; Astheimer *et al.*, 1995; Romero *et al.*, 1997; Remage-Healey and Romero, 2000). These studies have noted marked variation of the corticosterone response between birds but have not determined if the observed corticosterone responses were repeatable in the same bird.

Under the same conditions, different individuals can have a range of corticosterone responses to an identical stressor. Vleck *et al.* (2000) reported that individual Adelie penguins (*Pygoscelis adeliae*) maintained distinct differences in basal corticosterone concentrations throughout the breeding season. The current study confirms that individual differences in corticosterone are repeatable within individuals (e.g. different quail can have low or high corticosterone concentrations in response to mechanical restraint (Satterlee and Johnson, 1988; Jones *et al.*, 2000)).

In the current study, plasma corticosterone concentrations increased in all but one quail after 15 minutes of handling. Similar results were reported for chickens (Littin and Cockrem, 2001). Initial and basal corticosterone concentrations were low and consistent with those reported in the previous chapter and by Satterlee and Johnson (1988). One bird had high initial corticosterone concentrations on all three blood sampling sessions, which declined after the initial sample in contrast to all other birds. This bird was sampled within one minute of being removed from a

cage, making it unlikely that the blood sampling procedure was the cause of such high basal corticosterone concentrations.

In the remainder of birds, corticosterone generally declined to initial concentrations between 15 and 30 minutes after the onset of handling and remained low between 30 and 120 minutes, as reported in the previous chapter and by Boyd (2000). Selecting birds for divergent corticosterone concentrations has been used in several studies to produce strains with low or high basal or adrenocortical responses to a stressor. Selection for low or high basal corticosterone concentrations or responses has been made in chickens (Edens and Siegel, 1975), turkeys (Brown and Nestor, 1973) and quail (Satterlee and Johnson, 1988). Such studies emphasize the genetic origin of variation of the adrenocortical response and differences in hypothalamic-pituitary adrenal (HPA) axis sensitivity to stressors between individuals. In the current study all birds were held and handled under identical conditions, with the observed variation especially that of bird no. 4 indicating inherent genetic differences in adrenocortical responsiveness within the birds. Variation of the adrenocortical response between individuals has been documented in a wide range of vertebrates, and the genetic factors controlling it are currently being studied in rodents (reviewed by Morméde *et al.*, 2003).

Cockrem and Silverin (2002a) reported that the corticosterone response decreased over three repeat bleed occasions in great tits, indicating possible habituation to handling. In that study, great tits were captured from a free-living situation. Repeated handling of chickens on successive days, a domesticated species, resulted in no change in the corticosterone response to handling (Beuving and Vonder, 1978; Littin and Cockrem, 2001). Quail in the current study had similar initial corticosterone concentrations for all three bleeds as reported for chickens handled on four occasions (Littin and Cockrem, 2001) and great tits on three occasions (Cockrem and Silverin, 2002a). In contrast, previous studies in chickens have also reported a reduction in basal corticosterone concentrations in chickens after one week of twice daily handling (Webb and Marshaly, 1984) and a reduction in the corticosterone response over three weeks with handling repeated ten times per week (Freeman and Manning, 1979).

4.4.2 Quantification of variation in corticosterone responses

Variation in the corticosterone response to handling between and within individual quail was quantified using the approach applied previously in our laboratory in chickens (Littin and Cockrem, 2001) and great tits (Cockrem and Silverin, 2002a). Mean corticosterone concentrations did not differ between quail at any of the sampling times, although differences between birds at 0 minutes were almost significant with high concentrations in one bird. This indicates that corticosterone concentrations were more consistent between birds during the corticosterone response than were basal concentrations, whereas peak concentrations were the most consistent. In chickens, corticosterone varied significantly at 15 and 40 minutes but not at 0 minutes (Littin and Cockrem, 2001). The ratios of the largest to the smallest mean corticosterone concentrations of individual birds were also more consistent between 15 and 120 minutes than at 0 min. Neither the total or corrected integrated corticosterone responses differed between birds, whereas both varied in chickens (Littin and Cockrem, 2001) and the total but not the corrected response varied between great tits (Cockrem and Silverin, 2002a). The results of the current experiment indicate that the corticosterone responses were more consistent between individual quail than great tits and chickens.

Variation between and within birds was also quantified for corticosterone concentrations and integrated responses using the method described by Cockrem and Silverin (2002a). The coefficients of variation between birds for corticosterone concentrations between 15 and 120 minutes were relatively consistent and all were lower than for concentrations at 0 minutes. These results are similar to those reported in great tits (Cockrem and Silverin, 2002a), and support our earlier findings that the corticosterone concentrations during the adrenocortical response are more consistent than initial corticosterone concentrations. The coefficient of variation within birds for corticosterone at 15 minutes was smaller than for concentrations before and after the peak, whereas in great tits initial concentrations varied most within birds (Cockrem and Silverin, 2002a), and in chickens the coefficients were largest at 0 and 15 min (Littin and Cockrem, 2001).

Calculating the coefficients of variation for all variables of the corticosterone response is a useful tool for quantifying the variation of the corticosterone responses between and within birds, and allows comparisons between species. These results showed that despite significant variation in the response within birds, variation in the response between birds is relatively consistent. There was considerably more variation in initial corticosterone concentrations, than in the response, as found for great tits (Cockrem and Silverin, 2002a) but not chickens (Littin and Cockrem, 2001).

4.4.3 Summary

This study was conducted to quantify variation within and between quail in their corticosterone responses to handling. Responses of individual birds were generally repeatable, as previously found in chickens (Littin and Cockrem, 2001) and great tits (Cockrem and Silverin, 2002a). The selection of lines of quail for low or high corticosterone responses (Satterlee *et al.*, 1988) indicates a genetic basis for variation in corticosterone responses. Heritable variation in corticosterone responses between birds could be due to variation in hypothalamic responses to a stimulus, variation in pituitary gland responses to hypothalamic releasing hormones (CRF, AVT and MT in birds), variation in adrenal gland responses to ACTH, or to variable responses at more than one level of the HPA-axis. Variation in pituitary and adrenal responses have been reported in other species (Carsia *et al.*, 1988; Astheimer *et al.*, 1992; Romero *et al.*, 1998a), but have not been investigated in quail. The study reported in the next chapter was therefore conducted to examine the sources of variation in corticosterone responses in quail.

Chapter 5

Adrenal and pituitary responsiveness in quail with differing corticosterone responses to mechanical restraint

5.1 Introduction

In Chapter 3 and 4, the size of the corticosterone response to mechanical restraint or handling was shown to vary significantly between individuals. Individual variation of the corticosterone response is thought to be partly due to differences at some level of the HPA-axis (Astheimer *et al.*, 1994; Romero *et al.*, 1998). In the present study, quail with low or high corticosterone responses to mechanical restraint were used to determine if the adrenal and pituitary levels of the HPA-axis differed in quail with divergent corticosterone responses.

In many free-living passerines, the corticosterone response to stressors change markedly during different stages of the life cycle (Romero *et al.*, 1997; Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero and Wingfield, 1999). However, the variation considered in this study is that which exists between birds irrespective of season, developmental or reproductive state. Variation in the adrenocortical response to a stressor exists between individual quail in a random-bred quail population such as the quail sourced for our previous studies. The selection of low and high adrenocortical response lines over many generations makes such lines an ideal model for testing hypotheses on the relationship between behavioural, physiological and genetic traits and the relationship between different aspects of

stress and the adrenocortical response (Jones *et al.*, 1992a; Jones *et al.*, 1994a; Satterlee and Jones, 1997; Jones *et al.*, 2000; Odeh *et al.*, 2003a; Odeh *et al.*, 2003b).

Despite the well established differences between individual quail in adrenocortical responsiveness to stressors, few studies have been conducted to identify the physiological basis of these differences. Changes in plasma corticosterone concentrations are the main indicator used to measure activation of the HPA-axis to a stressor. In the bird, corticosterone secretion is stimulated by adrenocorticotrophic hormone (ACTH) which is released from the anterior pituitary, which in turn is stimulated by corticotrophin-releasing factor (CRF) released from the hypothalamus (reviewed by Carsia and Harvey, 2000). The hypothalamic-hypophysiotrophic factors arginine vasotocin (AVT) and mesotocin (MT) also stimulate ACTH secretion or have a permissive role in the stimulation of ACTH by CRF (Castro *et al.*, 1986; Westerhof *et al.*, 1992; Romero and Wingfield, 1998). In turn, the release of many of these factors is influenced by feedback from glucocorticoids. Differences between birds in their corticosterone responses could be mediated at several levels of the HPA-axis, adrenal sensitivity to ACTH; pituitary sensitivity to CRF, arginine vasotocin (AVT) or mesotocin (MT); or the perception of a stressor.

The current experiments were conducted to determine the level or levels of the HPA-axis at which variation between birds in their corticosterone responses arise. Corticosterone responses to mechanical restraint were measured in 70 quail and the 20 birds with the lowest response (lowest 30%) and 20 birds with the highest response (top 30%) identified. The two different response groups were tested for differences in adrenal or pituitary responsiveness to doses of ACTH or CRF that stimulate a sub-maximal corticosterone response similar to that observed when birds undergo mechanical restraint. Corticosterone responses to ACTH and CRF treatment were measured in these birds to determine relationships between adrenal sensitivity to ACTH, pituitary sensitivity to CRF and the size of individual corticosterone responses to mechanical restraint. Ovine CRF was uses as avian CRF has not been sequenced and avian pituitary cells respond to ovine CRF (Carsia *et al.*, 1986). A synthetic adrenocorticotrophin (Synacthen™, synthetic peptide

corresponding to amino acids 1-24 from the N-terminal region of human ACTH) was used to test adrenal sensitivity; this form of ACTH stimulates corticosterone secretion in chickens (Littin and Cockrem, 2001) and ducks (Harvey *et al.*, 1980; Noirault *et al.*, 1999; Faure *et al.*, 2003).

5.2 Methods and materials

5.2.1 Animals and housing

5.2.1.1 Source and type of birds

Seventy 7 week-old male Japanese quail (*Coturnix coturnix japonica*) were purchased from a supplier (Canter Valley Farms, Christchurch) and held in quail rooms for 2 weeks before any manipulation. The birds had been raised under a long-day photoperiod (15L:9D) at air temperatures of 20 to 25°C in mixed sex groups. The 7 week-old birds were sexually mature at time of purchase with large cloacal glands and cloacal foam present.

5.2.1.2 Housing conditions

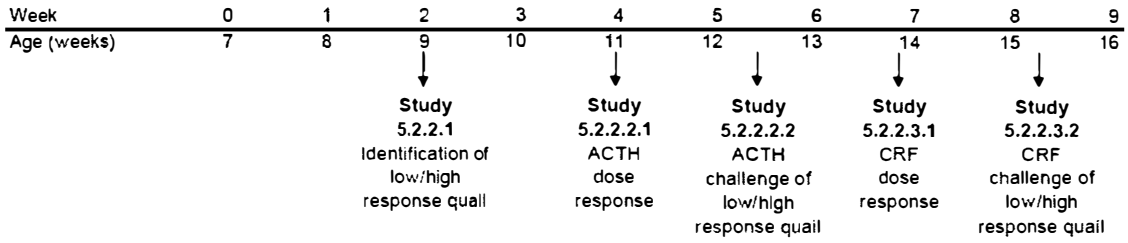
Each bird was identified with a numbered leg band. They were housed in individual cages measuring 20 cm (W) × 35 cm (D) × 24 cm (H) in animal rooms in the Veterinary Science building. Quail were held on a long day photoperiod (16L:8D; lights on from 0900 – 0100 h). An extractor fan provided ventilation for each room. A temperature control unit enabled the air temperature to be maintained at 20°C. Light in each room was provided by two 75W incandescent light bulbs controlled by a 24 hour/7 day time switch (HPM Excel Light Switch and Timer, Cat XL770T). The light intensity in the quail rooms was 130 lux in cages at the top, and 15 lux in cages near the floor of the room (Minolta Illuminance meter).

5.2.1.3 Food and water

Quail were provided with fresh water and food (Harvey Farms quail layer mash) *ab libitum*.

5.2.2 Experimental design

Timeline of Chapter Five treatments:



5.2.2.1 Identification of birds with low or high corticosterone responses to mechanical restraint in individual quail

The adrenocortical responses of 70 nine week old male Japanese quail were determined by subjecting each bird to 15 minutes of immobilisation by mechanical restraint in a metal cage using the method described in Chapter 4. Blood samples were collected immediately after the bird was removed from the quail room (0 min), and at 15 and 30 min). Corticosterone concentrations were measured in all blood samples by radioimmunoassay. Twenty birds with small corticosterone responses (< 3 ng/ml increase between 0 and 15 min) and 20 birds with high responses (> 5 ng/ml increase between 0 and 15 min) were identified and held for 14 days before use in further experiments. Quail with corticosterone responses < 3 and > 5 ng/ml between 0 and 15 min were chosen as the 20 lowest versus 20 highest responders to mechanical restraint respectively.

5.2.2.2 Relationship between the adrenocortical response and adrenal sensitivity to ACTH

5.2.2.2.1 Relationship between plasma corticosterone and dose of ACTH

Forty male Japanese quail were randomly selected from the pool of 70 quail and were assigned into five treatment groups (n=8 per group). The group allocation and known corticosterone response of each bird (as identified in Section 5.2.2.1) was checked to avoid placing a large proportion of low or high corticosterone response birds into one group. The corticosterone response the birds in each group s iden These groups received a single 100 μl injection of either saline or 0.05, 0.10, 0.20 or 0.50 IU of ACTH (Synacthen® depot, Novartis Pharmaceuticals, Auckland) into the jugular vein. Stock Synacthen® depot was diluted in a isotonic saline vehicle. A blood sample was taken at 0900 h (0 min, immediately before the injection) and

then further samples were taken 15, 30, 60 and 120 min after the injection. Plasma concentrations of corticosterone were measured in all blood samples. The birds were then rested for at least 10 days before further studies.

5.2.2.2.2 Relationship between a sub-maximal dose of ACTH and plasma corticosterone concentrations in low and high response quail

Synacthen® at a dose of 0.10 IU was selected as it caused a submaximal corticosterone response as shown in Section 5.2.2.2.1. Quail in the low and high response groups were given a single 100 μl injection of saline (n=6) or 0.10 IU of ACTH (Synacthen® immediate, Novartis Pharmaceuticals, Auckland) in 100 μl (n=14) of saline into the jugular vein. A blood sample was taken at 0900 h (0 min, immediately before the injection) and then further samples were taken at 15, 30, 60 and 120 min after the injection. Plasma concentrations of corticosterone were measured in all blood samples. The birds were then rested for at least 10 days before further studies.

5.2.2.3 Relationship between the adrenocortical response and pituitary sensitivity to CRF

5.2.2.3.1 Relationship between plasma corticosterone and dose of CRF

Forty male Japanese quail were randomly selected from the pool of 70 quail and were assigned into five treatment groups (n=8 per group). The group allocation and known corticosterone response of each bird (as identified in Section 5.2.2.1) was checked to avoid placing a large proportion of low or high corticosterone response birds into one group. These groups received either a single 100 μl injection of saline or 2, 4, 6 or 8 μg of CRF (synthetic ovine corticotrophin releasing factor C-3167, Sigma Chemical) dissolved in 100 μl of saline into the jugular vein. A blood sample was taken at 0900 h (0 min, immediately before the injection) and then further samples were taken at 15, 30, 60 and 120 min after the injection. Plasma concentrations of corticosterone were measured in all blood samples. The birds were then rested for at least 10 days before further studies.

5.2.2.3.2 Relationship between a sub-maximal dose of CRF and plasma corticosterone concentrations in low and high adrenocortical response quail

CRF at a dose of 6 μg was selected as it was the only dose that stimulated a consistent corticosterone response in all birds as shown in Section 5.2.2.3.1. Quail in the low and high response groups were given a single 100 μl injection of saline (n=6) or 6 μg of CRF (synthetic ovine corticotrophin releasing factor C-3167, Sigma Chemical) in 100 μl (n=14) of saline into the jugular vein. A blood sample was taken at 0900 h (0 min, immediately before the injection) and then further samples were taken at 15, 30, 60 and 120 min after the injection. Plasma concentrations of corticosterone were measured in all blood samples.

The experiments were conducted under a protocol approved by the Massey University Animal Ethics Committee.

5.2.3 Data collection

5.2.3.1 Corticosterone administration

ACTH (Synacthen® depot, Novartis Pharmaceuticals, Auckland) and CRF (synthetic ovine corticotrophin releasing factor C-3167, Sigma Chemical) solutions were prepared on the day of the experiment and then kept on a magnetic stirrer (Chiltern Scientific) at 4°C. Stock solutions were dissolved or mixed in 0.9% saline. ACTH and CRF solutions were administered with 1 ml insulin syringes and needles.

5.2.3.2 Tissue and blood samples

Blood samples were collected by venipuncture of the brachial vein in the wing with a 25 g needle, with 200 μl of blood collected into heparinised capillary tubes. All samples were collected within 1.5 minutes from the time the bird was removed from the cage. The blood was stored on ice until centrifuged at 2 000 g for 15 minutes at 4°C (Heraeus Christ 5000S refrigerated centrifuge). The plasma was removed and stored at -20°C until assayed.

5.2.3.3 Corticosterone assay

Corticosterone concentrations in quail plasma were measured by radioimmunoassay using an ICN ImmunoChem™ Double Antibody Corticosterone ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA). Samples were assayed in duplicate in five assays. Plasma was thawed and centifuged in a 1.5 ml Eppendorf tube at 14 000 g for 5 min (IEC Micromax ventilated microcentrifuge OM3590) to separate clear plasma from lipid within the plasma sample. A 10 μl aliquot of plasma was incubated with 100 μl of antibody (anti-corticosterone Cat # 07-120113) and 100 μl of iodinated corticosterone (approximately 5 000 cpm; ^{125}I-corticosterone Cat # 07-120121) at room temperature (22-25°C) for 2 hours. After incubation, 100 μl of precipitant solution (Cat # 07-166624) was added to all tubes and mixed using a Chiltern Scientific SS70 orbital shaker for approximately 5 s. Tubes were then centrifuged at 4 500 g for 15 min at 4°C and the supernatant aspirated. The precipitates were counted (3 min per sample) in a LKB Wallac 1261 Multigamma Gamma Counter.

The limit of sensitivity of the radioimmunoassay, defined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curve and expressed as corticosterone concentration in plasma, was 0.80 ng/ml. Serial dilutions of quail plasma in diluent buffer were parallel to the corticosterone standard curve (n=3). Recovery of ^{125}I-corticosterone added to quail plasma was 90.9 ± 3.8%, 99.2 ± 2.5%, and 90.4 ± 6.6% for three samples. Solutions of corticosterone in PBSG were prepared at concentrations that gave approximately 20, 50, and 80% binding on the standard curve. These solutions were used as high, medium and low quality control samples in every assay. Intra-assay coefficients of variation have yet to be determined. Inter-assay coefficients of variation in all 6 assays were 11.3, 10.5, and 9.9%.

The cross-reactions of the antibody with steroids tested by ICN Biomedicals were corticosterone (100.00%), desoxycorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.03%), progesterone (0.02%), androstenedione (0.01%), 5α-dihydrotestosterone (0.01%), cholesterol, dehyrdroepiandrosterone, dehyrdroepiandrosterone-sulphate, 11-desoxycortisol, 20α-dihydroprogesterone,

estrone, oestradiol-17α, oestradiol-17β, oestriol, pregnenolone, 17α-hydroxypregnenolone, and 17α-hydroxyprogesterone (all < 0.01%).

5.2.4 Calculation of the area under the corticosterone curve

The area under the curve for plasma corticosterone concentrations versus time was calculated for each bird, using the method described in Chapter Two. The 'total integrated response' (Cockrem and Silverin, 2002a) is a measure of the amount of corticosterone secreted during the sampling period and is expressed as ng/ml·min. Differences in basal corticosterone concentrations between birds were corrected by calculating a second measure called the 'corrected integrated response' by multiplying the corticosterone concentration at Time 0 by the duration of experiment and subtracting this from the total integrated response area. The corrected integrated response was only calculated for the period of the main response between 0 and 30 min, and not for birds where 15 and 30 min corticosterone concentrations fell below initial values. Areas under the curve were calculated using the trapezoid rule using GraphPad Prism 4.0 (GraphPad Software Inc.).

5.2.5 Statistical analyses

All results are presented as the mean ± SE (standard error) unless it was more appropriate to present individual data. All the data were examined for normality using Shapiro-Wilks tests and for equality of variance using Levene's homogeneity of variance test. Hormone data were $\log_{10}$ transformed to avoid heteroscedasticity.

If variances were homogenous then groups were compared with repeated measures two-way analyses of variance (ANOVA) with time and treatment as the grouping factors. Comparisons between times within each treatment and between treatments for each time were examined with post-hoc repeated measures contrasts. Data not complying with normality or variance criteria were compared using a Kruskal-Wallis non-parametric test followed by Mann-Whitney U independent group comparisons tests, or Friedman's test for repeat measures data.

All comparisons of means were conducting using SPSS™ 11.0 (SPSS Inc., Illinois) or Systat™ 8.0 (SPSS Inc., Illinois).

5.3 Results

5.3.1 Identifying low and high corticosterone responses to mechanical restraint

The adrenocortical response to 15 min of mechanical restraint varied between birds (Figure 5.1a). Initial corticosterone concentrations ranged from 1.19 to 8.86 ng/ml. Thereafter concentrations remained relatively constant in some birds but increased (2.95 – 17.99 ng/ml) in others after 15 min of mechanical restraint. Corticosterone concentrations had declined by 30 min in most birds, although it remained elevated or increased above baseline values in a few birds. Birds that showed low or high responses at 15 min were identified for a low (n=20) and high response (n=20) groups (Figure 5.1b-c).

There was a significant effect of mechanical restraint on corticosterone concentrations ($F_{2,209}$ = 37.374, $p < 0.001$; Figure 5.2a). Corticosterone concentrations were initially low (5.20 ± 0.20 ng/ml), increased at 15 min (9.52 ± 0.43 ng/ml) then decreased 30 min after the initial sample (6.60 ± 0.34 ng/ml) and remained higher than initial values (post-hoc Bonferroni test, $p = 0.007$). A separate analysis of birds selected for low and high corticosterone responses showed a significant effect of time ($F_{2,76}$ = 85.394, $p < 0.001$; Figure 5.2a) and group ($F_{1,38}$ = 30.499,$p < 0.001$), and a significant interaction between time and group ($F_{2,76}$ = 59.555, $p < 0.001$). Corticosterone concentrations in low response birds did not change over 30 min. Initial corticosterone concentrations did not differ between low and high birds ($F_{1,38}$ = 0.767, $p = 0.387$). Corticosterone concentrations increased in high response birds after mechanical restraint and were significantly higher than low response birds at 15 min ($F_{1,38}$ = 119.385, $p < 0.001$), then declined after 15 min and were higher than but did not differ significantly from low response birds at 30 min ($F_{1,38}$ = 3.958, $p = 0.054$).

Total and corrected integrated corticosterone responses of the high response group were greater than that of the low response group ($F_{1,39}$ = 55.563, $p < 0.001$ and $F_{1,27}$ = 48.656, $p < 0.001$ respectively; Figure 5.2b).

5.3.2 Relationship between the adrenocortical response and adrenal sensitivity to ACTH

5.3.2.1 Relationship between plasma corticosterone and dose of ACTH

Initial corticosterone concentrations were low in all groups and ranged from 1.07 – 7.24 ng/ml (Figure 5.3). Corticosterone concentrations remained low and relatively constant in all birds injected with saline (Figure 5.3a). There were increases in corticosterone concentrations between 0 and 15 min in 50% (4/8) of birds injected with the lowest dose (0.05 IU) of ACTH (Figure 5.3b). Corticosterone concentrations in these birds had largely declined to baseline by 60 min after the birds were first removed from their cages and remained relatively low at 120 min. Treatment with the next highest dose (0.10 IU) of ACTH stimulated a strong corticosterone response in 75% (6/8) of birds (Figure 5.3c). Corticosterone concentrations at 15 min varied between 5.64 and 14.00 ng/ml and then declined to baseline between 15 and 30 min in 88% of birds and remained low from 60 to 120 min. Treatment with 0.20 IU of ACTH stimulated a strong response in all birds, with corticosterone concentrations increasing to 7.49 – 19.37 ng/ml at 15 min, then declining at 30 min and remaining low thereafter (Figure 5.4d). The highest ACTH treatment (0.50 IU) stimulated the largest responses (Figure 5.3e), with corticosterone concentrations increasing in all birds to 10.72 – 25.50 ng/ml at 15 min. Corticosterone declined to initial concentrations in all but two birds by 30 min. Corticosterone continued to increase in one bird and was still high in another bird at 30 min, but declined at 60 min in both birds.

Corticosterone responses varied between ACTH dose and between time, with a significant interaction between dose and time (see Table 5.1 for all statistics for this analysis). Corticosterone concentrations were initially low in all five groups (control- and ACTH-treated) of birds (Figure 5.4a). Mean corticosterone concentrations in birds injected with saline remained low for the duration of the experiment. All doses of ACTH caused mean plasma corticosterone concentrations

to increase between 0 and 15 min, with a dose response relationship (Figure 5.4b). Corticosterone concentrations at 15 min in birds treated with 0.10, 0.20 or 0.50 IU per bird did not differ significantly from each other, but were significantly greater in the 0.05 IU group. Corticosterone declined and did not differ from initial concentrations between 15 and 30 min in all ACTH treated groups, and remained low and relatively constant at 60 and 120 min. Corticosterone at 15 min was no greater for birds treated with 0.50 IU than 0.20 IU, indicating that the maximum corticosterone response to an ACTH injection had been reached. The 0.10 IU dose resulted in a corticosterone response most similar to birds subjected to mechanical restraint.

There were significant differences between the groups in the total integrated corticosterone response between the groups (Figure 5.5; see Table 5.2 for all statistics for this analysis). The total integrated response increased in dose dependent manner, with the responses to 0.20 and 0.50 IU/bird of ACTH significantly larger than in birds injected with saline. The corrected corticosterone response varied between groups, and also increased in a dose dependent manner with a significant difference between the saline treated and 0.50 IU ACTH groups.

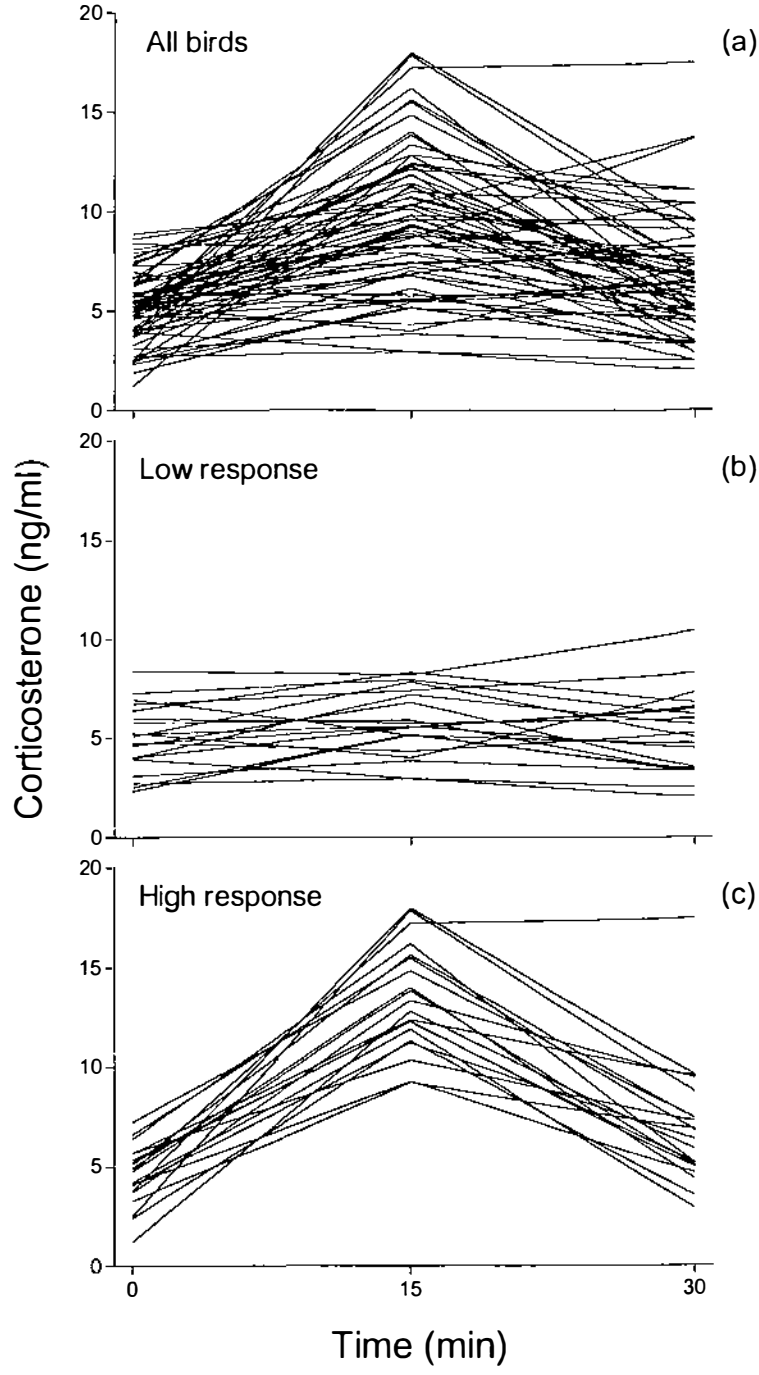


Figure 5.1 Plasma corticosterone in individual male quail immobilised by mechanical restraint for 15 min for (a) all birds (n=70), (b) birds with a low response to mechanical restraint (n=20), and (c) birds with a high response to handling (n=20). Results are plotted as raw data.

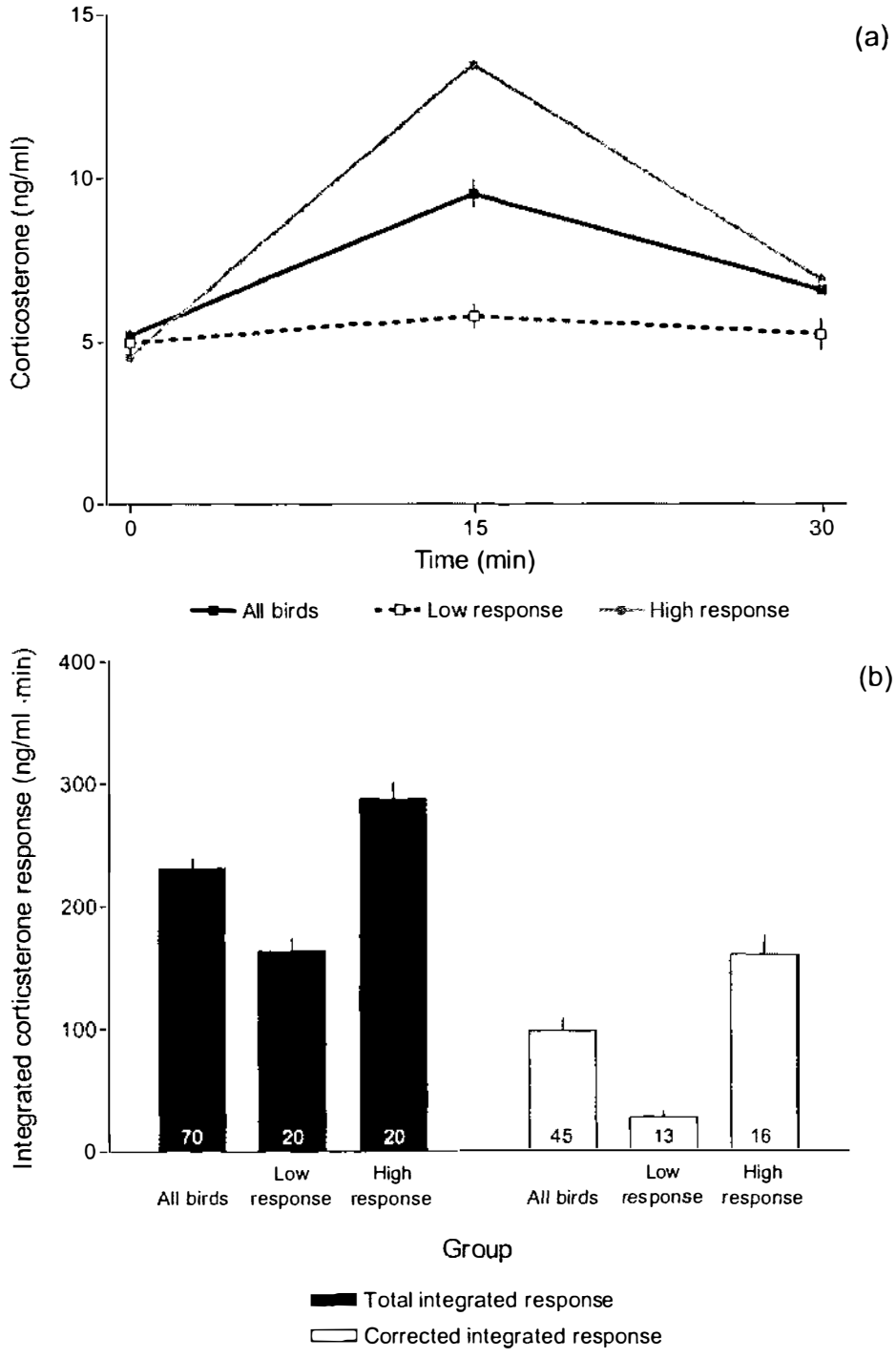


Figure 5.2 Mean plasma corticosterone concentrations (a) and total and corrected integrated corticosterone responses (*b*) in male quail immobilised by mechanical restraint for 15 min. Total integrated corticosterone responses were calculated from the areas under the corticosterone vs time curves for each bird, while corrected responses were calculated between 0 and 30 min. The number of birds per group are inset inside each column. Results are plotted as means ± standard error.

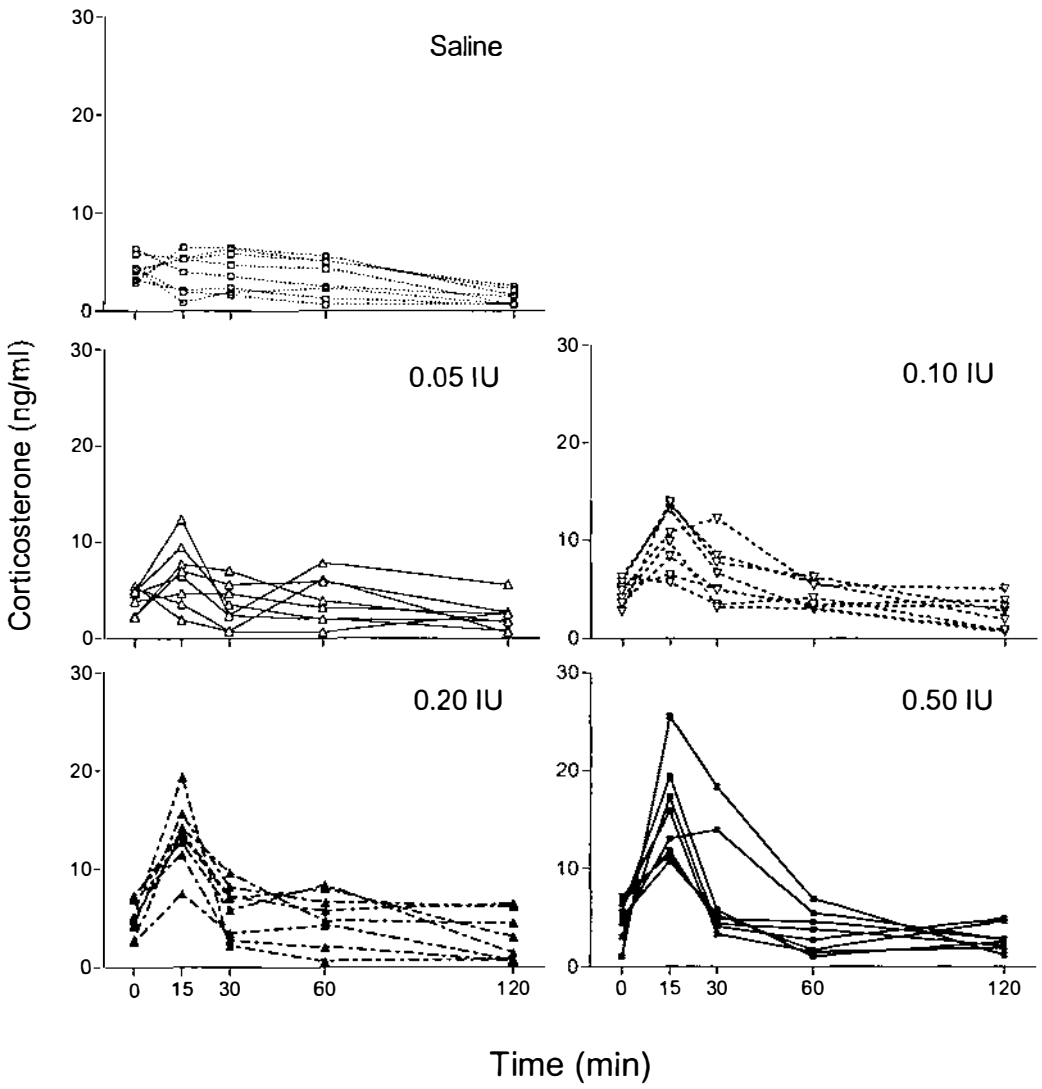


Figure 5.3 Plasma corticosterone concentrations in individual male quail that received a single injection of saline or 0.05, 0.10, 0.20, or 0.50 IU ACTH/bird immediately after the 0 min blood sample. Results are plotted as raw data.

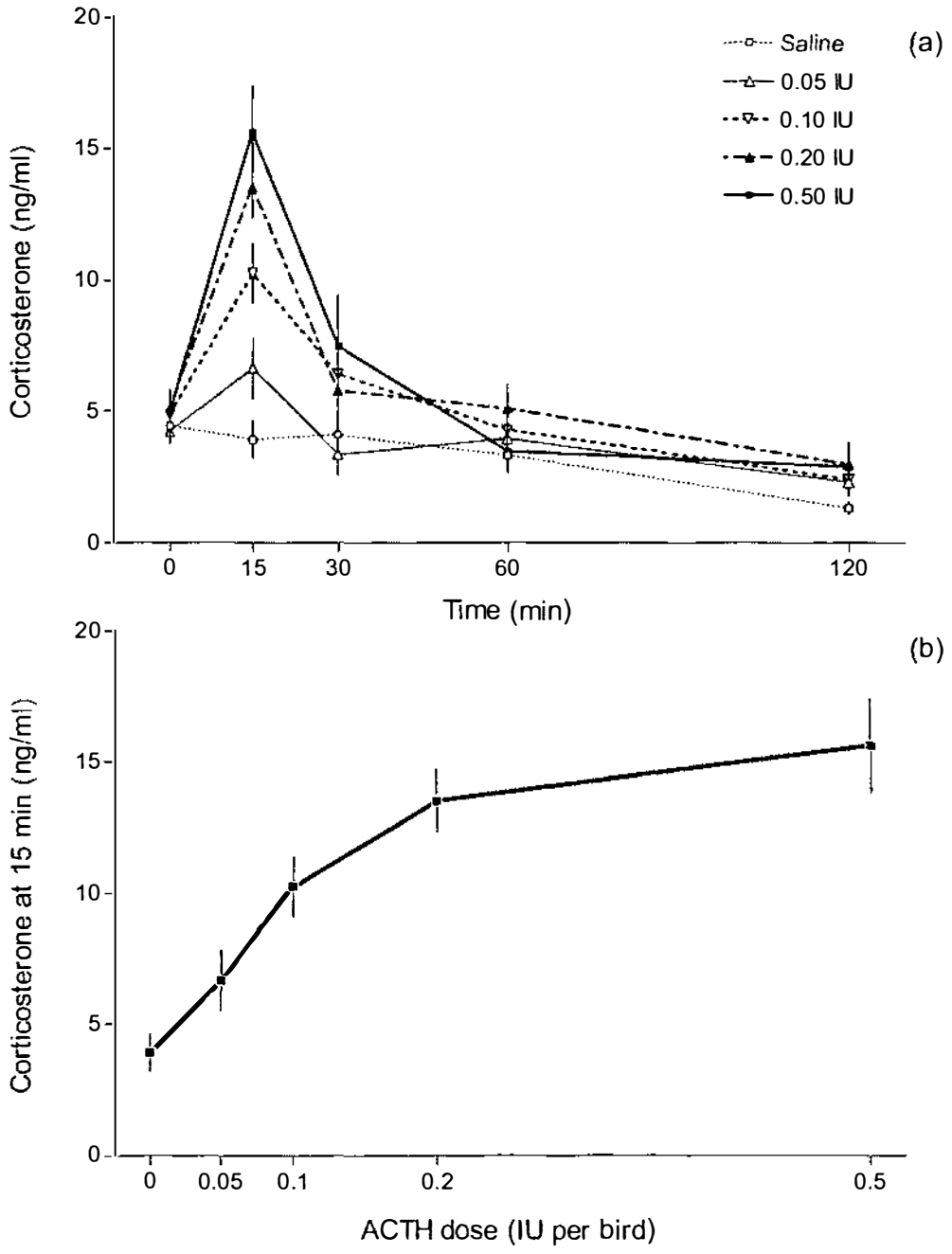


Figure 5.4 Mean plasma corticosterone concentrations in male quail that received a single injection of ACTH at a dose of 0.05, 0.10, 0.20 or 0.50 IU/bird (a); and mean plasma corticosterone concentrations 15 min after an injection of saline or ACTH (b). Results are plotted as means ± standard error.

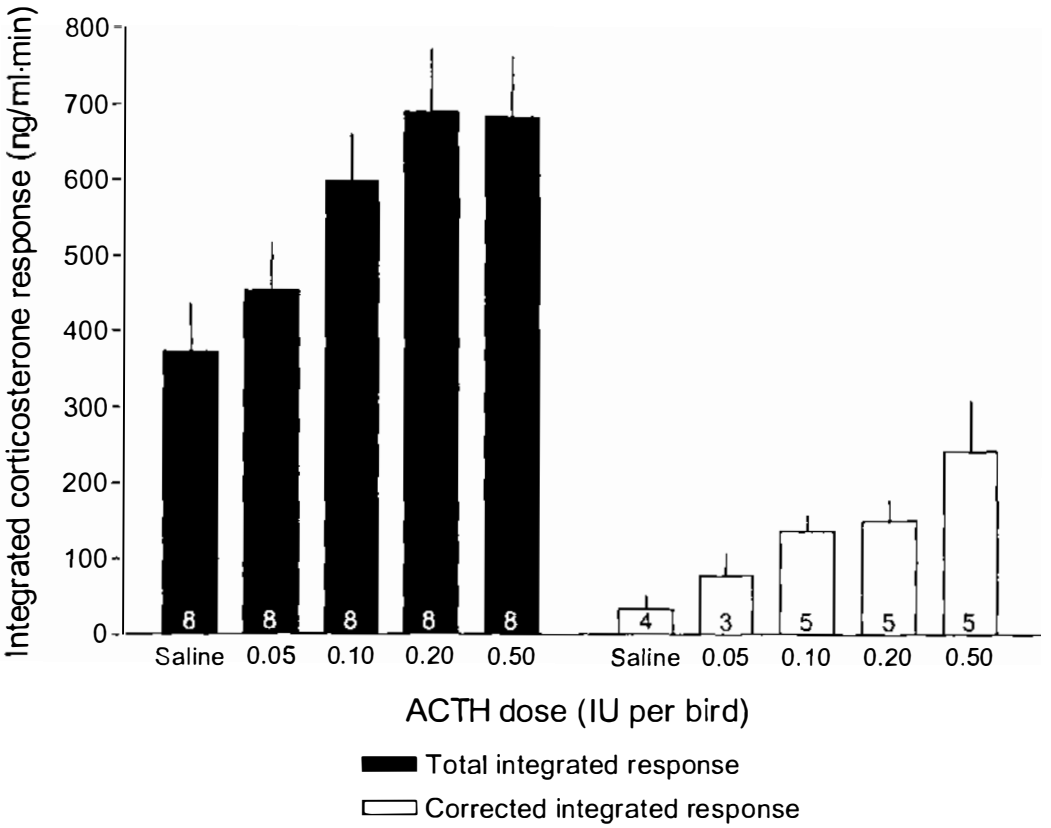


Figure 5.5 Total and corrected corticosterone responses in quail treated with saline or ACTH (Synacthen™) at doses of 0.05, 0.10, 0.20 or 0.50 IU/bird. Total integrated corticosterone responses were calculated from the areas under the corticosterone vs time curves for each bird, while corrected responses were calculated between 0 and 30 min. The number of birds per group are inset inside each column. Results are plotted as means ± standard error.

Table 5.1 Two-way repeated measures ANOVA for plasma corticosterone concentrations in male quail treated with ACTH.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Dose		4,340	4,35	0.006	**
Time		41.144	4,140	< 0.001	**
Interaction of dose and time		2.113	16,140	0.012	*
Comparisons within dose for each dose					
0 vs 15 min	Saline	1.184	1,35	0.284	
	0.05 IU	2.400	1,35	0.130	
	0.10 IU	9.093	1,35	0.005	**
	0.20 IU	17.032	1,35	< 0.001	**
	0.50 IU	25.736	1,35	< 0.001	**
15 vs 30 min	Saline	0.285	1,35	0.597	
	0.05 IU	20.553	1,35	< 0.001	**
	0.10 IU	8.121	1,35	0.007	**
	0.20 IU	27.690	1,35	< 0.001	**
	0.50 IU	24.708	1,35	< 0.001	**
30 vs 60 min	Saline	2.037	1,35	0.162	
	0.05 IU	0.787	1,35	0.381	
	0.10 IU	3.001	1,35	0.092	
	0.20 IU	1.487	1,35	0.231	
	0.50 IU	13.691	1,35	0.001	**
60 vs 120 min	Saline	8.445	1,35	0.006	**
	0.05 IU	2.979	1,35	0.093	
	0.10 IU	6.882	1,35	0.013	*
	0.20 IU	5.207	1,35	0.029	*
	0.50 IU	0.086	1,35	0.772	
0 vs 30 min	Saline	0.301	1,35	0.587	
	0.05 IU	1.919	1,35	0.175	
	0.10 IU	0.539	1,35	0.468	
	0.20 IU	0.050	1,35	0.825	
	0.50 IU	1.286	1,35	0.264	
0 vs 60 min	Saline	2.122	1,35	0.154	
	0.05 IU	0.584	1,35	0.450	
	0.10 IU	0.171	1,35	0.682	
	0.20 IU	0.331	1,35	0.569	
	0.50 IU	1.700	1,35	0.201	
0 vs 120 min	Saline	20.291	1,35	< 0.001	**
	0.05 IU	6.582	1,35	0.015	*
	0.10 IU	9.515	1,35	0.004	**
	0.20 IU	8.516	1,35	0.006	**
	0.50 IU	2.937	1,35	0.095	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 5.1 cont Two-way repeated measures ANOVA for plasma corticosterone in male quail treated with ACTH.

Comparisons between doses for each time		*F*	*df*	*p*	
0 min	Saline vs 0.05 IU	0.114	1,35	0.738	
	Saline vs 0.10 IU	0.203	1,35	0.655	
	Saline vs 0.20 IU	0.349	1,35	0.559	
	Saline vs 0.50 IU	0.013	1,35	0.911	
	0.05 vs 0.10 IU	0.620	1,35	0.436	
	0.05 vs 0.20 IU	0.862	1,35	0.360	
	0.05 vs 0.50 IU	0.203	1,35	0.655	
	0.10 vs 0.20 IU	0.020	1,35	0.889	
	0.10 vs 0.50 IU	0.114	1,35	0.738	
	0.20 vs 0.50 IU	0.229	1,35	0.636	
15 min	Saline vs 0.05 IU	5.741	1,35	0.022	*
	Saline vs 0.10 IU	20.823	1,35	< 0.001	**
	Saline vs 0.20 IU	33.820	1,35	< 0.001	**
	Saline vs 0.50 IU	40.568	1,35	< 0.001	**
	0.05 vs 0.10 IU	4.697	1,35	0.037	*
	0.05 vs 0.20 IU	11.693	1,35	0.002	**
	0.05 vs 0.50 IU	15.787	1,35	< 0.001	**
	0.10 vs 0.20 IU	1.598	1,35	0.219	
	0.10 vs 0.50 IU	3.262	1,35	0.080	
	0.20 vs 0.50 IU	0.307	1,35	0.583	
30 min	Saline vs 0.05 IU	1.133	1,35	0.294	
	Saline vs 0.10 IU	2.521	1,35	0.121	
	Saline vs 0.20 IU	1.363	1,35	0.251	
	Saline vs 0.50 IU	3.115	1,35	0.086	
	0.05 vs 0.10 IU	7.034	1,35	0.012	*
	0.05 vs 0.20 IU	4.982	1,35	0.032	
	0.05 vs 0.50 IU	8.007	1,35	0.008	**
	0.10 vs 0.20 IU	0.177	1,35	0.677	
	0.10 vs 0.50 IU	0.031	1,35	0.860	
	0.20 vs 0.50 IU	0.357	1,35	0.554	
60 min	Saline vs 0.05 IU	0.181	1,35	0.673	
	Saline vs 0.10 IU	1.414	1,35	0.242	
	Saline vs 0.20 IU	1.265	1,35	0.268	
	Saline vs 0.50 IU	0.040	1,35	0.842	
	0.05 vs 0.10 IU	0.583	1,35	0.450	
	0.05 vs 0.20 IU	0.489	1,35	0.489	
	0.05 vs 0.50 IU	0.051	1,35	0.823	
	0.10 vs 0.20 IU	0.004	1,35	0.949	
	0.10 vs 0.50 IU	0.977	1,35	0.330	
	0.20 vs 0.50 IU	0.854	1,35	0.362	
120 min	Saline vs 0.05 IU	1.917	1,35	0.175	
	Saline vs 0.10 IU	1.984	1,35	0.168	
	Saline vs 0.20 IU	2.636	1,35	0.113	
	Saline vs 0.50 IU	5.437	1,35	0.026	*
	0.05 vs 0.10 IU	0.001	1,35	0.981	
	0.05 vs 0.20 IU	0.057	1,35	0.813	
	0.05 vs 0.50 IU	0.897	1,35	0.350	
	0.10 vs 0.20 IU	0.046	1,35	0.831	
	0.10 vs 0.50 IU	0.852	1,35	0.362	
	0.20 vs 0.50 IU	0.502	1,35	0.483	

Table 5.2 One-way ANOVA for the integrated corticosterone responses in quail treated with ACTH.

Comparisons between doses		*F*	*df*	*p*	
Integrated corticosterone response					
Total		4.087	4,35	0.008	**
Corrected		3.715	4,17	0.024	*
Bonferroni post-hoc comparisons between groups					
		Total response		Corrected response	
		df	*p*	*df*	*p*
Saline vs	0.05 IU	1,35	1.000	1,17	1.000
	0.10 IU	1,35	0.289	1,17	0.918
	0.20 IU	1,35	0.029 *	1,17	0.588
	0.50 IU	1,35	0.034 *	1,17	0.021 *
0.05 vs	0.10 IU	1,35	1.000	1,17	1.000
	0.20 IU	1,35	0.222	1,17	1.000
	0.50 IU	1,35	0.259	1,17	0.177
0.10 vs	0.20 IU	1,35	1.000	1,17	1.000
	0.50 IU	1,35	1.000	1,17	0.685
0.20 vs	0.50 IU	1,35	1.000	1,17	1.000

5.3.2.2 Relationship between a sub-maximal dose (0.10 IU/bird) of ACTH and plasma corticosterone concentrations in low and high response quail

Corticosterone concentrations were low in four of six birds in both the low and high corticosterone response groups injected with saline, and remained relatively constant throughout the period of sampling in these birds (Figure 5.6). The initial corticosterone concentration was elevated (8.33 – 10.65 ng/ml) in two birds in each group. Corticosterone remained relatively unchanged or declined after the saline injection and did not differ between groups at 60 min. Initial corticosterone concentrations varied substantially in both the low and high groups before ACTH injection, ranging from 1.38 – 11.62 ng/ml (Figure 5.6). The administration of ACTH resulted in similar increases in corticosterone in both the low and high response groups. Corticosterone concentrations increased between 0 and 15 min, declined between 15 and 30 min, then generally continued to decline and remained low and relatively constant between 30 and 60 min.

Corticosterone varied between time, but not between groups, with a significant

interaction between group and time (see Table 5.3 for all statistics for this analysis). Corticosterone concentrations at 0 min did not differ between saline and ACTH treated birds, or low and high response groups (Figure 5.7a). There was no significant change in plasma corticosterone concentrations in the low and high response birds injected with saline during the sampling period. Corticosterone concentrations in low and high response birds injected with 0.10 IU/bird ACTH increased to peak values by 15 min (11.83 ± 3.05 ng/ml and 11.45 ± 3.06 ng/ml respectively), declined between 15 and 60 min then remained low for the remainder of the experiment (Figure 5.7a). Concentrations at 30 min were greater than initial concentrations, but generally did not differ from initial corticosterone concentrations at 60 and 120 min. Corticosterone did not differ at anytime between the low and high response birds that had been treated with ACTH.

There were significant differences in the total integrated corticosterone response between groups (Table 5.4; Figure 5.7b). The corrected integrated response (0-30 min) was significantly greater for ACTH than saline treated low or high response birds.

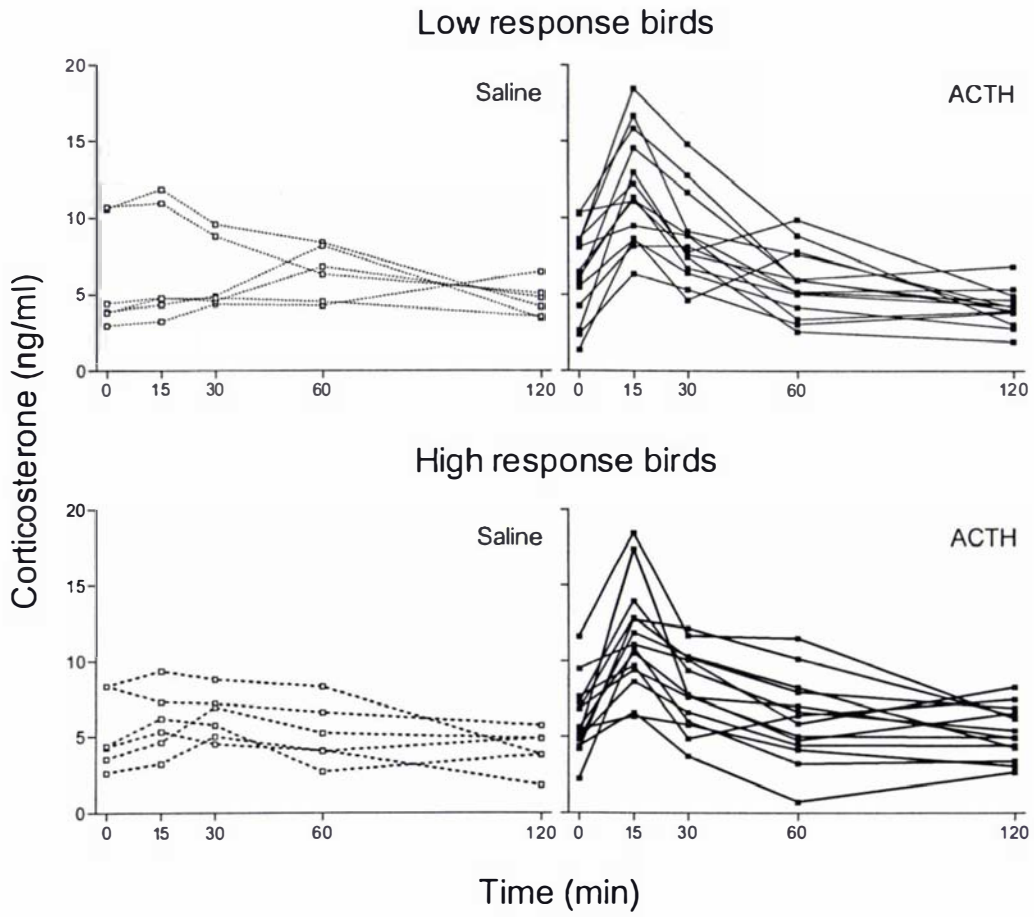


Figure 5.6 Plasma corticosterone in individual male quail with low or high responses to mechanical restraint. Birds were injected with saline (n=6) or 0.10 IU ACTH/bird (Synacthen™; n=14). Results are plotted as raw data.

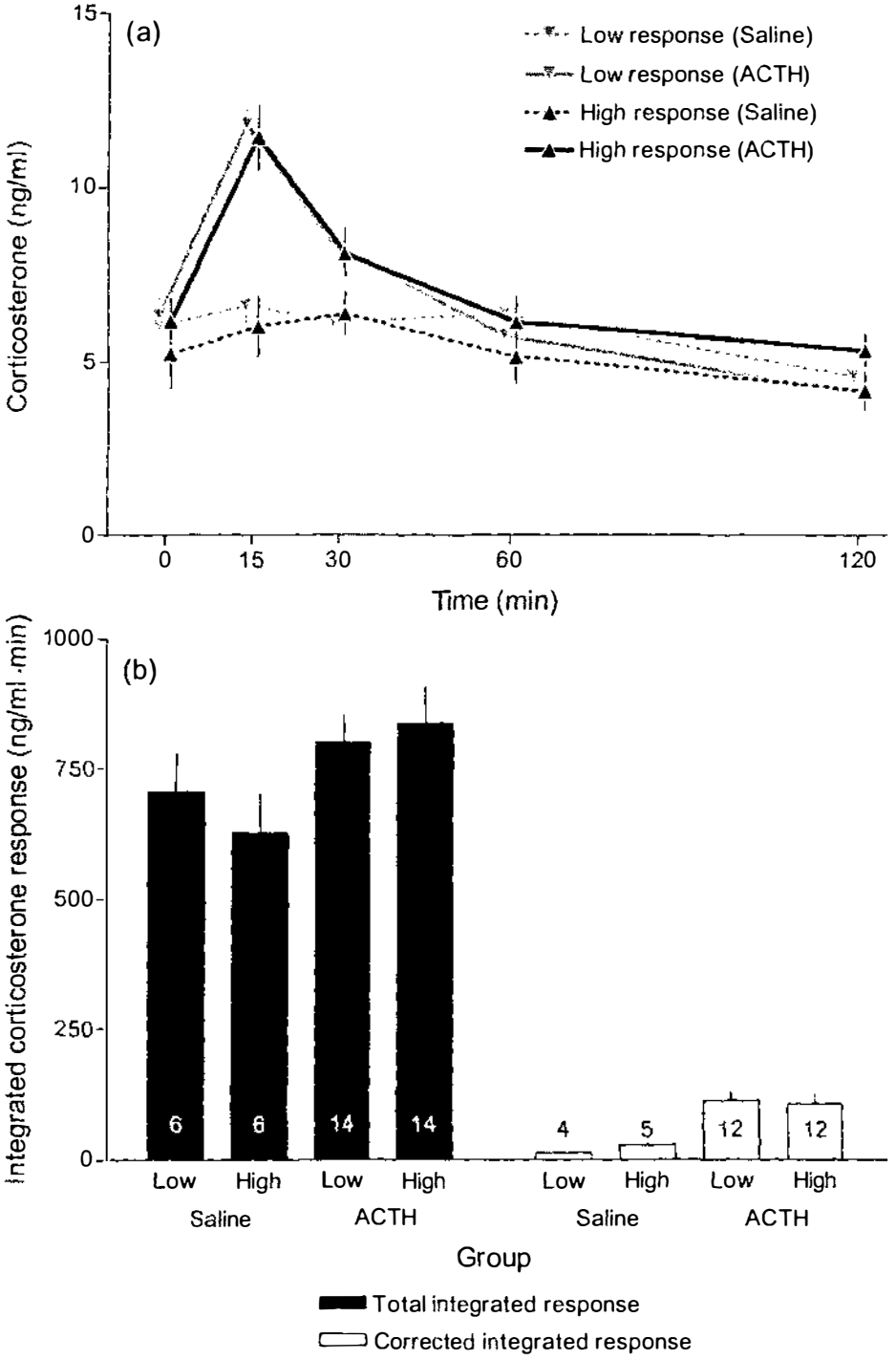


Figure 5.7 Mean plasma corticosterone concentrations (a); and the total and corrected integrated corticosterone responses (b) in male quail selected for low or high responses to mechanical restraint that were treated with saline (n=6) or 0.10 IU ACTH/bird (Synacthen™; n=14). Total integrated corticosterone responses were calculated from the areas under the corticosterone vs time curves for each bird, while corrected responses were calculated between 0 and 30 min. The number of birds per group are inset inside each column Results are plotted as means ± standard error.

Table 5.3 Two-way repeated measures ANOVA for corticosterone concentrations in low and high response quail treated with 0.10 IU ACTH/bird.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Group		1.502	3,36	0.230	
Time		21.234	4,144	< 0.001	**
Interaction of group and time		2.752	12,144	0.003	**
Comparisons within group for each group					
0 vs 15 min	Low (saline)	0.405	1,36	0.528	
	Low (ACTH)	45.480	1,36	< 0.001	**
	High (saline)	1.125	1,36	0.296	
	High (ACTH)	36.596	1,36	< 0.001	**
15 vs 30 min	Low (saline)	0.002	1,36	0.967	
	Low (ACTH)	28.040	1,36	< 0.001	**
	High (saline)	0.971	1,36	0.331	
	High (ACTH)	33.398	1,36	< 0.001	**
30 vs 60 min	Low (saline)	0.127	1,36	0.724	
	Low (ACTH)	16.046	1,36	< 0.001	**
	High (saline)	2.304	1,36	0.138	
	High (ACTH)	12.204	1,36	0.001	**
60 vs 120 min	Low (saline)	3.079	1,36	0.088	
	Low (ACTH)	6.470	1,36	0.015	*
	High (saline)	1.320	1,36	0.258	
	High (ACTH)	0.180	1,36	0.674	
0 vs 30 min	Low (saline)	0.373	1,36	0.545	
	Low (ACTH)	13.616	1,36	0.001	**
	High (saline)	2.626	1,36	0.114	
	High (ACTH)	7.406	1,36	0.010	*
0 vs 60 min	Low (saline)	0.486	1,36	0.490	
	Low (ACTH)	0.040	1,36	0.842	
	High (saline)	0.007	1,36	0.933	
	High (ACTH)	0.285	1,36	0.596	
0 vs 120 min	Low (saline)	0.639	1,36	0.429	
	Low (ACTH)	6.165	1,36	0.018	*
	High (saline)	0.864	1,36	0.359	
	High (ACTH)	0.925	1,36	0.343	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 5.3 cont Two-way repeated measures ANOVA for corticosterone concentrations in low and high response quail treated with 0.10 IU ACTH/bird.

Comparisons between groups for each time		*F*	*df*	*p*
0 min	Low (saline) vs Low (ACTH)	0.038	1,36	0.847
	Low (saline) vs High (saline)	0.121	1,36	0.730
	Low (ACTH) vs High (ACTH)	0.041	1,36	0.841
	High (saline) vs High (ACTH)	0.581	1,36	0.451
15 min	Low (saline) vs Low (ACTH)	14.486	1,36	0.001 **
	Low (saline) vs High (saline)	0.028	1,36	0.868
	Low (ACTH) vs High (ACTH)	0.065	1,36	0.800
	High (saline) vs High (ACTH)	14.486	1,36	0.001 **
30 min	Low (saline) vs Low (ACTH)	4.714	1,36	0.037 *
	Low (saline) vs High (saline)	0.107	1,36	0.745
	Low (ACTH) vs High (ACTH)	0.274	1,36	0.604
	High (saline) vs High (ACTH)	1.899	1,36	0.177
60 min	Low (saline) vs Low (ACTH)	0.314	1,36	0.579
	Low (saline) vs High (saline)	0.687	1,36	0.413
	Low (ACTH) vs High (ACTH)	0.003	1,36	0.959
	High (saline) vs High (ACTH)	0.145	1,36	0.706
120 min	Low (saline) vs Low (ACTH)	0.573	1,36	0.454
	Low (saline) vs High (saline)	0.470	1,36	0.497
	Low (ACTH) vs High (ACTH)	3.871	1,36	0.057
	High (saline) vs High (ACTH)	2.492	1,36	0.123

Table 5.4 One-way ANOVA for the integrated corticosterone responses in low and high response quail treated with 0.10 IU ACTH/bird.

Comparisons between doses	*F*	*df*	*p*
Integrated corticosterone response			
Total	1.383	3,36	0.264
Corrected	13.879	3,29	< 0.001 **

Bonferroni post-hoc comparisons between groups

		Total response		Corrected response	
		df	*p*	*df*	*p*
Low (saline) vs	Low (ACTH)	1,36	1.000	1,29	< 0.001 **
	High (saline)	1,36	1.000	1,29	0.572
Low (ACTH) vs	High (ACTH)	1,36	1.000	1,29	1.000
High (saline) vs	High (ACTH)	1,36	0.442	1,29	0.013 *

5.3.3 Relationship between the adrenocortical response and pituitary sensitivity to CRF

5.3.3.1 Relationship between plasma corticosterone and dose of CRF

Initial corticosterone concentrations varied between birds and ranged from 0.76 – 8.44 ng/ml (Figure 5.8). Corticosterone concentrations remained relatively constant in all but one of the saline treated birds during the study. Corticosterone concentrations increased between 0 and 15 min in birds injected with 2 μg CRF/bird, although the response varied and some birds did not respond to treatment (Figure 5.8b). Corticosterone concentrations generally declined between 15 and 30 min and remained low and relatively constant for the rest of the treatment. There was no consistent pattern to corticosterone concentrations in birds injected with 4 μg CRF/bird. Two birds showed large increases in corticosterone concentrations between 0 and 15 min while there was little change in the other six birds after treatment (Figure 5.8c). Treatment with 6 μg CRF/bird resulted in large increases in corticosterone to 6.60 – 14.24 ng/ml in 75% of birds at 15 min. Corticosterone increased to a lesser extent in the remaining birds (Figure 5.8d). Corticosterone declined to initial concentrations in 75% of birds between 15 and 30 min and remained low, while it remained elevated in two birds at 60 and 120 min. Treatment with 8 μg CRF/bird did not result in a clear increase in corticosterone concentration (Figure 5.8e), with increases in three birds but and little change in the other five birds.

Corticosterone concentrations varied between time but not between doses, with a significant interaction between dose and time (see Table 5.5 for all statistics for this analysis). Initial corticosterone concentrations were low and did not differ between the five groups (Figure 5.9a). Mean corticosterone concentrations in birds injected with saline remained low for the duration of the experiment, and the 2, 4, and 8 μg groups did not differ from the saline group at any of the sampling times. Treatment with 6 μg CRF was the only treatment that stimulated a significant corticosterone response (Figure 5.9a). Treatment with 6 μg CRF/bird induced a significant increase in mean plasma corticosterone concentrations between 0 and 15 min. Corticosterone concentrations at 15 min were 10.74 ± 3.80 ng/ml and were significantly higher than any of the other groups, then declined between 15 and

30 min and were no different from the other groups for the remainder of the study. The 6 μg CRF dose resulted in a net increase in corticosterone concentrations of approximately 6 ng/ml; an increase similar to that caused by 0.10 IU of ACTH or mechanical restraint.

There were no differences between groups in the total or corrected integrated corticosterone response between the groups (Figure 5.9b; see Table 5.6 for all statistics for this analysis).

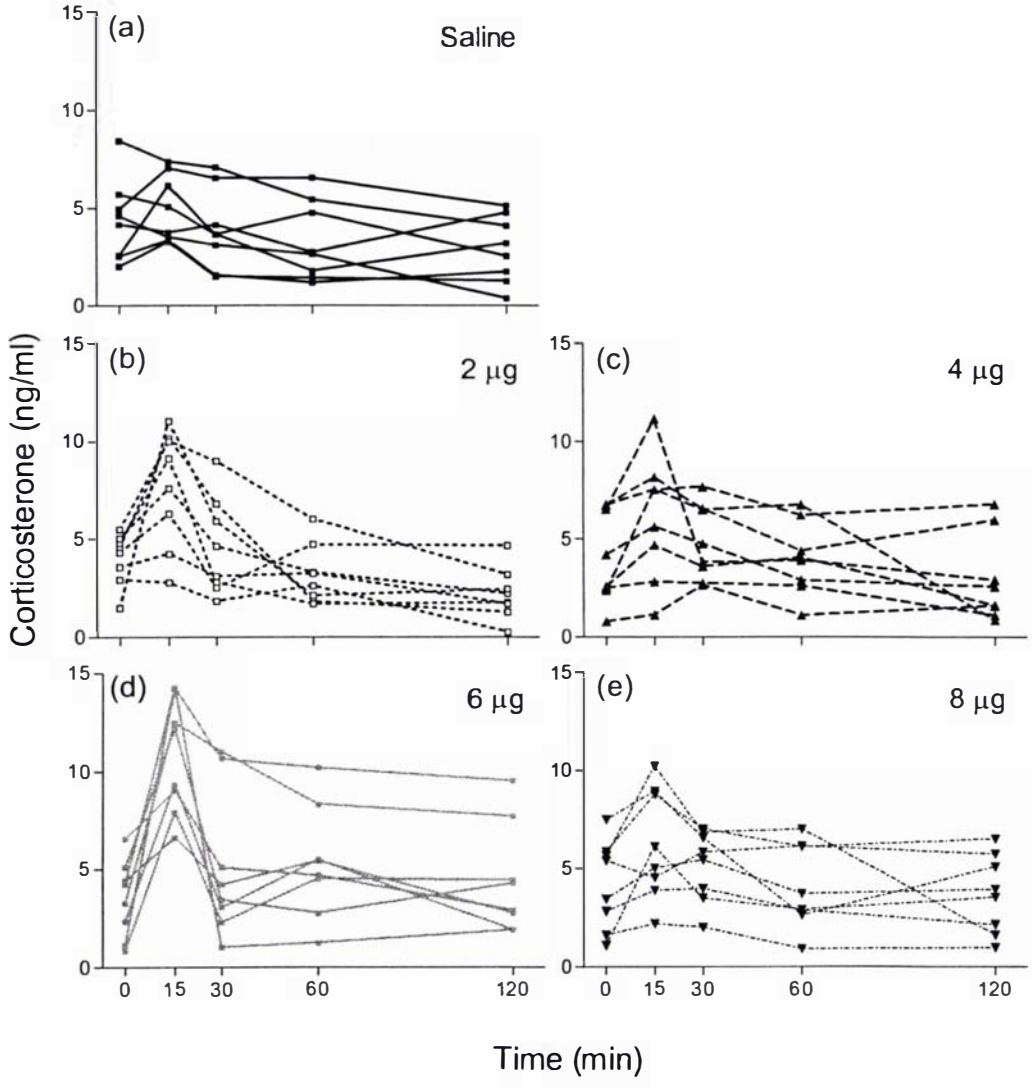


Figure 5.8 Plasma corticosterone concentrations in individual male quail that received a single injection of saline or 2, 4, 6, or 8 μg CRF/bird (n=8) immediately after the 0 min blood sample. Results are plotted as raw data.

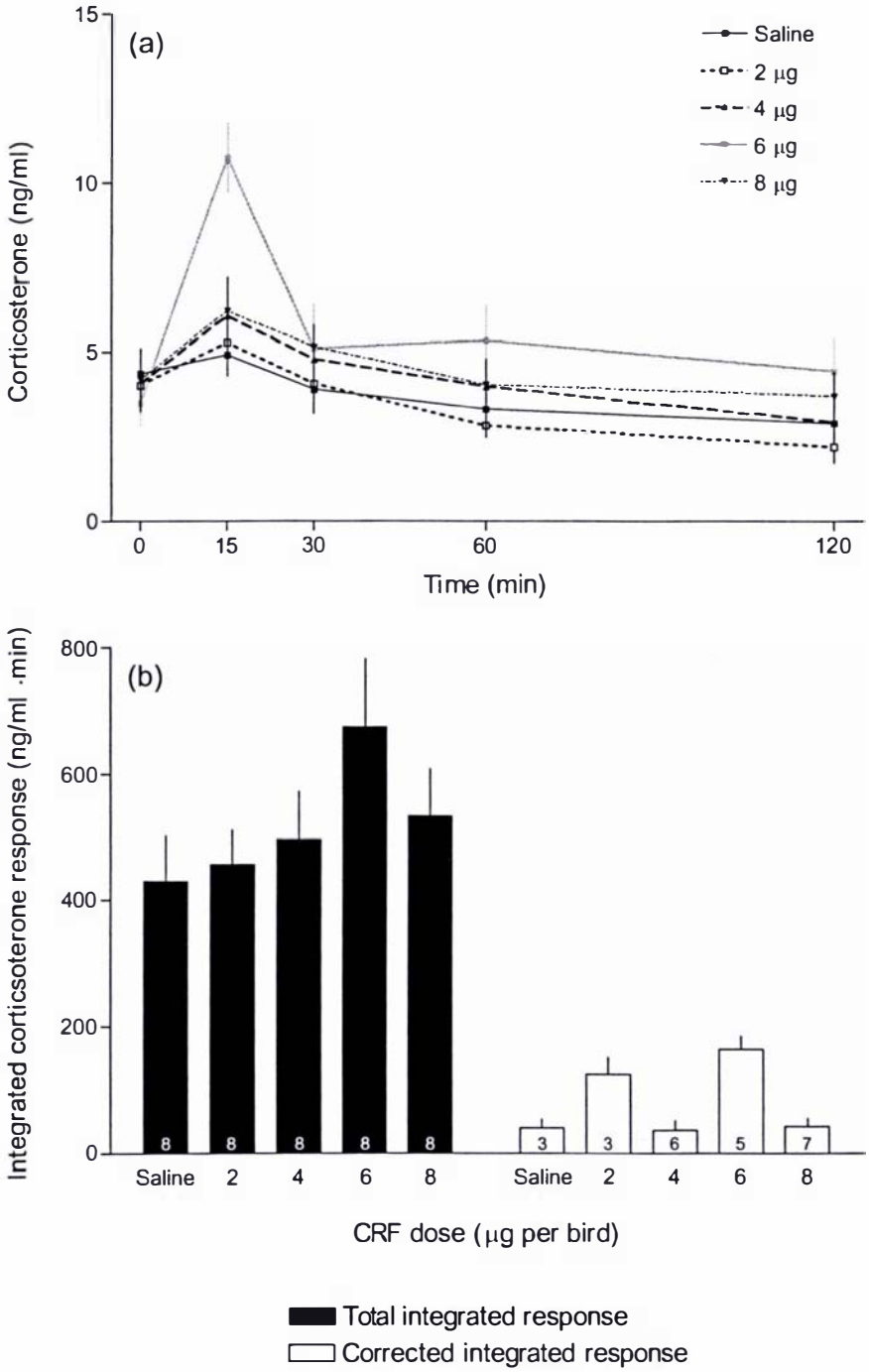


Figure 5.9 Mean plasma corticosterone concentrations (a); and the total and corrected integrated corticosterone responses (b) in male quail treated with saline (n=8) or 2, 4, 6, or 8 μg CRF/bird (n=8). Total integrated corticosterone responses were calculated from the areas under the corticosterone vs time curves for each bird, while corrected responses were calculated between 0 and 30 min. The number of birds per group are inset inside each column. Results are plotted as means ± standard error.

Table 5.5 Two-way repeated measures ANOVA for corticosterone concentrations in male quail treated with CRF.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Dose		1.292	4,35	0.292	
Time		34.915	4,140	< 0.001	**
Interaction of dose and time		3.266	16,140	< 0.001	**
Comparisons within dose for each dose					
0 vs 15 min	Saline	0.377	1,35	0.543	
	2 μg	15.803	1,35	< 0.001	**
	4 μg	4.858	1,35	0.034	*
	6 μg	63.129	1,35	< 0.001	**
	8 μg	4.940	1,35	0.033	*
15 vs 30 min	Saline	1.443	1,35	0.238	
	2 μg	12.945	1,35	0.001	**
	4 μg	2.268	1,35	0.141	
	6 μg	43.489	1,35	< 0.001	**
	8 μg	1.528	1,35	0.225	
30 vs 60 min	Saline	1.102	1,35	0.301	
	2 μg	6.070	1,35	0.019	*
	4 μg	2.037	1,35	0.162	
	6 μg	0.174	1,35	0.679	
	8 μg	3.921	1,35	0.056	
60 vs 120 min	Saline	0.412	1,35	0.525	
	2 μg	2.330	1,35	0.136	
	4 μg	2.599	1,35	0.116	
	6 μg	1.853	1,35	0.182	
	8 μg	0.258	1,35	0.615	
0 vs 30 min	Saline	0.347	1,35	0.560	
	2 μg	0.486	1,35	0.490	
	4 μg	0.835	1,35	0.367	
	6 μg	4.419	1,35	0.049	*
	8 μg	1.504	1,35	0.228	
0 vs 60 min	Saline	1.735	1,35	0.196	
	2 μg	1.068	1,35	0.309	
	4 μg	0.009	1,35	0.926	
	6 μg	5.328	1,35	0.027	*
	8 μg	0.029	1,35	0.865	
0 vs 120 min	Saline	3.910	1,35	0.056	
	2 μg	6.011	1,35	0.019	*
	4 μg	2.309	1,35	0.138	
	6 μg	1.630	1,35	0.210	
	8 μg	0.398	1,35	0.532	

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 5.5 cont Two-way repeated measures ANOVA for corticosterone concentrations in male quail treated with CRF.

Comparisons between doses for each time		*F*	*df*	*p*	
0 min	Saline vs 2 μg	0.118	1,35	0.733	
	Saline vs 4 μg	0.092	1,35	0.763	
	Saline vs 6 μg	0.751	1,35	0.392	
	Saline vs 8 μg	0.035	1,35	0.853	
	2 vs 4 μg	0.002	1,35	0.969	
	2 vs 6 μg	0.274	1,35	0.604	
	2 vs 8 μg	0.025	1,35	0.876	
	4 vs 6 μg	0.317	1,35	0.577	
	4 vs 8 μg	0.014	1,35	0.907	
	6 vs 8 μg	0.463	1,35	0.501	
15 min	Saline vs 2 μg	3.841	1,35	0.058	
	Saline vs 4 μg	0.678	1,35	0.416	
	Saline vs 6 μg	17.540	1,35	< 0.001	**
	Saline vs 8 μg	0.850	1,35	0.363	
	2 vs 4 μg	1.291	1,35	0.264	
	2 vs 6 μg	4.965	1,35	0.032	*
	2 vs 8 μg	1.077	1,35	0.307	
	4 vs 6 μg	11.320	1,35	0.002	**
	4 vs 8 μg	0.010	1,35	0.922	
	6 vs 8 μg	10.666	1,35	0.002	**
30 min	Saline vs 2 μg	0.288	1,35	0.595	
	Saline vs 4 μg	0.500	1,35	0.484	
	Saline vs 6 μg	0.925	1,35	0.343	
	Saline vs 8 μg	1.003	1,35	0.323	
	2 vs 4 μg	0.029	1,35	0.866	
	2 vs 6 μg	0.180	1,35	0.674	
	2 vs 8 μg	0.216	1,35	0.645	
	4 vs 6 μg	0.065	1,35	0.801	
	4 vs 8 μg	0.087	1,35	0.770	
	6 vs 8 μg	0.002	1,35	0.968	
60 min	Saline vs 2 μg	0.013	1,35	0.909	
	Saline vs 4 μg	0.401	1,35	0.531	
	Saline vs 6 μg	3.645	1,35	0.064	
	Saline vs 8 μg	0.473	1,35	0.496	
	2 vs 4 μg	0.560	1,35	0.459	
	2 vs 6 μg	4.098	1,35	0.051	
	2 vs 8 μg	0.645	1,35	0.427	
	4 vs 6 μg	1.628	1,35	0.210	
	4 vs 8 μg	0.003	1,35	0.957	
	6 vs 8 μg	1.491	1,35	0.230	
120 min	Saline vs 2 μg	0.462	1,35	0.501	
	Saline vs 4 μg	0.001	1,35	0.975	
	Saline vs 6 μg	2.237	1,35	0.144	
	Saline vs 8 μg	0.622	1,35	0.436	
	2 vs 4 μg	0.506	1,35	0.482	
	2 vs 6 μg	4.731	1,35	0.036	*
	2 vs 8 μg	2.155	1,35	0.151	
	4 vs 6 μg	2.143	1,35	0.152	
	4 vs 8 μg	0.573	1,35	0.454	
	6 vs 8 μg	0.500	1,35	0.484	

Table 5.6 One-way ANOVA for the integrated corticosterone responses in quail treated with CRF.

Comparisons between doses		F	df	p
Integrated corticosterone response				
Total		1.480	4,35	0.229
Corrected		3.757	4,19	0.020 *
Bonferroni post-hoc comparisons between groups				
		Total response		Corrected response
		df	p	df	p
Saline vs	2 μg	1,35	1.000	1,19	1.000
	4 μg	1,35	1.000	1,19	1.000
	6 μg	1,35	0.349	1,19	0.569
	8 μg	1,35	1.000	1,19	1.000
2 vs	4 μg	1,35	1.000	1,19	0.447
	6 μg	1,35	0.585	1,19	1.000
	8 μg	1,35	1.000	1,19	0.352
4 vs	6 μg	1,35	1.000	1,19	0.079
	8 μg	1,35	1.000	1,19	1.000
6 vs	8 μg	1,35	1.000	1,19	0.053

5.3.3.2 Relationship between a sub-maximal dose of CRF and plasma corticosterone concentrations in low and high response quail

Initial corticosterone concentrations across all four groups (saline- vs. CRF-treated low and high corticosterone responders to 15 min of mechanical restraint) ranged between 1.04 and 10.93 ng/ml (Figure 5.10). Corticosterone concentrations fluctuated and changed up to 3-4 fold in low and high response groups injected with saline, and did not differ between any of the groups at 0 min (Figure 5.11a). Administration of CRF did not affect corticosterone concentrations in low or high response groups and there was no consistent temporal pattern to secretion in either group. Corticosterone varied between time but not between treatment, with a significant interaction between treatment and time (Figure 5.11a; see Table 5.7 for statistical analysis). There was no corticosterone response to the administration of CRF in either low or high response groups. Corticosterone in saline and CRF treated low response groups did not change significantly during the sampling period. Corticosterone in saline and CRF-treated high response groups remained relatively constant between 0 and 30 min, then gradually declined between 30 and 120 min to be lower than initial concentrations at 120 min. There were no differences in either total or corrected integrated corticosterone response between the groups (Table 5.8; Figure 5.11b).

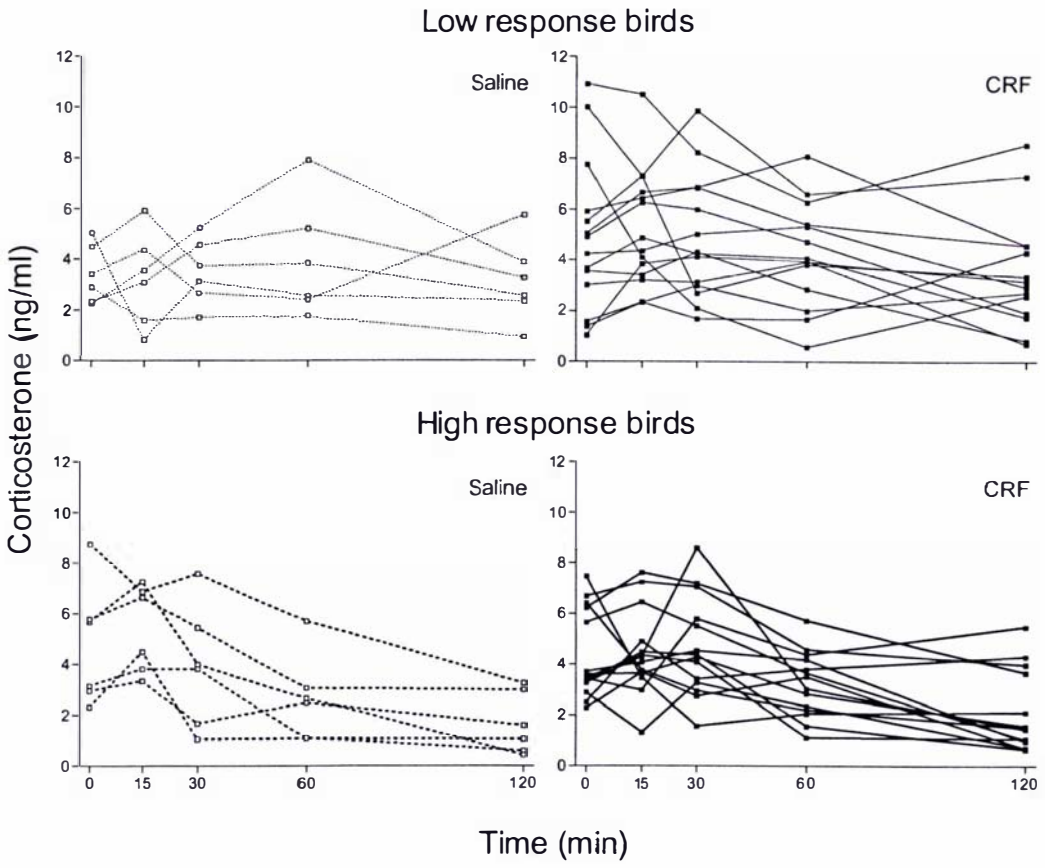


Figure 5.10 Plasma corticosterone in individual male quail selected for low or high responses to mechanical restraint. Birds were injected with saline (n=6) or 6 μg of CRF (n=14). Results are plotted as raw data.

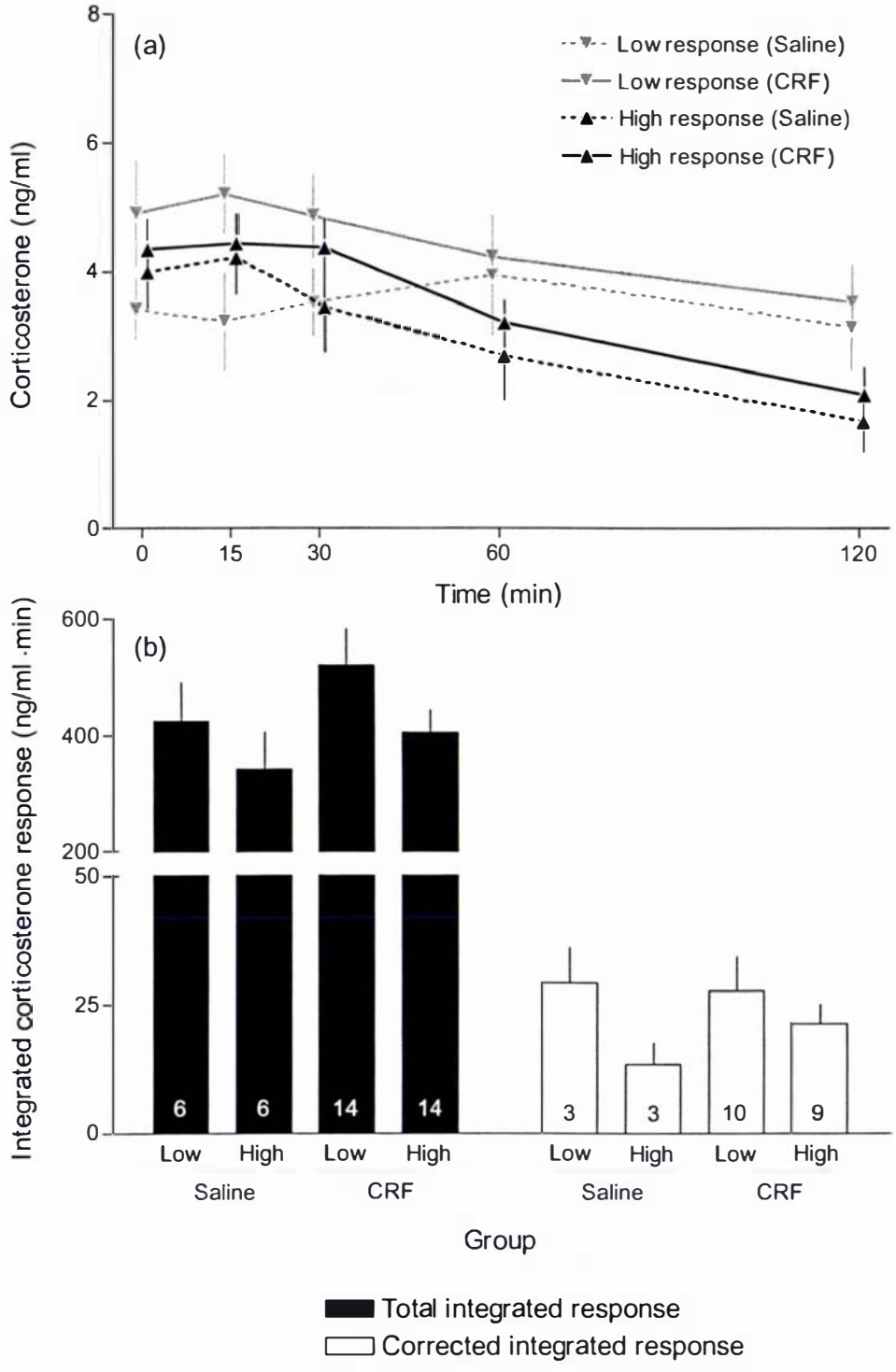


Figure 5.11 Mean plasma corticosterone concentrations (a); and the total and corrected integrated corticosterone responses (b) in male quail selected for low or high responses to mechanical restraint that were treated with saline (n=6) or 6 μg CRF/bird (n=14). Total integrated corticosterone responses were calculated from the areas under the corticosterone vs time curves for each bird, while corrected responses were calculated between 0 and 30 min. The number of birds per group are inset inside each column. Results are plotted as means ± standard error.

Table 5.7 Two-way repeated measures ANOVA for corticosterone concentrations in low and high response quail treated with 6 μg CRF/bird.

		Plasma corticosterone			
Effect		*F*	*df*	*p*	
Treatment		1.317	3,36	0.284	
Time		12.855	4,144	< 0.001	**
Interaction of treatment and time		1.853	12,144	0.045	*
Comparisons within treatment for each treatment					
0 vs 15 min	Low (saline)	0.956	1,36	0.335	
	Low (CRF)	1.670	1,36	0.204	
	High (saline)	0.090	1,36	0.765	
	High (CRF)	0.018	1,36	0.893	
15 vs 30 min	Low (saline)	1.429	1,36	0.240	
	Low (CRF)	0.612	1,36	0.439	
	High (saline)	2.916	1,36	0.096	
	High (CRF)	0.005	1,36	0.945	
30 vs 60 min	Low (saline)	0.071	1,36	0.791	
	Low (CRF)	2.703	1,36	0.109	
	High (saline)	2.420	1,36	0.129	
	High (CRF)	8.552	1,36	0.006	**
60 vs 120 min	Low (saline)	0.677	1,36	0.416	
	Low (CRF)	1.438	1,36	0.238	
	High (saline)	3.807	1,36	0.059	
	High (CRF)	9.895	1,36	0.003	**
0 vs 30 min	Low (saline)	0.004	1,36	0.950	
	Low (CRF)	0.258	1,36	0.615	
	High (saline)	1.026	1,36	0.318	
	High (CRF)	0.004	1,36	0.951	
0 vs 60 min	Low (saline)	0.044	1,36	0.835	
	Low (CRF)	0.303	1,36	0.585	
	High (saline)	3.100	1,36	0.087	
	High (CRF)	2.814	1,36	0.102	
0 vs 120 min	Low (saline)	0.357	1,36	0.554	
	Low (CRF)	2.953	1,36	0.094	
	High (saline)	13.336	1,36	0.001	**
	High (CRF)	22.456	1,36	< 0.001	**

Note. The first three rows show the results of the two-way repeated measures ANOVA for the main effects and their interaction. The remaining rows show the results of *post-hoc* comparisons of times for each treatment and comparisons of treatments at each time. Significant differences ($p < 0.05$) are marked with a single asterisk, while highly significant differences ($p < 0.01$) are marked with a double asterisk.

Table 5.7 cont Two-way repeated measures ANOVA for corticosterone concentrations in low and high response quail treated with 6 μg CRF/bird.

Comparisons between doses for each time		*F*	*df*	*p*	
0 min	Low (saline) vs Low (CRF)	0.656	1,36	0.423	
	Low (saline) vs High (saline)	0.263	1,36	0.611	
	Low (saline) vs High (CRF)	0.754	1,36	0.391	
	Low (CRF) vs High (saline)	0.041	1,36	0.840	
	Low (CRF) vs High (CRF)	0.006	1,36	0.940	
	High (saline) vs High (CRF)	0.069	1,36	0.795	
15 min	Low (saline) vs Low (CRF)	6.106	1,36	0.018	*
	Low (saline) vs High (saline)	2.248	1,36	0.143	
	Low (saline) vs High (CRF)	3.507	1,36	0.069	
	Low (CRF) vs High (saline)	0.486	1,36	0.490	
	Low (CRF) vs High (CRF)	0.597	1,36	0.445	
	High (saline) vs High (CRF)	0.010	1,36	0.922	
30 min	Low (saline) vs Low (CRF)	1.288	1,36	0.264	
	Low (saline) vs High (saline)	0.145	1,36	0.705	
	Low (saline) vs High (CRF)	0.810	1,36	0.374	
	Low (CRF) vs High (saline)	2.516	1,36	0.121	
	Low (CRF) vs High (CRF)	0.092	1,36	0.764	
	High (saline) vs High (CRF)	1.826	1,36	0.185	
60 min	Low (saline) vs Low (CRF)	0.018	1,36	0.894	
	Low (saline) vs High (saline)	1.548	1,36	0.221	
	Low (saline) vs High (CRF)	0.320	1,36	0.575	
	Low (CRF) vs High (saline)	2.580	1,36	0.117	
	Low (CRF) vs High (CRF)	0.816	1,36	0.372	
	High (saline) vs High (CRF)	0.821	1,36	0.371	
120 min	Low (saline) vs Low (CRF)	0.020	1,36	0.889	
	Low (saline) vs High (saline)	3.245	1,36	0.080	
	Low (saline) vs High (CRF)	2.191	1,36	0.148	
	Low (CRF) vs High (saline)	5.162	1,36	0.029	*
	Low (CRF) vs High (CRF)	4.378	1,36	0.044	*
	High (saline) vs High (CRF)	0.424	1,36	0.519	

Table 5.8 One-way ANOVA for the integrated corticosterone responses in low and high response quail treated with 6 μg CRF.

Comparisons between doses		*F*	*df*	*p*
Integrated corticosterone response				
Total		1.678	3,36	0.189
Corrected		0.809	3,21	0.503

Bonferroni post-hoc comparisons between groups

		Total response		Corrected response	
		df	*p*	*df*	*p*
Low (saline) vs	Low (CRF)	1,36	1.000	1,21	1.000
	High (saline)	1,36	1.000	1,21	1.000
	High (CRF)	1,36	1.000	1,21	1.000
Low (CRF) vs	High (saline)	1,36	0.312	1,21	1.000
	High (CRF)	1,36	0.614	1,21	1.000
High (saline) vs	High (CRF)	1,36	1.000	1,21	1.000

5.4 Discussion

Mechanical restraint causing immobilisation was a suitable stressor for identifying differences in the adrenocortical response of individual quail, allowing the identification of birds for low and high response groups. There was a positive dose-dependent relationship between ACTH and plasma corticosterone concentrations. The corticosterone response to a sub-maximal dose of ACTH did not differ between low and high response quail, showing there was no difference in adrenal sensitivity between the two response groups. Corticosterone responses to CRF were inconsistent, with only one of the four doses initiating clear responses. However, when this dose was given to low and high response birds it did not elicit a response in either group. This finding is similar to results reported in snow buntings (*Plectrophenax nivalis*) (Romero *et al.*, 1998b) and Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*) (Romero and Wingfield, 1999) where high doses of ovine CRF failed to affect corticosterone concentrations.

5.4.1 ACTH dose response

After treatment with ACTH plasma corticosterone concentrations increased in a dose dependent manner after 15 minutes and returned to basal after 30 minutes.

ACTH treatment has been shown to stimulate an increase in corticosterone concentrations in chickens (Beuving and Vonder, 1978; Beuving and Vonder, 1986; Littin and Cockrem, 2001), ducks (Harvey *et al.*, 1980; Noirault *et al.*, 1999; Faure *et al.*, 2003), and a range of passerine species (Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero and Wingfield, 1998; Wilson and Holberton, 2001). Carsia *et al.* (1988) showed that corticosterone secretion increased in Japanese quail adrenocortical cells incubated with ACTH. Beuving and Vonder (1986) reported that a dose of 0.1 IU/kg porcine ACTH initiated a sub-maximal corticosterone response in chickens. In many of these studies large doses of ACTH resulted in a maximal corticosterone response or a prolonged elevation of corticosterone. Studies conducted on passerines (Romero *et al.*, 1998a; Romero *et al.*, 1998b; Romero and Wingfield, 1998) focused mainly on the adrenal sensitivity to ACTH by stimulating a maximal corticosterone response at different stages of the lifecycle.

The smallest dose of ACTH (0.05 IU; 0.2 IU/kg body weight) resulted in a small but significant increase in corticosterone concentrations 15 minutes after administration. Peak corticosterone concentrations at 15 min increased with doses of 0.10, 0.20 and 0.50 IU (0.4, 0.8 and 2.0 IU/kg respectively). A dose of 0.8 IU/kg generally stimulated a maximal corticosterone response by 15 minutes. Beuving and Vonder (1986) found that a dose of 0.5 IU/kg stimulated a maximal response in chickens after 25 minutes, while Noirault *et al.* (1999) reported that corticosterone concentrations in ducks peaked 10 minutes after an injection of 0.125 IU/kg. Single reports of adrenal responsiveness to ACTH in a species are not sufficient to determine whether there are differences between species in these responses, as there are differences in the type of ACTH, age of the birds, species, sexes, and environmental conditions, all making direct comparisons difficult.

Unlike previous studies which have focussed on the adrenocortical potential to secrete corticosterone in response to ACTH, the present study was designed to identify a dose that stimulated a sub-maximal corticosterone response. In the current study a dose of 0.05 IU/bird (0.2 IU/kg) stimulated a small increase (approximately 2 ng/ml) in corticosterone, which was smaller than the response of birds to mechanical restraint. A maximal response was generally elicited from birds

given doses of 0.20 and 0.50 IU ACTH (0.8 and 2.0 IU/kg respectively), with little variation between individuals in the corticosterone response between these two doses. The 0.10 IU (0.4 IU/kg) dose resulted in an increase in corticosterone concentrations of approximately 5 ng/ml after 15 minutes. This was similar to the response of birds to mechanical restraint and, more importantly, there was a wide range of corticosterone responses as seen in responses to mechanical restraint.

5.4.2 Adrenal sensitivity to ACTH in birds selected for low or high responses

There was no difference in adrenal sensitivity to ACTH between Japanese quail selected for low or high responses to mechanical restraint. These results differ from the *in vitro* results of Carsia *et al.* (1988) who worked with adrenocortical cells from random-bred quail and from quail selected for a high corticosterone response to immobilization. Carsia *et al.* (1988) showed that maximal ACTH-induced corticosterone production by cells from the high response quail was greater than that of the random-bred quail.

The injection of ACTH stimulated an increase in corticosterone concentrations in both low and high response groups, with net increases in corticosterone at 15 minutes of 5.5 and 5.3 ng/ml respectively. This compares with an increase of 4.3 ng/ml in quail that were mechanically restrained. Plasma corticosterone declined after the peak at 15 minutes and returned to basal at 60 minutes.

As shown in the dose response study, the peak in corticosterone concentrations at 15 minutes varied between birds, with similar variation in the low and high response groups. However, the mean corticosterone response to 0.10 IU ACTH was the same in low and high response birds, indicating that differences between birds in their corticosterone responses to mechanical restraint were not due to differences in adrenal sensitivity to ACTH.

5.4.3 CRF dose response

Intrajugular injection of 2 to 8 μg CRF (8, 16, 24 or 32 μg/kg body weight) stimulated an increase in plasma corticosterone in some birds after 15 minutes. However, the increase in corticosterone was not consistent and there was no dose-dependent response. The 6 μg (24 μg/kg) dose was the only one to stimulate a significant increase in corticosterone.

It is well established that CRF stimulates ACTH secretion from the pituitary in birds, as shown by the incubation of ovine CRF with duck (Castro *et al.*, 1986) or chicken (Carsia *et al.*, 1986) pituitary cells *in vitro*. CRF stimulates corticosterone secretion from the adrenal gland via ACTH, so changes in corticosterone do not directly reflect the effect of CRF on ACTH secretion. However, the absence of differences between birds with low and high corticosterone responses to mechanical restraint in terms of their adrenal sensitivity to ACTH indicates that corticosterone responses to CRF are likely to reflect differences in pituitary sensitivity to CRF or CRF production. Westerhof *et al.* (1992) showed that ovine CRF treatment caused corticosterone concentrations to increase in a dose dependent manner in racing pigeons (*Columba livia domestica*), with doses of 10 and 100 μg/kg stimulating a significant increase in corticosterone concentrations 15, 30 and 60 minutes after the intravenous injection. In the present study corticosterone declined and returned to basal between 15 and 30 minutes in quail treated with 6 μg of CRF (24 μg/kg), whereas Westerhof *et al.* (1992) reported that corticosterone continued to increase between 15 and 60 minutes after the CRF treatment before declining to basal at 120 minutes.

In the current study, birds treated with 2 or 6 μg CRF (8 and 24 μg/kg) showed the most consistent corticosterone responses to CRF, although only the 6 μg/bird dose produced a significant response. The absence of a consistent and significant corticosterone response in birds treated with 8 μg CRF may indicate possible down-regulation of CRF-responsiveness of the anterior pituitary gland. The inconsistent corticosterone responses might have resulted from the use of these birds in three previous mechanical restraint or ACTH treatment studies. The range of responses

to CRF administration between the doses, and the odd responses by single individuals or groups could reflect an effect of repeated manipulations. Jones *et al.*, (2000) found experience-dependent sensitisation of the adrenocortical response in high stress line Japanese quail. It is possible that the corticosterone responses of some individuals be sensitive to repeated handling associated with blood sampling and the injection rather than CRF.

In Japanese quail, Launay *et al.* (1993) reported a slight but significant increase in corticosterone 30 minutes after a single injection of 15 μg/kg of human CRF. Ovine CRF was used in the present study, and has been used successfully to stimulate an increase in corticosterone release in a variety of avian species (Castro *et al.*, 1986; Carsia *et al.*, 1986; Westerhof *et al.*, 1992; Romero *et al.*, 1998b). The 6 μg/bird CRF dose stimulated a 7 ng/ml increase in corticosterone between 0 and 15 minutes, compared with a 5 ng/ml increase in response to 0.10 IU ACTH. However, although there was more variation between birds in their responses to the ACTH dose than the CRF dose, the 6 μg dose of CRF was used to treat low and high response groups since it was the only CRF dose to stimulate a significant corticosterone response.

5.4.4 Pituitary sensitivity to CRF in birds selected for low and high responses

Male quail with a low or high corticosterone responses to mechanical restraint were given an intrajugular injection of saline or 6 μg CRF/bird (24 μg/kg body weight). Treatment with CRF did not stimulate a corticosterone response in either the low or high response groups. The absence of a corticosterone response from the CRF treated birds contrasted with the CRF dose response study which showed a clear response to 6 μg CRF/bird.

The corticosterone response to CRF treatment was similar in birds that had been previously treated with saline or a high dose of CRF in the previous CRF dose response study. This suggests that the absence of any significant effect of CRF on corticosterone secretion in low and high response to mechanical restraint quail was

not due to previous treatment with CRF. It is more likely that the CRF was ineffective, or that the response to CRF was affected by the three previous sampling sessions experienced by the birds. Corticosterone concentrations at 0 minutes were higher and more variable than had been previous measured and this confounded the results. These quail were 15 weeks old and had been used in up to six previous experiments. It is possible that the birds were responding before being picked up when people were entering the quail rooms. This could be due to their previous experiences of being handled and blood sampled several times over the previous 8 weeks, as seen in the response in domestic fowl which habituate to repeated injections of corticotrophin (Freeman *et al.*, 1979). The results of the present study can also be compared with those previously reported in passerines where responses to with an ovine CRF dose selected to saturate the pituitary have been inconsistent (Romero *et al.*, 1998a; Romero *et al.*, 1998d; Romero and Wingfield, 1999).

5.4.5 Summary

The aim of this study was to determine if individual variation of the corticosterone response is caused by differences in adrenal or pituitary sensitivity to ACTH or CRF respectively, in Japanese quail selected for low or high corticosterone responses to mechanical restraint. Adrenal sensitivity to ACTH did not differ between quail with low or high corticosterone responses, while CRF treatment did not stimulate corticosterone responses consistently.

The stress response initiates physiological and behavioural responses that allow a bird to adapt to the source of the stressor, with some individuals better able to respond to stressors than others. There is marked variation of the corticosterone response between birds (Wingfield *et al.*, 1992; Wingfield, 1994; Astheimer *et al.*, 1995) and this variation exists due in part, to differences in the HPA-axis (Carsia *et al.*, 1988) and higher centres in the brain. In mammals, exposure to stressful stimuli has been shown to alter an individual's subsequent responses (Beylin and Shors, 2003), and it is likely that a similar mechanism exists in birds. There is also a genetic basis to corticosterone responses, as shown by the selection of quail for low

or high responses (Satterlee and Johnson, 1988). In this study, adrenal sensitivity to ACTH did not differ between low and high response quail indicating that the difference in sensitivity to a pharmacological dose of ACTH found by Carsia *et al.* (1988) may have a negligible if any influence on variation of the corticosterone response to a stressor. The present data indicate that individual variation is caused at a site higher than the adrenal gland.

The use of ovine CRF to assess pituitary sensitivity in low or high corticosterone response quail produced inconsistent results. Ovine and human CRF have been used in a variety of bird species to stimulate ACTH and corticosterone secretion with mixed success (Westerhof *et al.*, 1992; Romero and Wingfield, 1998). Despite the sensitivity of some birds to ovine and human CRF indicating the existence of an avian equivalent, an avian CRF has yet to be characterised. There is some evidence that AVT and MT are necessary for the action of CRF (Westerhof *et al.*, 1992; Romero and Wingfield, 1998), although the stimulation of ACTH secretion has been inconsistent with both AVT and MT in isolation and when used in combination with CRF.

The inconsistent responses to CRF meant that the role of pituitary gland sensitivity to CRF in contributing to variation between birds in their corticosterone responses could not be assessed. The inconsistent responses might have resulted from reusing birds from the earlier mechanical restraint, ACTH, and CRF dose response studies. This problem could be overcome in a future study by choosing birds with low or high responses to mechanical restraint from a new group of quail, thereby avoiding the reuse of birds in the ACTH study. Alternatively, establishing a colony of quail and breeding for low or high corticosterone responses to mechanical restraint as done in the Satterlee laboratory would produce completely naive birds for CRF treatment. It was not possible to conduct such studies for the present thesis research. However, the current study did show that adrenal sensitivity to a physiological dose of ACTH does not differ between quail selected for low or high corticosterone responses to mechanical restraint, indicating that individual variation is mediated at the pituitary gland or at high brain centres responsible for activating the HPA-axis.

Chapter 6

General discussion

The aims of the research described in this dissertation were to determine the effects of different stressors on corticosterone and sex steroid secretion in Japanese quail, and to investigate the neuroendocrine basis of variation between birds in their responses to stressors. The size of corticosterone and testosterone responses to stressors were dependent on the type and duration of the stimuli. Manual and mechanical restraint elicited larger corticosterone responses than other stressors, whilst the size of the corticosterone response increased with increasing duration of exposure to stressors. Manual and mechanical restraint also had the greatest inhibitory effect on testosterone secretion. This inhibitory effect on testosterone occurred independently of any change in LH secretion. This is one of the first reports of testosterone secretion in birds being inhibited by a single, short period of handling, and has implications for future studies in domestic and free-living birds where sex steroid concentrations are measured in birds that may already have been handled.

The effect that a stressor has on the reproductive system is influenced by an individual's perception of the stressor and the magnitude of the response to the stressor. The magnitude of the response of a bird to a stressor has a genetic basis, and is also influenced by the bird's previous experience of stressors. These factors differ between individuals and lead to variation in the stress response between individuals. The corticosterone response differed markedly between individual random-bred Japanese quail in this study, and between individuals of the strains of

quail selected for low or high stress responses by Satterlee and Johnson (1988). Individual differences in the ability to respond to stressors may contribute to variation in breeding success and survival (reviewed by Sapolsky and Wingfield, 2003). There has been considerable interest in corticosterone responses in free-living birds in relation to environmental conditions and latitude (Wingfield, 1994; Romero *et al.*, 1998a). However, little research has been conducted on how individual variation of the corticosterone response arises. In the present study, it was found that variation between birds in their corticosterone responses was caused by differences in adrenal sensitivity to ACTH. This indicates that individual variation is due to individual differences in pituitary sensitivity to CRF, AVT, MT or by differences at a higher level in the brain. The importance of these findings will be discussed in greater detail in the remainder of this chapter, and suggestions will be made for future potential areas of study.

Japanese quail were selected as a model species due to their availability and the large amount of previous research conducted overseas. There are no previous reports of experimental work with Japanese quail in New Zealand, so a series of pilot studies, not included in this dissertation, were conducted to identify the conditions that either stimulated gonadal regression or prevented gonadal growth as a prerequisite to studies of the effects of corticosterone on the control of the reproductive system.

In Chapter 2, a single subcutaneous 1.2 mg corticosterone injection was used to increase plasma corticosterone to pharmacological concentrations to determine if corticosterone treatment inhibited sex steroid secretion. LH concentrations in males were unaffected by corticosterone treatment showing that a short (< 30 min) elevation in corticosterone did not influence LH secretion from the pituitary. Handling associated with the blood collection and injection caused a decline in plasma testosterone that masked any effect of corticosterone treatment. Eskeland and Blom (1979) and Johnson (1981) had previously reported a decline in testosterone after repeated handling at 5 minute intervals. Less frequent blood sampling and similar doses of corticosterone have been used in Japanese quail and caused a decline in testosterone secretion (Boyd, 2000). Corticosterone is also

known to inhibit several enzymes involved in testicular steroidogenesis, causing a decrease in testosterone secretion in isolated rat leydig cells (Bambino and Hsueh, 1981, Mann and Orr, 1990; Orr and Mann 1992, Monder *et al.*, 1994). It is proposed that corticosterone acts in a similar fashion in the Japanese quail testes, and the increase in corticosterone associated with repeated handling accounts for the decline in testosterone secretion.

While a variety of stimuli were shown to stimulate a corticosterone response, manual and mechanical restraint were the most effective in stimulating an increase in plasma corticosterone and a decrease in testosterone secretion. A single period of manual or mechanical restraint caused a decrease in testosterone for more than 2 hours. In contrast, there is a single report of an increase in testosterone secretion following 10 minutes of mechanical restraint (Heiblum *et al.*, 2000). Mechanical restraint was the preferable method for characterising individual variation in the adrenocortical response in our quail and was comparable to the method used by Satterlee and Johnson (1988). Mechanical restraint was logistically easy to apply making it preferable over the intermittent handling method used previously for quail (Boyd, 2000) and chickens (Littin and Cockrem, 2001).

Individual variation of the corticosterone response to immobilisation by manual or mechanical restraint has been reported in a range of avian species (Boyd, 2000; Littin and Cockrem, 2001; Cockrem and Silverin, 2002a). Free-living birds also differ in their stress response to an identical stressor. This variation, influenced by differences in body condition and social status may be related to variation in survival and reproductive performance (reviewed by Wingfield and Sapolsky, 2003). In previous studies, quail have been selected for divergent corticosterone responses and these strains have been used to study the relationship between the adrenocortical response and behavioural, physiological and genetic traits (Satterlee and Johnson, 1988; Jones *et al.*, 1992a; Jones *et al.*, 1994a; Satterlee and Jones, 1997; Jones *et al.*, 2000; Odeh *et al.*, 2003a; Odeh *et al.*, 2003b). However, few studies have specifically addressed the exact causes of individual variation of the corticosterone response.

The corticosterone response varies between individuals in response to a range of different stressors including immobilisation (Satterlee and Johnson, 1988), handling (Littin and Cockrem, 2001; Cockrem and Silverin, 2002a), low temperature (Brown and Nestor, 1973), or social stress (Gross and Siegel, 1985). Variation could arise at the CNS or at some level of the HPA-axis, or a combination of both. The CNS plays an important part in the magnitude and duration of the HPA response activated in an individual. Following activation of the HPA-axis, differences in pituitary and adrenal sensitivity to products of the HPA-axis are also likely to influence the corticosterone response.

The general pattern of the corticosterone response for an individual was found to be repeatable when quail were handled on three occasions 10 days apart. A single corticosterone response from a bird therefore can be used as a measure of that bird's responsiveness to the stressor. In previous studies, quail have been selected for low or high corticosterone responses based on the response to a stressor on one occasion. This study confirms that single corticosterone responses of quail can be used as a basis for selection of birds with low or high corticosterone responses.

ACTH injections have been used previously to assess adrenal sensitivity to ACTH (Romero *et al.*, 1998a; Romero and Wingfield, 1998; Romero *et al.*, 1998b). ACTH treatment has also been used to determine adrenocortical cell sensitivity *in vitro* in random-bred quail and quail selected for a high corticosterone response to immobilisation (Carsia *et al.*, 1988). In the current study, an *i.v.* injection of ACTH at a dose of 0.10 IU/bird stimulated a range of corticosterone responses that was similar to the range of responses to mechanical restraint. The 0.10 IU injection of ACTH was equally effective in stimulating a corticosterone response in birds with low or high corticosterone responses to mechanical restraint. This showed that individual variation in the corticosterone response to the restraint stressor is not regulated by adrenal sensitivity to ACTH. In contrast, Carsia *et al.* (1988) showed that the adrenocortical cells of quail selected for a high stress response to mechanical restraint were more sensitive to ACTH than those of random-bred quail. However, isolated adrenocortical cells of high stress quail only differed in their response to pharmacological doses of ACTH and basal corticosterone secretion

in cells from high response birds did not differ from that of cells from random-bred birds. Carsia *et al.* (1988) proposed that adrenocortical cells in random-bred and high response quail are equally sensitive to ACTH, but that the cells of the high stress line are more able to couple ACTH with transmembrane signals and steroidogenic enzymes associated with corticosterone synthesis. In the current study, birds were treated with a sub-maximal dose of ACTH at physiological concentrations, resulting in similar corticosterone responses in low and high response quail. This suggests that the *in vitro* difference in response to ACTH found by Carsia *et al.* (1988) does not contribute to the variation *in vivo.*

The avian pituitary gland has been shown to be responsive to ovine CRF both *in vitro* and *in vivo* in a variety of species (Carsia *et al.*, 1986; Castro *et al.*, 1986; Westerhof *et al.*, 1992; Launay *et al.*, 1993; Romero *et al.*, 1998b). Treating quail with a single i.v. injection of oCRF stimulated an increase in corticosterone secretion, indicating that ACTH secretion from the anterior pituitary gland was responsive to CRF. However, CRF-treatment of quail with low or high corticosterone responses to mechanical restraint was ineffective, in contrast to the CRF dose response study where some quail were responsive to a dose of 6 μg CRF/bird. The absence of any response to CRF meant it was not possible to determine whether the variation in responses to mechanical restraint was due to variation in pituitary responsiveness to CRF. Previous studies (Romero *et al.*, 1998b; Romero and Wingfield, 1999) have shown CRF treatment to be inconsistent, particularly where changes in corticosterone concentrations are used to assess ACTH secretion. It is possible that other factors are required in conjunction with CRF treatment to cause a repeatable and robust increase in corticosterone secretion. Treatment with CRF may need to be preceded, accompanied, or followed by treatment with AVT or MT. CRF treatment *in vivo* in combination with AVT or MT have been shown to be effective in causing an increase in plasma corticosterone via ACTH in a pigeons, quail and passerines (Westerhof *et al.*, 1992; Romero *et al.*, 1998b).

Further studies are necessary to examine the pituitary gland as a site where individual variation of the corticosterone response may be regulated. CRF treatment of low or high response quail needs to be repeated to assess the

responsiveness of the pituitary gland to CRF in quail which show a low or high response to mechanical restraint. Such a study would include AVT and MT treatment in isolation or in combination with CRF as well as the measurement of plasma ACTH concentrations.

If the pituitary glands of low and high response quail did not differ in their responsiveness to physiological doses of CRF, AVT or MT *in vivo*, the responses of pituitary cells isolated from low and high response quail to pharmacological doses of CRF, AVT, or MT could differ *in vitro*. As discussed earlier, Carsia *et al.* (1988) showed that maximal ACTH-induced corticosterone production differed between quail with divergent corticosterone responses to mechanical restraint, and a similar difference may exist in pituitary cells of low or high response quail. Any such differences may have little effect *in vivo* at physiological concentrations of the hypothalamo-hypophysiotrophic factors.

The CNS is an area that has received little attention in birds when assessing the causes of individual variation in stress responses. Stressors have been shown to activate different regions of the brain in quail (Takeuchi *et al.*, 1996) and the degree of activation and activation of different regions are likely to vary between individuals exposed to the same stressor. The activation of the endocrine stress response is via the CNS, and differences in the measured corticosterone response between individuals exposed to the same stressor may reflect differences in the activation of neurons in the CNS.

Individual variation in the corticosterone response to mechanical restraint is likely to be mediated by differential activation of the regions of the brain involved with the initial stress response. Immunoreactivity of the immediate early gene c-*fos* can be used to identify the activation of neurons in the CNS in response to photoperiodic (Meddle and Follett, 1995), reproductive (Meddle *et al.*, 1999) and stressor stimuli in Japanese quail (Takeuchi *et al.*, 1996). The distribution and intensity of c-*fos* immunoreactivity has been used in mammals and birds to determine the neural pathways activated by different stimuli. Immobilisation stimulates an increase in *fos*-like immunoreactivity in the lateral preoptic area,

median preoptic, paraventricular, arcuate, dorsomedial hypothalamic nuclei and lateral hypothalamus (Briski and Gillen, 2001). Measuring c-*fos* expression in quail selected for low or high corticosterone responses to mechanical restraint would show if the activated neural pathways differed between the two strains. Locations of c-*fos* protein expression could be identified in low or high corticosterone response quail using either an antibody raised against c-*fos* or probes for c-*fos* mRNA for *in situ* hybridisation. Once the precise location of stressor-induced c-*fos* expression in the quail hypothalamus was determined, c-*fos* mRNA expression could be quantified using a c-*fos* mRNA quantitative competitive polymerase chain reaction (PCR) established and validated for quail hypothalamic tissue during a recent visit to a collaborator's laboratory.

In order to understand the physiological basis of individual variation in the corticosterone response it is necessary to understand the neural and endocrine mechanisms that generate a corticosterone response following exposure to a stressor. The results of the current study (summarised in Figure 6) show that the adrenocortical responses of Japanese quail differ in relation to the type and duration of the stressor and show that the response to a standardised stressor is generally repeatable for an individual quail. Differences in adrenocortical responses between individuals are likely to have a genetic or neurological basis and to be modified by experience. The precise locations in the HPA-axis that mediate variation of the response are not known, although the results of this study indicate that the locations are the pituitary gland and higher centres in the brain. Therefore, future work with quail that have divergent corticosterone responses should include an examination of pituitary gland sensitivity to hypothalamo-hypophysial factors and the quantification of differences in neuronal activation in brains regions associated with the stress response.

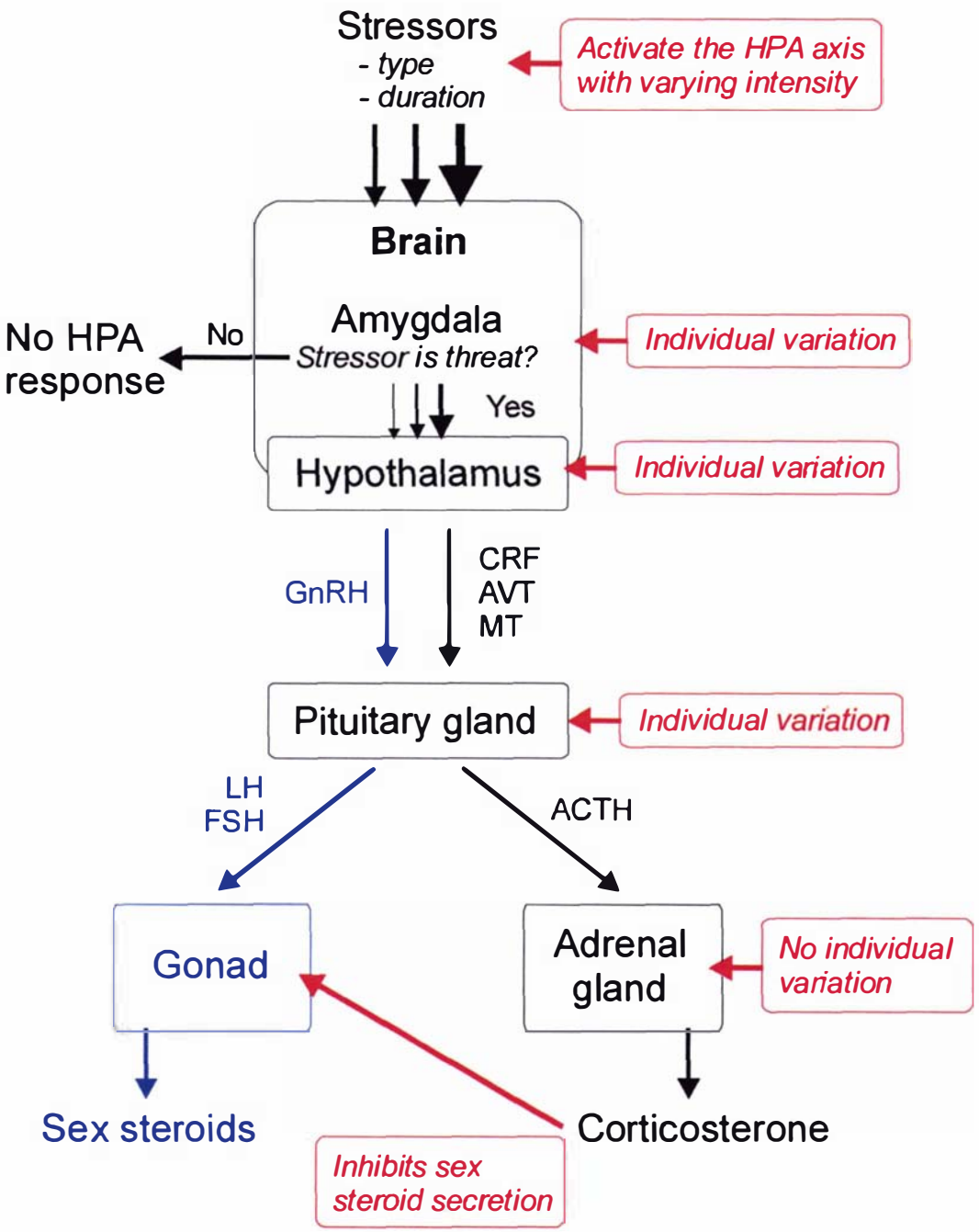


Figure 6 Schematic representation of the stress response in Japanese quail. An acute stressor activates the hypothalamic-pituitary-adrenal axis causing an increase in corticosterone secretion. The findings of this thesis (red) were that the type and duration of the stressor affect the size of the corticosterone response; the increase in corticosterone during a response inhibits sex steroid secretion from the gonads; and individual variation of the corticosterone response is not caused by individual differences in adrenal sensitivity to ACTH, but is likely to be mediated at the pituitary gland or the brain.

Literature cited

Adkins-Regan, E. (1999). Foam produced by male Coturnix quail: what is its function? *The Auk* **116**: 184-193.

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

Ahima, R. S. and Harlan, R. E. (1992). Glucocorticoid receptors in LHRH neurons. *Neuroendocrinology* **56**: 845-850.

Akana, S. F. and Dallman, M. F. (1997). Chronic cold in adrenalectomized, corticosterone (b)-treated rats—facilitated corticotropin responses to acute restraint emerge as b increases. *Endocrinology* **138**: 3249–3258.

Alexander, B. and Wolfson, A. (1970). Prolactin and sexual maturation in the Japanese quail, *Coturnix coturnix japonica. Poultry Science* **49**: 632-640.

Almeida, O. F. X. (1982). Hypothalamic secretion of thyrotrophin releasing hormone is decreased in male Japanese quail exposed to long daily photoperiods. *Journal of Endocrinology* **92**: 261-265.

Arrington, L. C., Ringer, R. K., and Worlford, J. H. (1969). Effect of pinealectomy of coturnix quail reared under non-stimulatory photoperiods. *Poultry Science* **48**: 454-459.

Astheimer, L. B., Buttemer, W. A., and Wingfield, J. C. (1995). Seasonal and acute changes in adrenocortical responsiveness in an arctic-breeding bird. *Hormones and Behavior* **29**: 442-457.

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

Astheimer, L. E., Buttemer, W. A., and Wingfield, J. C. (1994). Gender and seasonal differences in the adrenocortical response to ACTH challenge in an Arctic passerine, *Zonotrichia leucophrys gambelii. General and Comparative Endocrinology* **94**: 33-43.

Attardi, B., Tsujii, T., Friedman, R., Zeng, Z., Roberts, J. L., Dellovade, T. L., Pfaff, D. W., Chandran, U. R., Sullivan, M. W., and DeFranco, D. B. (1997). Glucocorticoid repression of gonadotropin-releasing hormone gene expression and secretion in morphologically distinct subpopulations of GT1-7 cells. *Molecular and Cellular Endocrinology* **131**: 241-255.

Balthazart, J., Massa, R., and Negri-Cesi, P. (1979). Photoperiodic control of testosterone metabolism, plasma gonadotrophins, cloacal gland growth, and reproductive behavior in the Japanese quail. *General and Comparative Endocrinology* **39**: 222-235.

Bambino, T. H. and Hsueh, A. J. W. (1981). Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis *in vivo* and *in vitro*. *Endocrinology* **108**: 2142-2148.

Behan, D. P., DeSouza, E. B., Lowry, P. J., Potter, E., Sawshenko, P., and Vale, W. W. (1995). Corticotropin releasing factor (CRF) binding protein: a novel regulator of CRF and related peptides. *Frontiers in Neuroendocrinology* **16**: 362-382.

Beuving, G. and Vonder, G. (1977). Daily rhythm of corticosterone in laying hens and the influence of egg laying. *Journal of Reproduction and Fertility* **51**: 169-173.

Beuving, G. and Vonder, G. M. (1978). Effect of stressing factors on corticosterone levels in the plasma of laying hens. *General and Comparative Endocrinology* **35**: 153-159.

Beuving, G. and Vonder, G. M. A. (1986). Comparison of the adrenal sensitivity to ACTH of laying hens with immobilization and plasma baseline levels of corticosterone. *General and Comparative Endocrinology* **62**: 353-358.

Beylin, A. V. and Shors, T. J. (2003). Glucocorticoids are necessary for enhancing the acquisition of associative memories after acute stressful experience. *Hormones and Behavior* **43**: 124-131.

Boissin, J. and Assenmacher, I. (1970). Circadian rhythms in adrenal cortical activity in the quail. *Journal of Interdisciplinary Cycle Research* **1**: 251-265.

Bonaz, B. and Rivest, S. (1998). Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. *American Journal of Physiology* **275**: R1438-R1449.

Boswell, T., Hall, M. R., and Goldsmith, A. R. (1995). Testosterone is secreted extragonadally by European quail maintained on short days. *Physiological Zoology* **68**: 967-984.

Boulakoud, M. S. and Goldsmith, A. R. (1991). Thyroxine treatment induces changes in hypothalamic gonadotrophin- releasing hormone characteristic of photorefractoriness in starlings (*Sturnus vulgaris*). *General and Comparative Endocrinology* **82**: 78-85.

Boulakoud, M. S., Ivings, W. E., and Goldsmith, A. R. (1991). Thyroxine treatment prevents the development of photosensitivity in European starlings (*Sturnus vulgaris*). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **161**: 516-520.

Boyd, R. A. (2000). Corticosterone responses to handling and effects of corticosterone injections in the Japanese quail (*Coturnix coturnix japonica*). Unpublished MSc Thesis, Massey University, Palmerston North, New Zealand.

Brain, P. C., Onagbesan, O. M., Peddies, M. J., and Taylor, T. G. (1988). Changes in plasma concentrations of reproductive steroids in female Japanese quail (*Coturnix coturnix japonica*) raised on long or short photoperiods. *General and Comparative Endocrinology* **69**: 174-180.

Brann, D. W. and Mahesh, V. B. (1991). Role of corticosteroids in female reproduction. *FASEB Journal* **5**: 2691-2698.

Breuner, C. W., Greenberg, A. L., and Wingfield, J. C. (1998). Noninvasive corticosterone treatment rapidly increases activity in Gambel's White-crowned sparrows *(Zonotrichia leucophrys gambelii). General and Comparative Endocrinology* **111**: 386-394.

Briski, K. and Gillen, E. (2001). Differential distribution of *fos* expression within the male rat preoptic area and hypothalamus in response to physical vs. psychological stress. *Brain Research Bulletin* **55**: 401-408.

Brown, K. I. and Nestor, K. E. (1973). Some physiological responses of turkeys selected for high and low adrenal responses to cold stress. *Poultry Science* **52**: 1948-1954.

Buntin, J. D., Lea, R. W., and Figge, G. R. (1988). Reductions in plasma LH concentration and testicular weight in ring doves following intracranial injection of prolactin or growth hormone. *Journal of Endocrinology* **118**: 33-40.

Buntin, J. D., Halawani, M. E., Ottinger, M. A., Fan, Y., and Fivizzani, A. J. (1998). An analysis of sex and breeding stage differences in prolactin binding activity in brain and hypothalamic GnRH concentration in Wilson's phalarope, a sex role-reversed species. *General and Comparative Endocrinology* **109**: 119-132.

Burke, W. H. and Papkoff, H. L. P. B. G. A. (1979). Preparation and properties of luteinizing hormone (LH) subunits from the turkey (*Meleagris gallopavo*) and their recombination with subunits of ovine LH. *General and Comparative Endocrinology* **37**: 501-507.

Calogero, A. E., Burrello, N., Bosboom, A. M. J., Garofalo, M. R., Weber, R. F. A., and D'Agata, R. (1999). Glucocorticoids inhibit gonadotropin-releasing hormone by acting directly at the hypothalamic level. *Journal of Endocrinological Investigation* **22**: 666-670.

Campbell, T., Lin, S., DeVries, C., and Lambert, K. (2003). Coping strategies in male and female rats exposed to multiple stressors. *Physiology and Behavior* **78**: 495-504.

Camper, P. M. and Burke, W. H. (1977). The effect of prolactin on reproductive function in female Japanese quail (*Coturnix coturnix japonica*). *Poultry Science* **56**: 1130-1134.

Carlisle, A. J., Mitchell, M. A., Hunter, R. R., Duggan, J. A., and Randall, J. M. (1998). Physiological responses of broiler chickens to the vibrations experienced during road transport. *British Poultry Science* **39**: S48-S60.

Carsia, R. V., Weber, H., and Perez Jr., F. M. (1986). Corticotropin-releasing factor stimulates the release of adrenocorticotropin from domestic fowl pituitary cells. *Endocrinology* **118**: 143-148.

Carsia, R. V., Weber, H., and Satterlee, D. G. (1988). Steroidogenic properties of isolated adrenocortical cells from Japanese quail selected for high serum corticosterone response to immobilization. *Domestic Animal Endocrinology* **5**: 231-240.

Carsia, R. V. and Harvey, S. (2000). Adrenals. *In* "Sturkie's Avian Physiology, Fifth Edition" (C. G. Whittow, Ed.), pp489-537. Academic Press, San Diego.

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

Chadwick, D. J., Marsh, J., and Ackerill, K. (1993). Corticotropin Releasing Factor. John Wiley & Sons Inc, West Sussex.

Chaturvedi, C. M., Dubey, L., and Phillips, D. (1992). Influence of different photoperiods on development of gonad, cloacal gland and circulating thyroid hormone in male Japanese quail *Coturnix coturnix japonica*. *Indian Journal of Experimental Biology* **30**: 680-684.

Chrousos, G. (1998). Editorial: Ultradian, circadian, and stress-related hypothalamic-pituitary-adrenal axis activity - a dynamic digital-to-analog modulation. *Endocrinology* **139**: 437-440.

Cockrem, J. F. (1990). Circadian rhythms in Antarctica Penguins. *In* "Penguin Biology" (L. S. Davis and J. T. Darby, Eds.). Academic Press, Sydney.

Cockrem, J. F. (1995). Timing of seasonal breeding in birds, with particular reference to New Zealand birds. *Reproduction, Fertility, and Development* **7**: 1-19.

Cockrem, J. F. and Silverin, B. (2002a). Variation within and between Birds in Corticosterone Responses of Great Tits (*Parus major*). *General and Comparative Endocrinology* **125**: 197-206.

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

Connolly, P. B. and Callard, I. P. (1987). Steroids modulate the release of luetinizing hormone from quail pituitary cells. *General and Comparative Endocrinology* 68: 466-472.

Daley, C. A., Sakurai, H., Adams, B. M., and Adams, T. E. (1999). Effect of stress-like concentrations of cortisol on gonadotroph function in orchidectomized sheep. *Biology of Reproduction* 60: 158-163.

Davies, D. T., Goulden, L. P., Follett, B. K., and Brown, N. L. (1976). Testosterone feedback on luteinizing hormone (LH) secretion during a photoperiodically induced breeding cycle in Japanese quail. *General and Comparative Endocrinology* **30**: 477-486.

Davies, D. T. and Follett, B. K. (1980). Neuroendocrine regulation of gonadotrophin-releasing hormone secretion in the Japanese quail. *General and Comparative Endocrinology* **40**: 220-225.

Davison, T. F., Scanes, C. G., Harvey, S., and Flack, I. H. (1980). The effect of an injection of corticotrophin on plasma concentrations of corticosterone, growth hormone and prolactin in two strains of domestic fowl. *British Poultry Science* **21**: 287-293.

Davison, T. F., Freeman, B. M., and Rea, J. (1985). Effects of continuous treatment with synthetic ACTH1-24 or corticosterone on immature *Gallus domesticus*. *General and Comparative Endocrinology* **59**: 416-423.

Dawson, A., Goldsmith, A. R., Nicholls, T. J., and Follett, B. K. (1986). Endocrine changes associated with the termination of photorefractoriness by short daylengths and thyroidectomy in starlings (*Sturnus vulgaris*). *Journal of Endocrinology* **110**: 73-79.

Day, T. A., Buller, K. M., Xu, Y., Dayas, C. V., and Crane, J. W. (1999). Separation of neural pathways mediating HPA-axis responses to emotional and physical stressors. *In* "Control Mechanisms of Stress and Emotion: Neuroendocrine-Based Studies" (H. Yamashita, J. W. Funder, J. G. Verbalis, Y. Ueta, and Y. Endo, Eds.). Elsevier Science, Amsterdam.

DeFranco, D. B., Attardi, B., and Chandran, U. R. (1994). Glucocorticoid receptor-mediated repression of GnRH gene expression in a hypothalamic GnRH-secreting neuronal cell line. *In* "Brain Corticosteriod Receptors: Studies on the Mechanism, Function, and Neurotoxicity of Corticosteroid Action" (E. R. de Kloet, E. C. Azmitia, and P. W. Lan, Eds.), Vol. 746, pp473-475. The New York Academy of Sciences, New York.

Delville, Y., Sulon, J., Hendrick, J. C., and Balthazart, J. (1984). Effect of the presence of females on the pituitary-testicular activity in male Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology* **55**: 295-305.

Delville, Y., Sulon, J., and Balthazart, J. (1985). Hormonal correlates of gonadal regression and spontaneous recovery in Japanese quail exposed to short day-lengths. *Archives Internationales de Physiologie et de Biochimie* **93**: 123-133.

Deviche, P., Heyns, W., Balthazart, J., and Hendrick, J. C. (1979). Inhibition oh LH plasma levels by corticosterone administration in the male duckling (*Anas platyrhynchos*). *IRCS Medical Science* **7**: 622.

Deviche, P., Heyns, W., Balthazart, J., and Hendrick, J. C. (1980). Relationships between the pituitary-adrenal and -gonadal axes in the male domestic duck. *General and Comparative Endocrinology* **40**: 332.

Deviche, P., Massa, R., Bottoni, L., and Hendrick, J.-C. (1982). Effect of corticosterone on the hypothalamic-pituitary-gonadal system of male Japanese quail exposed to either short or long photoperiods. *Journal of Endocrinology* **95**: 165-173.

Deviche, P. (1983). Interactions between adrenal function and reproduction in male birds. *In* "Avian Endocrinology: Environmental and Ecological Perspectives" (S. Mikami, Ed.), pp243-254. Springer-Verlag, Berlin.

Deviche, P. and Small, T. (2001). Photoperiodic control of seasonal reproduction: neuroendocrine mechanisms and adaptations. *In* "Avian Endocrinology" (A. Dawson and C. M. Chaturvedi, Eds.), pp113-139. Narosa Publishing House, New Dehli, India .

Edens, F. W. and Siegel, H. S. (1975). Adrenal response in high and low ACTH response lines of chickens during acute heat stress. *General and Comparative Endocrinology* **25**: 64-73.

El Halawani, M. E., Silsby, J. L., Youngren, O. M. and Phillips, R. E. (1991). Exogenous prolactin delays photo-induced sexual maturity and suppresses ovariectomy-induced luteinizing hormone secretion in the turkey (*Meleagris gallopavo*). *Biology of Reproduction* **44**: 420-424.

Eskeland, B. and Blom, A. K. (1979). Plasma corticosteroid levels in laying hens. Effect of two different blood sampling techniques and of rough handling of the animals. *Acta Veterinaria Scandinavica* **20**: 270-275.

Etches, R. J. and Cunningham, F. J. (1976). The effect of pregnenolone, progesterone, deoxycorticosterone or corticosterone on the time of ovulation and oviposition in the hen. *British Poultry Science* **17**: 637-642.

Etches, R. J. (1976). A radioimmunoassay for corticosterone and its application to the measurement of stress in poultry. *Steroids* **28**: 763-773.

Etches, R. J., Croze, F., and Duke, C. E. (1981). Plasma concentrations of luteinizing hormone, progesterone, testosterone and estradiol in follicular and peripheral venous plasma during the ovulation cycle of the hen. *In* "Recent Advances in Avian Endocrinology. Advances in Physiological Sciences Vol. 33", pp89-98. Budapest Press, Budapest.

Etches, R. J. and Croze, F. (1983). Plasma concentrations of LH, progesterone, and corticosterone during ACTH- and corticosterone-induced ovulation in the hen (*Gallus domesticus*). *General and Comparative Endocrinology* **50**: 359-365.

Etches, R. J., Williams, J. B., and Rzasa, J. (1984). Effects of corticosterone and dietary changes in the hen on ovarian function, plasma LH and steroids and the response to exogenous LH-RH. *Journal of Reproduction and Fertility* **70**: 121-130.

Etches, R. J. (1990). The ovulatory cycle of the hen. *CRC Critical Reviews in Poultry Biology* **2**: 293-318.

Faure, J. M., Val-Laillet, D., Guy, G., Bernadet, M. D., and Guémené, D. (2003). Fear and stress reactions in two species of duck and their hybrid. *Hormones and Behavior* **43**: 568-572.

Follett, B. K. and Farner, D. S. (1966). The effects of the daily photoperiod on gonadal growth, neurohypophyseal hormone content, and neurosecretion in the hypothalamohypophyseal system of the Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology* **7**: 111-124.

Follett, B. K. and Sharp, P. J. (1969). Circadian rhythmicity in photoperiodically induced gonadotrophin release and gonadal growth in the quail. *Nature* **223**: 968-971.

Follett, B. K., Davies, D. T., and Gledhill, B. (1977). Photoperiodic control of reproduction in Japanese quail: changes in gonadotrophin secretion on the first day of induction and their pharmacological blockade. *Journal of Endocrinology* **74**: 449-460.

Follett, B. K. and Maung, S. L. (1978). Rate of testicular maturation, in relation to gonadotrophin and testosterone levels, in quail exposed to various artificial photoperiods and to natural daylengths. *Journal of Endocrinology* **78**: 267-280.

Follett, B. K. (1981). The stimulation of luteinizing hormone and follicle-stimulating hormone secretion in quail with complete and skeleton photoperiods. *General and Comparative Endocrinology* **45**: 306-316.

Follett, B. K. (1984). Birds. *In* "Marshall's Physiology of Reproduction: Volume 1" (G. E. Lamming, Ed.), pp283-350. Churchill Livingston, Edinburgh.

Follett, B. K. and Nicholls, T. J. (1984). Photorefractoriness in Japanese Quail: Possible involvement of the thyroid gland. *Journal of Experimental Zoology* **232**: 573-580.

Follett, B. K., Nicholls, T. J., and Mayes. C. R. (1988). Thyroxine can mimic photoperiodically induced gonadal growth in Japanese quail. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **157**: 829-835.

Follett, B. K. and Pearce-Kelly, A. (1990). Photoperiodic control of the termination of reproduction in Japanese quail (*Coturnix coturnix japonica*). *Proceedings of the Royal Society of London* **242**: 225-230.

Foster, R. G., Plowman, G., Goldsmith, A. R., and Follett, B. K. (1987). Immunohistochemical demonstration of marked changes in the LHRH system of photosensitive and photorefractory European starlings (*Sturnus vulgaris*). *Journal of Endocrinology* **115**: 211-220.

Foster, R. G., Panzica, G. C., Parry, D. M., and Viglietti-Panzica, C. (1988). Immunocytochemical studies on the LHRH system of the Japanese quail: influence by photoperiod and aspects of sexual differentiation. *Cell and Tissue Research* **253**: 327-335.

Freeman, B. M., Manning, A. C., and Flack, I. H. (1979). Habituation by the immature fowl in response to repeated injections of corticotrophin. *British Poultry Science* **20**: 391-399.

Freeman, B. M. and Manning, A. C. (1979). Stressor effects of handling on the immature fowl. *Research in Veterinary Science* **26**: 223-226.

Freeman, B. M. and Flack, I. H. (1980). Effects of handling on plasma-corticosterone concentrations in the immature domestic fowl. *Comparative Biochemistry and Physiology A-Physiology* **66**: 77-81.

Gibson, W. R., Follett, B. K., and Gledhill, B. (1975). Plasma levels of luteinizing hormone in gonadectomized Japanese quail exposed to short or to long daylengths. *Journal of Endocrinology* **64**: 87-101.

Gilles, G., Linton, E., and Lowry, P. (1982). Corticotropin-releasing activity of the new CRF is potentiated several times by vasopressin. *Nature* **305**: 355-357.

Gledhill, B. and Follett, B. K. (1976). Diurnal variation and the episodic release of plasma gonadotrophins in Japanese quail during a photoperiodically induced gonadal cycle. *Journal of Endocrinology* **71**: 245-257.

Goldsmith, A. R. and Hall, M. (1980). Prolactin concentrations in the pituitary gland and plasma of Japanese quail in relation to photoperiodically induced sexual maturation and egg laying. *General and Comparative Endocrinology* **42**: 449-454.

Goldsmith, A. R. and Nicholls, T. J. (1984). Thyroxine induces photorefractoriness and stimulates prolactin secretion in European starlings (*Sturnus vulgaris*). *Endocrinology* **101**: R1-R3.

Goldsmith, A. R., Nicholls, T. J., and Plowman, G. (1985). Thyroxine treatment facilitates prolactin secretion and induces a state of photorefractoriness in thyroidectomized starlings. *Journal of Endocrinology* **104**: 99-103.

Goldsmith, A. R., Ivings, W. E., Pearce-Kelly, A. S., Parry, D. M., Plowman, G., Nicholls, T. J., and Follett, B. K. (1989). Photoperiodic control of the development of the LHRH neurosecretory system of European starlings (*Sturnus vulgaris*) during puberty and the onset of photorefractoriness. *Journal of Endocrinology* **122**: 255-268.

Gross, W. B. and Siegel, P. B. (1985). Selective breeding for corticosterone response to social stress. *Poultry Science* **64**: 2230-2233.

Guyomarc'h, C. and Guyomarc'h, J. C. (1984). The influence of social factors on the onset of egg production in Japanese quail (*Coturnix coturnix japonica*). *Biology of Behaviour* **9**: 333-342.

Guyomarc'h, C. and Guyomarc'h, J. C. (1989). Stimulation of sexual development in female Japanese quail by male song: influence of ecoethological variables. *Biology of Behaviour* **14**: 52-65.

Hall, T. R., Harvey, S., and Chadwick, A. (1986). Control of prolactin secretion in birds: a review. *General and Comparative Endocrinology* **62**: 171-184.

Harvey, S., Merry, B. J., and Phillips, J. G. (1980). Infleunce of stress on the secretion of corticosterone in the duck (*Anas platyrhynchos)*. *Journal of Endocrinology* **87**: 161-171.

Harvey, S. P. J. G., Rees, A., and Hall, T. R. (1984). Stress and adrenal function. *Journal of Experimental Zoology* **232**: 633-645.

Hatanaka, F. and Wada, M. (1988). Mechanism controlling photostimulated luteinizing hormone secretion is different from preovulatory luteinizing hormone surge in Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology* **70**: 101-108.

Hattori, A., Ishii, S., and Wada, M. (1986). Effects of two kinds of chicken luteinizing hormone-releasing hormone (LH-RH), mammalian LH-RH and its analogs on the release of LH and FSH in Japanese quail and chicken. *General and Comparative Endocrinology* **64**: 446-455.

Hattori, M. and Wakabayashi, K. (1979). Isoelectric focusing and gel filtration studies on the heterogeneity of avian pituitary luteinizing hormone. *General and Comparative Endocrinology* **39**: 215-221.

Hayashi, H., Imai, K., and Imai, K. (1991). Characterization of chicken ACTH and α-MSH: the primary sequence of chicken ACTH is more similar to xenopus ACTH than to other avian ACTH. *General and Comparative Endocrinology* **82**: 434-443.

Heiblum, R., Arnon, E., Gvaryahu, G., Robinzon, B., and Snapir, N. (2000). Short-term stress increases testosterone secretion from testes in male domestic fowl. *General and Comparative Endocrinology* **120**: 55-66.

Hennessy, M. B. and Levine, S. (1978). Sensitive pituitary-adrenal responsiveness to varying intensities of psychological stimulation. *Physiology and Behavior* **21**: 295-297.

Hennessy, M. B., Heybach, J. P., Vernikos, J., and Levine, S. (1979). Plasma corticosterone concentrations sensitively reflect levels of stimulus intensity in the rat. *Physiology and Behavior* **22**: 821-825.

Herold, M., Brezinschek, H. P., Gruschwitz, M., Dietrich, H., and Wick, G. (1992). Investigation of ACTH responses of chickens with autoimmune disease. *General and Comparative Endocrinology* **55**: 188-198.

Homma, K., Wilson, W. O., and Siopes, T. D. (1972). Eyes have a role in photoperiodic control of sexual activity of Coturnix. *Science* **178**: 421-423.

Johnson, A. L. (1981). Comparison of three serial blood sampling techniques on plasma hormone concentrations in the laying hen. *Poultry Science* 60: 2322-2327.

Johnson, A. L. (2000). Reproduction in the Female. *In* "Sturkie's Avian Physiology" (G. C. Whittow, Ed.), pp569-596. Academic Press, San Diego, California.

Jones, R. B., Beuving, G., and Blokhuis, H. J. (1988). Tonic immobility and heterophil/lymphocyte responses of domestic fowl to corticosterone infusion. *Physiology and Behavior* **42**: 249-253.

Jones, R. B., Satterlee, D. G., and Ryder, F. H. (1992a). Fear and distress in Japanese quail chicks of two lines genetically selected for low or high adrenocortical response to immobilization stress. *Hormones and Behavior* **26** : 385-393.

Jones, R. B., Satterlee, D. G., and Ryder, F. H. (1992b). Research note: open-field behavior of Japanese quail chicks genetically selected for low or high plasma corticosterone response to immobilization stress. *Poultry Science* **71**: 1403-1407.

Jones, R. B., Satterlee, D. G., and Ryder, F. H. (1994a). Fear of humans in Japanese quail selected for low or high adrenocortical response. *Physiology and Behavior* **56**: 379-383.

Jones, R. B., Mills, A. D., Faure, J.-M., and Williams, J. B. (1994b). Restraint, fear, and distress in Japanese quail genetically selected for long or short tonic immobility reactions. *Physiology and Behavior* **56**: 529-534.

Jones, R. B. (1996). Fear and adaptability in poultry: Insights, implications and imperative. *World's Poultry Science Journal* **52**: 131-174.

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

Juss, T. S. (1993). Neuroendocrine and neural changes associated with the photoperiodic control of reproduction. *In* "Avian Endocrinology" (P. J. Sharp, Ed.), pp47-60. Journal of Endocrinology, Bristol.

Józsa, R., Vigh, S., Schally, A. V., and Mess, B. (1984). Localization of corticotrophin-releasing factor -containing neurons in the brain of domestic fowl. *Cell and Tissue Research* **236**: 245-248.

Kato, M. and Konishi, T. (1968). The effect of light and temperature on the testicular growth of Japanese quail. *Poultry Science* **47**: 1052-1056.

Kikuchi, M. and Ishii, S. (1989). Radioiodination of chicken luteinizing hormone without affecting receptor binding potency. *Biology of Reproduction* **41**: 1047-1054.

King, J. and Follett, B. K. (1997). c-*Fos* expression in the putative avian suprachiasmatic nucleus. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology* **180**: 541-551.

King, J. A. and Millar, R. P. (1982). Structure of chicken hypothalamic luteinizing hormone-releasing hormone: II isolation and characterisation. *Journal of Biological Chemistry* **257**: 10729-10732.

Kirby, J. D. and Froman, D. P. (2000). Reproduction in Male Birds. *In* "Sturkie's Avian Physiology" (G. C. Whittow, Ed.), pp597-615. Academic Press, San Diego, California.

Knight, P. G., Cunningham, F. J., and Gladwell, R. T. (1983). Concentrations of immunoreactive luteinizing hormone releasing hormone in discrete brain regions of the cockerel: effects of castration and testosterone replacement therapy. *Journal of Endocrinology* 96: 471-480.

Kobayashi, M. and Ishii, S. (2002). Effects of starvation on gonadotropin and thyrotropin subunit mRNA levels and plasma hormone levels in the male Japanese quail (*Coturnix coturnix japonica*). *Zoological Science* **19**: 331-342.

Konakchieva, R., Mitev, Y., Osborne, A., and Patchev, V. (1997). Chronic melatonin treatment and the hypothalamic-pituitary-adrenal axis in the rat: Attenuation of the secretory response to stress and effects on hypothalamic neuropeptide content and release. *Biology of the Cell* **89**: 587-596.

Konishi, T., Ishiguro, T., and Kato, M. (1965). Photoperiodic studies on the testicular development of Japanese quail. *Zoological Magazine* **74**: 54-62.

Kovács, K., Péczely, P., and Pethes, G. (1983). Daily fluctuations of peripheral metabolism of corticosterone in male Japanese quails: a dynamic approach to the

development of plasma corticosterone daily rhythms. *Comparative Biochemistry and Physiology. A: Comparative Physiology* **75**: 467-469.

Kovács, K. and Péczely, P. (1983). Phase shifts in circadian rhythmicity of total, free corticosterone and transcortine plasma levels in hypothyroid male Japanese quails. *General and Comparative Endocrinology* **50**: 483-489.

Kovács, K. and Péczely, P. (1991). Plasma adrenocorticotropin in domestic geese: effects of ether stress and endocrine manipulations. *General and Comparative Endocrinology* **84**: 192-198.

Kovács, K. J., Westphal, H. M., and Peczely, P. (1989). Distribution of glucocorticoid receptor-like immunoreactivity in the brain, and its relation to CRF and ACTH immunoreactivity in the hypothalamus of the Japanese quail, *Coturnix coturnix japonica. Brain Research* **505**: 239-245.

Launay, F., Mills, A. D., Faure, J.-M., and Williams, J. B. (1993). Effects of CRF on isolated Japanese quail selected for fearfulness and for sociality. *Physiology and Behavior* **54**: 111-118.

Levin, N. and Roberts, J. L. (1991). Positive regulation of propiomelanocortin gene expression in corticotropes and melanotropes. *Frontiers in Neuroendocrinology* **12**: 1-22.

Li, Z. Z. and Burke, W. H. (1987). Influence of 12 hours of sound stimuli on gonad development and plasma luteinizing hormone in Japanese quail (*Coturnix coturnix japonica*) exposed to 6 hours of daily light. *Poultry Science* **66**: 1045-1052.

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

Lovejoy, D. A. and Balment, R. J. (1999). Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. *General and Comparative Endocrinology* **115**: 1-22.

Ma, X.-M., Levy, A., and Lightman, S. L. (1997). Rapid changes in heteronuclear RNA for arginine vasopressin but not for corticotropin releasing hormone in response to acute corticosterone administration. *Journal of Neuroendocrinology* **9**: 723-728.

Mann, D. R. and Orr, T. E. (1990). Effect of restraint stress on gonadal prooopionmelanocortin peptides and the pituitary-testicular axis in rats. *Life Sciences* **46**: 1601-1609.

Martin, J. T., Balthazart, J., and Burke, W. J. (1984). Adrenocortical hormones and reproductive function in Japanese quail. *Journal of Steroid Biochemistry* **20**: 1561.

Massa, R., Davies, D. T., and Bottoni, L. (1980). Cloacal gland of the Japanese quail: androgen dependence and metabolism of testosterone. *Journal of Endocrinology* **84**: 223-230.

McFarland, L. Z., Mather, F. B., and Wilson, W. O. (1964). Testicular response to gonadotropin in intermittent light in coturnix reared under limited light. *American Journal of Veterinary Research* **25**: 1531-1534.

McIlroy, P. J., Kocsis, J. F., Weber, H., and Carsia, R. V. (1999). Dietary protein restriction stress in the domestic fowl (*Gallus gallus domesticus*) alters adrenocorticotropin-transmembranous signalling and corticosterone negative feedback in adrenal steroidogenic cells. *General and Comparative Endocrinology* **113**: 255-266.

McNabb, F. M. A. (1988). Peripheral thyroid hormone dynamics in precocial and altricial avian development. *American Zoologist* **28**: 427-440.

Meddle, S. L. and Follett, B. K. (1995). Photoperiodic activation of a *fos*-like immunoreactive protein in neurones within the tuberal hypothalamus of Japanese quail . *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology* **176**: 79-89.

Meddle, S. L., Foidart, A., Wingfield, J. C., Ramenofsky, M., and Balthazart, J. (1999). Effects of sexual interactions with a male on *fos*-like immunoreactivity in the female quail brain. *Journal of Neuroendocrinology* **11**: 771-784.

Mikami, S. and Yamada, S. (1984). Immunohistochemistry of the hypothalamic neuropeptides and anterior pituitary cells in the Japanese quail. *Journal of Experimental Zoology* **232**: 405-417.

Mikami, S.-I. (1986). Immunocytochemistry of the avian hypothalamus and adenohypophysis. *International Review of Cytology* **103**: 189-248.

Mikami, S.-I., Yamada, S., Hasegawa, Y., and Miyamato, K. (1988). Localization of avian LHRH-immunoreactive neurons in the hypothalamus of the domestic fowl, *Gallus domesticus*, and the Japanese quail, *Coturnix coturnix*. *Cell and Tissue Research* **251**: 51-58.

Millam, J. R., El Halawani, M. E., and Burke, W. H. (1985). Effect of cyclic sound cues on sexual development in nonphotostimulated Japanese quail. *Poultry Science* **64**: 169-175.

Millar, R. P., de L. Milton, R. C., Follett, B. K., and King, J. A. (1986). Receptor binding and gonadotropin-releasing activity of a novel chicken gonadotropin-releasing hormone [His^5, Trp^7, Tyr^8]-GnRH and a $D\text{-}Arg^6$ and analog. *Endocrinology* **119**: 224-231.

Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H. (1984). Identification of the second gonadotropin-releasing hormone in chicken

hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 3874-3878.

Monder, C., Miroff, Y., Marandici, A., and Hardy, M. P. (1994). 11β-Hydroxysteroid dehydrogenase alleviates glucocorticoid-mediated inhibition of steroidogenesis in rat Leydig cells. *Endocrinology* **134**: 1199-1204.

Moore, I. T. and Jessop, T. S. (2003). Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Hormones and Behavior* **43**: 39-47.

Morméde, P., Courvoisier, H., Ramos, A., Marissal-Arvy, N., Ousova, O., Déautés, C., Duclos, M., Chaouloff, F., and M.-P. Moisan (2003). Molecular genetic approaches to investigate individual variations in behavioral and neuroendocrine stress responses. *Psychoneuroendocrinology* **27**: 563-583.

Murton, R. K. and Westwood, N. J. (1977). Avian Breeding Cycles. Oxford University Press.

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

Nicholls, T. J., Goldsmith, A. R., and Dawson, A. (1988). Photorefractoriness in birds and comparison with mammals. *Physiological Reviews* **68**: 133-175.

Noirault, J., Guémené, D., Guy, G., and Faure, J. M. (1999). Corticosterone plasma corticosterone in male mule ducks: effects of sampling sites, repeated samplings and ACTH injections. *British Poultry Science* **40**: 304-308.

Odeh, F. M., Cadd, G. G., and Satterlee, D. G. (2003a). Genetic characterization of stress responsiveness in Japanese quail. 1. Analyses of line effects and combining abilities by diallel crosses. *Poultry Science* **82**: 25-30.

Odeh, F. M., Cadd, G. G., and Satterlee, D. G. (2003b). Genetic characterization of stress responsiveness in Japanese quail. 2. Analyses of maternal effects, additive sex linkage effects, heterosis, and heritability by diallel crosses. *Poultry Science* **82**: 31-35.

Oishi, T. and Konishi, T. (1978). Effects of photoperiod and temperature on testicular and thyroid activity of the Japanese quail. *General and Comparative Endocrinology* **36**: 250-254.

Oishi, T. and Konishi, T. (1983). Variations in the photoperiodic cloacal response of Japanese quail: association with testes weight and feather color. *General and Comparative Endocrinology* **50**: 1-10.

Orr, T. E. and Mann, D. R. (1990). Effects of restraint stress on plasma LH and testosterone concentrations, leydig cell LH/HCG receptors, and *in vitro* testicular steroidogenesis in adult rats. *Hormones and Behavior* **24**: 324-341.

Orr, T. E. and Mann, D. R. (1992). Role of glucocorticoids in the stress-induced suppression of testicular steroidogenesis in adult male rats. *Hormones and Behavior* **26**: 350-363.

Ottinger, M. A. and Brinkley, H. J. (1979). Testosterone and sex related physical characteristics during the maturation of the male Japanese quail (*Coturnix coturnix japonica*). *Biology of Reproduction* **20**: 905-909.

Owens, M. J. and Nemeroff, C. B. (1991). Physiology and pharmacology of corticotropin-releasing factor. *Pharmacological Reviews* **43**: 425-473.

Panzica, G. C. and Viglietti-Panzica, C. (1999). Gonadal steroid-dependent neuronal circuitries in avian limbic and preoptic regions. *European Journal of Morphology* **37**: 112-116.

Panzica, G. C., Balthazart, J., Pessatti, M. and Viglietti-Panzica, C. (2002). The parvocellular vasotocin system of Japanese quail: A developmental and adult model for the study of influences of gonadal hormones on sexually differentiated and behaviorally relevant neural circuits. *Environmental Health Perspectives* **110**: 423-428.

Petitte, J. N. and Etches, R. J. (1988). The effect of corticosterone on the photoperiodic response of immature hens. *General and Comparative Endocrinology* **69**: 424-430.

Petitte, J. N. and Etches, R. J. (1989). The effect of corticosterone on the response of the ovary to pregnant mare's serum gonadotrophin in sexually immature pullets. *General and Comparative Endocrinology* **74**: 377-384.

Petitte, J. N. and Etches, R. J. (1991). Daily infusion of corticosterone and reproductive function in the domestic hen (*Gallus domesticus*). *General and Comparative Endocrinology* **83**: 397-405.

Phillips, J. G., Butler, P. J., and Sharp, P. J. (1985). The reproductive system and its functions. *In* "Physiological Strategies in Avian Biology", pp112-139. Blackie and Son., Glasgow.

Plotsky, P. M., Cunningham, E. T. Jr., and Widmaier, E. P. (1989). Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocrine Reviews* **10**: 437-458.

Radke, W., Albasi, C., and Harvey, S. (1984). Dietary sodium and adrenocortical activity in ducks (*Anas playrhnchos*) and chickens (*Gallus domesticus*). *General and Comparative Endocrinology* **56**: 121-129.

Radke, W., Albasi, C., Rees, A., and Harvey, S. (1985). Dietary sodium and adrenocortical activity in ducks (*Anas playrhnchos*) and chickens (*Gallus domesticus*). *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* **82**: 285-288.

Reinert, B. D. and Wilson, F. E. (1996a). Thyroid dysfunction and thyroxine-dependent programming of photoinduced ovarian growth in American tree sparrows (*Spizella arborea*). *General and Comparative Endocrinology* **103**: 71-81.

Reinert, B. D. and Wilson, F. E. (1996b). The thyroid and the hypothalamic-pituitary-ovarian axis in American tree sparrows (*Spizella arborea*). *General and Comparative Endocrinology* **103**: 60-70.

Remage-Healey, L. and Romero, L. M. (2000). Daily and seasonal variation in response to stress in captive starlings (*Sturnus vulgaris*): glucose. *General and Comparative Endocrinology* **119**: 60-68.

Rivier, C. and Plotsky, P. M. (1986). Mediation by corticotropin-releasing factor (CRF) of adenohypophysial hormone secretion. *Annual Review of Physiology* **48**: 475-494.

Rivier, C. and Rivest, S. (1991). Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. *Biology of Reproduction* **45**: 523-532.

Robinson, J. E. and Follett, B. K. (1982). Photoperiodism in Japanese quail: the termination of seasonal breeding by photorefractoriness. *Proceedings of the Royal Society of London* **215**: 95-116.

Romero, L. M., Ramenofsky, M., and Wingfield, J. C. (1997). Season and migration alters the corticosterone response to capture and handling in an Arctic migrant, the white-crowned sparrow (*Zonotrichia leucophrys gambelii*). *Comparative Biochemistry and Physiology. Part C: Pharmacology, Toxicology and Endocrinology* **116**: 171-177.

Romero, L. M., Soma, K. K., and Wingfield, J. C. (1998a). Changes in pituitary and adrenal sensitivities allow the snow bunting (*Plectrophenax nivalis*), an arctic-breeding song bird, to modulate corticosterone release seasonally. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **168**: 353-358.

Romero, L. M., Soma, K. K., and Wingfield, J. C. (1998b). Hypothalamic-pituitary-adrenal axis changes allow seasonal modulation of corticosterone in a bird. *American Journal of Physiology* **274**: R1338-R1344.

Romero, L. M., Soma, K. K., and Wingfield, J. C. (1998c). The hypothalamus and adrenal regulate modulation of corticosterone release in redpolls (*Carduelis flammea*-an arctic-breeding song bird). *General and Comparative Endocrinology* **109**: 347-355.

Romero, L. M., Dean, S. C., and Wingfield, J. C. (1998d). Neurally active stress peptide inhibits territorial defense in wild birds. *Hormones and Behavior* **34**: 239-247.

Romero, L. M. and Wingfield, J. C. (1998). Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's white-crowned sparrows (*Zonotrichia*

leucophrys gambelii). *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology* **119**: 31-36.

Romero, L. M. and Wingfield, J. C. (1999). Alterations in hypothalamic-pituitary-adrenal function associated with captivity in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*). *Comparative Biochemistry and Physiology. B: Comparative Biochemistry and Molecular Biology* **122**: 13-20.

Romero, L. M. and Remage-Healey, L. (2000). Daily and seasonal variation in response to stress in captive starlings (*Sturnus vulgaris*): corticosterone. *General and Comparative Endocrinology* **119**: 52-59.

Rozenboim, F. E., Tabibzadeh, J. L., and El Halawani, M. E. (1993). Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotrophin releasing hormone I and II content and anterior pituitary VIP receptors in laying turkey hens. *Biology of Reproduction* **48**: 1246-1250.

Sachs, B. D. (1967). Photoperiodic control of the cloacal gland of the Japanese quail. *Science* **157**: 201-203.

Sakai, H. and Ishii, S. (1983). Radioimmunoassay of avian FSH. *In* "Avian Endocrinology: Environmental and Ecological Perspectives" (S. Mikami, Ed.), pp125-134. Springer-Verlag, Berlin.

Satterlee, D. G., Jacobs-Perry, L. A., and Truax, R. E. (1983). Circulating corticosterone response of wild-type Japanese quail to albino quail intrusion. *Poultry Science* **62**: 310-313.

Satterlee, D. G. and Johnson, W. A. (1988). Selection of Japanese quail for contrasting blood corticosterone response to immobilization. *Poultry Science* **67**: 25-32.

Satterlee, D. G. and Jones, R. B. (1997). Ease of capture in Japanese quail of two lines divergently selected for adrenocortical response to immobilization. *Poultry Science* **76**: 469-471.

Satterlee, D. G., Marin, R. H., and Jones, R. B. (2002). Selection of Japanese quail for reduced adrenocortical responsiveness accelerates puberty in males. *Poultry Science* **81**: 1071-1076.

Scanes, C. G. (2000). Introduction to Endocrinology: Pituitary Gland. *In* "Sturkie's Avian Physiology" (G. C. Whittow, Ed.), pp437-460. Academic Press, San Diego, California.

Schiml-Webb, P. A., Temple, J. L., and Rissman, E. F. (2001). Glucocorticoids affect gonadotropin-releasing hormone immunoreactivity in musk shrew brain. *General and Comparative Endocrinology* **123**: 163-169.

Schwabl, H. (1999). Developmental changes and among-sibling variation of corticosterone levels in an altricial avian species. *General and Comparative Endocrinology* **116**: 403-408.

Sharp, P. J., Dawson, A., and Lea, R. W. (1998). Control of luteinizing hormone and prolactin secretion in birds. *Comparative Biochemistry and Physiology. Part C: Pharmacology, Toxicology and Endocrinology* **119**: 275-282.

Sharp, P. J. and Blache, D. (2003). A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long- and short-day photoperiods. *Canadian Journal of Physiology and Pharmacology* **81**: 350-358.

Siegel, H. S. (1971). Adrenals, stress and the environment. *Worlds Poultry Science Journal* **27**: 327-349.

Siegel, H. S. (1995). Stress, strains and resistance. British Poultry Science 36: 3-22.

Silverin, B. (1997). Annual cycles in willow tits *Parus montanus*. *In* "Frontiers in Environmental and Metabolic Endocrinology" (S. L. Maitra, Ed.), pp161-170. The University of Burdwan, India.

Silverin, B. (1998). Stress responses in birds. *Poultry and Avian Biology Reviews* **9**: 153-168.

Silverin, B., Kikuchi, M., and Ishii, S. (1999). Effect of season and photoperiod on FSH in male Great Tits. *General and Comparative Endocrinology* **113**: 457-463.

Siopes, T. D. and Wilson, W. O. (1974). Extraocular modification of photoreception in intact and pinealectomized coturnix. *Poultry Science* **53**: 2035-2041.

Siopes, T. D. and Wilson, W. O. (1975). The cloacal gland - an external indicator of testicular development in coturnix. *Poultry Science* **54**: 1225-1229.

Siopes, T. D. and Wilson, W. O. (1980). Participation of the eyes in the photosexual response of Japanese quail (*Coturnix coturnix japonica*). *Biology of Reproduction* **23**: 352-357.

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

Smith, R. F. and Dobson, H. (2002). Hormonal interactions within the hypothalamus and pituitary with respect to stress and reproduction in sheep. *Domestic Animal Endocrinology* **23**: 75-85.

Speiss, J., Dautzenberg, F. M., Sydow, S., Hauger, R. L., Rühmann, A., Blank, T., and Radulovic, J. (1998). Molecular properties of the CRF receptor. *Trends in Endocrinology and Metabolism* **9**: 140-145.

Spinedi, E., Giacomani, M., Jacquier, M. C., and Gaillard, R. C. (1991). Changes in hypothalamo--corticotrope axis after bilateral adrenalectomy: evidence for a median eminence site of glucocorticoid action. *Neuroendocrinology* **53**: 160-170.

Stein, G. S. and Bacon, W. L. (1976). Effect of photoperiod upon age and maintenance of sexual development in female *Coturnix coturnix japonica*. *Poultry Science* **55**: 1214-1218.

Takeuchi, H., Yazaki, Y., Matsushima, T., and Aoki, K. (1996). Expression of *fos*-like immunoreactivity in the brain of quail chick emitting the isolation-induced distress calls. *Neuroscience Letters* **220**: 191-194 [Abstract].

Talbot, R. T. and Sharp, P. J. (1994). A radioimmunoassay for recombinant-derived chicken prolactin suitable for the measurement of prolactin in other avian species. *General and Comparative Endocrinology* **96**: 361-369.

Tanabe, Y., Nakamura, T., Fujioka, K., and Doi, O. (1979). Production and secretion of sex steroid hormones by the testes, the ovary, and the adrenal glands of embryonic and young chickens (*Gallus domesticus*). *General and Comparative Endocrinology* **39**: 26-33.

Tanabe, Y., Ogawa, T., and Nakamura, T. (1981). The effect of short-term starvation on pituitary and plasma LH, plasma estradiol and progesterone, and on pituitary response to LH-RH in the laying hen (*Gallus domesticus*). *General and Comparative Endocrinology* **43**: 392-398.

Tanabe, Y., Saito, N., and Nakamura, T. (1986). Ontogenetic steroidogenesis by testes, ovary, and adrenals of embryonic and postembryonic chickens (*Gallus domesticus*). *General and Comparative Endocrinology* **63**: 456-463.

Tanaka, K., Mather, F. B., Wilson, W. D., and McFarland, L. Z. (1964). Effect of photoperiod on early growth of gonads and on potency of gonadotrophins of the anterior pituitary in Coturnix. *In* "Canadian Federation of Biological Societies Meeting Proceedings", pp662-665. Canadian Federation of Biological Societies, Ottawa.

Tanaka, K., Mather, F. B., Wilson, W. O., and McFarland, L. Z. (1965). Effect of photoperiod on early growth of gonads and on potency of gonadotropins in the anterior pituitary of coturnix. *Poultry Science* **44**: 662-665.

Teitsma, C. A., Anglade, I., Lethimonieer, C., LeDrean, G., Saligaut, D., Ducouret, B., and Kah, O. (1999). Glucocorticoid receptor immunoreactivity in neurons and pituitary cells implicated in reproductive functions in rainbow trout: A double immunohistochemical study. *Biology of Reproduction* **60**: 642-650.

Tilbrook, A. J., Turner, A. I., and Clarke, I. J. (2000). Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of Reproduction* **5**: 105-113.

Tilbrook, A. J., Turner, A. I., and Clarke, I. J. (2002). Stress and reproduction: Central mechanisms and sex differences in non-rodent species. *Stress* **5**: 83-100.

Tsuyoshi, H. and Wada, M. (1992). Termination of LH secretion in Japanese quail due to high- and low-temperature cycles and short daily photoperiods. *General and Comparative Endocrinology* **85**: 424-429.

Urbanski, H. F. and Follett, B. K. (1982a). Sexual differentiation of the photoperiodic response in Japanese quail. *Journal of Endocrinology* **92**: 279-282.

Urbanski, H. F. and Follett, B. K. (1982b). Photoperiodic modulation of gonadotrophin secretion in the castrated Japanese quail. *Journal of Endocrinology* **92**: 73-83.

Van de Kar, L. D. and Blair, M. L. (1999). Forebrain pathways mediating stress-induced hormone secretion. *Frontiers in Neuroendocrinology* **20**: 1–48.

Van Lier, E., Regueiro, M., Perez-Clariget, R., Andersson, H., Kindahl, H., and Forsberg, M. (1999). Effects of adrenocorticotrophin (ACTH) and progesterone on luteinising hormone (LH) secretion in recently castrated rams. *Animal Reproduction Science* **55**: 115-126.

Van Tienhoven, A. (1961). The effect of massive doses of corticotropin and of corticosterone on ovulation of the chicken (*Gallus domesticus). Acta Endocrinologica* **38**: 407-412.

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

Wada, M. (1979). Photoperiodic control of LH secretion in Japanese quail with special reference to the photoinducible stage. *General and Comparative Endocrinology* **39**: 141-149.

Wada, M., Hatanaka, F., Tsuyoshi, H., and Sonoda, Y. (1990). Temperature modulation of photoperiodically induced LH secretion and its termination in Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology* **80**: 465-472.

Wada, M., Akimoto, R., and Tsuyoshi, H. (1992). Annual changes in levels of plasma LH and size of cloacal protrusion in Japanese quail (*Coturnix coturnix japonica*) housed in outdoor cages under natural conditions. *General and Comparative Endocrinology* **85**: 415-423.

Wada, M. (1993). Low temperature and short days together induced thyroid activation and suppression of LH release in Japanese quail. *General and Comparative Endocrinology* **90**: 355-363.

Wada, M., Shimizu, T., Kobayashi, S., Yatani, A., Sandaiji, Y., Ishikawa, T., and Takemure, E. (1999). Behavioral and hormonal basis of polygynous breeding in male bush warblers (*Cettia diphone*). *General and Comparative Endocrinology* **116**: 422-432.

Wakabayashi, S., Kikuchi, M., Wada, M., Sakai, H., and Ishii, S. (1992). Induction of ovarian growth and ovulation by administration of a chicken gonadotrophin preparation to Japanese quail kept under a short-day regimen. *British Poultry Science* **33**: 847-858.

Webb, M. L. and Marshaly, M. M. (1984). Effect of adaptation to handling on the circulating corticosterone concentration of laying hens. *British Poultry Science* **25**: 425-427.

Westerhof, I., Lumeij, J. T., Mol, J. A., van den Brom, W. E., and Rijnberk, A. (1992). *In vivo* studies on the effects of ovine corticotrophin-releasing hormone, arginine vasotocin, arginine vasopressin, and haloperidol on adrenocortical function in the racing pigeon (*Columba livia domestica*). *General and Comparative Endocrinology* **88**: 76-82.

Williams, J. B., Etches, R. J., and Rzasa, J. (1985). Induction of a pause in laying by corticosterone infusion or dietary alterations: effects on the reproductive system, food consumption and body weight. *British Poultry Science* **26**: 25-34.

Wilson, C. M. and Holberton, R. L. (2001). An alternative method for delivering adrenocorticotropin hormone in birds. *General and Comparative Endocrinology* **122**: 349-353.

Wilson, E. K., Rogler, J. C., and Erb, R. E. (1979). Effect of sexual experience, location, malnutrition, and repeated sampling on concentrations of testosterone in blood plasma of *Gallus domesticus* roosters. *Poultry Science* **58**: 718-786.

Wilson, F. E. and Follett, B. K. (1975). Corticosterone-induced gonadosuppression in photostimulated tree sparrows. *Life Sciences* **17**: 1451-1456.

Wilson, F. E. (1985). Androgen feedback-dependent and independent control of photoinduced LH secretion in male tree sparrows (*Spizella arborea*). *Journal of Endocrinology* **105**: 141-152.

Wilson, F. E. (1986). A testosterone-independent reduction in net photoperiodic drive triggers photorefractoriness in male tree sparrows (*Spizella arborea*). *Journal of Endocrinology* **109**: 133-137.

Wilson, F. E. (1989). Extraocular control of photorefractiveness in American tree sparrows (*Spizella arborea*). *Biology of Reproduction* **41**: 111-116.

Wilson, F. E. (1990a). Extraocular control of seasonal reproduction in female tree sparrows (*Spizella arborea*). *General and Comparative Endocrinology* **77**: 397-402.

Wilson, F. E. (1990b). On the recovery of photosensitivity in two passerine species, American tree sparrows (*Spizella arborea*) and Harris' sparrows (*Zonotrichia querula*). *General and Comparative Endocrinology* **79**: 283-290.

Wilson, F. E. (1990c). The drive on luteinizing hormone secretion in castrated tree sparrows (*Spizella arborea*) exposed to short days is daylength dependent. *General and Comparative Endocrinology* **77**: 484-488.

Wilson, F. E. and Reinert, B. D. (1993). The thyroid and photoperiodic control of seasonal reproduction in American tree sparrows (*Spizella arborea*). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **163**: 563-573.

Wilson, S. C. and Sharp, P. J. (1975). Episodic release of luteinizing hormone in the domestic fowl. *Journal of Endocrinology* **64**: 77-86.

Wilson, S. C. and Sharp, P. J. (1976). Effects of androgens, oestrogens and deoxycorticosterone acetate on plasma concentrations of luteinizing hormone in laying hens. *Journal of Endocrinology* **69**: 93-102.

Wilson, W. O. and Huang, R. H. (1962). A comparison of the time of ovipositing for coturnix and chicken. *Poultry Science* **41**: 1843-1845.

Wilson, W. O., Mather, F. B., and Tanaka, K. (1965). Maintenance of egg production in Coturnix following reduction in photoperiod. *Poultry Science* **44**: 1299-1302.

Wingfield, J. C. (1983). Environmental and endocrine control of avian reproduction: an ecological perspective. *In* "Avian Endocrinology: Environmental and Ecological Perspectives" (S. Mikami, Ed.), pp265-288. Springer-Verlag, Berlin.

Wingfield, J. C., Matt, K. S., and Farner, D. S. (1984). Physiologic properties of steroid hormone-binding proteins in avian blood. *General and Comparative Endocrinology* **53**: 281-292.

Wingfield, J. C. and Silverin, B. (1986). Effects of corticosterone on territorial behavior of free-living male song sparrows, *Melospiza melodia*. *Hormones and Behavior* **20**: 405-417.

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

Wingfield, J. C. (1994). Modulation of the adrenocortical response to stress in birds. *In* "Perspectives in comparative endocrinology" (S. Tobe, Ed.), pp520-528. National Research Council of Canada, Toronto.

Wingfield, J. C., Hahn, T. P., Wada, M., and Schoech, S. J. (1997a). Effects of daylength and temperature on gonadal development, body mass, and fat depots in

white-crowned sparrows, *Zonotrichia leucophrys pugetensis*. *General and Comparative Endocrinology* **107**: 44-62.

Wingfield, J. C., Breuner, C., and Jacobs, J. (1997b). Corticosterone and behavioral response to unpredictable events. *In* "Perspectives in Avian Endocrinology" (S. Harvey and R. J. Etches, Eds.). Journal of Endocrinology Ltd., Bristol.

Wingfield, J. C., Jacobs, J., and Hillgarth, N. (1997c). Ecological constraints and the evolution of hormone-behavior interrelationships. *Annals of the New York Academy of Science* **807**: 22-41.

Wingfield, J. C., Maney, D. L., Breuner, C. W., Jacobs, J. D., Lynn, S., Ramenofsky, M., and Richardson, R. D. (1998). Ecological bases of hormone-behavior interactions: The "Emergency life history stage". *American Zoologist* **38**: 191-206.

Wingfield, J. C., Ramos-Fernandez, G., Nuñez-de la Mora, A., and Drummond, H. (1999). The effects of an "El Niño" southern oscillation event on reproduction in male and female Blue-footed Boobies, *Sula nebouxii*. *General and Comparative Endocrinology* **114**: 163-172.

Wingfield, J. C. and Romero, L. M. (2001). Adrenocortical responses to stress and their modulation in free-living vertebrates. *In* "Handbook of Physiology: The Endocrine System" (B. S. McEwen and H. M. Goodman, Eds.), Vol. 4, pp211-234. Oxford University Press, New York.

Wingfield, J. C. and Sapolsky, R. M. (2003). Reproduction and resistance to stress: when and how. *Journal of Neuroendocrinology* **15**: 711-724.

Woodard, A. E. and Mather, F. B. (1964). The timing of ovulation, movement of the ovum through the oviduct, pigmentation and shell deposition in the Japanese quail. *Poultry Science* **43**: 1427-1432.

Yasuo, S.; Watanabe, M.; Okabayashi, N., Ebihara, S., and Yoshimura, T. (2003). Circadian clock genes and photoperiodism: comprehensive analysis of clock gene expression in the mediobasal hypothalamus, the suprachiasmatic nucleus, and the pineal gland of Japanese quail under various light schedules. *Endocrinology* **144**: 3742-3748

Yu, J., Xie, L. Y., and Abou-Samra, A. B. (1996). Molecular cloning of a type A chicken corticotropin releasing factor receptor with high affinity for urotensin-I. *Endocrinology* **137**: 192-197.

Zachariasen, R. and Newcomer, W. (1971). Phenylethanolamine-N-methyl transferase activity in the avian adrenal following immobilization or adrenocorticotropin. *General and Comparative Endocrinology* **23**: 193-198.

Zelena, D., Mergl, Z., Földes, A., Kovács, K. J., Tóth, Z., and Makara, G. B. (2003). Role of hypothalamic inputs in maintaining pituitary-adrenal responsiveness in repeated restraint. *American Journal of Physiology* **285**: E1110-E1117.