# Corticosterone, fear behaviour and plasma corticosterone responses to stressors in Japanese quail

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# Abstract

Stress responses involve activation of the hypothalamo-pituitary-adrenal (HPA) axis and the secretion of glucocorticoid hormones, and can help animals cope with changes in their environments. Corticosterone is the primary glucocorticoid in birds and has metabolic actions, and can affect behaviour and reproduction. Stimuli that activate the HPA axis are called stressors, and stressors can be classified as either physical or emotional. When animals respond to emotional stressors they also experience fear, and the magnitude of a corticosterone response to an emotional stressor is thought to be related to the degree of fearfulness that is experienced by a bird. The great majority of studies have measured plasma corticosterone responses of birds to emotional rather than physical stressors, and corticosterone responses to emotional stressors are assumed to reflect the responsiveness of the HPA axis of birds to stressors in general. The aims of the research described in this thesis were to determine the effects of corticosterone on fear behaviour, reproductive function, and plasma corticosterone responses to stressors in Japanese quail (Coturnix coturnix japonica), both during and after treatment, and to examine if plasma corticosterone responses to similar emotional stressors, and to different types of emotional and physical stressors are related in individual quail.

Plasma corticosterone concentrations were significantly higher in quail treated with corticosterone in their drinking water compared with controls during a 21 day treatment period, and concentrations remained elevated eight days after treatment ended. Corticosterone had little or no effect on the fearfulness of quail in tonic immobility, novel object or open field tests of fear behaviour. Body weight, food intake, egg production and egg weight were significantly lower in some corticosterone treatment groups than in controls during treatment and for up to 22 days after treatment ended. Corticosterone concentrations in quail were generally unaffected after 24 h of fasting, so the effects of elevated plasma corticosterone on corticosterone responses to a natural stressor in quail could not be determined. Corticosterone had marked effects on the physiology of quail for several weeks after treatment ended, suggesting that chronic elevations in plasma corticosterone resulting from climate change or human disturbance could have negative affects in birds even after exposure to a stressor ends.

There were significant positive relationships between the magnitudes of plasma corticosterone responses to the emotional stressors of 15, 30 or 60 min handling followed by 45, 30 or 0 min confinement respectively in individual Japanese quail. Plasma corticosterone responses to 15 min handling followed by 45 min confinement are commonly measured in domesticated species of birds, and the findings of the present study suggest that magnitudes of responses to this standardised stressor may reflect the responsiveness of the HPA axis of birds to emotional stressors in general. Treatment with insulin and treatment with lipopolysaccharide (LPS) were shown to be physical stressors in quail, and doses and blood sampling times determined in insulin and LPS dose-response tests were used in a study of plasma corticosterone responses to emotional and physical stressors in quail. There were no relationships between corticosterone responses of individual birds subjected to emotional (handling) and physical (insulin and LPS) stressors, whereas there were significant correlations in responses of the birds to the two physical stressors. These results suggest that corticosterone responses of birds to standardised emotional stressors such as handling and confinement in domesticated species or capture and restraint in free-living species may not reflect the responsiveness of the HPA axis of birds to stressors in general. Given that quail displayed consistent individual differences in their plasma corticosterone responses to emotional stressors and to physical stressors, but magnitudes of corticosterone responses to both classes of stressor were unrelated in individual quail, these findings suggest that birds may possess at least two quite distinct stress responses to help them cope with changes in their environments.

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# 1. General introduction

Animals may experience potentially harmful events each day. A change could occur within the body, such as a fall in blood glucose, or an animal may experience a situation in its environment, such as a lack of food, that requires physiological and behavioural responses in order to survive. The stress response is an adaptive response that is initiated when animals experience stressors, where stressors are stimuli that pose physical challenges or are perceived as threats (Chrousos, 1998; Cockrem, 2007; Jankord and Herman, 2008). Stress responses involve activation of the hypothalamo-pituitary-adrenal (HPA) axis and the secretion of glucocorticoid hormones (Selye, 1974; Holmes and Phillips, 1976). In most mammals and teleost fish the predominant glucocorticoid is cortisol, whilst in some mammals (e.g. rats) and all non-mammalian tetrapods, including birds, the primary glucocorticoid is corticosterone (Holmes and Phillips, 1976). Glucocorticoids can help animals to avoid the potentially deleterious effects of changes in their environments. The secretion of corticosterone during stress in birds, for example, can increase gluconeogenesis to restore normal blood glucose concentrations (Wingfield and Kitaysky, 2002), or can increase locomotor activity to promote food searching behaviour (Wingfield and Ramenofsky, 1997).

A variety of experimental approaches can be used to study stress and the effects of stress in birds. One approach to studying stress is to expose birds to a stimulus that poses a physical challenge, such as bacterial infection, or to a stimulus that might be perceived as a threat, such as an unfamiliar situation (Herman et al., 2003). The stimulus could potentially initiate an endogenous corticosterone stress response in the bird. An approach to studying the effects of stress is to treat birds with exogenous corticosterone to simulate the rise in plasma corticosterone concentrations that occur during stress. Blood samples can be collected from birds during these studies and plasma corticosterone concentrations can subsequently be measured by radioimmunoassay. Corticosterone concentrations can then be used to estimate the level of stress birds are experiencing, or be related to physiological or behavioural changes that occur within birds. Both experimental approaches were used in the current study of

corticosterone, fear behaviour and plasma corticosterone responses to stressors in Japanese quail (Coturnix coturnix japonica).

This first introductory chapter reviews the literature that is relevant to the thesis as a whole, and discusses the biological significance of the main questions of the thesis. Chapter one concludes with an outline of the thesis. Each of the six experimental chapters has an introduction and discussion that cover material specific to that chapter. A general discussion integrates the findings of the experimental chapters and identifies future research possibilities. Cited literature appears in a list of references following the general discussion, and appendix figures and tables are included at the end of the thesis.

## 1.1 Stress, stressors and the hypothalamo-pituitary-adrenal axis

Stress means different things to different people and so there is no universally accepted definition of the term stress. Few people could argue however, that the founder of the biological concept of stress was Hans Selye. In a one-page document in the journal Nature, Selye formulated the idea of the General Adaptation Syndrome, or GAS (Selye, 1936). In studies of rats (Rattus norvegicus), Selye observed that a diverse array of external stimuli such as exposure to cold, surgical injury, or excessive muscular exercise, produced a syndrome in the animal that was common to all noxious agents. Selye concluded that the nature of the damaging agent was unimportant; instead, he noted that the physiological consequences of the stimuli within the body were a response to "damage as such". Selye suggested that there were three phases to the GAS; the alarm, resistance and exhaustion phases. In his book "Stress without distress", Selye expanded on his concept of stress, suggesting that stress involved activation of the hypothalamo-pituitary-adrenal axis and the sympathetic nervous system (Selye, 1974). In the same decade that Selye proposed his idea of the GAS, Walter Cannon released a book named "The wisdom of the body". Cannon proposed the concept of homeostasis to account for the "coordinated physiological processes which maintain most of the steady states in the organism" (Cannon, 1932). The concept of homeostasis has since been incorporated into a biological definition of stress by some scientists, with stress often referred to as "a state of threatened homeostasis" (Chrousos, 1998). Given that there is some disagreement as to the biological meaning of stress, it is

important to begin by providing the definition of stress that applies to the current study. Here, an animal is considered to be in a state of stress when the hypothalamo-pituitary-adrenal (HPA) axis is activated in response to a stressor, and a stress response only occurs when there is an increase in glucocorticoid secretion. Stress responses may also involve activation of the sympathetic nervous system (SNS) and the secretion of catecholamines (Selye, 1974; Silverin, 1998; Sapolsky et al., 2000). The SNS component of a stress response is often referred to as the "fight or flight " response (Cannon, 1929), may last only for a few seconds (Wingfield et al., 1998), and is comparable to the alarm phase of Selye's GAS. Increased activity of the SNS is not a definitive indicator of stress, however, as catecholamines may also be secreted in response to stimuli that are not stressors. Catecholamine secretion increased significantly in homing pigeons (Columba livia domestica) during a 60-80 min flight, for example, whereas plasma corticosterone concentrations in pigeons after the flight were no different than corticosterone concentrations before the flight (Viswanathan et al., 1987). It was, therefore, concluded that pigeons did not experience stress during the 60-80 min flight.

In the current study, stimuli that pose physical challenges to animals and initiate stress responses through disturbance of physical or chemical tissue parameters are called physical stressors, whilst stimuli that are perceived as a threat following comparison with innate predispositions or previous experience and initiate stress responses are called emotional stressors (Day et al., 1999; Jankord and Herman, 2008). The separation of stressors into two distinct categories has widespread support (Li et al., 1996; Herman and Cullinan, 1997; Day et al., 1999; Dayas et al., 2001; Reyes et al., 2003; Wittmann, 2008), although names for the two types of stressor vary. The names used here are consistent with those of Day et al. (1999), although physical stressors can also be called physiological, homeostatic, systemic, somatic, bottom-up or interoceptive stressors, and emotional stressors are also referred to as psychological, processive, neurogenic, exteroceptive, top-down, psychogenic or cognitive stressors (DiMicco et al., 2002; Walker et al., 2002; Reyes et al., 2003; Pecoraro et al., 2006; Jankord and Herman, 2008). Potential physical stressors include immune challenge, haemorrhage and treatment with insulin, whilst emotional stressors can include handling by a human, restraint, novel environments and the sight of a potential predator (Dayas et al., 2001; Herman et al., 2003).

Irrespective of the type of stressor an animal is responding to, however, all stress responses involve activation of the HPA axis. When an animal responds to a stressor corticotropin-releasing factor (CRF) and arginine vasotocin (AVT, birds) or arginine vasopressin (AVP, mammals) are secreted from parvocellular and magnocellular neurons respectively, of the paraventricular nucleus (PVN) of the hypothalamus. CRF travels in the portal blood system to the anterior pituitary gland, where it stimulates the secretion of adrenocorticotropic hormone (ACTH) by corticotroph cells. ACTH is then carried in the blood to the adrenal glands, where it stimulates the synthesis and secretion of glucocorticoids (Lopez et al., 1999; Tachibana et al., 2004).

There is considerable interest in the neurocircuitry involved in the generation of stress responses to different categories of stressor, with a variety of interpretations of the data available from studies in mammals. One view is that physical stressors are relayed directly to the hypothalamic PVN, probably via brainstem catecholaminergic projections, whilst responses to emotional stressors are integrated through limbic forebrain regions, including the amygdala, hippocampus and prefrontal cortex (Herman and Cullinan, 1997). An alternative view is that physical stressors are processed by the central nucleus of the amygdala whilst responses to emotional stressors are mediated primarily through the medial nucleus of the amygdala (Day et al., 1999). A study of rats found that physical stressors, such as immune challenge or haemorrhage, primarily activated cells of the central nucleus of the amygdala, whilst emotional stressors, including restraint or noise, recruited cells of the medial nucleus of the amygdala (Dayas et al., 2001). Physical stressors also activated different medullary noradrenergic cell groups to emotional stressors, and it was suggested that "neural activation footprints" could be used to categories stressors (Dayas et al., 2001). It is also reported that exposure to a physical stressor (treatment with lipopolysaccharide) and to an emotional stressor (30 min restraint) recruit few genes in common in the hypothalamic PVN of rats (Reyes et al., 2003). Thus, although the exact mechanisms remain unclear, there is sufficient empirical evidence to suggest that the neurocircuitry involved in the generation of stress responses differs for physical and emotional stressors.

While most people consider stress to have mainly negative consequences, stress responses can actually help animals cope with changes in their environment (Reeder and Kramer, 2005). Increased glucocorticoid secretion in an animal during a severe storm

or when it is attacked by a predator, for example, can mobilise energy to help the individual move away from the source of danger (Wingfield, 2003). Stress responses that involve transient or short-term increases in glucocorticoid secretion are often referred to as acute stress responses. The acute glucocorticoid stress response is believed to be adaptive (Wingfield, 1994; Wingfield et al., 1998; Breuner et al., 2008), and the early stages of this response can be equated with the resistance phase of Selye's GAS (Selye, 1936). It is suggested that a "good" acute stress response might involve relatively low initial blood glucocorticoid concentrations that reach maximum concentrations within minutes of exposure to a stressor, with glucocorticoids quickly returning to initial concentrations after exposure to a stressor ends (Breuner et al., 2008). An acute increase in glucocorticoid secretion lasting minutes to hours can have a range of effects in animals that include suppression of reproductive behaviour (without regression of the reproductive system), regulation of the immune system, and an increase in gluconeogenesis and foraging behaviour (Wingfield, 1994; Wingfield et al., 1998). Glucocorticoids also play a major role in recovery from an acute stress response. HPA axis activity can be reduced, for example, via negative feedback mechanisms when glucocorticoids bind to high affinity mineralocorticoid receptors (MRs) and low affinity glucocorticoid receptors (GRs) in various regions of the brain, including the hippocampus, lateral septum and hypothalamus (de Kloet, 2000; Tilbrook and Clarke, 2006). Attenuation of the acute stress response, therefore, limits the potentially deleterious effects of glucocorticoid excess. In contrast to acute stress, chronic stress can occur in animals when they experience a series of stressors, or are constantly exposed to a stressor, resulting in blood glucocorticoid concentrations that are frequently elevated above initial concentrations for days or even weeks. Chronic glucocorticoid secretion in animals can be equated with the exhaustion phase of Selye's GAS, and can lead to inhibition of the reproductive system, suppression of the immune system, severe protein loss, neuronal cell death, and suppression of growth (Wingfield, 1994; Wingfield et al., 1998). Thus, while acute glucocorticoid stress responses are considered to be adaptive, chronic stress is generally non-adaptive, eventually leading to death in some instances (Tsigos and Chrousos, 2002; Tilbrook and Clarke, 2006).

## 1.2 Studying stress and the effects of stress in birds

Given the adaptive value of the acute glucocorticoid stress response, the potentially

deleterious consequences of chronic stress, and the myriad actions of glucocorticoids, studies of stress and the effects of stress in birds are important from both ecological and production perspectives (Mench, 1991; Wingfield, 1994; Silverin, 1998; Cockrem, 2007; Breuner et al., 2008). Two commonly used approaches to studying stress and the effects of stress in birds are to characterise corticosterone responses to stressors, and to treat birds with exogenous corticosterone.

### 1.2.1 Corticosterone responses to stressors in birds

A corticosterone response involves activation of the HPA axis in response to a stressor, and the magnitude and duration of corticosterone responses can be used to estimate the level of stress birds are experiencing. Responses to stressors can be characterised by measuring corticosterone concentrations in blood or faecal samples collected from birds after exposure to a stressor (Fraisse and Cockrem, 2006). Corticosterone responses to a range of stressors have been extensively studied in both domesticated and free-living species of birds, where stressors can include fasting (Harvey et al., 1983), social isolation (Mills et al., 1993) and presentation of a novel object in the home cage (Richard et al., 2008). In order to measure and then compare the magnitude and duration of corticosterone responses between individuals, populations or species, birds are normally subjected to a standardised artificial emotional stressor. The stressor most widely used in free-living species is capture followed by restraint (Wingfield, 1994; Silverin, 1998), whilst stressors commonly used in domesticated birds are mechanical restraint (Jones and Satterlee, 1996) or repeated handling followed by confinement (Fraisse and Cockrem, 2006). Free-living birds are either caught in nets or trapped, whereas captive and domesticated birds are removed from their aviaries or cages. An initial blood sample is collected, then birds are held in a cloth bag or box, mechanically restrained or handled repeatedly for up to 60 min, and further blood samples are taken. It is thought that birds perceive these procedures as a predation event, and a robust corticosterone response is often initiated (Wingfield, 1994; Silverin, 1998). The magnitude and duration of a corticosterone response can depend on the type and severity of a stressor (Harvey et al., 1984; Canoine et al., 2002), and may also vary with the time of year or breeding stage (Romero, 2002), migratory status (Romero et al., 1997; Holberton, 1999), body condition (Heath and Dufty, 1998), habitat (Partecke et al., 2006), gender (Astheimer et al., 1994) and age (Blas et al., 2006).

Plasma corticosterone responses to capture followed by restraint in a cloth bag or box for 30 to 60 min have been extensively studied in many free-living species of birds. Corticosterone usually rises steadily for 30 min and may continue to increase throughout a 60 min period of restraint. Mean corticosterone concentrations immediately after capture are usually low (5 to 10 ng/ml), and maximum mean corticosterone concentrations can range from 20 to 100 ng/ml or more (Silverin, 1998; Landys-Ciannelli et al., 2002; Adams et al., 2005). Plasma corticosterone responses to repeated handling (pick up and put down) for 15 min followed by confinement in a cardboard box for 45 min have been measured in domesticated Japanese quail and chickens (Gallus domesticus; Littin and Cockrem, 2001; Fraisse and Cockrem, 2006; Hull et al., 2007). Corticosterone typically rises at 15 min, declines at 30-40 min and remains relatively constant thereafter. Mean corticosterone concentrations in birds immediately after removal from their cages are usually 1 to 3 ng/ml (Kovacs et al., 1983; Korte et al., 1997; Littin and Cockrem, 2001; Fraisse and Cockrem, 2006; Hull et al., 2007). Maximum mean corticosterone concentrations after 15 min handling are <15 ng/ml, and are markedly lower than maximum corticosterone concentrations in many free-living species following capture and restraint in a cloth bag or box.

There are marked differences between individual birds in their corticosterone responses to stressors. In studies of domesticated species (chickens; Littin and Cockrem, 2001), and captive (great tits, Parus major; Cockrem and Silverin, 2002b) or wild (Adelie penguins, Pygoscelis adeliae; Cockrem et al., 2009) free-living species, corticosterone responses of birds exposed to the same stressor differ in magnitude and duration. Differences in corticosterone responses between birds lead to questions about how variation in corticosterone responses arises and why variation exists. Variation in corticosterone responses may result from differences in the way a stressor is perceived, or from differences in the functioning of the HPA axis, and both genetics and previous experience may influence the response (Satterlee and Johnson, 1988; Cockrem, 2007). But what is the functional significance of individual variation in corticosterone responses? It has been suggested that birds with relatively low corticosterone responses might have greater fitness (survival and reproduction) in constant environments, whilst birds with higher corticosterone responses might have greater fitness in changing environments (Cockrem, 2005). Individual variation in corticosterone responses to stressors is an area of intense interest, given the significance of the stress response in

relation to the evolution and ecology of species (Williams, 2008). Whilst there are marked differences between individual birds in their corticosterone responses to stressors, the responses of individuals exposed to the same emotional stressor on more than one occasion are generally repeatable (Littin and Cockrem, 2001; Cockrem and Silverin, 2002b; Wada et al., 2008; Angelier et al., 2009).

The great majority of studies of stress in birds have measured plasma corticosterone responses of individuals to just one type of artificial stressor and only on one occasion. Natural stressors that birds experience in the wild may vary in type, duration, and intensity, however, and measuring corticosterone responses to stressors on single occasions really only provides a "snapshot" of HPA axis activity within individuals or groups of birds. Indeed, measuring corticosterone responses of birds to stressors on single occasions gives no indication of whether or not these measurements reflect the phenotype of the sampled individuals or sampling error (Williams, 2008; Wingfield et al., 2008). Characterising the responses of birds to one or more stressors in natural environments is difficult, given that the same birds need to be captured on two or more occasions. Domesticated species such as chickens and quail, captive free-living species (i.e. great tits), and flightless free-living species like penguins, however, are easier to repeatedly subject to stressors. Indeed, plasma corticosterone responses to handling and confinement or capture and restraint have been measured on three or four occasions in these species. Chickens had greater mean corticosterone concentrations after 15 min handling on the first occasion compared with three subsequent occasions on which corticosterone concentrations were similar (Littin and Cockrem, 2001), and corticosterone concentrations at 30 min were similar on three occasions in great tits (Cockrem and Silverin, 2002b) and Adelie penguins (Cockrem et al., 2009). However, these studies do not provide any knowledge of how birds may respond to similar emotional stressors. Do the magnitudes and durations of plasma corticosterone responses increase in birds as the duration of a stressor increases, and are the responses to similar emotional stressors related in individual birds? The current study describes an experiment that was designed to address these questions (see section 1.7).

Plasma corticosterone responses to emotional stressors like capture followed by restraint or repeated handling followed by confinement have been extensively studied in free-living and domesticated species of birds. It is important to note, however, that these are

not natural situations for birds; rather they are artificial situations that have been designed by humans to simulate a predation event (Wingfield, 1994; Silverin, 1998), a situation that could occur in a bird's natural environment. Responses to natural stressors are more difficult to measure than responses to artificial stressors, particularly in wild birds (Cockrem, 2007). One of the major assumptions of avian stress endocrinology is that standardised emotional stressors reflect the responsiveness of the HPA axis of birds to stressors in general, and hence, to more natural stressors (Breuner et al., 2008). This assumption has never been tested, however, and there are no data to confirm that plasma corticosterone responses to capture followed by restraint or handling followed by confinement reflect the responsiveness of the HPA axis of birds to physical stressors such as immune challenge or treatment with insulin. Do corticosterone responses to standardised emotional stressors reflect the responsiveness of the HPA axis of birds to physical stressors, and hence, to stressors in general? A potential answer to this important question was investigated in the present study (see section 1.7).

### 1.2.2 Treatment of birds with corticosterone

Treatment of birds with exogenous corticosterone can simulate the rise in plasma corticosterone concentrations that occur during stress, and can be used to address important questions about the effects of stress in birds. Neural pathways involved in the generation of stress responses, and described in section 1.1, are not responsible for the increase in plasma corticosterone concentrations in birds treated with corticosterone. The HPA axes of birds treated with corticosterone are therefore not activated, and such birds are not in a state of stress per se. Treatment with corticosterone is nonetheless widely used to study the effects of stress in birds, and different treatment methods can be used to simulate the increases in plasma corticosterone concentrations that occur during acute or chronic stress. Plasma corticosterone concentrations can be experimentally elevated for minutes to hours to simulate the rise in corticosterone concentrations that occur when birds experience acute stress. Plasma corticosterone in birds can be increased for minutes to hours by applying corticosterone to the skin (Busch et al., 2008b), injecting corticosterone into mealworms before ingestion (Breuner et al., 1998; Lohmus et al., 2006), or by single injections of corticosterone (Lin et al., 2004b). Other treatment methods can be used to increase plasma corticosterone

concentrations for hours to days to simulate the rise in corticosterone concentrations that occur when birds experience chronic stress. Plasma corticosterone can be elevated for hours to days by implanting birds with subcutaneous or intraperitoneal implants or pellets containing corticosterone (Etches et al., 1984; Davison et al., 1985; Williamson and Davison, 1987; Jones et al., 1988; Donker and Beuving, 1989; Petitte and Etches, 1989; Horton et al., 2007), or by treating birds with corticosterone in their food (Davison et al., 1983; Lin et al., 2004a) or drinking water (Post et al., 2003; Hull et al., 2007; Shini et al., 2008). Treating birds with corticosterone in their drinking water is a particularly effective, non-invasive method of simulating the increase in plasma corticosterone concentrations that occur during stress. The increase in plasma corticosterone concentrations that occur in birds after single drinks of treated water simulate the rise in corticosterone that occurs during acute stress, whilst multiple drinks of treated water over a period of days simulates the rise in corticosterone concentrations that occur during chronic stress.

Chronic stress and its potentially deleterious effects in animals are described in section 1.1. But what environmental factors can lead to chronic stress in birds in their natural habitats? Studies in free-living species of birds report that predation risk, human disturbance and poor habitat quality are some of the factors that can lead to elevated plasma corticosterone concentrations within individuals. For example, male tropical stonechats (Saxicola torquata axillaris) that occupy territories that also contain predatory fiscal shrikes (Lanius collaris) had higher plasma corticosterone concentrations than male stonechats that occupy territories free of shrikes (Scheuerlein et al., 2001); faecal corticosterone concentrations in male spotted owls (Strix occidentalis caurina) were higher in birds that had home ranges closer to intensively logged forest areas compared to birds that occupy home ranges in less disturbed areas of the forest (Wasser et al., 1997); and American stonechats (Setophaga ruticilla) in poorer habitats had higher plasma corticosterone concentrations than individuals in better quality habitats (Marra and Holberton, 1998). Studies where domesticated or free-living species of birds are treated with corticosterone provide a useful method for investigating the effects of chronic stress on parameters such as reproductive function and body weight. For example, treatment of chickens or Japanese quail with corticosterone for seven days or more can induce ovarian regression and decrease oviduct weights (Etches et al., 1984; Hull et al., 2007), and interrupted ovulation following treatment with corticosterone

results in decreased egg production in hens (Petitte and Etches, 1991). Treatment with corticosterone-filled silastic implants can decrease parental behaviours in pied flycatchers (Ficedula hypoleuca; Silverin, 1986), and can reduce territorial behaviours in male song sparrows (Melospiza melodia; Wingfield and Silverin, 1986). A further consequence of chronic stress in birds can be a reduction in body weight (Wingfield, 1994; Wingfield et al., 1998). Glucocorticoids such as corticosterone promote gluconeogenesis, and protein from flight muscles is thought to provide the primary substrate for gluconeogenesis (Gray et al., 1990; Astheimer et al., 1992). Although extensive research has provided important information about how birds are affected by the elevated plasma corticosterone concentrations that occur during chronic stress, it is unknown if, or how long it takes, for birds to recover from chronic stress. Can reproductive function and body weight in birds be permanently affected by chronic stress, or can birds recover from the deleterious consequences of sustained elevations in plasma corticosterone concentrations? An experiment was designed to investigate these questions and is presented in the current thesis (see section 1.7).

Individual birds can experience more than one stressor at a time. A fall in blood glucose concentration that activates the HPA axis, for example, may coincide with the sight of a potential predator, and knowledge of how elevated plasma corticosterone concentrations affect responses of birds to subsequent stressors is important from an evolutionary perspective. Corticosterone has negative feedback effects on the HPA axis (see section 1.1), and negative feedback during a corticosterone response could potentially dampen responsiveness of the HPA axis to subsequent stressors. This possibility could be investigated by characterising the responses of birds to stressors during a period of corticosterone treatment. Indeed, a previous study in our laboratory measured plasma corticosterone responses to 15 min handling followed by 45 min confinement in quail treated with corticosterone in their drinking water (Hull et al., 2007). Corticosterone responses in some treated birds were reduced compared to controls, suggesting that corticosterone treatment may disrupt the responses of birds to a handling and confinement stressor. Clearly, however, further work is required to delineate the effects of elevated plasma corticosterone on corticosterone responses to stressors in birds, and the current study describes an experiment that was designed to address this matter (see section 1.7).

## 1.3 Actions of corticosterone in birds

Corticosterone is the primary glucocorticoid secreted by the adrenal glands during stress in birds. The adrenals are paired glands that lie anterior and medial to the kidneys and consist of adrenocortical and chromaffin tissue (Carsia and Harvey, 2000). Corticosterone is secreted by adrenocortical tissue whilst chromaffin tissue secretes catecholamines. Corticosterone is a steroid hormone that is synthesised from cholesterol via a pathway mediated by cytochrome P-450 enzymes, and is metabolised in the liver and intestines (Vylitová et al., 1998; Rosol et al., 2001). Around 90% of corticosterone in circulation may be bound to corticosteroid binding globulin (CBG). Unbound or free corticosterone is suggested to be the biologically active form, and CBG is thought to regulate the availability and clearance of corticosterone (Breuner and Orchinik, 2002). Corticosterone exerts its effects in birds by binding to intracellular mineralocorticoid (MR) and glucocorticoid (GR) receptors, or by binding to membrane bound receptors (mGR). MRs have the highest affinity for corticosterone whilst mGRs have the lowest affinity, such that unequal proportions of the receptor types are bound at different corticosterone concentrations (Breuner and Orchinik, 2001; Landys et al., 2006).

The primary metabolic actions of corticosterone are to regulate carbohydrate and lipid metabolism. Corticosterone stimulates gluconeogenesis and lipogenesis (Marsh, 1992). The primary substrate for gluconeogenesis is probably muscle protein, and treatment with corticosterone causes significant atrophy of flight muscles in passerines (Gray et al., 1990; Astheimer et al., 2000), and an increase in the production of uric acid, a by-product of amino acid degradation, in chickens (Davison et al., 1983; Saadoun et al., 1987) and Japanese quail (De la Cruz et al., 1981). Treatment with corticosterone can increase plasma glucose concentrations in birds (Davison et al., 1985; Dong et al., 2007; Yuan et al., 2008). An increase in fat deposition in the abdomen and liver often occurs after corticosterone treatment and has been shown in passerines (Gray et al., 1990), chickens (Kafri et al., 1988; Hayashi et al., 1994) and Japanese quail (Bray, 1993). Alterations in protein and fat metabolism following treatment with corticosterone often, but not always, lead to a reduction in body weight gain (Silverin, 1986; Wingfield and Silverin, 1986; Bray, 1993; Hull et al., 2007; Busch et al., 2008a). Corticosterone is generally considered to have a stimulatory effect on food intake and feeding behaviour

in birds (Petitte and Etches, 1991; Nasir et al., 1999; El-Lethey et al., 2001; Kitaysky et al., 2003; Lohmus et al., 2006), although treatment with corticosterone may decrease or have no effect on food intake in both domesticated and free-living species (Simon, 1984; Gray et al., 1990; Hayashi et al., 1994).

Corticosterone influences several types of behaviours in birds. Treatment with corticosterone can increase begging behaviour in black-legged kittiwake chicks (Rissa tridactyla; Kitaysky et al., 2001) and house sparrow nestlings (Passer domesticus; Loiseau et al., 2008), and can increase the efficiency of behaviours associated with food caching and retrieval in mountain chickadees (Poecile gambeli; Pravosudov, 2003). There is an inverted U-shaped relationship between corticosterone dose and perch hopping behaviour in Gambel's white-crowned sparrows (Zonotrichia leucophrys gambelii), although this effect on locomotor activity varies with the photoperiod (Breuner et al., 1998; Breuner and Wingfield, 2000). Corticosterone can also reduce parental and territorial behaviours in free-living birds (Silverin, 1986; Wingfield and Silverin, 1986). Relationships between corticosterone and fear behaviour in Japanese quail will be discussed in more detail in section 1.4.

The HPA axis interacts with the immune system at several levels (Marsh, 1992), and treatment of birds with corticosterone can affect immune parameters. For example, corticosterone can decrease the size of lymphoid organs, including the bursa of Fabricius, thymus and spleen (Gross et al., 1980; Donker and Beuving, 1989; Hull et al., 2007), and can increase the heterophil to lymphocyte ratio in birds (Gross and Siegel, 1983; Jones et al., 1988; Donker and Beuving, 1989). Treatment with corticosterone can reduce both humoral and cell-mediated immune responses in chickens (El-Lethey et al., 2003; Post et al., 2003), and the immune response to phytohaemagglutinin (PHA) challenge in house sparrow nestlings (Loiseau et al., 2008).

Corticosterone may act at all levels of the hypothalamo-pituitary-gonadal (HPG) axis to alter reproductive function in birds (Pottinger, 1999). Treatment of laying hens with corticosterone can decrease plasma concentrations of luteinising hormone (LH), which may result from reduced secretion of gonadotropin-releasing hormone by the hypothalamus (Etches et al., 1984; Williams et al., 1985; Petitte and Etches, 1988). Corticosterone treatment can induce ovarian regression and decrease oviduct weights in

chickens and Japanese quail, which may be a consequence of decreased plasma LH concentrations (Etches et al., 1984; Williams et al., 1985; Hull et al., 2007). Interrupted ovulation following treatment with corticosterone has also been reported in hens, resulting in decreased egg production (Moudgal et al., 1991; Petitte and Etches, 1991). Treatment of bobwhite quail (Colinus virginianus) with corticosterone decreased ovary and oviduct weights in female birds, and reduced testis weights and sperm production in male birds (Cain and Lien, 1985).

## 1.4 Fear and fearfulness in birds

Animals generate physiological and behavioural responses to help cope with changes in their environments. Relatively benign environmental stimuli may require minor adjustments in physiology and behaviour in order to maintain homeostasis, such as a slight change in wind direction prompting an animal to move into shelter. Other stimuli may require more robust responses in order for animals to survive. Two types of stimuli that animals may encounter are threatening stimuli and stimuli that pose physical challenges. Threatening stimuli are stimuli from the environment that are likely to cause damage or danger and can elicit a state of fear and a fear response in animals. Fear can therefore be defined as a state or situation in which an animal is responding to an environmental threat (LeDoux, 1996; Rodrigues et al., 2009). Fear responses help animals to avoid the potentially deleterious consequences of exposure to danger, and involve both behavioural and physiological adjustments. The amygdala is a region of the brain responsible for detecting and responding to threatening stimuli, and fear arousal is one of the most potent activators of the HPA axis (Davis, 1997; Rodrigues et al., 2009). Auditory, visual, somatosensory, gustatory and olfactory stimuli are processed by the sensory thalamus and cortex, and information about threatening stimuli is sent to the amygdala (Rodrigues et al., 2009). The amygdala has downstream projections to the hypothalamus, and during a fear response amygdaloid signalling leads to activation of the HPA axis by stimulating the secretion of CRF by the hypothalamic paraventricular nucleus (Rodrigues et al., 2009). When a threatening stimulus leads to a state of fear in an animal and the HPA axis is activated, then the stimulus can be called an emotional stressor. Activation of the HPA axis in response to an emotional stressor, therefore, is synonymous with a fear response (LeDoux, 1996; Fendt and Fanselow, 1999; Labar and LeDoux, 2001; Walker et al., 2003). In contrast, when animals

experience stimuli that pose physical challenges and the HPA axis is activated, the stimulus can be called a physical stressor. An animal responding to a physical stressor is responding to an actual physical challenge, rather than a threat, so an animal responding to a physical stressor will not be in a state of fear. An animal in a state of fear, therefore, will also be generating a stress response, whereas an animal generating a stress response will not necessarily be in a state of fear.

The term fear can be used to describe both the experience (i.e. state of fear) and expression (i.e. fear behaviour) of an emotional event (Davis, 1997). Although the experience of fear cannot be measured directly, the expression of fear behaviour can be used to infer an animal's fear state, also known as its level of fearfulness. Changes in freezing, scanning and vigilance behaviours for example, can potentially be used as measures of fear behaviour (Davis, 1997). The tonic immobility test is a widely used measure of fear in birds, where tonic immobility is an "unlearned response easily induced by brief manual restraint in which an animal remains still and exhibits reduced responsiveness to external stimulation" (Jones, 1986). Tonic immobility may represent the final stage in anti-predator behaviour, and the duration of tonic immobility is considered to be proportional to fearfulness (Gallup, 1974). Other fear behaviour tests used in birds are open field tests, and to a lesser extent, novel object tests (Forkman et al., 2007). Studies of fear have invariably used domesticated rather than free-living species of birds, and have focused on finding methods to reduce the potentially detrimental effects of heightened fear on welfare and performance in poultry (Jones, 1996; Satterlee and Jones, 1997). In an ecological context, however, fear behaviour has evolved to help birds avoid danger, and so there is interest in fearfulness in birds from an evolutionary perspective as well.

Although relationships between fearfulness and the HPA axis have not been studied in wild free-living species of birds, knowledge of how birds respond to threatening events in their natural environments can be gained from studies in domesticated species like the Japanese quail. One interesting finding from studies in quail is that when birds are subjected to an emotional stressor their levels of fearfulness increase in subsequent behaviour tests of fear. For example, quail restrained for 5 min, either 0 or 55 min before tonic immobility or open field testing showed higher levels of fearfulness than control birds left undisturbed before behaviour testing (Satterlee et al., 1993; Satterlee

and Marin, 2006). When birds experience emotional stressors in their natural habitats, do their levels of fearfulness also increase, and what might this mean? Corticosterone is the primary glucocorticoid secreted when birds respond to emotional stressors such as restraint (Carsia and Harvey, 2000), and these findings raise important questions about whether or not elevated plasma corticosterone concentrations were responsible for the subsequent increase in fearfulness in these quail. Corticosterone secretion is considered to help animals cope with threatening situations, primarily through increasing mobilisation of stored energy (Wingfield and Kitaysky, 2002), but can corticosterone also potentiate fear behaviour in birds? A study designed to address this question is presented in the current thesis (see section 1.7).

## 1.5 Japanese quail

### 1.5.1 General characteristics and terminology

Japanese quail belong to the order Galliformes and the family Phasianidae (Minvielle, 2004), and were domesticated in about the eleventh century from wild populations in Eastern Asia, where some populations still remain (Hinshaw et al., 1969; Chang et al., 2005). Domesticated Japanese quail retain wild-type plumage, although they have a variety of other colourations, including white plumage (Mills et al., 1997). Japanese quail are sexually dimorphic in plumage and body size, with females larger than males. Quail are an important species for meat and egg production as they are relatively inexpensive to maintain, have rapid growth and early sexual maturity, and females can lay up to 300 eggs a year (Hinshaw et al., 1969; Baumgartner, 1994). Quail are important in research (Mills et al., 1997; Minvielle, 2004), and are commercially available in New Zealand as meat birds. Chickens have also been domesticated, from wild populations of red junglefowl, and are bred for meat and egg production (Al-Nasser et al., 2007). The great majority of studies of Japanese quail and chickens have used domesticated rather than free-living populations of birds, and here both species will be considered "domesticated species of birds" unless otherwise stated. "Free-living species of birds" refers to species that normally occur in the wild but may also be held in captivity for experimental purposes.

### 1.5.2 Japanese quail selection experiments

Lines of Japanese quail have been genetically selected for contrasting corticosterone

responses (Satterlee and Johnson, 1988), and for divergent tonic immobility responses (Mills and Faure, 1991). Activity of the HPA axis and fearfulness, therefore, both have genetic components in birds. The selection criterion for corticosterone responses was the plasma corticosterone concentrations after 4-10 min immobilisation in a metal crush cage. Quail with relatively low and high corticosterone responses are called low and high stress quail respectively (Satterlee and Johnson, 1988). The selection criterion for tonic immobility responses was the duration of tonic immobility (Mills and Faure, 1991). Artificially selected lines of Japanese quail were originally developed to research methods of reducing the potentially detrimental effects of heightened stress and fear in production species (Jones and Hocking, 1999). Stress and fear in poultry, for example, can lead to injury, pain or even death; feather pecking behaviour; reduced egg production; and decreased growth (Jones, 1996), and finding methods of reducing stress and fear in poultry is important from both economic and animal welfare perspectives. Indeed, extensive studies in quail from the two selection experiments have produced some interesting findings. For example, low stress quail not only have lower corticosterone responses to emotional stressors than high stress quail, they also show lower levels of fearfulness than high stress quail in a range of behaviour tests (Jones et al., 1992a; Jones, 1996). Low stress quail also have a range of desirable production traits, such as reduced osteoporosis and developmental instability (Satterlee and Roberts, 1990; Satterlee et al., 2000), and earlier sexual maturity in both sexes (Marin et al., 2002; Satterlee et al., 2002). Similarly, quail in the short tonic immobility response line consistently show lower levels of fearfulness than quail in the long tonic immobility response line (Jones, 1996). In apparent contradiction to findings in low and high stress birds, however, quail in the short tonic immobility response line show higher corticosterone responses to emotional stressors than quail in the long tonic immobility response line (Hazard et al., 2008).

### 1.5.3 Japanese quail used in the present study

Japanese quail are used for research in our laboratory and are widely used in other laboratories. Quail for our research are purchased at five weeks of age from Canter Valley Farm near Christchurch, where they are raised as meat birds in mixed-sex groups in wood shaving-covered floor pens. The quail used in the present study can be considered “unselected” quail, in the sense that they have not been artificially selected

on the basis of low or high plasma corticosterone responses to an emotional stressor, or selected for short or long tonic immobility responses like the selected quail described in section 1.5.2. Previous studies in our laboratory have investigated stress in birds by characterising corticosterone responses to different stressors in quail, and studies have examined the effects of stress in birds by measuring physiological parameters in quail treated with corticosterone in their drinking water. Quail immobilised by mechanical restraint for 15 min had mean plasma corticosterone concentrations between 5 and 14 ng/ml in a study that characterised corticosterone responses to stressors (unpub. data). Quail treated with corticosterone in their drinking water had mean plasma corticosterone concentrations between 7 and 20 ng/ml (Hull et al., 2007). In a different population of quail, mean corticosterone was between 5 and 25 ng/ml following 10-120 min of continuous mechanical restraint (Hazard et al., 2008). Treating quail with corticosterone in their drinking water, therefore, is an effective method of experimentally elevating plasma corticosterone concentrations to within the physiological range for quail. A standardised stressor for quail has also been developed in our laboratory, and involves handling birds for 15 min and then confining them in a cardboard box for 45 min. Blood samples are taken at 0, 15, 30 and 60 min for measurement of corticosterone concentrations. Handling followed by confinement is an emotional stressor, and a fear response occurs when animals experience emotional stressors (see section 1.4). Quail can display marked corticosterone responses to the emotional stressors of mechanical restraint and handling followed by confinement (see Hull et al., 2007), so it is apparent that a state of fear can be elicited in our quail.

## 1.6 Avian personalities

Marked differences between individuals in their physiological (e.g. magnitude of corticosterone response to handling followed by confinement) and behavioural (e.g. duration of tonic immobility) responses are discussed in sections 1.2.1 and 1.5.2. There is, however, considerable experimental evidence to suggest that the responses of individual animals can be more or less consistent in a variety of environmental situations (Koolhaas et al., 1999; Sih et al., 2004; Bell, 2007; Reale et al., 2007; Cockrem et al., 2009). It is widely considered that animals, like humans, also have personalities, where personalities are suites of correlated behaviours that are consistent across different situations and over time (Carere and Eens, 2005). Although

personalities vary along a continuum, some individuals of a species are relatively bold, aggressive, uninhibited and active, and can be considered to have proactive personalities, whilst others are relatively shy, submissive and inactive, and can be considered to have reactive personalities (Cockrem, 2007). Avian personalities have been extensively studied in great tits selected on behavioural responses in novel environment and novel object tests (Groothuis and Carere, 2005). Birds are classified as either fast or slow explorers on the basis of their latencies to explore novel environments and approach novel objects, and fast and slow great tits are considered to have proactive and reactive personalities respectively (Carere et al., 2005). Consistent differences in the behavioural and physiological responses of birds in the two lines have been identified. Compared to slow exploring great tits for example, fast explorers show greater risk taking behaviour (Van Oers et al., 2004), attack intruders and approach females sooner (Carere et al., 2005), and have lower HPA axis activity during social challenges (Carere et al., 2003).

Although relationships between the HPA axis and fear behaviour in low and high stress Japanese quail have traditionally been approached from a production perspective (see section 1.5.2), these selection lines can be viewed in an ecological context if the concept of avian personalities is considered. Indeed, the concept of avian personalities has recently been applied to low and high stress Japanese quail (Cockrem, 2007). Low stress quail consistently show lower corticosterone responses to emotional stressors and lower levels of fearfulness and are suggested to have proactive personalities, whilst high stress quail have higher corticosterone responses to emotional stressors and higher levels of fearfulness and are suggested to have reactive personalities. It is thought that birds with proactive personalities may be more successful in constant environments, whilst birds with reactive personalities may be more successful in changing environments (Cockrem, 2005). Although significant progress has been made in understanding avian personalities in selected populations of birds like the great tits described here and in the Japanese quail described in section 1.5.2, birds are not artificially selected on behavioural responses in novel environment and novel object tests, or on plasma corticosterone responses to brief mechanical restraint in their natural environments. Do birds from populations that have not been artificially selected for such traits demonstrate consistent levels of fearfulness across a range of environmental situations, and do the birds with relatively low or high plasma corticosterone responses

to emotional stressors also show relatively low or high levels of fearfulness respectively? These important questions were addressed in studies described in the present thesis (see section 1.7).

## 1.7 Outline of thesis

The overall aims of the research described in this dissertation were to determine the effects of corticosterone on fear behaviour, reproductive function, and plasma corticosterone responses to stressors in Japanese quail, both during and after treatment, and to examine if plasma corticosterone responses to similar emotional stressors, and to different types of emotional and physical stressors are related in individual quail.

Transient, or acute, increases in plasma corticosterone concentrations for minutes to hours are thought to be beneficial in animals, whereas sustained, or chronic, elevations in plasma corticosterone for days or weeks can have detrimental effects (Breuner et al., 2008). Although the potentially deleterious effects of elevated plasma corticosterone concentrations in birds are well known, there is no knowledge of how birds might recover from chronically elevated plasma corticosterone concentrations. Plasma corticosterone increases when birds experience stressors, and many of the stressors birds will experience in the foreseeable future will be anthropogenic in origin. A combination of climate change and human disturbance, otherwise known as global change, could lead to chronic stress in individuals of many species of birds with devastating consequences (Wingfield, 2008). Stress responses and fear behaviour can help birds to cope in changing environments, and knowledge of how elevated plasma corticosterone might affect responses to stressors and fear behaviour is crucial to understand how birds might be affected by global change. Research on avian personalities has explored the relationship between hypothalamo-pituitary-adrenal (HPA) axis activity and fearfulness in quail that have been artificially selected on plasma corticosterone responses to emotional stressors. It is proposed that quail of a low stress line with lower corticosterone responses and lower levels of fearfulness have proactive personalities, whilst quail of a high stress line with higher corticosterone responses and higher levels of fearfulness have reactive personalities (Cockrem, 2007). It is unknown, however, if quail from unselected populations also show consistent levels of fearfulness in a range of behaviour tests, or if HPA axis activity and fearfulness are related in unselected quail.

Of all the studies of plasma corticosterone responses to stressors in birds the proportion that measured responses of individuals to two or more stressors, or to physical stressors, is strikingly low. Indeed, the great majority of studies have measured corticosterone responses of birds to standardised artificial emotional stressors on just one occasion. A major assumption of avian stress endocrinology is that corticosterone responses to standardised emotional stressors reflect the responsiveness of the HPA axis of birds to stressors in general (Breuner et al., 2008), but there are no data to confirm this assumption. Furthermore, whilst there are marked individual differences between birds in their corticosterone responses to a single stressor, it is unknown if measuring the responsiveness of the HPA axis of birds on a single occasion truly reflects the phenotype of the sampled individuals. Studies of plasma corticosterone responses of birds to two or more stressors are clearly required to determine if consistent individual differences exist in the corticosterone responses of birds to different types of stressors.

The main questions addressed in the present study of Japanese quail were:

1. Is corticosterone responsible for stressor-induced increases in fear behaviour in quail?
2. Do individuals from a population of quail not artificially selected on low or high plasma corticosterone responses to an emotional stressor show consistently low or high levels of fearfulness in a range of behaviour tests?
3. Is reproductive function and body weight in quail permanently affected by chronic corticosterone treatment?
4. Do elevated plasma corticosterone concentrations alter corticosterone responses of quail to a subsequent fasting stressor?
5. Are plasma corticosterone responses to similar emotional stressors related in individual quail?
6. Can treatment with insulin or lipopolysaccharide be physical stressors in quail?
7. Do plasma corticosterone responses to an artificial emotional stressor reflect the responsiveness of the HPA axis of quail to physical stressors, and hence, to stressors in general?
8. Are magnitudes of plasma corticosterone responses to an emotional stressor related to levels of fearfulness in individuals from a population of quail not artificially selected on low or high plasma corticosterone responses to an emotional stressor?

Chapter's two to four present results from an experiment in which behavioural and physiological data were collected from groups of laying quail treated with corticosterone in their drinking water for 21 days. Chapter two describes relationships between corticosterone and behaviour in quail, presents results on the consistency of responses of quail in behaviour tests of fear, and addresses questions one and two. Chapter three describes plasma corticosterone profiles in control and treated birds and the effects of corticosterone in laying quail both during and after treatment, and addresses question three. Control and treated quail were subjected to 24 h of fasting to investigate the effects of elevated plasma corticosterone concentrations on corticosterone responses to a potential stressor. This study addresses question four and the results are presented in Chapter four.

Chapter's five to seven describe experiments in which plasma corticosterone responses to different stressors were characterised in adult quail. Corticosterone responses of quail to 15, 30 and 60 min handling followed by confinement for 45, 30 or 0 min respectively were measured to address question five and this study is described in Chapter five. Responses of quail to treatment with insulin or treatment with lipopolysaccharide (LPS) were measured to investigate if these treatments could be physical stressors in quail. This study addresses question six and the results are presented in Chapter six. Questions seven and eight were addressed in Chapter seven. Corticosterone responses of individual quail to emotional (handling) and physical (treatment with insulin or treatment with LPS) stressors were characterised to determine if responses of birds to the two classes of stressor are related, and behavioural responses in novel object tests of fear behaviour were examined for comparison with plasma corticosterone responses to handling and confinement in individual quail.

# 2. Relationships between corticosterone and behaviour, and repeatability and reliability of behaviour tests of fear in Japanese quail

## 2.1 Introduction

Stimuli that pose physical challenges to animals and initiate stress responses through disturbance of physical or chemical tissue parameters are called physical stressors, whilst stimuli that are perceived as a threat following comparison with innate predispositions or previous experience and initiate stress responses are called emotional stressors (Day et al., 1999; Jankord and Herman, 2008). Stress responses involve activation of the hypothalamo-pituitary-adrenal (HPA) axis and the secretion of glucocorticoids (Selye, 1974). When birds respond to emotional stressors with activation the HPA axis they also experience fear, and fear can be defined as a state or situation in which an animal is responding to an environmental threat (LeDoux, 1996; Rodrigues et al., 2009). Although fear cannot be measured directly, behavioural variables considered to be associated with fear can be measured. An animal in a state of fear might express avoidance behaviours, flight or escape behaviours, or assume immobility postures, such as crouching or freezing (Jones, 1996), and the expression of such behaviours can be measured in controlled tests. These measurements can then be used to infer the degree of fearfulness of animals, where individuals that show behaviours consistent with higher levels of fearfulness are considered to be in a higher state of fear than individuals that show lower levels of fearfulness. Behaviour tests of fear typically measure responses of subjects to novel or startling stimuli, and two tests commonly used in birds are the novel object and open field tests (Jones, 1996; Miller et al., 2006). Birds that take longer to pass or peck a novel object are considered to have higher levels of fearfulness than birds that take shorter times (Miller et al., 2005). Similarly, silent, inactive birds that defaecate less in an open field test are considered to be more fearful than active birds (Gallup et al., 1976; Jones and Mills, 1983). The tonic immobility test is also widely used as a measure of fear in birds, where individuals more susceptible to tonic immobility induction and with longer durations of tonic immobility are considered to be more fearful (Gallup, 1979; Jones, 1986). Although fear is difficult

to measure, studies of fearfulness in birds are important from animal welfare and production perspectives, and in relation to understanding personality in animals (Boissy, 1995; Rushen et al., 1999; Carere et al., 2005; Miller et al., 2005; Cockrem, 2007).

Corticosterone is the primary glucocorticoid secreted when birds respond to stressors (Carsia and Harvey, 2000), and Japanese quail (Coturnix coturnix japonica) that experienced emotional stressors 0 or 55 min before tonic immobility or open field testing showed higher levels of fearfulness than control quail left undisturbed before behaviour testing (Satterlee et al., 1993; Satterlee and Marin, 2006). These results raise the question of whether or not elevated plasma corticosterone concentrations were responsible for the stressor-induced increases in fear behaviour in these quail. Exposure to an emotional stressor activates neural pathways involved in the generation of stress responses (see Day et al., 1999), whereas treatment of birds with corticosterone does not activate these pathways. Instead, treatment of birds with corticosterone simulates the rise in plasma corticosterone concentrations that occur during stress, and any measured effects on fear behaviour can be ascribed to the actions of corticosterone, rather than the experience of stress per se. The present study was conducted, therefore, to determine if corticosterone is responsible for the stressor-induced increases in fearfulness reported in other populations of quail. Japanese quail were treated with corticosterone at three different concentrations in their drinking water for 21 days. The concentrations of corticosterone were chosen to elevate mean plasma corticosterone in treated birds to within the physiological range for quail. Between day 6 and 17 of corticosterone treatment, the behaviours of treated and untreated birds were measured in the tonic immobility test on two occasions, and in novel object and open field tests. There is evidence that social behaviour in birds is also related to activity of the HPA axis (Jones et al., 2002). The runway test has been used in studies of sociality in birds, where birds that spend more time closer to conspecifics are considered to be more social (Suarez and Gallup, 1983). The behavioural responses of quail in a runway test of sociality were, therefore, also measured in the present study during corticosterone treatment. Blood samples were collected for measurement of plasma corticosterone concentrations, and daily corticosterone intakes were calculated for individual birds, in order to determine relationships between corticosterone and behaviour in tests of fear and sociality in quail.

Avian personalities have been extensively studied, particularly in great tits (Parus major), and can be defined as suites of correlated behaviours that are consistent across different situations and over time (Carere and Eens, 2005; Groothuis and Carere, 2005). Personalities vary along a continuum, but individuals can be classified as having either proactive or reactive personalities. It has been proposed that birds with proactive personalities might be less fearful than birds with reactive personalities (Cockrem, 2007). If birds are to be classified into personality groups on the basis of relative levels of fearfulness, then behavioural responses of individual birds in tests designed to measure fear should be consistent. In other words, if a bird showed relatively low levels of fearfulness in one behaviour test of fear, then the same bird should show similar levels of fearfulness if it were subjected to the same test again, and it should also show relatively low levels of fearfulness in similar tests. Studies in quail and chickens (Gallus domesticus) have shown that individual birds can consistently express low or high levels of fearfulness in a range of behaviour tests (Mills and Faure, 1986; Jones, 1987; Jones, 1996). In the present study, the effects of corticosterone treatment on the consistency of behavioural responses in quail were investigated by using various statistical analyses to calculate the repeatability of the tonic immobility test, and the reliability of the three behaviour tests of fear in treated and untreated birds.

## 2.2 Materials and methods

### 2.2.1 Animals and husbandry

Wild-type female Japanese quail were purchased at five weeks of age from our usual supplier (Canter Valley Farm, Christchurch). The birds had been raised in mixed sex groups under a long day photoperiod (15 h light: 9 h dark) at air temperatures between 20 and $25^0$C. Each bird was identified with a coloured, numbered leg band and housed in an individual cage measuring 35 x 20 x 24 cm (length x width x height). Each cage had individual troughs in which fresh food (NRM meat bird crumbles) and water were provided ad libitum. Quail were held on a long day photoperiod (16 h light: 8 h dark; lights on from 0600 to 2200 h). An extractor fan provided ventilation for each room and a Carrier temperature control unit maintained the air temperature at $20 \pm 2^0$C. Light was provided by two 75W incandescent light bulbs controlled by a 24 h/seven day time switch (HPM Excel Light Switch and Timer). Quail had four weeks to acclimatise to housing conditions and were nine weeks of age when the experiment began.

### 2.2.2 Experimental design

Quail were randomly assigned to four groups of 20 birds on day -8 (day -8 was 8 days before corticosterone treatment began). All quail used in this study were part of a larger experiment in which additional data were collected in the week before corticosterone treatment began (day -7 to day -1), during the three week treatment period (day 0 to day 20), and on the day after treatment ended (day 21). Corticosterone treatment began at 0800 h on day 0. Corticosterone (Sigma) was dissolved in 99% ethanol and then diluted in water to concentrations of 0.0077, 0.0154 and 0.0231 mg/ml. Previous work in our laboratory had shown that daily corticosterone intakes were approximately 0.4, 0.8 and 1.2 mg/bird in quail drinking water containing corticosterone at these concentrations. Groups of quail were provided with water (controls) or with water containing 0.0077, 0.0154 or 0.0231 mg/ml of corticosterone. A measured volume of water was added to the individual water trough of each bird at the beginning of the treatment period. Water troughs were located on the outside of cages, and birds had access to water through a small opening in their cage. The volume of water added each day was recorded so the total volume consumed over the treatment period and the mean daily water intake could be calculated. Mean daily water intakes were multiplied by corticosterone concentrations to give mean daily corticosterone consumptions for each bird. Daily water intakes were greater than expected, so beginning at 1700 h on day 4 of treatment the concentrations of corticosterone were reduced to 0.0058, 0.0115 and 0.0173 mg/ml, respectively, in order to maintain the planned daily corticosterone intakes. The relative corticosterone intakes of quail remained similar when corticosterone concentrations in the drinking water were reduced on day 4 of treatment. Birds were assigned to treatment groups on the basis of their calculated daily corticosterone intakes between 1700 h on day 4 and when treatment finished at the end of day 20. The groups were 0 (control group, N = 18), 0.31-0.60 (N = 20), 0.61-0.90 (N = 13), 0.91-1.50 (N = 19) or >1.51 (N = 6) mg corticosterone/bird/day. Two control birds and two treated birds died during treatment and were not included in these groups.

### 2.2.3 Tests of fear behaviour

#### 2.2.3.1 Tonic immobility

Tonic immobility tests were performed on days 6 and 16 of corticosterone treatment. Birds were removed from their cages and carried to an adjacent room for the tests, which were conducted between 0800 and 1100 h. The test procedure was similar to that

used by Jones et al., (1994a). Each bird was picked up individually and placed on its back in a U-shaped polystyrene cradle 25 x 20 x 10 cm (length x width x height). The cradle was 7 cm wide and 5 cm deep at its lowest point, and was covered with several layers of cloth. The experimenter placed one hand over the bird's head and the other hand over the bird's sternum and restrained it for 15 s. The number of inductions (15 s periods of restraint) required to enter tonic immobility lasting at least 15 s and the duration of tonic immobility (length of time for bird to right itself) were recorded by the experimenter who sat approximately 1 m in front of the bird. If a bird did not enter tonic immobility after five attempts, a score of 0 s was given for duration. If a bird did not right itself after a period of 10 min, the bird was given a maximum score of 600 s for duration.

#### 2.2.3.2 Novel object

Behavioural responses of quail to a novel object were measured once on days 10 or 11 of corticosterone treatment. Birds were tested in their home cages between 1200 and 1600 h. Cardboard partitions placed between cages 24 h before tests prevented visual contact between neighbouring birds. Tests began when the experimenter hung a novel object (plastic ball 7 cm in diameter, coloured with red and white vertical stripes) from the middle of the roof of the test bird's cage. The experimenter then stood silent and motionless in front of the cage at a distance of 1 m. The latency for the bird to first pass the novel object, the number of passes the bird made of the novel object, and the number of pecks the bird made at the novel object were recorded over a 5 min period.

#### 2.2.3.3 Open field

Birds were taken individually from their cages and tested in a separate room on days 16 or 17 of corticosterone treatment. Tests were conducted between 0800 and 1600 h. The test procedure was based on methods described by Jones and Faure (1982) and Jones et al., (1992b). The open field consisted of a ring of galvanised sheet steel, 1 m in diameter and 45 cm high on a grey painted concrete floor. Birds were taken from their cage and placed in the centre of the open field. The experimenter then sat silent and motionless in full view of the bird and recorded the latency to first step, the time spent walking, the latency to vocalise, and the number of defaecations during the 10 min test period. All faeces were removed from the floor before the next bird was tested.

### 2.2.4 Runway tests of sociality

The runway test was used to measure sociality of birds on day 12 of corticosterone treatment. Tests were conducted between 0800 and 1700 h and followed the methods of Jones et al., (2002). The walls of the runway apparatus were unpainted wood and measured 200 x 30 x 30 cm (length x width x height). The runway had a wire mesh floor and was suspended 50 cm above the grey painted concrete floor of the test room. A wire mesh partition 30 cm from one end of the runway delineated the goal box. At the opposite end of the runway there was a second, removable wire mesh partition 20 cm from the end of the runway that delineated the start box. The two age-matched stimulus female quail were placed in the goal box and the test bird was placed in the start box and allowed 30 s to acclimatise. The 10 min test began when the wire mesh divider separating the start box from the runway was lifted and the test bird was free to walk along the runway. The experimenter stood behind the start box and recorded the latency for the test bird to leave the start box, the time for the test bird to reach 50 cm from the start box, the time for the test bird to reach within 20 cm of the goal box, the number of entries the test bird made within 20 cm of the goal box, and the time the test bird spent within 20 cm of the goal box.

### 2.2.5 Blood sample collection

Blood samples were collected between 1230 and 1500 h on days 7, 14 and 21. Blood samples were collected by puncture of a wing vein followed by withdrawal of up to 200 µl of blood into heparinised capillary tubes. All blood samples were collected in a separate room within one min from the time the bird was removed from its cage. Blood samples were kept cool on ice for up to 3 h until centrifugation at 2 000 rpm for 10 min at $10^0$C (Heraeus Christ Cryofuge 5000S). Plasma was removed after centrifugation and frozen at $-20^0$C. Plasma corticosterone concentrations were subsequently measured by radioimmunoassay.

### 2.2.6 Experimental procedures

Additional data that were collected and are presented in other chapters were: Chapter three; blood samples for the measurement of corticosterone were collected on day -1, and body weight and cloacal diameter were measured on days -1, 7, and 14. Food intake was measured over 24 h between day -6 and -5, and between day 8 and day 9. Egg production and egg weight were recorded or measured daily. A fasting study is

presented in Chapter four; quail were fasted for 24 h, beginning between 1230 and 1430 h on day 14. Blood samples were collected at the end of the fast and 3 h later, body weight was measured at the end of the fast, and food intake was measured during the 3 h following the fast.

### 2.2.7 Corticosterone radioimmunoassay

Each plasma sample was thawed, transferred to a 1.5 ml microcentrifuge tube and spun for 10 min at 18 000 g to separate lipids from plasma. Clear plasma from below the lipid layer was transferred to another tube and diluted in phosphate buffer with saline and gelatine (PBSG) for the measurement of corticosterone by radioimmunoassay using an assay validated for the measurement of corticosterone in quail plasma (Hull et al., 2007). Iodinated corticosterone, antiserum against corticosterone and a second antibody were obtained from MP Biomedicals. The sensitivity of the corticosterone assay was determined as the hormone concentration at the mean - 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity, expressed as ng steroid/ml plasma, was 0.35 ng/ml. Solutions of corticosterone in PBSG at concentrations that gave approximately 80, 50 and 20% binding on the standard curve were used as low, medium and high quality controls in every assay. The intra-assay coefficients of variation were 8.8%, 4.3%, and 13.5% and the inter-assay coefficients of variation 16.0%, 8.3% and 16.9% for low, medium and high solutions respectively.

### 2.2.8 Statistics

One bird from the lowest treatment group and one bird from the highest treatment group expressed sickness behaviour before and during the novel object and open field tests, and these birds were excluded from statistical analyses involving these two tests. A high corticosterone value for one control bird was identified as an outlier using Grubb's test (Barnett and Lewis, 1994), and this bird was excluded from analyses of plasma corticosterone. Statistical analyses were performed using Systat (Systat Software) and GraphPad Prism (GraphPad Software Inc). Levene's tests indicated that variances were not homogeneous for some behaviour variables, so non parametric statistics were used for all behaviours. Relationships between pairs of behaviour variables in the first tonic immobility, novel object, open field and runway tests were determined with Pearson correlations as part of principal components analysis (see below). Data are presented as mean ± standard error (S.E.).

### 2.2.9 Principal components analysis

Principal components analysis (PCA) was used to examine relationships between behavioural variables in the first tonic immobility, novel object, open field and runway tests. This approach has been used in studies of feather pecking behaviour in chickens (Van Hierden et al., 2002), tonic immobility and open field behaviour in Japanese quail (Jones et al., 1991), and behaviour of calves (Bos taurus) in open field and novel object tests (Van Reenen et al., 2005). Principal components are linear combinations of the original variables, and principal components analysis can summarise many variables into a few components that account for variance in the data set. Principal components analyses were performed on the Pearson correlation matrix for each combination of variables, with principal components with eigenvalues ≥1 retained for further analyses. Components were rotated after the initial analysis using varimax (orthogonal) rotation, and component loadings and the percentage of total variance explained by each component were calculated. Component loadings are bivariate correlations between behavioural variables and principal components, and behavioural variables with loadings ≥0.5 were considered to have a high loading on a given component. Coefficients generated during PCA for each behavioural variable were then multiplied by values of each behavioural variable and then summed to give PCA component scores for individual birds.

### 2.2.10 Fear score ranks

Individual fear score ranks were calculated for the first tonic immobility, novel object and open field tests following methods used for chickens (Jones and Mills, 1983; Jones, 1987; Jones, 1988) and Japanese quail (Mills and Faure, 1986). The ranks were calculated by ranking birds from the least fearful to most fearful for each variable within each test (Jones and Mills, 1983). Values of each variable were ranked from low to high or from high to low depending on the predicted relationship of each variable with fear. The ranks for variables within a test were summed to give a fear score for each bird for each behaviour test, so a low score corresponds to relatively low fearfulness and a high score to high fearfulness. The fear scores were then ranked from low to high to give fear score ranks (least to most fearful) for birds in each behaviour test.

The relationships with fear of each variable in the tonic immobility test were assigned as follows (Jones, 1988): number of inductions negative relationship so birds were

arranged in order from high to low numbers of inductions; duration of tonic immobility positive relationship. The relationships with fear of each variable in the novel object test were assigned as follows: latency to first pass positive relationship so birds were arranged in order from low to high latencies; number of passes negative relationship so birds were arranged in order from high to low numbers of passes; number of pecks negative relationship. Fear score ranks have not previously been calculated using all variables measured in novel object tests performed in the present study. The relationships of each variable with fear were assigned with reference to Miller et al., (2005) who considered that long latencies to approach and to make contact with a novel object are usually taken to indicate high fearfulness, and to Jones (1987; 1988) who gave birds that pecked a novel object lower scores than birds that avoided the object and who considered that a high score indicated a high level of fearfulness. The relationships with fear of each variable in the open field test were assigned as follows (Gallup et al., 1976; Jones and Mills, 1983; Mills and Faure, 1986): latency to first step positive relationship so birds were arranged in order from low to high latencies; time spent walking negative relationship so birds were arranged in order from high to low times; latency to vocalise positive relationship; number of defaecations negative relationship. The relationship between the number of defaecations and fear was taken to be negative (Gallup et al., 1976; Faure et al., 1983; Suarez and Gallup, 1983), although Jones and Merry (1988) considered the relationship to be positive.

### 2.2.11 Effects of corticosterone treatment on behaviour

Individual PCA component scores for each behaviour test and the three fear behaviour tests combined were calculated with coefficients generated from PCA that analysed control and treated birds together. Individual fear score ranks were calculated using control and treated birds together. Kruskal-Wallis one way ANOVAs and Mann-Whitney U tests were then used to compare behaviour variables, PCA component scores and fear score ranks across groups.

### 2.2.12 Relationships between corticosterone and behaviour

A mean value was calculated for each bird for corticosterone concentrations on days 7, 14 and 21. Corticosterone concentrations on each day were also ranked within control and treated birds, then used to calculate an overall corticosterone rank for each bird for the three days. Corticosterone concentrations on the day after corticosterone treatment

ended (day 21) were similar to concentrations on day 14 of treatment (see Fig. 3.1), so were included in calculations of corticosterone variables. Individual PCA component scores for each behaviour test and for the three fear behaviour tests combined were calculated with coefficients generated from PCA that analysed control and treated birds separately. Individual fear score ranks were calculated using control and treated birds separately. Relationships between corticosterone variables (mean corticosterone concentration over days 7, 14 and 21; corticosterone rank over days 7, 14 and 21) and behaviour variables, PCA component scores and fear score ranks were determined with Spearman correlations.

### 2.2.13 Repeatability of tonic immobility behaviour test

Individual fear score ranks were calculated for control and treated birds separately for the first and second tonic immobility tests. Comparisons between mean values of behaviour variables in the first and second tonic immobility tests were made using two tailed t-tests, for control and treated birds separately. Relationships between behaviour variables and fear score ranks for control and treated birds in the first and second tonic immobility tests were determined with Pearson correlations.

Statistical repeatability is a measure that describes the proportion of variance in a variable that occurs among rather than within individuals. Repeatability for a variable can be calculated from a one way analysis of variance in which repeatability, r, is given by the formula: $r = s2A / ( s2 + s2A )$ where s2A is the among-groups variance component and s2 is the within-group variance component. These variance components are calculated from the mean squares in the analysis of variance as $s2 = MSW$ and $s2A = (MSA - MSW)/n0$, where n0 is a coefficient related to the sample size per group in the analysis of variance. Statistical repeatabilities of variables in the first and second tonic immobility tests were calculated by the method of Lessells and Boag (1987). This method has been used to calculate repeatabilities for exploratory and risk-taking behaviour in great tits (Parus major; Dingemanse et al., 2002; Van Oers et al., 2004).

### 2.2.14 Reliability of three tests of fear behaviour

The reliability of different tests for the measurement of fear has been addressed by determining relationships between measures from different tests. Jones (1987) reported correlations between fear score ranks in pairs of tests (two novel object tests and tonic

immobility) in chickens. Jones and Mills (1983) and Mills and Faure (1986) used the Kendall's coefficient of concordance (W) test to examine the degree of correlation between responses of individual birds in open field, tonic immobility, hole-in-wall and response-to-bell tests in chickens and quail. The W test was used to calculate the degree of correlation of fear score ranks for the first tonic immobility, novel object and open field tests for control and treated birds separately.

## 2.3 Results

### 2.3.1 Relationships between pairs of behaviour variables and principal components analysis

Just four of the 72 correlations between pairs of the nine behaviour variables in the three tests of fear were significant in control and treated birds (Table 2.1 and 2.2, see Appendix Fig. 2.1 to 2.4). There were positive correlations in control birds between the duration of tonic immobility and latency to first step in the open field, and between the number of pecks at the novel object and number of defaecations in the open field. However, if a control bird with a value of 600 s for the duration of tonic immobility and latency to first step in the open field test is excluded from analysis, then the relationship between these two variables is not significant ($r = 0.317$, $p = 0.216$). Similarly, if the control bird that made 6 pecks at the novel object and defaecated twice in the open field is excluded from the analysis then the relationship between these two variables is not significant ($r = 0.369$, $p = 0.145$). There was an inverse relationship between the latency to first step and time spent walking in the open field in treated birds, and a positive relationship between the time spent walking and number of defaecations in the open field. Correlations between pairs of variables in the runway test were positive for relationships between the latency to leave the start box and the times to reach 50 cm from the start box and to reach within 20 cm of the goal box (Table 2.3, see Appendix Fig. 2.5 to 2.7). The times to reach 50 cm from the start box and to reach within 20 cm of the goal box were also positively related. There were negative correlations with the number of entries within 20 cm of the goal box and the latency to leave the start box and the times to reach 50 cm from the start box and to reach within 20 cm of the goal box. There were also negative correlations with the time spent within 20 cm of the goal box and the latency to leave the start box and the times to reach 50 cm from the start box and to reach within 20 cm of the goal box. There was a positive relationship between

the number of entries within 20 cm of the goal box and the time spent within 20 cm of the goal box for control but not treated birds.

A principal components analysis using the two measures of tonic immobility produced one principal component that accounted for 60.3% of the total variance for control birds and 62.9% for treated birds. An analysis using the three measures in the novel object test produced one principal component that accounted for 55.3% of total variance for control birds and 39.9% for treated birds. An analysis using the four measures in the open field test produced two principal components that accounted for 71.7% of total variance for control birds and 64.8% for treated birds. When all nine variables for the first tonic immobility, novel object and open field tests were used in a principal components analysis four components were identified that accounted for 83.8% of the total variance for control birds and 60.0% for treated birds (Table 2.4 and 2.5). The analysis using the five measures in the runway test produced one principal component that accounted for 76.6% of total variance for control birds and 65.8% for treated birds.

There were single principal components for the tonic immobility, novel object and runway tests. The first component in the open field test most likely represents locomotor activity or exploratory behaviour as the latency to the first step had a high positive loading and the time spent walking a high negative loading in control birds (Fig. 2.1). Birds that took a long time to begin walking in the open field therefore tended to spend less time walking, as expected. The latency to vocalise and number of defaecations had high positive loadings on the second component in control birds. The latency to vocalise and number of defaecations are considered to have positive and negative relationships with fear respectively, so the second component is unlikely to represent fear in control birds. The component loadings for treated birds differed from those for control birds (Fig. 2.1). The first component potentially represents fear, as the latency to the first step had a high positive loading and the time spent walking a high negative loading, as for control birds, and there was a high negative loading for the number of defaecations in treated birds.

PCA of behavioural variables from the tonic immobility, novel object and open field tests combined in control birds revealed that no component was measuring fear across the three tests (Table 2.4). The first component for the tonic immobility, novel object

and open field tests combined could potentially be inversely related to fear with respect to the open field test in treated birds, but there is no evidence to suggest this component is measuring fear in the tonic immobility and novel object tests (Table 2.5).

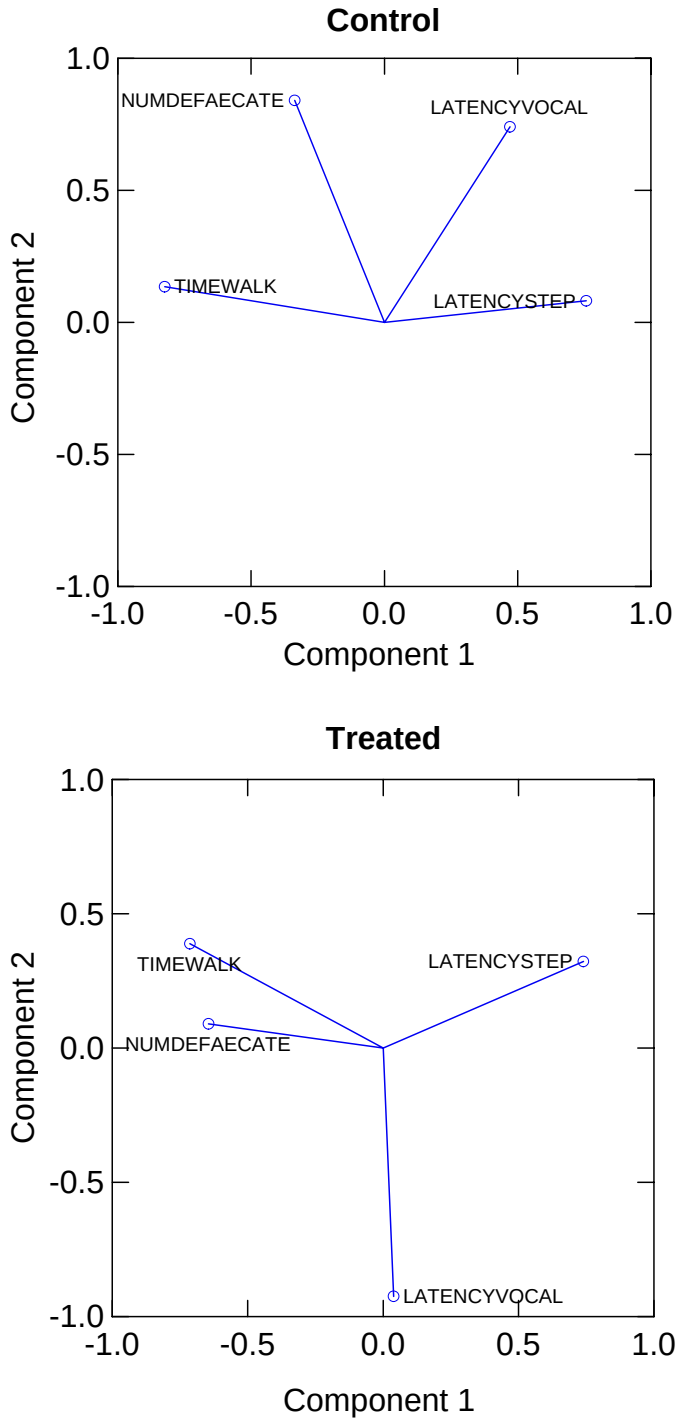


**Figure 2.1.** Distributions of behavioural variables in relation to the first two principal components of principal components analyses of open field variables in control quail and in quail treated with corticosterone. Labels indicate the mean positions of variables in relation to the first two components. Each variable has a specific loading on the X- and Y-axis. The label abbreviations are LATENCYSTEP = latency to first step; TIMEWALK = time spent walking; LATENCYVOCAL = latency to vocalise; NUMDEFAECATE = number of defaecations. Control, N = 18; treated, N = 56.

**Table 2.1.** Pearson correlation coefficients between pairs of the nine behaviour variables measured in the first tonic immobility, novel object and open field tests in control quail, N = 18. *p < 0.05, **p < 0.01, ***p < 0.001.

| | Tonic immobility test | | Novel object test | | | Open field test | | |
|---|---|---|---|---|---|---|---|---|
| Behavioural variable | Number of inductions | Duration | Latency to first pass | Number of passes | Number of pecks | Latency to first step | Time spent walking | Latency to vocalise |
| Tonic immobility test | | | | | | | | |
| Duration | -0.206 | | | | | | | |
| Novel object test | | | | | | | | |
| Latency to first pass | 0.016 | -0.058 | | | | | | |
| Number of passes | -0.161 | -0.099 | -0.456 | | | | | |
| Number of pecks | -0.404 | -0.125 | -0.218 | 0.298 | | | | |
| Open field test | | | | | | | | |
| Latency to first step | -0.110 | 0.958*** | -0.087 | -0.191 | -0.111 | | | |
| Time spent walking | -0.086 | -0.285 | 0.086 | -0.341 | 0.014 | -0.362 | | |
| Latency to vocalise | -0.246 | 0.142 | 0.460 | -0.304 | 0.330 | 0.237 | -0.244 | |
| Number of defaecations | -0.417 | -0.200 | -0.184 | -0.045 | 0.685** | -0.109 | 0.279 | 0.272 |

**Table 2.2.** Pearson correlation coefficients between pairs of the nine behaviour variables measured in the first tonic immobility, novel object and open field tests in quail treated with corticosterone, N = 56. *p < 0.05, **p < 0.01, ***p < 0.001.

| | Tonic immobility test | | Novel object test | | | Open field test | | |
|---|---|---|---|---|---|---|---|---|
| Behavioural variable | Number of inductions | Duration | Latency to first pass | Number of passes | Number of pecks | Latency to first step | Time spent walking | Latency to vocalise |
| Tonic immobility test | | | | | | | | |
| Duration | -0.259 | | | | | | | |
| Novel object test | | | | | | | | |
| Latency to first pass | -0.142 | -0.066 | | | | | | |
| Number of passes | 0.104 | 0.156 | -0.098 | | | | | |
| Number of pecks | 0.186 | -0.107 | -0.040 | 0.146 | | | | |
| Open field test | | | | | | | | |
| Latency to first step | -0.123 | 0.114 | -0.035 | -0.144 | -0.065 | | | |
| Time spent walking | 0.148 | -0.095 | -0.161 | 0.115 | 0.008 | -0.282* | | |
| Latency to vocalise | 0.049 | -0.096 | 0.092 | -0.050 | 0.041 | -0.070 | -0.224 | |
| Number of defaecations | -0.079 | -0.002 | -0.019 | 0.088 | 0.064 | -0.169 | 0.267* | -0.051 |

**Table 2.3.** Pearson correlation coefficients between the five behaviour variables measured in runway tests in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. *p < 0.05, **p < 0.01, ***p < 0.001.

| Behavioural variable | Time to reach 50 cm from start box | Time to reach within 20 cm of goal box | Number of entries within 20 cm of goal box | Time spent within 20 cm of goal box |
|---|---|---|---|---|
| Control birds | | | | |
| Latency to leave start box | 0.693** | 0.689** | -0.499* | -0.576* |
| Time to reach 50 cm from start box | | 0.994*** | -0.705** | -0.800*** |
| Time to reach within 20 cm of goal box | | | -0.715*** | -0.807*** |
| Number of entries within 20 cm of goal box | | | | 0.510* |
| Treated birds | | | | |
| Latency to leave start box | 0.694*** | 0.580*** | -0.276* | -0.503*** |
| Time to reach 50 cm from start box | | 0.882*** | -0.321* | -0.735*** |
| Time to reach within 20 cm of goal box | | | -0.419** | -0.852*** |
| Number of entries within 20 cm of goal box | | | | 0.130 |

**Table 2.4.** Loadings on the first four components extracted by principal components analysis (PCA), after varimax (orthogonal) rotation, of behavioural variables measured in the first tonic immobility, novel object and open field tests in control quail, N = 18.

| Behavioural variable | Component | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Tonic immobility test | | | | |
| Number of inductions | 0.221 | -0.702* | 0.002 | 0.031 |
| Duration | -0.977* | -0.021 | 0.009 | 0.075 |
| Novel object test | | | | |
| Latency to first pass | 0.111 | -0.201 | -0.857* | -0.116 |
| Number of passes | 0.210 | 0.167 | 0.591* | 0.646* |
| Number of pecks | 0.160 | 0.869* | 0.042 | 0.203 |
| Open field test | | | | |
| Latency to first step | -0.978* | -0.002 | -0.060 | 0.100 |
| Time spent walking | 0.268 | 0.134 | 0.091 | -0.891* |
| Latency to vocalise | -0.161 | 0.409 | -0.806* | 0.236 |
| Number of defaecations | 0.134 | 0.861* | -0.001 | -0.241 |
| Eigenvalue | 2.371 | 2.199 | 1.838 | 1.137 |
| Variance explained (%) | 26.347 | 24.431 | 20.419 | 12.634 |

*Variable considered to have a high loading for a component (loading ≥ 0.500)

**Table 2.5.** Loadings on the first four components extracted by principal components analysis (PCA), after varimax (orthogonal) rotation, of behavioural variables measured in the first tonic immobility, novel object and open field tests in quail treated with corticosterone, N = 56.

| Behavioural variable | Component | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Tonic immobility test | | | | |
| Number of inductions | -0.060 | -0.687* | 0.200 | 0.383 |
| Duration | -0.120 | 0.796* | 0.153 | 0.170 |
| Novel object test | | | | |
| Latency to first pass | 0.143 | 0.091 | -0.680* | -0.252 |
| Number of passes | 0.199 | 0.287 | 0.136 | 0.731* |
| Number of pecks | 0.010 | -0.225 | -0.104 | 0.635* |
| Open field test | | | | |
| Latency to first step | -0.671* | 0.196 | 0.172 | -0.198 |
| Time spent walking | 0.647* | -0.208 | 0.485 | -0.034 |
| Latency to vocalise | -0.112 | -0.161 | -0.685* | 0.217 |
| Number of defaecations | 0.709* | 0.167 | 0.012 | 0.005 |
| Eigenvalue | 1.730 | 1.392 | 1.178 | 1.100 |
| Variance explained (%) | 19.226 | 15.470 | 13.087 | 12.220 |

*Variable considered to have a high loading for a component (loading ≥ 0.500)

### 2.3.2 Effects of corticosterone treatment on behaviour

Variables measured in the tonic immobility and novel object tests did not differ significantly between groups (Fig. 2.2, see Appendix Table 2.1 for Kruskal-Wallis one way ANOVA statistics; see Fig. 3.1 for mean plasma corticosterone concentrations for each group). There were no differences between groups that received corticosterone treatment and the control group (see Appendix Table 2.4 for Mann-Whitney statistics) for tonic immobility variables, or for novel object variables with the exception of the latency to first pass. This latency appeared to be shorter in groups of birds treated with corticosterone than in control birds (Fig. 2.2), although was significantly shorter only in the 0.31-0.60 mg corticosterone/bird/day group ($U = 246.5$, $p = 0.019$). The high mean value for latency to first pass in the control group is partly due to a single outlier with a maximum score of 300 s for this variable. However, when statistics were performed with the outlier removed there was still a significant difference in the latency to first pass between the control group and 0.31-0.60 mg corticosterone/bird/day treatment group ($U = 227.5$, $p = 0.032$).

The number of defaecations differed between groups in the open field test ($K_4 = 15.01$, $p = 0.005$; see Fig. 2.2), with more defaecations by birds receiving the highest amount of corticosterone than by control birds ($U = 16.0$, $p = 0.016$). There were no other significant differences between groups in variables in the open field test or in the runway test (see Appendix Tables 2.1 and 2.4), except for a greater time spent within 20 cm of the goal box by birds receiving 0.91-1.50 mg corticosterone/bird/day compared with control birds ($U = 103.0$, $p = 0.038$).

There were no differences between groups in PCA component scores for individual tests or PCA component scores from the three fear behaviour tests combined. Fear score ranks in the three tests did not differ between groups (see Appendix Table 2.2 and 2.3 for statistics). The PCA component 1 score in the runway test differed between the control and 0.91-1.50 mg corticosterone/bird/day treatment group ($U = 238.0$, $p = 0.042$), but there were no other significant differences between treatment and control groups for fear score ranks or PCA component scores (see Appendix Table 2.5 and 2.6).

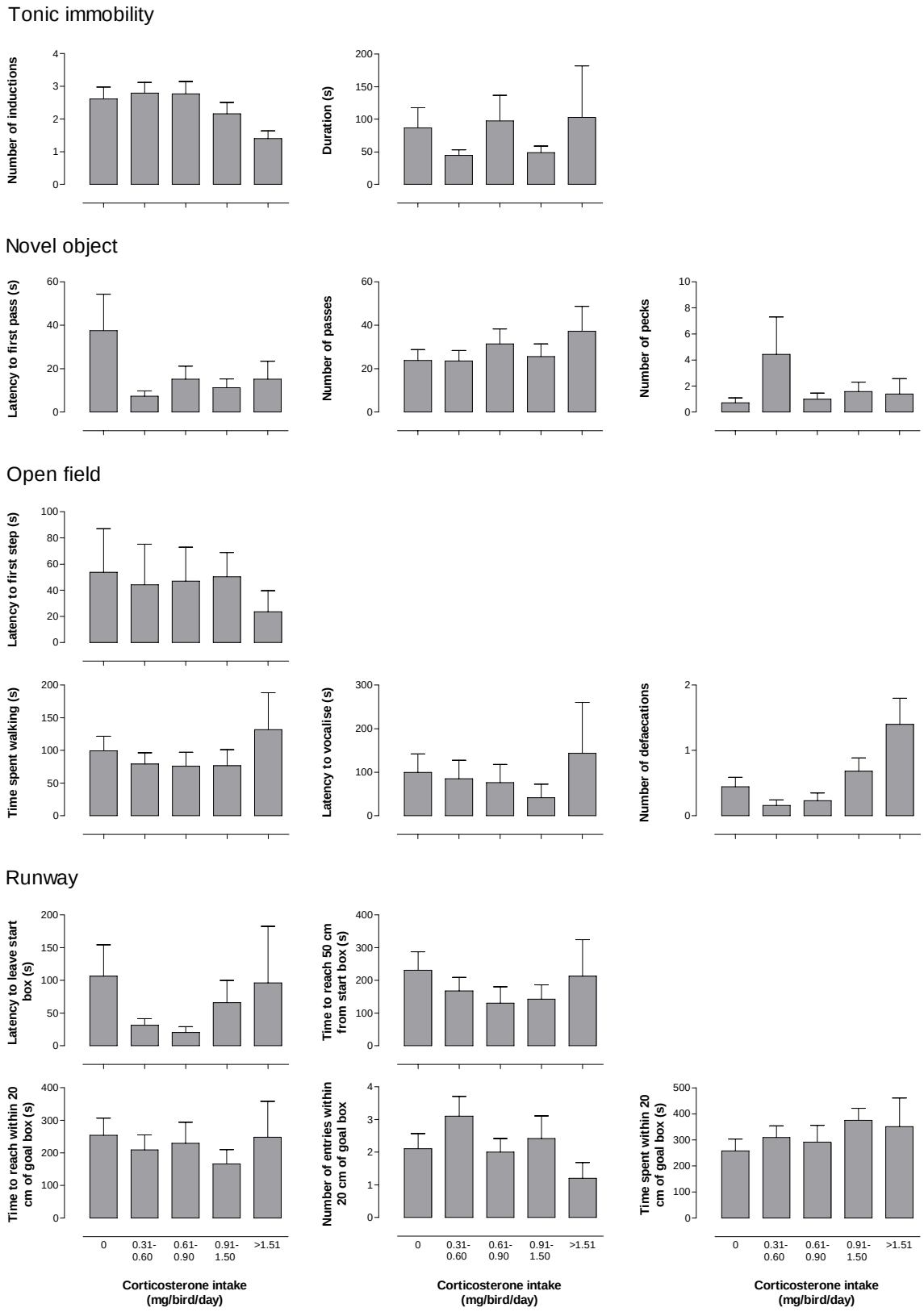


**Figure 2.2.** Effects of corticosterone treatment on all variables in the four tests of behaviour in quail. Control (0), N = 18; 0.31-0.60, N = 19; 0.61-0.90, N = 13; 0.91-1.50, N = 19; >1.51, N = 5. Data are presented as mean ± S.E.

### 2.3.3 Relationships between corticosterone and behaviour

For treated birds there were three significant correlations when Spearman correlations were calculated between mean plasma corticosterone concentrations over days 7, 14 and 21 and behaviour variables in the four behaviour tests, and between corticosterone ranks over the three days and behaviour variables (see Appendix Table 2.7 and Appendix Fig. 2.8). The significant correlations were between time spent walking in the open field test and mean corticosterone ($r = -0.308$, $p = 0.021$), time spent walking in the open field test and corticosterone rank ($r = -0.347$, $p = 0.009$) and number of defaecations in the open field test and corticosterone rank ($r = 0.304$, $p = 0.023$). Correlations were also performed between the corticosterone variables and PCA component scores for individual behaviour tests and for the three fear behaviour tests combined, and between corticosterone variables and fear score ranks (see Appendix Table 2.8 and Appendix Fig. 2.9). Four of these correlations were significant for treated birds (PCA component 1 score for open field test and mean corticosterone, $r = 0.269$, $p = 0.045$; PCA component 1 score for the three fear behaviour tests combined and mean corticosterone, $r = -0.304$, $p = 0.023$; PCA component 1 score for the open field test and corticosterone rank, $r = 0.308$, $p = 0.021$; PCA component 1 score for the three fear behaviour tests combined and corticosterone rank, $r = -0.332$, $p = 0.012$).

### 2.3.4 Repeatability of tonic immobility behaviour test

The numbers of inductions and the duration of tonic immobility in control and treated quail did not change when birds were retested after 10 days (Table 2.6). The numbers of inductions for individual birds in the two tests were significantly correlated, but there was no significant correlation between the duration of tonic immobility in the first and second tests (Table 2.7). Calculated fear score ranks for individual birds in the two tests were significantly correlated for control but not treated birds. Correlations between tests for both behaviour measures were higher in control than treated birds. Statistical repeatabilities were also higher in control birds (Table 2.8).

**Table 2.6.** Mean values and statistics for behaviour variables from first and second tonic immobility tests in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 58. *Indicates a significant result ($p < 0.05$).

| | First test | | Second test | | |
|---|---|---|---|---|---|
| | Mean | S.E. | Mean | S.E. | p |
| Control birds | | | | | |
| Number of inductions | 2.61 | 0.37 | 2.44 | 0.36 | 0.748 |
| Duration | 86.78 | 31.11 | 129.33 | 35.04 | 0.370 |
| Treated birds | | | | | |
| Number of inductions | 2.50 | 0.19 | 2.48 | 0.21 | 0.952 |
| Duration | 65.88 | 12.25 | 83.76 | 15.04 | 0.359 |

**Table 2.7.** Pearson correlation coefficients between variables in first and second tonic immobility tests in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 58. *Indicates a significant result ($p < 0.05$).

| | r | df | p |
|---|---|---|---|
| Control birds | | | |
| Number of inductions | 0.499 | 16 | 0.035* |
| Duration | 0.105 | 16 | 0.678 |
| Fear score rank[1] | 0.534 | 16 | 0.023* |
| | | | |
| Treated birds | | | |
| Number of inductions | 0.341 | 56 | 0.009* |
| Duration | 0.057 | 56 | 0.670 |
| Fear score rank[1] | 0.253 | 56 | 0.055 |

[1]Fear score ranks calculated for first and second tonic immobility tests separately.

**Table 2.8.** Repeatabilities (r) and statistics of behaviour variables from first and second tonic immobility tests in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 58. *Indicates a significant result ($p < 0.05$).

| | r | S.E. | Lower 95 % CI | Upper 95 % CI | p |
|---|---|---|---|---|---|
| Control birds | | | | | |
| Number of inductions | 0.515 | 0.173 | 0.088 | 0.784 | 0.011* |
| Duration | 0.106 | 0.233 | -0.358 | 0.533 | 0.328 |
| Treated birds | | | | | |
| Number of inductions | -0.353 | 0.066 | -0.575 | -0.083 | 0.995 |
| Duration | -0.012 | 0.076 | -0.289 | 0.267 | 0.532 |

### 2.3.5 Reliability of three tests of fear behaviour

The degree of correlation between the fear score ranks for individual birds in the first tonic immobility, novel object and open field behaviour tests was calculated using the Kendall's coefficients of concordance (W) test. The fear ranks assigned to individual birds in the three tests were not significantly correlated (control birds $W = 0.002$, $\chi^2 = 0.083$, $p = 0.959$; treated birds $W = 0.011$, $\chi^2 = 1.187$ $p = 0.552$).

## 2.4 Discussion

This is the first study to examine the effects of corticosterone treatment on levels of fearfulness and sociality in Japanese quail. Corticosterone treatment had little or no effect on the fearfulness of birds in tonic immobility, novel object and open field tests of fear, and in a runway test of sociality. Plasma corticosterone variables were in most cases not significantly correlated with variables measured in behaviour tests. Behavioural responses in the tonic immobility test appeared to be less consistent in treated than untreated birds. Fear score ranks for individual birds in different tests of fearfulness were not correlated, in contrast to findings in previous studies of quail and chickens. The results of the present study suggest that elevated plasma corticosterone concentrations are not responsible for the stressor-induced increases in fearfulness that have been reported in other populations of quail.

Behaviour variables measured in the first tonic immobility test did not differ between the control group and any of the groups treated with corticosterone. The effects of corticosterone treatment on tonic immobility responses in laying hens implanted with subcutaneous minipumps containing either a polyethylene glycol vehicle (control) or corticosterone were investigated by Jones et al., (1988). The tonic immobility responses of control and treated birds were measured 4 and 11 days after implantation, when plasma corticosterone concentrations in treated birds were significantly higher than in controls. On both occasions the number of alert head movements during tonic immobility was significantly lower, and the duration of tonic immobility was significantly longer in birds receiving corticosterone than in controls, but there was no effect of corticosterone on the latency to the first head movement during tonic immobility, or on the number of inductions required to attain tonic immobility. El-lethey et al., (2001) also reported that chickens fed corticosterone had longer durations

of tonic immobility than untreated control chickens. At the time of tonic immobility testing however, mean plasma corticosterone concentrations in treated chickens were only slightly higher than initial values, whereas corticosterone in treated quail in the present study was at the mid to upper end of the physiological range. Glucocorticoids exert their effects by binding to type I mineralocorticoid receptors (MRs) or type II glucocorticoid receptors (GRs) in the cytoplasm, or to membrane-bound receptors (Herbert et al., 2006). MRs have around a ten-fold higher affinity for glucocorticoids than GRs, such that MRs are saturated at lower glucocorticoid concentrations, with the proportion of bound GRs increasing with successively higher concentrations of glucocorticoids (Herbert et al., 2006). Differential receptor occupancy is one mechanism by which varying concentrations of corticosterone could have alternative effects on the duration of tonic immobility in birds (Koolhaas et al., 1999; Romero, 2004). In support of the possible dose-dependent effects of corticosterone, perch hopping was higher than in controls in white-crowned sparrows (Zonotrichia leucophrys gambelii) fed mealworms containing intermediate, but not high, concentrations of corticosterone (Breuner et al., 1998).

Jones et al., (1988) and El-Lethey et al., (2001) provide no solid explanation of how corticosterone might act to increase durations of tonic immobility in chickens, although Jones et al., (1988) did suggest that exogenous corticosterone either had a direct effect on fear behaviour or acted via negative feedback effects on the HPA axis. The negative feedback effects of glucocorticoids at various levels of the HPA axis are well known. Corticotropin-releasing factor (CRF) is integral in initiating the HPA axis response to stressors, for example, and glucocorticoids can act at the paraventricular nucleus of the hypothalamus to inhibit CRF secretion (de Kloet, 2000; Tilbrook and Clarke, 2006). CRF is also secreted from extrahypothalamic brain regions such as the amygdala during fear responses, and CRF is integral in sustaining fear (Schulkin et al., 2005). Recent evidence from studies in mammals suggests that feedforward actions of glucocorticoids on CRF neurons in brain regions such as the amygdala may be capable of maintaining heightened fear (Schulkin et al., 2005). These mechanisms have not been studied in birds, however, and the mechanism in which corticosterone increased durations of tonic immobility in chickens remains unknown.

In the present study, just one of the seven behaviour variables measured in the novel object or open field tests differed between groups (control and corticosterone treatment). There was a significant difference between groups in the number of defaecations in the open field test, but only birds in the highest treatment group made significantly more defaecations than controls. In contrast, the two lowest treatment groups made fewer defaecations than controls. Although the latency to first pass the novel object was significantly less in the lowest treatment group than in controls, the three highest treatment groups did not differ from controls in this variable. Given that variables measured in tonic immobility tests also did not differ between control and treated birds, there is little evidence that treatment with corticosterone increased the fearfulness of quail in the present study. There is also no evidence of corticosterone treatment affecting sociality in quail, as measured by the responses of birds in runway tests. In agreement with the corticosterone intake data, there were no strong correlations between either of the two corticosterone variables (mean plasma corticosterone concentrations over days 7, 14 and 21; mean corticosterone ranks over days 7, 14 and 21) and individual or derived behaviour variables measured in the three behaviour tests of fear. Quail subjected to the emotional stressor of restraint either 0 or 55 min prior to tonic immobility or open field testing showed higher levels of fearfulness than quail left undisturbed before behaviour testing (Satterlee et al., 1993; Satterlee and Marin, 2006), and the results of the current study suggest that elevated plasma corticosterone concentrations are not responsible for such stressor-induced increases in fearfulness in quail. The amygdala is a region of the brain responsible for detecting and responding to threatening stimuli (Davis, 1997; Rodrigues et al., 2009). Stimuli that are perceived as threatening and activate the HPA axis are called emotional stressors, and the amygdala is stimulated when birds experience emotional stressors like restraint. The amygdala is integral in initiating fear responses (Rodrigues et al., 2009) and it is possible that stimulation of the amygdala during restraint, rather than an elevation in plasma corticosterone, is responsible for the stressor-induced increases in fearfulness reported in other populations of quail.

Three statistical methods were used to examine the repeatability of the tonic immobility test in order to determine the consistency of the behavioural responses of quail in a test of fear. Although mean values of tonic immobility behaviour variables did not change from the first test to the second test for both control and corticosterone-treated birds, the

correlation of fear score ranks between the two tests was relatively strong and significant in control birds only. Statistical repeatability for the two tonic immobility tests was also higher in untreated than treated birds. These results are analogous to the findings of a study in chickens. Jones (1989) measured tonic immobility responses in groups of birds before and after one of three treatments lasting 72 h: one group had free access to food and acted as a control, while the other groups were either fasted, or were fasted and exposed to inaccessible food. Correlations between tonic immobility duration for the first and second tests were significant for birds fed ad libitum, but not for birds in the other two treatments. Food deprivation can be a stressor in chickens (Scanes et al., 1980; Harvey et al., 1983), and it may be that elevated plasma corticosterone in birds during fasting acted similarly to experimentally elevated corticosterone in the current study, and disrupted the responses of birds in tonic immobility tests. Several other studies have measured the behavioural responses of chickens in tonic immobility tests on more than one occasion. The mean duration of tonic immobility decreased with age (Hocking et al., 2001) or habituation in some strains (Gentle et al., 1985). In chickens subjected to the tonic immobility test on 3, 4, or 13 different occasions however, there was a high degree of correlation between the ranks for duration of tonic immobility, as measured by Kendall's coefficients of concordance (Jones, 1988; Hocking et al., 2001; Ghareeb et al., 2008).

In the present study, there were no correlations between fear score ranks assigned to individual birds in the first tonic immobility, novel object or open field tests. This result was the same for control birds and for birds treated with corticosterone. In other words, a bird that had a high fear score rank in the tonic immobility test for example, did not necessarily have a high fear score rank in the novel object or open field tests. In contrast, significant correlations in the fear score ranks assigned to individual birds in three or four different behaviour tests of fear have been reported in both chickens and quail (Jones and Mills, 1983; Mills and Faure, 1986; Jones, 1987). In the present study, it might be that corticosterone treatment affected quail in a way that disrupted the behavioural responses of birds in different situations, although there was also variability in the levels of fearfulness shown by control birds.

The concept of avian personalities has recently been applied to low and high stress Japanese quail, with low stress quail suggested to have proactive personalities and high

stress quail suggested to have reactive personalities (Cockrem, 2007). Indeed low stress quail consistently show lower levels of fearfulness than high stress quail in a range of behaviour tests. The levels of fearfulness expressed by individual quail during behaviour tests in the present study were variable, however, and it appears that the study of fearfulness as a personality trait is more difficult in unselected than selected populations of quail (see Chapter eight for further discussion).

There is considerable experimental evidence to support a relationship between HPA axis activity and fearfulness in birds, and studies in quail from other populations report that exposure to an emotional stressor can increase fearfulness in quail. The present study examined if elevated plasma corticosterone concentrations can increase fear behaviour in quail, but corticosterone treatment did not appear to affect the levels of fearfulness of quail in tonic immobility, novel object and open field tests. It is suggested, therefore, that stimulation of the amygdala, rather than elevated plasma corticosterone concentrations, is responsible for increasing fearfulness in quail following exposure to an emotional stressor. There were no correlations between fear score ranks awarded to control and corticosterone-treated quail in the three behaviour tests of fear. In conclusion, it appears that corticosterone is not responsible for the stressor-induced increases in fearfulness that have been reported in other populations of quail and that the study of fearfulness as a personality trait is more difficult in unselected rather than selected populations of quail.

# 3. Effects of corticosterone treatment in laying Japanese quail

## 3.1 Introduction

Stressors are stimuli that initiate stress responses in animals, where stress responses involve activation of the hypothalamo-pituitary-adrenal (HPA) axis and the secretion of glucocorticoid hormones (Selye, 1974). Corticosterone is the primary glucocorticoid secreted during stress in birds, and corticosterone has a range of actions that can help birds cope with changes in their environments (Carsia and Harvey, 2000). Whilst short-term or acute stress responses that last for minutes to hours are thought to be adaptive, chronic stress that lasts for days or even weeks can have deleterious effects in birds, such as inhibiting reproductive function and reducing body weight (Wingfield, 1994; Wingfield et al., 1998; Breuner et al., 2008). Predation risk, human disturbance and poor habitat quality are environmental factors that can lead to chronic stress in birds (Wasser et al., 1997; Marra and Holberton, 1998; Scheuerlein et al., 2001), where plasma corticosterone concentrations are frequently elevated above initial concentrations for days or weeks. Treatment of birds with corticosterone can simulate the rise in plasma corticosterone concentrations that occur during chronic stress and several methods have been used to treat birds with corticosterone for seven days or more. Birds have been given subcutaneous or intraperitoneal implants or pellets containing corticosterone (Etches et al., 1984; Davison et al., 1985; Williamson and Davison, 1987; Jones et al., 1988; Donker and Beuving, 1989; Petitte and Etches, 1989; Horton et al., 2007), or they have been treated with corticosterone in their food (Davison et al., 1983; Lin et al., 2004a) or drinking water (Post et al., 2003; Hull et al., 2007; Shini et al., 2008). Plasma corticosterone in treated birds typically increases to maximum concentrations within the first four days. Corticosterone then decreases as treatment continues, but generally remains higher in treated than untreated birds until treatment ends. Plasma corticosterone was measured following corticosterone treatment in just one study, and had returned to initial concentrations in chickens (Gallus domesticus) two days after treatment ended (Davison et al., 1985).

Although the effects of chronic corticosterone treatment have been extensively studied in both domesticated and free-living species of birds, there are no data on how birds might recover from the potentially deleterious consequences of this treatment. Important questions can be asked about the long-term effects of chronic stress in birds in their natural environments by measuring physiological parameters in birds after corticosterone treatment has ended. Does the reproductive system and body weight of birds quickly recover from chronic stress, or do sustained elevations in plasma corticosterone concentrations have permanent effects on these parameters, such that survival and reproduction are reduced? The aim of the present study, therefore, was to determine the effects of chronic corticosterone treatment in laying Japanese quail (Coturnix coturnix japonica) both during treatment, and up to 22 days after treatment ended. Quail were treated with corticosterone in their drinking water for 21 days, and plasma corticosterone concentrations were measured during treatment, as well as one and eight days after treatment ended. Relationships of corticosterone with metabolism, reproduction and food intake in birds are well documented (Etches et al., 1984; Donker and Beuving, 1989; Gray et al., 1990; Hayashi et al., 1994; El-Lethey et al., 2001; Post et al., 2003; Hull et al., 2007), so body weight, cloacal diameter, egg production, egg weight and food intake were measured before, during and after treatment as physiological indicators of how corticosterone might affect laying quail. Corticosterone was delivered to quail at three different concentrations in their drinking water, so any dose-dependent effects of corticosterone on measured variables could be determined. Knowledge of the effects of chronic corticosterone treatment in quail will provide important new information on how birds may be affected by chronic stress in their natural environments.

## 3.2 Materials and methods

### 3.2.1 Animals and husbandry

Please refer to section 2.2.1 for details on animals and husbandry.

### 3.2.2 Experimental design

Eighty quail that had each laid at least three eggs in the previous week were randomly assigned to four groups of 20 birds on day -8 (day -8 was 8 days before corticosterone treatment began). Data were collected from all birds in the week before corticosterone

treatment began (day -7 to day -1), during the three week treatment period (day 0 to day 20), and in the three weeks after treatment ended (day 21 to day 41). Two measurements were made at the beginning of the fourth week after corticosterone treatment ended (day 42). Each day in this experiment began at 0800 h on one calendar day and ended at 0800 h the following calendar day, and corticosterone treatment began at 0800 h on day 0. Please refer to section 2.2.2 for further details on the method of corticosterone treatment. The groups were 0 (control group), 0.31-0.60, 0.61-0.90, 0.91-1.50 or >1.51 mg corticosterone/bird/day. Two control birds and four treated birds died during the experiment, so sample sizes differed between groups.

### 3.2.3 Experimental procedures

Blood samples were collected on days -1, 7, 14, 21 and 28, and body weight and cloacal diameter were measured on days -1, 7, 14, 21, 28, 35 and 42. Water intake was measured between 1700 h on day 4 and the end of day 20. Food intake was measured over 24 h between day -6 and day -5, day 8 and day 9, and between day 22 and day 23. Egg production and egg weight were recorded or measured daily from day -7 to day 41. Additional data were collected but are not presented here: behavioural responses were recorded during tonic immobility tests on day 6 and again on day 16; novel object tests on day 10 or 11; runway tests on day 12; open field tests on day 16 or 17 (see Chapter two). Quail were fasted for 24 h, beginning between 1230 and 1430 h on day 14. Blood samples were collected at the end of the fast and 3 h later, body weight was measured at the end of the fast, and food intake was measured over the 3 h following the fast (see Chapter four).

Please refer to section 2.2.5 for details on blood sample collection. Body weight (± 0.1 g) was measured using an electronic balance and cloacal diameter (± 0.1 mm) was measured using callipers. All blood samples and body weight and cloacal diameter measurements were taken between 1230 and 1500 h. Food intake measurements began between 1100 and 1400 h and ended 24 h later. Individual food troughs were removed, emptied, filled with a known amount of food, and replaced on the cage. Food troughs were located on the outside of cages, and birds had access to food through a small opening in their cage. The remaining food in each food trough was weighed (± 0.1 g) 24 h later. Daily egg production was measured by recording the presence or absence of

an egg in each cage at 0900 h. Egg weight (± 0.1 g) was measured with an electronic balance.

### 3.2.4 Corticosterone radioimmunoassay

Please refer to section 2.2.7 for details on corticosterone radioimmunoassay.

### 3.2.5 Statistics

A high corticosterone value for a control bird was identified as an outlier using Grubb's test (Barnett and Lewis, 1994), and this bird was excluded from analyses of plasma corticosterone. Statistical analyses were performed using Systat (Systat Software.). Plasma corticosterone concentrations were transformed to logarithms. Egg production was calculated for each week as the mean percentage of birds in the group that laid an egg each day that week. Egg weight was calculated for each week as the mean weight of all eggs laid in each group, using only data from birds that laid at least one egg every week. Levene's tests were conducted for all variables to check for homogeneity of variances. Changes in plasma corticosterone concentration, body weight and cloacal diameter were compared between groups using repeated measures ANOVAs with time (day) and group (control or corticosterone treatment) as the grouping factors. Post hoc comparisons were made between times for control and treatment groups and between control and treatment groups for each time using univariate F tests. Differences in water intake were compared between groups using one way ANOVA. Levene's tests showed that parametric statistics could not be used to analyse changes in food intake, egg production or egg weight. Instead, Kruskal-Wallis one way ANOVAs were used to compare food intake, egg production and egg weight between groups for each time, with pairwise comparisons between the control group and each treatment group performed with Mann-Whitney U tests. Friedman's one way repeated measures ANOVAs were used to compare food intake, egg production and egg weight between times for each group, with pairwise comparisons between times performed with Wilcoxon signed rank tests. Data are presented as mean ± standard error (S.E.).

## 3.3 Results

### 3.3.1 Plasma corticosterone

There were significant differences between groups and sampling times in plasma

corticosterone concentrations, and a significant interaction between groups and sampling times (two way repeated measures ANOVA $F_{4,69} = 40.327$, $p < 0.001$; $F_{4,276} = 209.217$, $p < 0.001$; $F_{16,276} = 8.319$, $p < 0.001$; see Appendix Table 3.1 for further statistics). Mean corticosterone increased slightly in control quail between day -1 and day 21 ($F_{1,69} = 26.485$, $p < 0.001$; Fig. 3.1, see Appendix Fig. 3.1 for individual plasma corticosterone concentrations), but not between day 21 and day 28 ($F_{1,69} = 0.146$, $p = 0.704$). Mean corticosterone increased in all treatment groups until day 14 of treatment (day -1 to day 14; $F_{1,69} > 67.000$, $p < 0.001$), but not between day 14 of treatment and day 21 ($F_{1,69} < 1.000$, $p \geq 0.340$). Corticosterone decreased between day 21 and day 28 in the 0.31-0.60 mg corticosterone/bird/day group, but not in the three highest treatment groups ($F = 5.189$, $p = 0.026$; $F_{1,69} < 1.400$, $p > 0.200$).

Initial mean plasma corticosterone did not differ between control and treatment groups on day -1 ($F_{1,69} < 1.800$, $p > 0.180$). Corticosterone was however higher in all treatment groups than in control birds one day after treatment ended (day 21; $F_{1,69} > 32.000$, $p < 0.001$) and eight days after treatment ended (day 28; $F_{1,69} > 25.00$, $p < 0.001$).

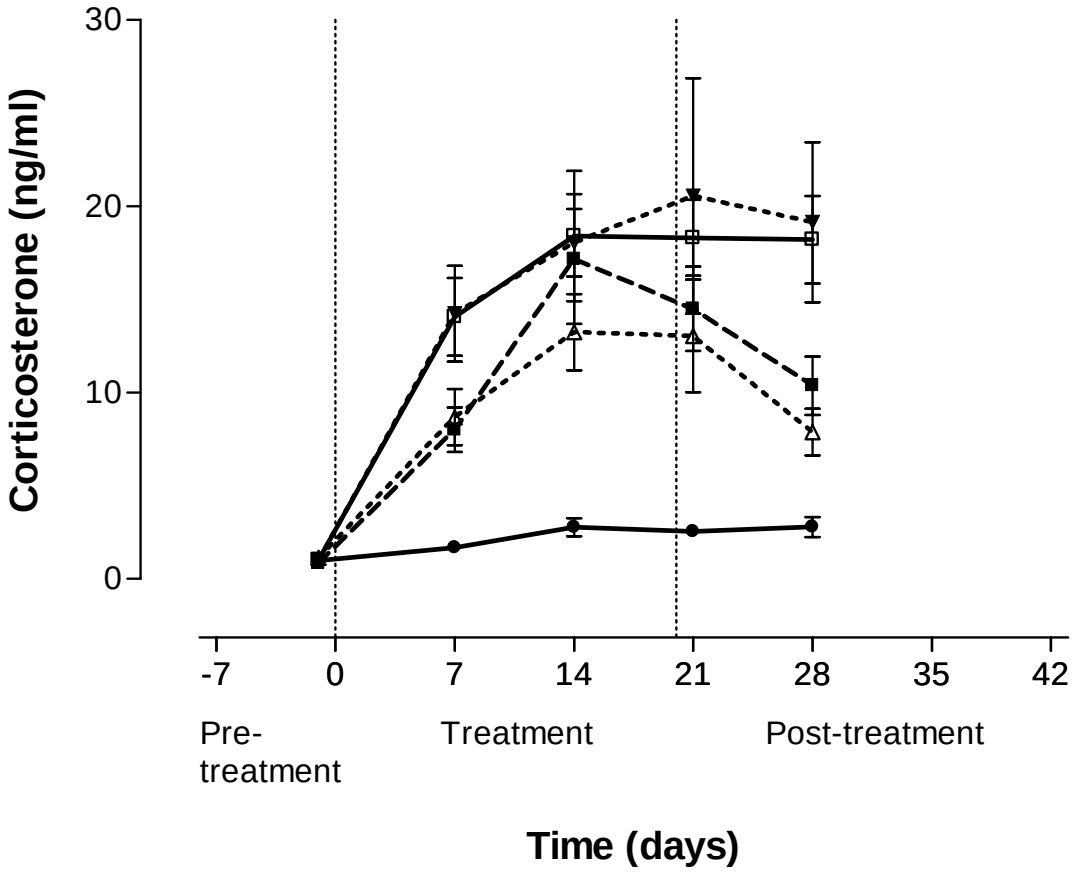


**Figure 3.1.** Plasma corticosterone concentrations in control quail and in quail treated with corticosterone. Control (—●—), N = 17; 0.31-0.60 mg corticosterone/bird/day (·····Δ·····), N = 20; 0.61-0.90 mg corticosterone/bird/day (- - - ■ - - -), N = 13; 0.91-1.50 mg corticosterone/bird/day (—□—), N = 19; >1.51 mg corticosterone/bird/day (·····▼·····), N = 5. Initial samples were collected one day before corticosterone treatment began, and vertical dotted lines denote the beginning (day 0) and end (day 20) of treatment. Data are presented as mean ± S.E.

### 3.3.2 Body weight

Differences in body weight between groups approached significance, and there was a significant effect of time, and a significant interaction between group and time (two way repeated measures ANOVA $F_{4,69} = 2.309$, $p = 0.066$; $F_{6,414} = 27.278$, $p < 0.001$; $F_{24,414} = 3.671$, $p < 0.001$; see Appendix Table 3.2 for further statistics). Mean body weight increased in control quail between day -1 and day 21, and between day 21 and day 42 ($F_{1,69} = 15.910$, $p < 0.001$; $F_{1,69} = 12.125$, $p = 0.001$; Fig. 3.2, see Appendix Fig. 3.2 for individual body weights). Body weight increased during the treatment period in the three lowest treatment groups (day -1 to day 21; $F_{1,69} > 12.000$, $p \leq 0.001$) but not in the >1.51 mg corticosterone/bird/day group (day -1 to day 21; $F_{1,69} = 0.519$, $p = 0.474$). Body weight continued to increase after treatment ended in the 0.31-0.60 and 0.61-0.90 mg corticosterone/bird/day groups (day 21 to day 42; $F_{1,69} = 18.767$, $p < 0.001$; $F_{1,69} = 7.661$, $p = 0.007$), but not in the 0.91-1.50 and >1.51 mg corticosterone/bird/day groups (day 21 to day 42; $F_{1,69} = 1.128$, $p = 0.292$; $F_{1,69} = 5.453$, $p = 0.067$).

Body weight did not differ between control and treatment groups before treatment began on day -1 ($F_{1,69} < 1.800$, $p > 0.180$) or on the day after treatment ended (day 21; $F_{1,69} < 1.500$, $p > 0.220$). At the beginning of the fourth week after treatment ended however, body weight was lower in the >1.51 mg corticosterone/bird/day group than in controls (day 42; $F_{1,69} = 10.326$, $p = 0.002$), whereas body weight in the three lowest treatment groups did not differ from controls ($F_{1,69} < 2.000$, $p > 0.160$).

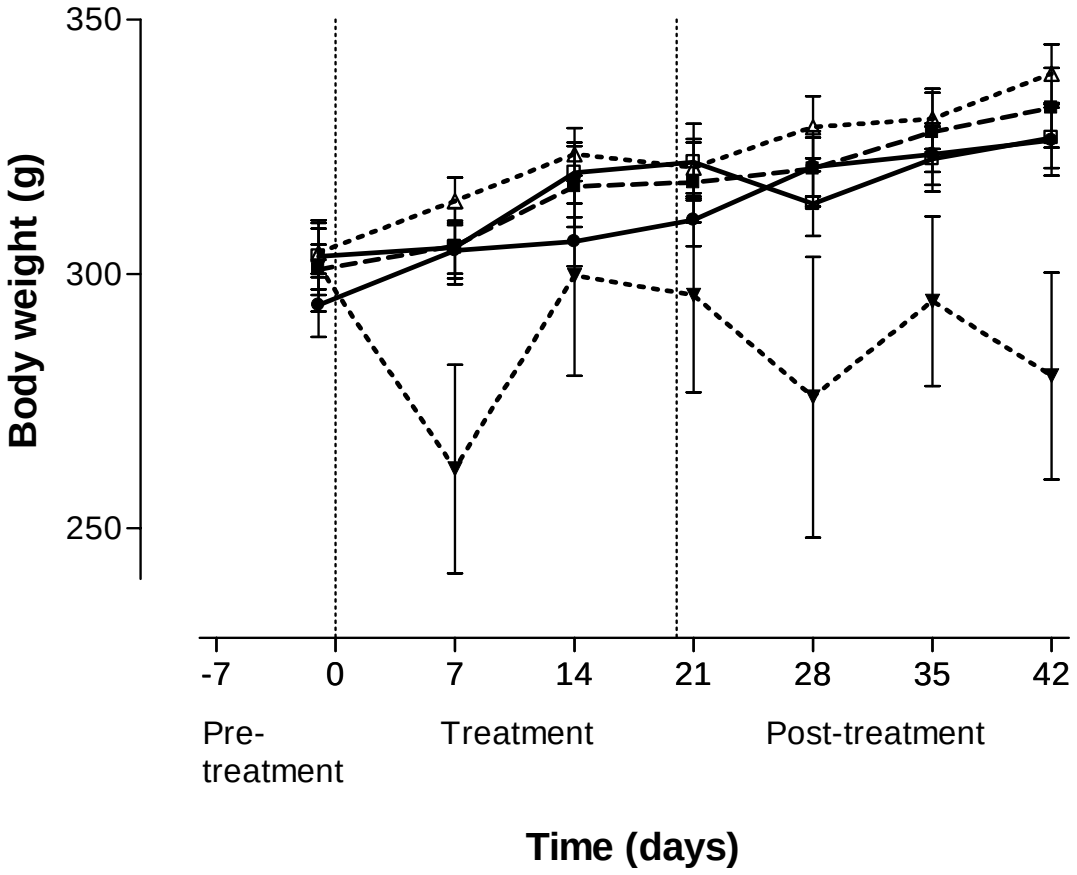


**Figure 3.2.** Body weights in control quail and in quail treated with corticosterone. Control (—●—), N = 18; 0.31-0.60 mg corticosterone/bird/day (·····Δ·····), N = 20; 0.61-0.90 mg corticosterone/bird/day (- - - ■ - - -), N = 13; 0.91-1.50 mg corticosterone/bird/day (—□—), N = 18; >1.51 mg corticosterone/bird/day (····· ▼ ·····), N = 5. Initial measurements were taken one day before corticosterone treatment began, and vertical dotted lines denote the beginning (day 0) and end (day 20) of treatment. Data are presented as mean ± S.E.

### 3.3.3 Cloacal diameter

There were no differences in cloacal diameter between groups, whilst there was a significant effect of time, but no interaction between group and time (two way repeated measures ANOVA $F_{4,69} = 1.392$, $p = 0.246$; $F_{6,414} = 13.654$, $p < 0.001$; $F_{24,414} = 1.352$, $p = 0.125$; see Appendix Table 3.3 for further statistics). Mean cloacal diameter did not change in control quail between day -1 and day 21, and decreased slightly between day 21 and day 42 (day -1 to day 21, $F_{1,69} = 3.741$, $p = 0.057$; day 21 to day 42; $F_{1,69} = 3.964$, $p = 0.050$; Fig. 3.3, see Appendix Fig. 3.3 for individual cloacal diameters). Cloacal diameter in treated quail did not change during the treatment period (day -1 to day 21; $F_{1,69} < 0.650$, $p \geq 0.440$). Cloacal diameter then decreased between day 21 and day 42 in the 0.61-0.90 and 0.91-1.50 mg corticosterone/bird/day groups ($F_{1,69} = 15.291$, $p < 0.001$; $F_{1,69} = 10.185$, $p = 0.002$) and did not change in the 0.31-0.60 and >1.51 mg corticosterone/bird/day groups ($F_{1,69} = 3.111$, $p = 0.082$; $F_{1,69} = 3.260$, $p = 0.075$).

Cloacal diameter did not differ between control and treatment groups before treatment began (day -1; $F_{1,69} < 0.250$, $p > 0.600$), on the day after treatment ended (day 21; $F_{1,69} < 3.200$, $p > 0.080$) or at the beginning of the fourth week after treatment ended (day 42; $F_{1,69} < 2.600$, $p > 0.100$).

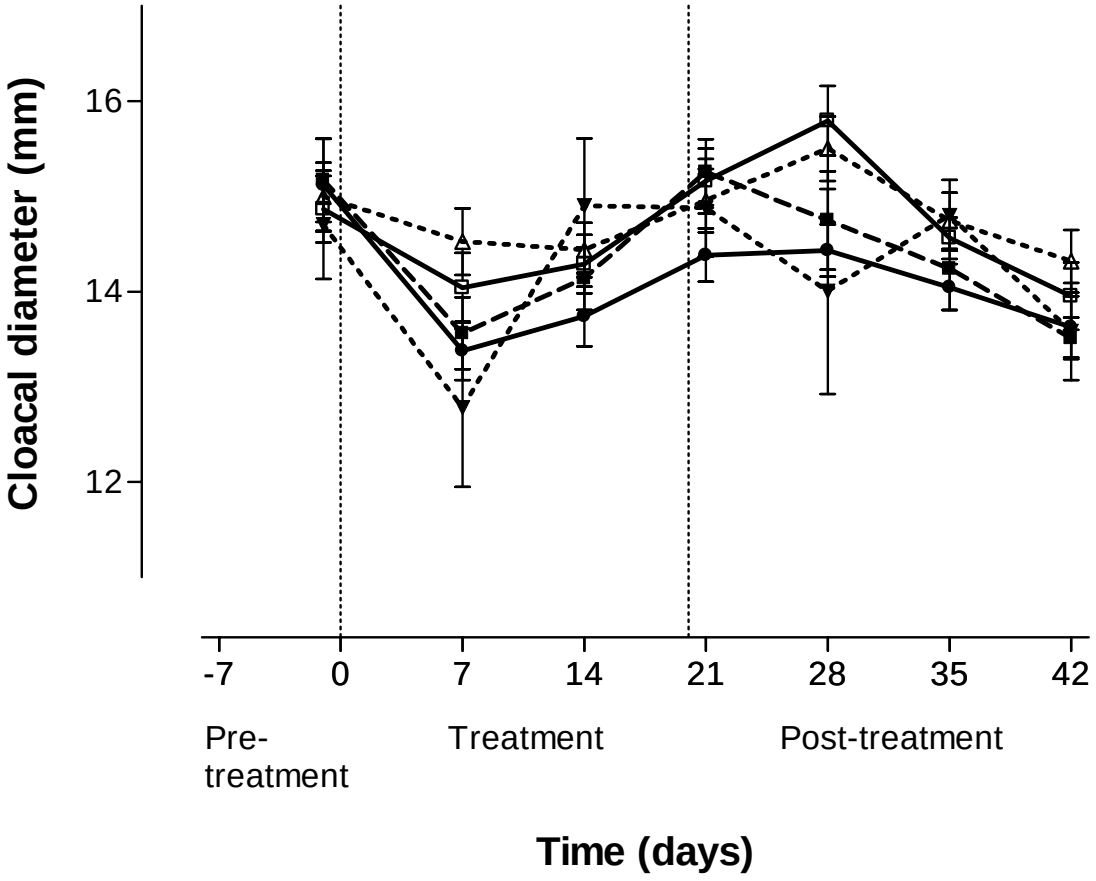


**Figure 3.3.** Cloacal diameters in control quail and in quail treated with corticosterone. Control (—●—), N = 18; 0.31-0.60 mg corticosterone/bird/day (·····Δ·····), N = 20; 0.61-0.90 mg corticosterone/bird/day (- - - ■ - - -), N = 13; 0.91-1.50 mg corticosterone/bird/day (—□—), N = 18; >1.51 mg corticosterone/bird/day (····· ▼ ·····), N = 5. Initial measurements were taken one day before corticosterone treatment began, and vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment. Data are presented as mean ± S.E.

### 3.3.4 Water intake

There were no differences in water intake during the treatment period between control birds and birds treated with corticosterone in their drinking water (one way ANOVA $F_{3,72} = 0.920$, $p = 0.436$).

### 3.3.5 Food intake

There were no differences between groups in food intake over 24 h before corticosterone treatment, but there were differences in food intake between groups during and after treatment (Kruskal-Wallis one way ANOVA day -6 to day -5; $K_4 = 3.506$, $p = 0.477$; day 8 to day 9; $K_4 = 10.900$, $p = 0.028$; day 22 to day 23; $K_4 = 48.226$, $p < 0.001$; see Appendix Table 3.4 for further statistics). Mean food intake was higher in the 0.31-0.60 and 0.91-1.50 mg corticosterone/bird/day groups than controls in the second week of treatment (day 8 to day 9; $U = 91.000$, $p = 0.009$; $U = 78.500$, $p = 0.005$; Fig. 3.4; see Appendix Fig. 3.4 for individual food intakes). Food intake was lower in the three highest treatment groups than in controls in the week after treatment ended (day 22 to day 23; $U > 90.000$, $p < 0.001$).

Differences in food intake between times were significant in the control group, and in all treatment groups. (Friedman's one way repeated measures ANOVA $F_2 = 19.694$, $p < 0.001$; $F_2 > 6.400$, $p \leq 0.041$; see Appendix Table 3.5 for further statistics). Food intake in control birds did not change between the first and second times (day -6 to day -5 vs day 8 to day 9; $Z = 1.633$, $p = 0.102$; Fig. 3.4), but increased between the second and third times (day 8 to day 9 vs day 22 to day 23; $Z = 3.158$, $p = 0.002$). Food intake in the 0.31-0.60 and 0.91-1.50 mg corticosterone/bird/day groups was higher in the second week of treatment than in the week before treatment (day -6 to day -5 vs day 8 to day 9; $Z = 3.211$, $p = 0.001$; $Z = 2.334$, $p = 0.020$), but there were no differences between these times in the 0.61-0.90 and >1.51 mg corticosterone/bird/day groups. Food intake in the three highest treatment groups was lower in the week after treatment ended than in the second week of treatment (day 8 to day 9 vs day 22 to day 23; $Z < -2.000$, $p < 0.045$). This difference approached significance in the lowest treatment group (day 8 to day 9 vs day 22 to day 23; $Z = -0.941$, $p = 0.052$).

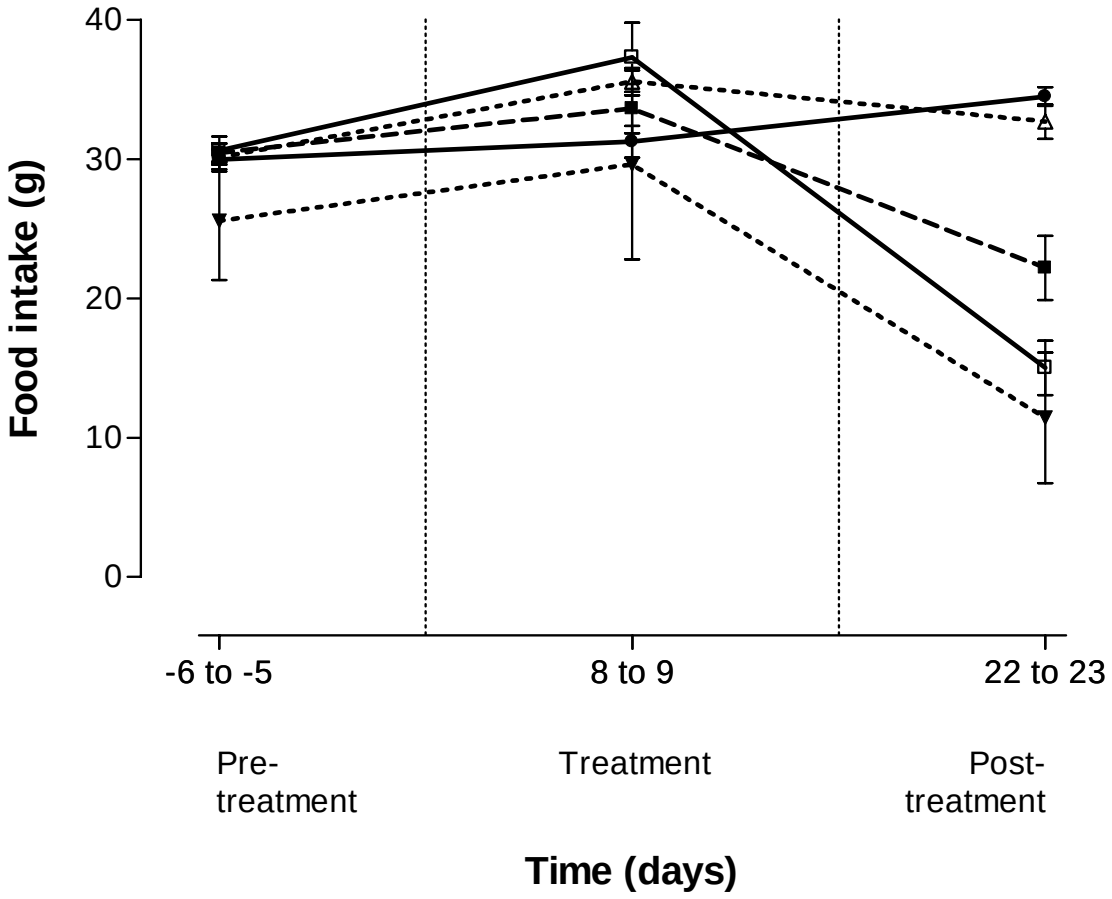


**Figure 3.4.** Food intakes over 24 h in control quail and in quail treated with corticosterone. Control (—●—), N = 18; 0.31-0.60 mg corticosterone/bird/day (·····Δ·····), N = 20; 0.61-0.90 mg corticosterone/bird/day (- - - ■ - - -), N = 13; 0.91-1.50 mg corticosterone/bird/day (—□—), N = 19; >1.51 mg corticosterone/bird/day (····· ▼ ·····), N = 5. Vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment. Data are presented as mean ± S.E.

### 3.3.6 Egg production

There were no differences between groups in egg production in the week before corticosterone treatment began, but egg production differed between groups in the third week of treatment, and in the third week after treatment ended (Kruskal-Wallis one way ANOVA day -7 to day -1; $K_4 = 6.369$, $p = 0.173$; day 14 to day 20; $K_4 = 82.233$, $p < 0.001$; day 35 to 41; $K_4 = 12.605$, $p = 0.013$; see Appendix Table 3.6 for further statistics). Egg production was lower in the three highest treatment groups than in controls in the third week of treatment (day 14 to day 20; $U > 3100.000$, $p < 0.001$), but not in the lowest treatment group (day 14 to day 20; $U = 8232.000$, $p = 0.178$; Fig. 3.5). Egg production did not differ between the three lowest treatment groups and controls in the third week after treatment ended (day 35 to day 41; $U < 9050.000$, $p > 0.350$), but was lower in the highest treatment group than in controls (day 35 to day 41; $U = 2523.500$, $p = 0.007$).

Differences in egg production between weeks were significant for the control group (Friedman's one way repeated measures ANOVA $F_6 = 13.369$, $p = 0.038$; see Appendix Table 3.7 for further statistics). There were differences in egg production between periods (before, during and after treatment) in the three highest treatment groups ($F_6 > 33.000$, $p < 0.001$; Fig. 3.5), but not in the lowest treatment group ($F_6 = 3.289$, $p = 0.772$). Egg production in control birds did not change between days -7 to -1 and days 14 to 20, but increased between days 14 to 20 and days 35 to 41 ($Z = -0.729$, $p = 0.466$; $Z = 4.131$, $p < 0.001$). Egg production in the three highest treatment groups was lower in the third week of treatment than in the week before treatment began (day -7 to day -1 vs day 14 to day 20; $Z < -4.000$, $p < 0.001$), but did not change in the lowest treatment group (day -7 to day -1 vs day 14 to day 20; $Z = -1.715$, $p = 0.086$). Egg production in all treatment groups was higher in the third week after treatment ended than in the third week of corticosterone treatment (day 14 to day 20 vs day 35 to day 41; $U > 2.400$, $p \leq 0.016$).

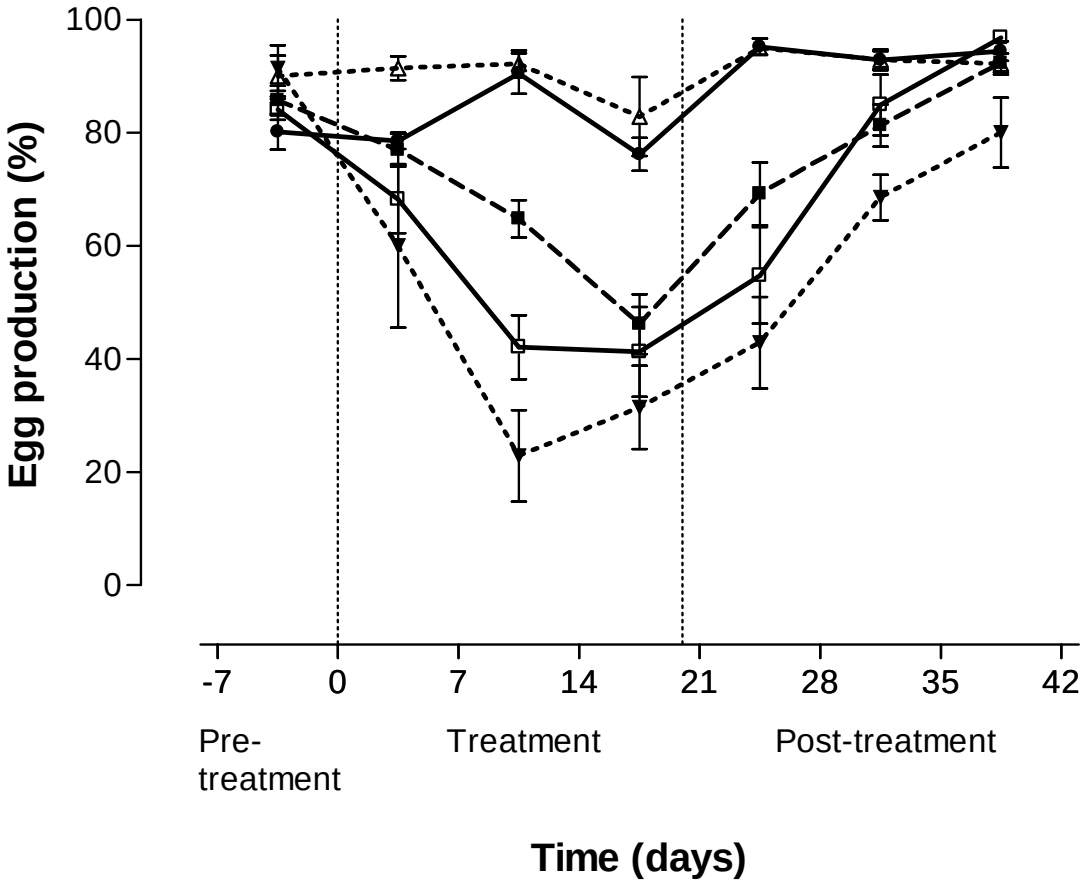


**Figure 3.5.** Egg production (calculated for each week as the mean percentage of birds in the group that laid an egg each day that week) in control quail and in quail treated with corticosterone. Control (—●—), N = 18; 0.31-0.60 mg corticosterone/bird/day (·····Δ·····), N = 20; 0.61-0.90 mg corticosterone/bird/day (- - - ■ - - -), N = 13; 0.91-1.50 mg corticosterone/bird/day (—□—), N = 18; >1.51 mg corticosterone/bird/day (·····▼·····), N = 5. Vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment. Data are presented as mean ± S.E. and are plotted at the midpoint of each week.

### 3.3.7 Egg weight

Differences in egg weight between groups were significant in the week before corticosterone treatment began, in the third week of treatment, and in the third week after treatment ended (Kruskal-Wallis one way ANOVA day -7 to day -1; $K_4 = 45.450$, $p < 0.001$; day 14 to day 20; $K_4 = 100.193$, $p < 0.001$; day 35 to day 41; $K_4 = 61.899$, $p < 0.001$; see Appendix Table 3.8 for further statistics). Egg weight was greater in the two lowest treatment groups than in controls in the week before treatment began (day -7 to day -1; $U = 3393.000$, $p < 0.001$; $U = 2492.000$, $p = 0.001$), but did not differ between the two highest treatment groups and controls (day -7 to day -1; $U = 3527.000$, $p = 0.182$; $U = 874.000$, $p = 0.538$; Fig. 3.6; see Appendix Fig. 3.5 for individual egg weights). Egg weight was greater in the lowest treatment group (day 14 to day 20; $U = 3234.500$, $p < 0.001$), and lower in the 0.91-1.50 mg corticosterone/bird/day group (day 14 to day 20; $U = 3227.000$, $p < 0.001$) than in controls in the third week of treatment, but did not differ between the 0.61-0.90 and >1.51 mg corticosterone/bird/day groups and controls (day 14 to day 20; $U = 2307.000$, $p = 0.055$; $U = 587.500$, $p = 0.131$). Egg weight was greater in the two lowest treatment groups than in controls in the third week after treatment ended (day 35 to day 41; $U = 3833.000$, $p < 0.001$; $U = 3427.000$, $p = 0.030$), but not in the two highest treatment groups (day 35 to day 41; $U = 4794.000$, $p = 0.873$; $U = 1384.000$, $p = 0.250$).

Differences in egg weight between weeks were significant for the control group, and the three lowest treatment groups (Friedman's one way repeated measures ANOVA $F_6 = 122.128$, $p < 0.001$; $F_6 > 45.000$, $p < 0.001$; see Appendix Table 3.9 for further statistics). There were insufficient data in the highest treatment group for Friedman's ANOVA. Egg weight in control birds increased between days -7 to -1 and days 14 to 20 ($Z = 3.770$, $p < 0.001$; Fig. 3.6), and continued to increase between days 14 to 20 and days 35 to 41 ($Z = 5.535$, $p < 0.001$). Egg weight in the lowest treatment group was greater in the third week of treatment than in the week before treatment began (day -7 to day -1 vs day 14 to day 20; $Z = 2.645$, $p = 0.008$), but was lower in the 0.61-0.90 and 0.91-1.50 mg corticosterone/bird/day groups in the third week of treatment than in the week before treatment (day -7 to day -1 vs day 14 to day 20; $Z = -2.749$, $p = 0.006$; $Z = -4.730$, $p < 0.001$). Egg weight in the highest treatment group in the third week of treatment did not differ from before treatment began (day -7 to day -1 vs day 14 to day 20; $Z = -0.654$, $p = 0.513$). Egg weight in all treatment groups was higher in the third

week after treatment ended than in the third week of treatment (day 14 to day 20 vs day 35 to day 41; $Z > 2.500$, $p \leq 0.009$).

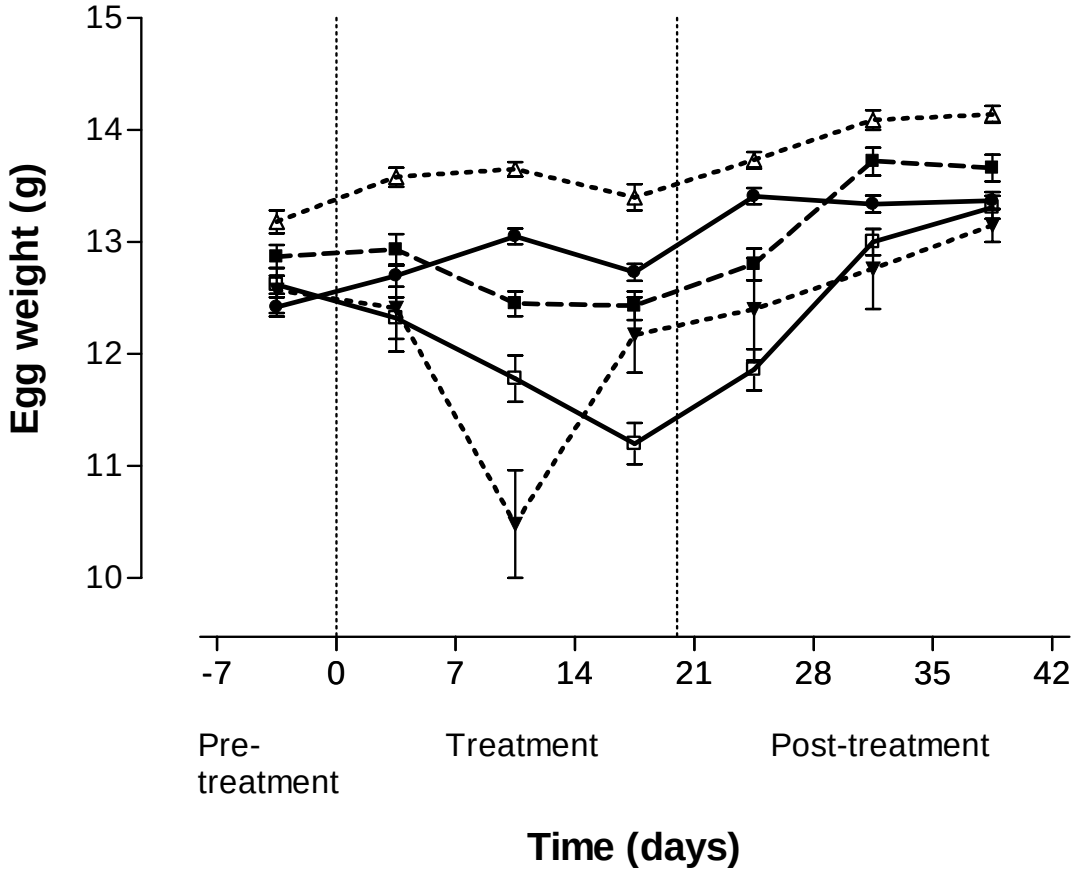


**Figure 3.6.** Egg weights (calculated for each week as the mean weight of all eggs laid in each group, using only data from birds that laid at least one egg every week) in control quail and in quail treated with corticosterone. Control (—●—), N = 17; 0.31-0.60 mg corticosterone/bird/day (·····Δ·····), N = 20; 0.61-0.90 mg corticosterone/bird/day (- - - ■ - - -), N = 11; 0.91-1.50 mg corticosterone/bird/day (—□—), N = 12; >1.51 mg corticosterone/bird/day (····· ▼ ·····), N = 3. Vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment. Data are presented as mean ± S.E. and are plotted at the midpoint of each week.

## 3.4 Discussion

Plasma corticosterone in Japanese quail treated with corticosterone in their drinking water for 21 days increased from around 1 ng/ml on the day before treatment began to maximum concentrations of 13-18 ng/ml on day 14 of treatment. Mean corticosterone concentrations did not change between day 14 of treatment and the day after treatment ended, and had decreased in only one of four treatment groups one week later. Body weight, food intake, egg production and egg weight were significantly lower in some treatment groups than in controls for up to 22 days following treatment. These findings confirm that corticosterone can affect laying quail for up to several weeks after treatment has ended.

Repeated handling can be a stressor in quail (Hull et al., 2007), and all quail were handled during the experiment for blood sample collection, weighing, measurement of cloacal diameter and for fear behaviour testing. The slight increase in mean plasma corticosterone in control birds between days -1 and 21 may result from handling. Corticosterone in treatment groups was 8-14 ng/ml on day seven of treatment, and was markedly elevated above controls for the remaining treatment period. Mean corticosterone concentrations in quail of the same stock were 5-14 ng/ml after 15 min immobilisation by mechanical restraint (unpub. data). Experimentally elevated corticosterone concentrations in the present study were therefore within the physiological range for quail. Corticosterone has previously been delivered in the drinking water to broiler chickens at approximate daily intakes of 0.7, 1.4 or 2.8 mg/bird/day (Post et al., 2003), to Japanese quail at daily intakes of 0.21-0.35, 0.44-0.58 or 0.68-0.94 mg/bird/day (Hull et al., 2007), and to brown Hyline hens at a concentration of 20 mg/l (Shini et al., 2008). Plasma corticosterone concentrations were higher in treated birds than in controls at or near the end of treatment in all studies, consistent with the present results, but blood samples were not collected in these studies after treatment ended.

In the present study, plasma corticosterone was markedly higher in treated birds than in untreated controls both one and eight days after corticosterone treatment ended. Corticosterone has a half-life of around 10 min in quail (Kovacs and Peczely, 1983) and a high metabolic clearance rate in birds (Carsia and Harvey, 2000). Elevated plasma

corticosterone concentrations in quail after treatment ended could have resulted from hyperactivity of the HPA axis (Sapolsky et al., 1984), or from release of corticosterone into the bloodstream by corticosteroid-binding globulin (Breuner and Orchinik, 2002). Alternatively, regeneration of corticosterone from inactive metabolites by hydroxysteroid dehydrogenases (Seckl and Walker, 2004; Kucka et al., 2006), or by release of lipid-soluble corticosterone from storage in adipose tissue, could explain the elevation in plasma corticosterone in treated birds up to eight days after treatment ended.

Although it is unclear how plasma corticosterone concentrations in treated quail remained elevated after a source of exogenous corticosterone had been removed, the present findings provide important insights into the long-term effects of chronic stress in laying birds and their offspring. There is considerable interest in how maternal steroid hormones deposited in eggs during egg formation may affect the phenotype of avian offspring (Schwabl, 1993; Groothuis et al., 2005; Breuner, 2008), and it is thought that concentrations of corticosterone in avian eggs may correlate with plasma corticosterone concentrations in female birds at the time of lay. Recently it was shown that corticosterone concentrations in the albumen of chicken eggs were positively correlated with plasma corticosterone concentrations in laying birds treated with corticosterone (Downing and Bryden, 2008), and that yolk corticosterone concentrations were significantly elevated above controls in eggs from Japanese quail subjected to chronic restraint stress (Okuliarova et al., 2010). Hayward and Wingfield (2004) treated female quail with corticosterone and found that offspring from treated birds had reduced growth rates and higher plasma corticosterone responses to restraint than offspring from control birds. These findings raise important questions about how stress in laying birds might affect the amount of corticosterone transferred to their eggs, and hence the development of their offspring. It is suggested that variations in concentrations of corticosterone in avian eggs can adjust the phenotype of the developing offspring to suit the environmental conditions at the time of lay (Okuliarova et al., 2010). Plasma corticosterone concentrations in laying quail in the present study remained elevated for up to eight days after chronic corticosterone treatment ended, and so the potential for laying birds to alter the development of their offspring in the wild may continue for many days after exposure to a stressor ends.

In hindsight blood samples for the measurement of corticosterone should have been collected from birds more than eight days after treatment ended. It was assumed, however, that plasma corticosterone would have returned to initial concentrations shortly after the source of exogenous corticosterone was removed. Egg production in the three highest treatment groups was significantly lower than in controls in the third week of treatment, then increased after treatment ended, and was the same as controls in all but the highest treatment group in the third week after treatment ended. The fall in egg production in the three highest treatment groups generally mirrored the rise in plasma corticosterone during the treatment period. It is suggested therefore, that plasma corticosterone in all treatment groups most likely returned to initial concentrations by the third or fourth week after treatment ended, when egg production was close to pre-treatment values. The findings of the present study are particularly interesting, and suggest that plasma corticosterone concentrations in birds that experience chronic stress in the wild may not return to initial values until some time after exposure to a stressor ends. This suggestion, however, is based on the assumption that elevated plasma corticosterone concentrations in quail after treatment ended were not an artefact of exogenous corticosterone treatment, but rather a result of elevated plasma corticosterone concentrations. Such a scenario may have consequences for the fitness (survival and reproduction) of individuals and species of free-living birds. For example, plasma corticosterone concentrations were negatively correlated with body condition and survival in Eurasian treecreeper (Certhia familiaris) chicks living in areas of forest with poor food supply (Suorsa et al., 2003), and the annual survival of cliff swallows (Petrochelidon pyrrhonota) sampled early in the breeding season generally declined with increasing endogenous plasma corticosterone concentrations (Brown et al., 2005). Studies in free-living species of birds, therefore, support the notion that elevated plasma corticosterone concentrations could have detrimental effects on the fitness of wild birds even after exposure to a stressor ends.

Chronic corticosterone treatment simulates the rise in plasma corticosterone concentrations that occur during chronic stress, and so provides a useful method for investigating the effects of chronic stress in birds. Treated quail ingested corticosterone each time they drank, and although corticosterone concentrations in individual birds treated with corticosterone fluctuated between sampling times, concentrations were consistently higher in treated birds than in untreated controls in the great majority of

cases. Transient increases in glucocorticoids are generally considered to be beneficial in animals, whereas sustained or chronic elevations in glucocorticoids can have deleterious effects (Wingfield, 1994; Wingfield et al., 1998). The results of the present study generally agree with this notion, although none of the negative effects of corticosterone treatment were evident in quail with the lowest corticosterone intakes, supporting previous findings of dose-dependent effects of corticosterone in birds (see Hayashi et al., 1994; Breuner et al., 1998; Hull et al., 2007). The effects of acute and chronic corticosterone treatment in birds can be compared if data from studies in free-living species are considered together with food intake data from the current study. For example, it is generally considered that acute increases in plasma corticosterone concentrations promote behaviours associated with self maintenance, such as increasing foraging behaviour (Wingfield 2003), and acute corticosterone treatment is reported to increase foraging intensity in red-eyed vireos (Vireo olivaceus; Lohmus et al., 2006). In the present study, however, mean food intake over 24 h was significantly lower in quail from the three highest corticosterone treatment groups than in controls, when measured one day after chronic corticosterone treatment ended. This finding supports the notion that chronic corticosterone treatment, and hence, chronic stress can have deleterious consequences in birds.

Mean body weight increased during corticosterone treatment in all but the highest treatment group. Corticosterone can increase the rate of protein breakdown in skeletal muscle of chickens in a dose-dependent manner (Hayashi et al., 1994), so increased protein degradation in the highest treatment group could account for the difference in body weight gain compared to other groups. It is possible that treatment with corticosterone also resulted in protein degradation in quail from the three lowest treatment groups, even though mean body weight in these birds increased during treatment. For example, song sparrows (Melospiza melodia) and pied flycatchers (Ficedula hypoleuca) lost protein from flight muscles during treatment with corticosterone-filled silastic implants, although an increase in fat deposition during treatment resulted in no overall change in body weight (Silverin, 1986; Wingfield and Silverin, 1986). Body weight continued to increase between days 21 and 42 in the two lowest treatment groups, and did not change between these times in the two highest treatment groups, suggesting that corticosterone affected body weight gain in some quail even after treatment ended. It is possible that such deleterious effects associated

with relatively high plasma corticosterone concentrations could reduce the fitness of birds in an ecological context. For example, plasma corticosterone concentrations in non-breeding American redstarts (Setophaga ruticilla) living in poorer habitats were negatively correlated with body condition during spring, and low body condition could result in delayed departure for migration, and hence, late arrival at breeding grounds (Marra and Holberton, 1998).

Changes in egg production and egg weight in the present study further illustrate how laying birds can be affected for up to several weeks after a source of exogenous corticosterone has been removed. Egg production in the highest treatment group for example, was significantly lower than in controls in the third week after treatment ended, and egg weight in the three highest treatment groups was lower than in controls in the week after corticosterone treatment ended (statistics not shown). Treatment of chickens or quail with corticosterone can reduce weights of the oviduct (Petitte and Etches, 1989; 1991; Hull et al., 2007) and ovary (Etches et al., 1984), and decrease plasma concentrations of luteinising hormone, progesterone and oestradiol (Etches et al., 1984; Petitte and Etches, 1989). Although there are no corticosterone data to support this claim, it is likely that plasma corticosterone concentrations gradually fell in the weeks after treatment ended, and at the same time normal reproductive function was slowly restored in laying quail, and egg production and egg weight in the three highest treatment groups returned to pre-treatment values. Whilst short-term elevations in plasma corticosterone concentrations during acute stress responses may temporarily suppress functions not needed for immediate survival (i.e. reproduction; Wingfield, 2003), the findings of the present study suggest that chronic elevations in plasma corticosterone can have long-term inhibitory effects on the reproductive system. Indeed, it is generally found that free-living birds with relatively low plasma corticosterone concentrations have higher annual reproductive success than birds with higher corticosterone concentrations, such that chronically elevated plasma corticosterone in wild birds can result in reduced fitness (Bonier et al., 2009; Busch and Hayward, 2009). For instance, the probability of successfully fledging a chick was negatively correlated with plasma corticosterone concentrations in black-browed albatrosses (Thallasarche melanophris; Angelier et al., 2007); food shortages resulted in elevated plasma corticosterone concentrations in the common murre (Uria aalge) which eventually led to a decrease in reproductive performance (Kitaysky et al., 2007); and

clutch initiation dates were positively correlated with plasma corticosterone concentrations in Florida scrub-jays (Aphelocoma caerulescens; Schoech et al., 2009). In the present study, egg production and egg weight in some groups of quail treated with corticosterone were lower than in untreated controls for up to three weeks after treatment ended. The current findings, therefore, suggest that any negative effects of chronic stress on the fitness of free-living birds may continue for some time after exposure to a stressor ends.

The potentially deleterious consequences of chronic corticosterone treatment in birds have been well studied, and data from the current study show for the first time that the detrimental effects of corticosterone, particularly on the reproductive system, can continue for up to several weeks after treatment has ended. For example, plasma corticosterone concentrations were elevated in Japanese quail during a 21 day corticosterone treatment period, and remained elevated eight days after treatment ended, and body weight gain and reproductive function were affected for up to 22 days after corticosterone treatment ended in the highest treatment groups. This study demonstrates that the effects of chronically elevated plasma corticosterone concentrations in birds in their natural environments may persist after exposure to a stressor ends.

# 4. Effects of corticosterone treatment on responses to fasting in Japanese quail

## 4.1 Introduction

Stressors are stimuli that activate the hypothalamo-pituitary-adrenal (HPA) axis and lead to the secretion of glucocorticoid hormones (Selye, 1974). Corticosterone is the primary glucocorticoid secreted in birds (Carsia and Harvey, 2000), and elevated plasma concentrations of corticosterone can be associated with increased protein breakdown and fat deposition (Gray et al., 1990; Hayashi et al., 1994), suppression of reproductive behaviour (Silverin et al., 1997) and heightened fear behaviour (Jones et al., 1988) and locomotor activity (Breuner et al., 1998). Corticosterone may also stimulate food intake in birds, although relationships between corticosterone and food intake remain unclear. Treatment of chickens (Gallus domesticus) with corticosterone for example, increased food intake in some studies (Siegel and Van Kampen, 1984; Nasir et al., 1999; El-Lethey et al., 2001), but not others (Davison et al., 1983; Simon, 1984; Williams et al., 1985; Kafri et al., 1988).

Fasting can be a potent stressor in birds (Freeman, 1985; Mench, 1991), and there are reports of significant increases in plasma corticosterone after 24 h of food deprivation in immature (Nir et al., 1975; Freeman et al., 1980; Harvey et al., 1983; Geris et al., 1999) and adult (Scanes et al., 1980) chickens. Corticosterone responses to fasting however, vary among individuals and may depend on energy reserves and past experience (Webster, 2003). Deprivation of food and water for 12 h elevated plasma corticosterone in mature Japanese quail (Coturnix coturnix japonica; Scott et al., 1983), but the effects of food withdrawal alone on corticosterone have not been investigated in quail.

The effect of corticosterone on food intake in birds may be dose-dependent. Food intake increased in chickens delivered higher doses of corticosterone but not in chickens given lower doses (Petitte and Etches, 1991; Covasa and Forbes, 1995), and corticosterone stimulated food intake in chickens fed high protein but not low protein diets (Bartov, 1985). Furthermore, delivery of corticosterone to white-crowned

sparrows (Zonotrichia leucophrys gambelii) did not affect food intake in otherwise unmanipulated birds (Astheimer et al., 1992). Following 24 h food deprivation however, the intensity of feeding was much greater in birds with corticosterone implants than in untreated controls, suggesting corticosterone may stimulate food intake in birds following a fast.

If birds experience a series of stressors or if they are continuously exposed to a stressor, then plasma corticosterone concentrations may remain elevated for days or even weeks. Predation risk, human disturbance and poor habitat quality are environmental factors that can elevate plasma corticosterone above initial concentrations in free-living birds (Wasser et al., 1997; Marra and Holberton, 1998; Scheuerlein et al., 2001), and important information about how birds respond to stressors in their natural environments can be gained from studies in domesticated species like Japanese quail. Do elevated plasma corticosterone concentrations alter the responsiveness of the HPA axis, so that birds show either reduced or exaggerated responses to subsequent stressors, and how might alterations in HPA axis responsiveness affect the ability of free-living birds to respond successfully to stressors? Treatment with corticosterone simulates the increase in plasma corticosterone concentrations that occur when birds experience stressors, and it has previously been found that corticosterone responses of birds to a handling or restraint stressor can be affected by this treatment (Hull et al., 2007; Muller et al., 2009). In the present study, corticosterone was delivered to Japanese quail in their drinking water to generate a range of plasma corticosterone concentrations. Birds were subjected to a 24 h fast then given free access to food for the following three hours. The aims of the study were to determine in quail the effects of corticosterone treatment on plasma corticosterone responses to the potential stressor of fasting, and to determine the effects of corticosterone on food intake immediately after a fast.

## 4.2 Materials and methods

### 4.2.1 Animals and husbandry

Please refer to section 2.2.1 for details on animals and husbandry.

### 4.2.2 Experimental design

Quail were randomly assigned to four groups of 20 birds on day -8 (day -8 was 8 days

before corticosterone treatment began). All quail in this study were part of a larger experiment in which additional data were collected in the week before corticosterone treatment began (day -7 to day -1) and during the three week treatment period (day 0 to day 20). Corticosterone treatment began at 0800 h on day 0, then quail were subjected to a 24 h fast beginning between 1230 and 1430 h on day 14 of corticosterone treatment. Please refer to section 2.2.2 for further details on the method of corticosterone treatment. The groups were 0 (control group, N = 18), 0.31-0.60 (N = 20), 0.61-0.90 (N = 13), 0.91-1.50 (N = 19) or >1.51 (N = 6) mg corticosterone/bird/day. Two control birds and two treated birds died before day 14 and were not included in these groups.

### 4.2.3 Experimental procedures

Blood samples were collected immediately before the 24 h fast began on day 14 (starting at 1230 h), after 24 h of fasting, and 3 h after food presentation. Birds were bled in the same order on each occasion. Body weight was measured immediately after each blood sample was collected at the beginning and end of the fast. Food intake was measured during the 3 h following the fast. Additional data were collected but are not presented here: blood samples for the measurement of corticosterone were collected on days -1 and 7, and body weight and cloacal diameter were measured on days -1 and 7, and cloacal diameter was measured on day 14. Food intake was measured over 24 h between day -6 and day -5, and between day 8 and day 9. Egg production and egg weight were recorded or measured daily (see Chapter three). Behavioural responses were recorded during the tonic immobility test on day 6; the novel object test on day 10 or 11; the runway test on day 12 (see Chapter two).

Please refer to section 2.2.5 for details on blood sample collection. Body weight (± 0.1 g) was measured using an electronic balance. Food intake measurements began when each quail was returned to its cage following blood sample collection after the fast. Individual empty food troughs were removed and filled with 30 g of food and placed back on the cage. Food troughs were located on the outside of cages, and birds had access to food through a small opening in their cage. The remaining food in each trough was weighed (± 0.1 g) 3 h later.

### 4.2.4 Corticosterone radioimmunoassay

Please refer to section 2.2.7 for details on corticosterone radioimmunoassay.

### 4.2.5 Statistics

A high corticosterone value for one bird in the lowest treatment group was identified as an outlier using Grubb's test (Barnett and Lewis, 1994), and this bird was omitted from statistical analyses. Statistical analyses were performed using Systat (Systat Software). Plasma corticosterone concentrations were transformed to logarithms, and Levene's tests confirmed homogeneity of variances for these data. Changes in plasma corticosterone concentrations and body weight were compared between groups using repeated measures ANOVAs with time (before and after 24 h of fasting, and 3 h after food presentation for corticosterone concentration; before and after 24 h of fasting for body weight) and group (control or corticosterone treatment) as the grouping factors. Post hoc comparisons were made between times for control and treatment groups and between control and treatment groups for each time using univariate F tests. Differences in food intake in the 3 h following food presentation were compared between groups using one way ANOVA. Post hoc comparisons were made between groups using univariate F tests. Data are presented as mean ± standard error (S.E.).

## 4.3 Results

### 4.3.1 Plasma corticosterone

Corticosterone concentrations in control birds before the fast varied from 0.79 to 7.37 ng/ml, whereas initial concentrations in birds receiving corticosterone in their drinking water ranged from 1.96 to 52.67 ng/ml (see Appendix Fig. 4.1 for individual corticosterone concentrations). There were significant differences between groups and sampling times in corticosterone concentrations, and a significant interaction between groups and sampling times (two way repeated measures ANOVA $F_{4,70} = 54.325$, $p < 0.001$; $F_{2,140} = 3.525$, $p = 0.032$; $F_{8,140} = 2.980$, $p = 0.004$; see Appendix Table 4.1 for further statistics). Corticosterone concentrations did not change in control birds after 24 h of fasting, or after 3 h of food presentation following the fast ($F_{1,70} = 1.370$, $p = 0.246$ and $F_{1,70} = 1.410$, $p = 0.239$; Fig. 4.1). Mean corticosterone decreased after 24 h of fasting then increased after 3 h of food presentation in the 0.31-0.60 mg corticosterone/bird/day treatment group ($F_{1,70} = 14.996$, $p < 0.001$; $F_{1,70} = 7.559$, $p = 0.008$). There were no changes in mean corticosterone concentrations in birds receiving 0.61-0.90 and >1.51 mg corticosterone/bird/day, whilst corticosterone did not change after fasting then increased 3 h later in birds receiving 0.91-1.50 mg

corticosterone/bird/day ($F_{1,70} = 0.189$, $p = 0.665$; $F_{1,70} = 8.780$, $p = 0.004$). Mean corticosterone was significantly lower in controls compared with treatment groups before ($F_{1,70} > 44.000$, $p < 0.001$) and after ($F_{1,70} > 22.000$, $p < 0.001$) the fast, and three hours following food presentation ($F_{1,70} > 63.000$, $p < 0.001$).

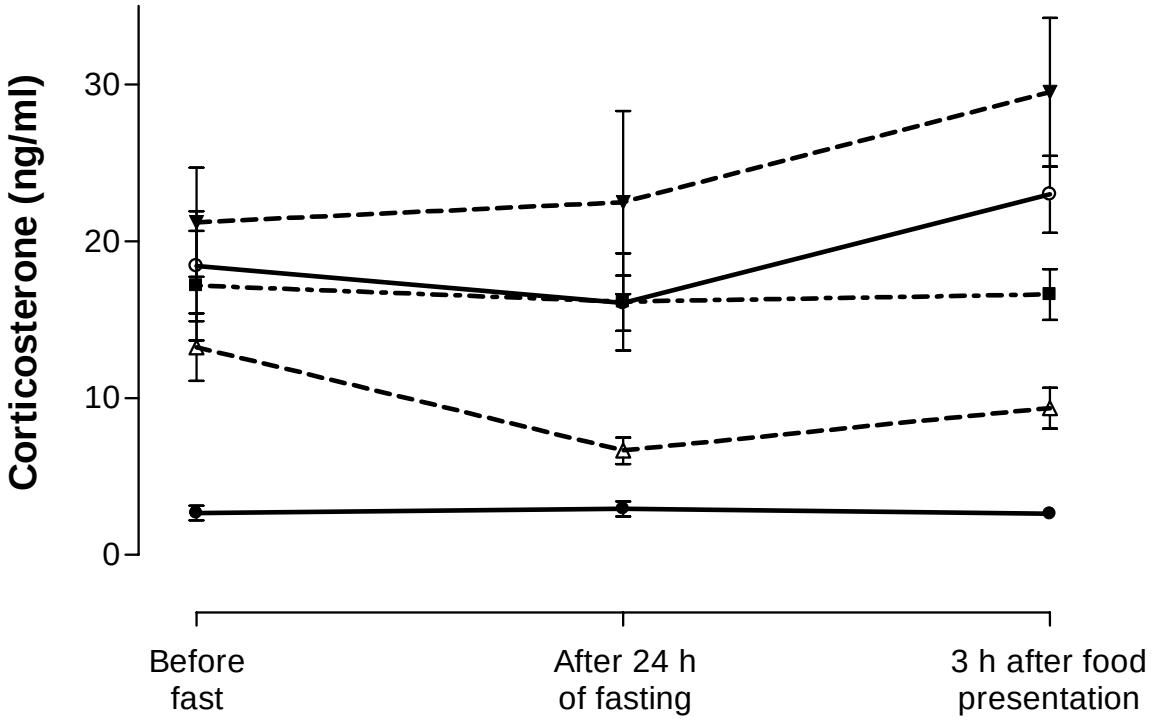


**Figure 4.1.** Plasma corticosterone concentrations before and after 24 h of fasting and 3 h after food presentation in control quail and in quail treated with corticosterone. Control (—●—), N = 18; 0.31-0.60 mg corticosterone/bird/day (---Δ---), N = 19; 0.61-0.90 mg corticosterone/bird/day (---■---), N = 13; 0.91-1.50 mg corticosterone/bird/day (—○—), N = 19; >1.51 mg corticosterone/bird/day (---▼---), N = 6. Data are presented as mean ± S.E.

### 4.3.2 Body weight

There was no overall effect of corticosterone treatment on body weight, but fasting significantly affected body weight and there was a significant interaction (two way repeated measures ANOVA $F_{4,70} = 2.329$, $p = 0.064$; $F_{1,70} = 1365.456$, $p < 0.001$; $F_{4,70} = 4.120$, $p = 0.005$; Fig. 4.2.; see Appendix Fig. 4.2 for individual body weights; see Appendix Table 4.2 for further statistics).

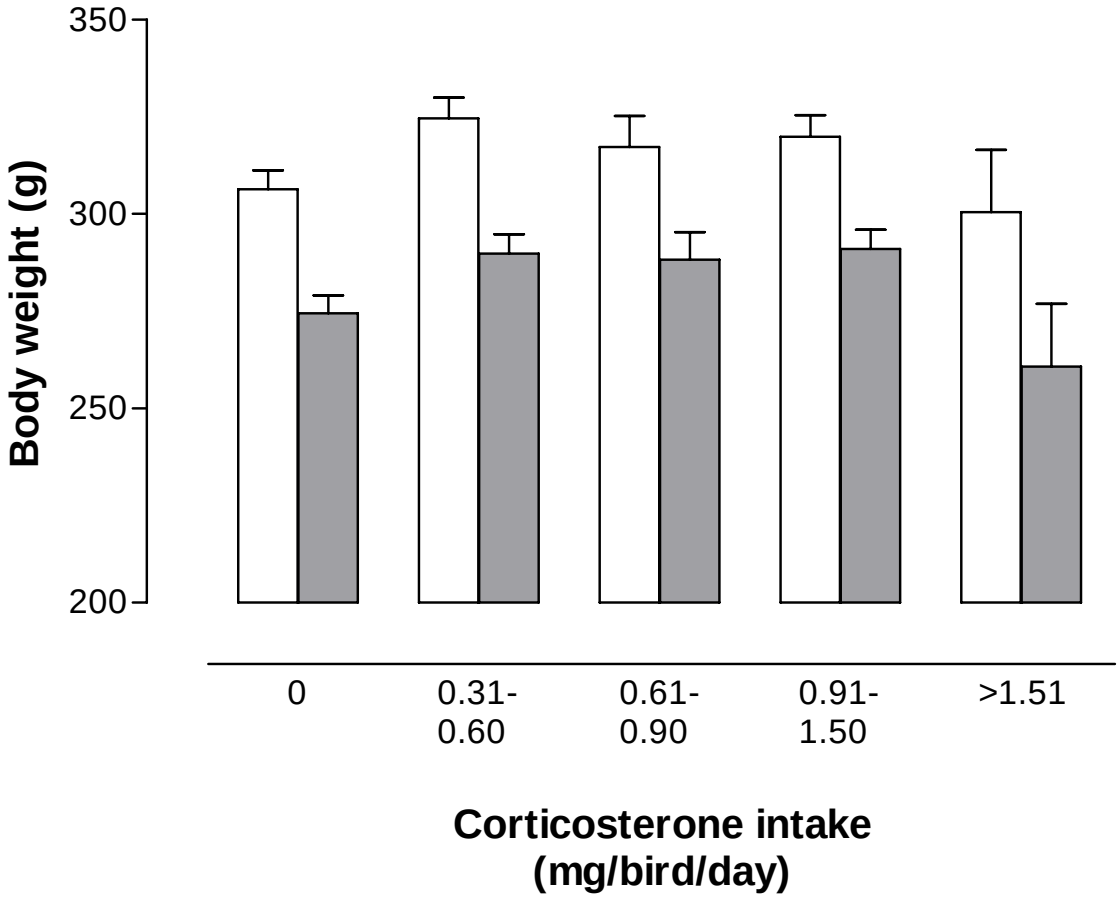


**Figure 4.2.** Body weights before (open bars) and after (shaded bars) 24 h of fasting in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6. Data are presented as mean ± S.E.

### 4.3.3 Food intake

Corticosterone treatment had a significant effect on food intake in the three hours following the fast (one way ANOVA $F_{4,70} = 6.642$, $p < 0.001$; see Appendix Fig. 4.3 for individual food intakes; see Appendix Table 4.3 for further statistics). Food intake was greater in all four treatment groups (8.6 ± 0.4, 8.2 ± 0.4, 8.0 ± 0.5 and 11.4 ± 1.2 g) than in untreated controls (6.8 ± 0.4 g; $F_{1,70} = 7.682$, $p = 0.007$; $F_{1,70} = 4.023$, $p = 0.049$; $F_{1,70} = 4.033$, $p = 0.048$; $F_{1,70} = 25.425$, $p < 0.001$; Fig. 4.3). Food intake did not differ amongst the three lowest treatment groups ($F_{1,70} < 0.600$, $p \geq 0.442$), but was greater in the highest treatment group than in each of the three lowest treatment groups ($F_{1,70} = 9.791$, $p = 0.003$; $F_{1,7} = 11.135$, $p = 0.001$; $F_{1,70} = 13.434$, $p < 0.001$).

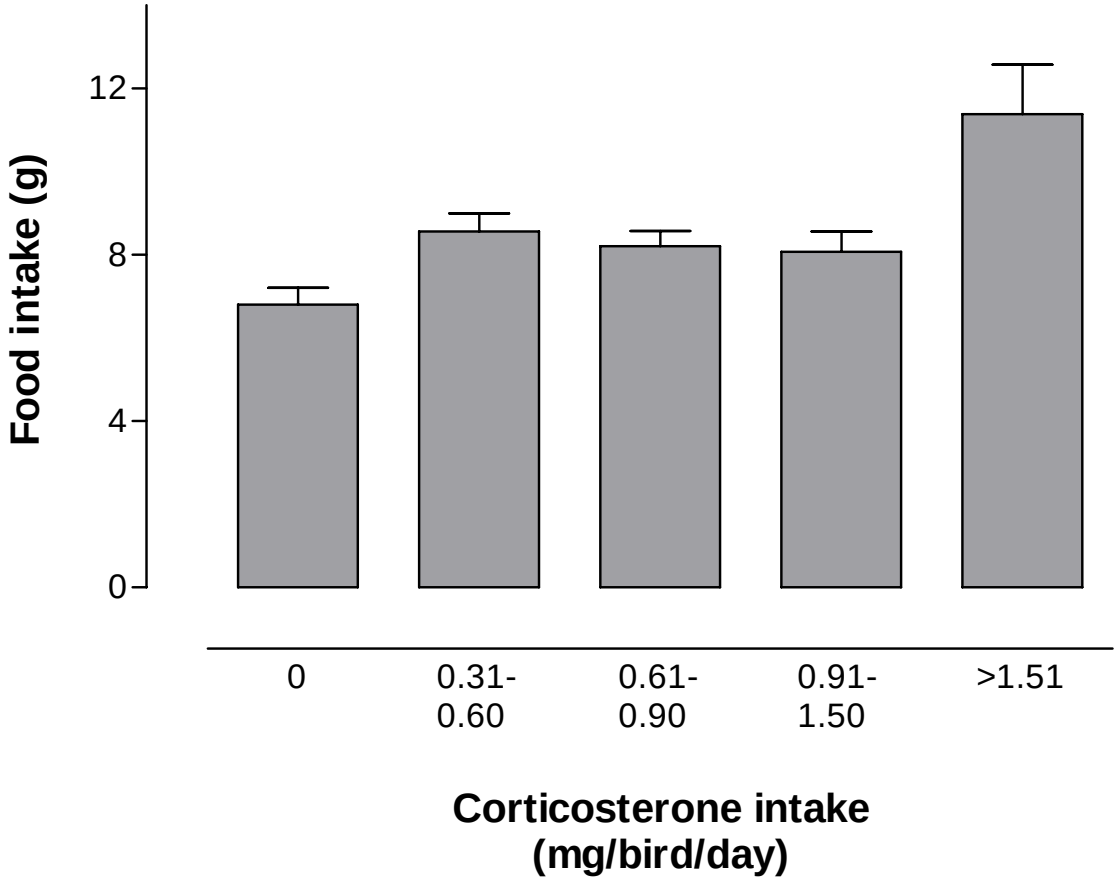


**Figure 4.3.** Food intakes in the 3 h after 24 h of fasting in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6. Data are presented as mean ± S.E.

## 4.4 Discussion

This is the first study to examine the effect of food deprivation alone on plasma corticosterone in Japanese quail. Corticosterone in control birds and in three out of four groups of birds treated with corticosterone did not change after 24 h of fasting. Food intake in the three hours following food presentation was, however, higher in birds treated with corticosterone than in untreated controls, suggesting that corticosterone may stimulate food intake following fasting in birds.

Physiological responses to natural fasting periods have been extensively studied in Antarctic penguins (e.g. emperor, Aptenodytes forsteri; and king penguins Aptenodytes patagonicus; Groscolas and Robin, 2001), with three phases of fasting described based on rates of body weight decline (Cherel et al., 1988a). The three phase concept of fasting has also been applied to domesticated geese (Anser anser; Le Maho et al., 1981), greater snow geese (Chen caerulescens atlantica; Boismenu et al., 1992) and chickens (Webster, 2003). Phase I of fasting lasts only a few days and is associated with stomach emptying and a rapid decrease in the rate of body weight loss. Phase II can last up to several months and is characterised by catabolism of fat stores, protein sparing and a constant rate of body weight decline. The third and final phase is defined by increased protein breakdown and rapid body weight loss (Cherel et al., 1988a). Plasma corticosterone concentrations in penguins during early phase II of fasting are similar to non-fasted birds (Robin et al., 1998; Groscolas and Robin, 2001), and presumably corticosterone in phase I is also relatively low (see Cockrem et al., 2006). Corticosterone during phase III of fasting is however markedly elevated above concentrations in phase II in emperor penguins (Robin et al., 1998), juvenile and adult king penguins (Le Ninan et al., 1988; Cherel et al., 1988b) and in Adelie penguins (Pygoscelis adeliae; Cockrem et al., 2006). Sartori et al., (1995) suggested, based on rates of body weight decline, that male Japanese quail have three phases of fasting, with phase I lasting 2-3 days. If this is the case, then our data are consistent with quail remaining in the first phase of fasting following 24 h food deprivation, with no elevation in plasma corticosterone. Plasma corticosterone concentrations however, can be significantly elevated above initial concentrations after 24 h of fasting in immature chickens (Scanes et al., 1980; Harvey et al., 1983) and small passerines (Astheimer et al., 1992). Furthermore, after 24 hour of fasting in pine siskins (Carduelis pinus), the

lightest and heaviest individuals had the highest and lowest corticosterone concentrations respectively (Astheimer et al., 1992). The HPA axis response of birds to food deprivation may depend on age and body condition, with increased corticosterone secretion after 24 h of fasting in immature chickens and small passerines consistent with entry of these birds into phase III of fasting. Indeed, emptying of the stomach would occur sooner in smaller birds, and smaller birds are also likely to have less fat reserves to maintain phase II of fasting. This suggestion is supported by studies investigating plasma corticosterone concentrations in chickens of different ages following 24 h of fasting. Freeman et al., (1983) found little evidence of increased corticosterone secretion during a 24 h fast in 16-week-old chickens weighing 1.0 to 1.5 kg, whereas three-week-old chickens weighing 0.15 to 0.20 kg had significantly increased plasma corticosterone 4 h after food withdrawal and maintained this elevation until the end of a 24 h fast (Freeman et al., 1980).

Mean food intake in the three hours following the 24 h fast was significantly greater in all four groups of quail treated with corticosterone than in untreated controls. Quail in the highest treatment group consumed almost 1.7 times the amount of food than control birds in three hours. Furthermore, quail in the highest corticosterone treatment group consumed significantly more food in the three hours following the fast than quail in each of the three lowest treatment groups, supporting the findings of previous studies of dose-dependent effects of corticosterone in birds (see Hayashi et al., 1994; Breuner et al., 1998). Previous studies of food intake in otherwise unmanipulated Japanese quail following treatment with corticosterone have given mixed results. Food intake in adult quail given corticosterone orally, was less but not significantly different from food intake in untreated controls, when measured over seven days of treatment (De la Cruz et al., 1981); there was no relationship between corticosterone dose and food intake over 24 h in immature quail given corticosterone in their drinking water (Hull et al., 2007); and single injections of corticosterone for seven days increased daily food consumption in immature male quail (Bray, 1993). It may be that effects of corticosterone on food intake in quail are more pronounced after fasting and less apparent in quail with free access to food. The results presented here are consistent with the findings of a study in white-crowned sparrows (Astheimer et al., 1992), which is the only other report to measure food intake immediately after a 24 h fast in birds treated with corticosterone.

The actions of exogenous corticosterone on food intake after fasting in corticosterone-treated birds may be mediated by neuropeptide Y (NPY). NPY is a neurotransmitter that stimulates food intake in birds (Kuenzel et al., 1987; Richardson et al., 1995; Denbow, 1999; Furuse, 2002), and fasting for 24 to 48 h can increase the activity of neurons expressing NPY mRNA in the hypothalamus of adult Japanese quail (Boswell et al., 2002) and can increase hypothalamic NPY content in chickens (Zhou et al., 2005). Moreover, studies in rats (Rattus norvegicus) report that corticosterone can potentiate NPY gene expression (Akabayashi et al., 1994) and elevate NPY-induced feeding (Stanley et al., 1989). A fasting-induced increase in NPY might have stimulated food intake in both control and treated birds when food was returned following the fast, with this effect heightened in birds with experimentally elevated plasma corticosterone concentrations. Corticotropin-releasing factor (CRF) is a powerful anorexigenic peptide in birds (Denbow et al., 1999; Richardson et al., 2000) and is subject to negative feedback by corticosterone. Increased food intake following the fast in birds with elevated corticosterone could also be due to suppression of CRF release.

Plasma corticosterone responses to handling followed by confinement in quail treated with corticosterone in their drinking water were measured in a previous study in our laboratory (Hull et al., 2007). Corticosterone responses in some treated birds were reduced compared to controls, suggesting that corticosterone treatment may disrupt the responses of birds to a handling stressor. In the present study, however, corticosterone in control birds and in three out of four groups of birds treated with corticosterone did not change after 24 h of fasting, so no conclusions can be drawn regarding how elevated plasma corticosterone concentrations affect corticosterone responses of birds to subsequent stressors. In contrast, the effects of corticosterone on food intake in quail give important insights into the role of glucocorticoids in helping birds cope with changes in their natural environments. Free-living birds may experience unpredictable periods of food deprivation following heavy snowfalls or during severe storms, and it has been suggested that corticosterone secretion in response to these events will promote behavioural and physiological changes that are needed for survival (Wingfield and Ramenofsky, 1997). Indeed, plasma corticosterone concentrations in ground-feeding dark-eyed juncos (Junco hyemalis) were significantly higher during heavy snowfall than before or after heavy snowfall (Rogers et al., 1993), and common diving

petrels (Pelecanoides urinatrix) had higher plasma corticosterone concentrations during a severe storm than in calmer weather before a storm (Smith et al., 1994). Furthermore, body weight in petrels captured during the storm was lower than in petrels captured in calmer weather, indicating that birds in the storm probably had reduced food consumption. Increased foraging activity and feeding (if food is located) would be highly adaptive following heavy snowfalls or severe storms (Astheimer et al., 1992), and the results of the present study support the notion that elevated plasma corticosterone concentrations, rather than the experience of fasting per se, can increase feeding behaviour in birds following unpredictable periods of food deprivation. Indeed, increased food intake should result in greater energy reserves in the form of fat depots, which could potentially increase the likelihood of survival should severe conditions persist (Rogers, et al., 1993).

In conclusion, plasma corticosterone in control and treated quail did not increase after 24 h of fasting, and the adult quail were most likely in phase I of fasting. Even though there was no increase in endogenous corticosterone secretion during the fast in quail, birds receiving exogenous corticosterone consumed more food in the three hours following the fast than controls. The results indicate that corticosterone may stimulate food intake in birds under certain conditions, such as when birds have access to food immediately after a period of food deprivation.

# 5. Plasma corticosterone responses to emotional stressors in Japanese quail

## 5.1 Introduction

Stressors are stimuli that generate stress responses in vertebrates. Stress responses involve activation of the hypothalamo-pituitary-adrenal (HPA) axis and the secretion of glucocorticoids (Selye, 1974). Stimuli that pose physical challenges to animals and initiate stress responses through disturbance of physical and chemical tissue parameters are called physical stressors, whilst stimuli that are perceived as a threat following comparison with innate predispositions or previous experience and initiate stress responses are called emotional stressors (Day et al., 1999). Potential physical stressors include immune challenge, haemorrhage and treatment with insulin, whilst emotional stressors can include handling by a human, restraint, novel environments and the sight of a predator (Dayas et al., 2001; Herman et al., 2003). Corticosterone is the major glucocorticoid in birds (Carsia and Harvey, 2000), and increased plasma corticosterone concentrations have been measured in some free-living birds following exposure to natural stressors, such as inclement weather (Wingfield et al., 1983; Smith et al., 1994) or the sight of a predator (Cockrem and Silverin, 2002a). In order to characterise and then compare corticosterone responses among individuals or groups of birds however, birds are normally subjected to a standardised emotional stressor. The standardised emotional stressor most widely used with free-living species is capture followed by restraint (Wingfield, 1994; Silverin, 1998), whilst standardised emotional stressors commonly used with domesticated birds are mechanical restraint (Jones and Satterlee, 1996) or repeated handling followed by confinement (Fraisse and Cockrem, 2006).

Short-term stress responses that involve increased secretion of glucocorticoids for minutes to hours are commonly referred to as acute glucocorticoid stress responses. The acute stress response is considered to be adaptive, with increased secretion of glucocorticoids promoting physiological and behavioural changes needed for survival (Wingfield, 1994; Wingfield et al., 1998; Breuner et al., 2008). There are marked differences between individual birds in their acute corticosterone stress responses to the

same emotional stressor, and there is recent interest in the functional significance of this variation (Cockrem, 2007; Williams, 2008; Cockrem et al. 2009). It has been suggested that birds with relatively low corticosterone responses might have greater fitness (survival and reproduction) in constant environments, whilst birds with higher corticosterone responses might have greater fitness in changing environments (Cockrem, 2005). The great majority of studies of stress in birds however, have measured corticosterone responses of individuals to just one type of emotional stressor and only on one occasion. Measuring corticosterone responses of birds to stressors on single occasions really only provides a "snapshot" of HPA axis activity, and gives no indication of whether or not these measurements truly reflect the phenotype of the sampled individuals (Williams, 2008). Whilst studies in both domesticated and free-living species of birds report that plasma corticosterone responses to the same emotional stressor are generally repeatable when measured on two (Wada et al., 2008; Angelier et al., 2009), three (Cockrem and Silverin, 2002b; Cockrem et al., 2009) or four occasions (Littin and Cockrem, 2001), it is unknown if corticosterone responses of individual birds to similar emotional stressors are related. Do individual birds consistently show relatively low or high plasma corticosterone responses to several similar emotional stressors? The present study in Japanese quail (Coturnix coturnix japonica) was conducted to address this question. Individual quail were subjected to three emotional stressors and plasma corticosterone responses were measured. The stressors used were 15 min handling followed by 45 min confinement in a cardboard box; 30 min handling followed by 30 min confinement in a cardboard box; and 60 min handling. Birds were handled for 15 min on three occasions, so the repeatability of corticosterone concentrations at 0 and 15 min, and the effects of handling occasion on corticosterone concentrations at 15 min could be determined. The present study was designed to provide important new information on the functional significance of individual variation in corticosterone responses in birds.

## 5.2 Materials and methods

### 5.2.1 Animals and husbandry

Wild-type male Japanese quail were purchased at five weeks of age from our usual supplier (Canter Valley Farm, Christchurch). Quail were given seven weeks to acclimatise to housing conditions and were 12 weeks of age when the experiment

began. Please refer to section 2.2.1 for further details on animals and husbandry.

### 5.2.2 Corticosterone responses to handling and confinement

Plasma corticosterone responses to handling and confinement were measured as previously described in quail (Hull et al., 2007) and chickens (Gallus domesticus; Littin and Cockrem, 2001; Fraisse and Cockrem, 2006). Fourteen quail were handled for 15, 30 and 60 min on separate occasions, with the order of handling time randomised for each bird. The order of handling of birds on each occasion was randomised. The second handling occasion was 14 days after the first occasion, and the third handling occasion was 12 days later. Birds were subjected to stressors between 0900 and 1200 h.

Birds were removed from their cages and an initial blood sample (0 min) was collected. Please refer to section 2.2.5 for further details on blood sample collection. Each bird was put into a cardboard box and then handled for 15, 30, or 60 min (gently lifted up, turned upside down, then replaced in the box). Each bird was picked up and put down at intervals of 15 s every 75 s throughout the designated handling time. Birds were normally picked up and put down six times during each 15 s handling interval. Birds were left individually in their cardboard boxes at the end of the handling time. Second blood samples were taken at 15 min, and then birds were placed back in the cardboard boxes. Subsequent samples were taken at 30 and 60 min, and birds were then returned to their cages.

### 5.2.3 Corticosterone radioimmunoassay

Please refer to section 2.2.7 for details on corticosterone radioimmunoassay.

### 5.2.4 Statistics

Statistical analyses were performed using Systat (Systat Software). Plasma corticosterone concentrations were transformed to logarithms, and Levene's tests confirmed homogeneity of variances for these data. Changes in plasma corticosterone concentrations were compared between handling times (15, 30 and 60 min) and handling occasions ($1^{st}$, $2^{nd}$ and $3^{rd}$) using repeated measures ANOVA with time (0, 15, 30 and 60 min), handling time and handling occasion as the grouping factors. Post hoc comparisons were made between times for each handling time, between handling times for each time and between handling occasions for 0 and 15 min using univariate F tests.

All quail were handled for 15 min on three occasions, and statistical repeatabilities for 0 and 15 min corticosterone concentrations were calculated by the method of Lessells and Boag (1987). The total areas under plasma corticosterone response curves (AUCs) were determined in GraphPad Prism (GraphPad Software Inc) using the trapezoid rule (Cockrem and Silverin, 2002b). Total AUCs were calculated separately for plasma corticosterone responses to 15 min handling followed by 45 min confinement, 30 min handling followed by 30 min confinement, and to 60 min handling. Birds were then ranked on their total AUCs for each stressor, where low and high ranks indicate relatively low and high total AUCs respectively. Relationships between ranks of total AUC calculated for each stressor were determined with Spearman correlations. Data are presented as individual points or as mean ± standard error (S.E.).

## 5.3 Results

### 5.3.1 Plasma corticosterone responses to handling and confinement

There were marked individual differences between birds in their corticosterone responses to handling and confinement (see Fig. 5.1 for individual corticosterone responses). The 14 birds were handled for 15 min on three occasions, then handling either ended, continued for 30 min in total or continued for 60 min in total. Corticosterone concentrations increased between 0 and 15 min in the great majority (80 %) of events when birds were handled during this period. Corticosterone declined between 15 and 30 min in 13 of the birds handled for 15 min, then continued to decline in nine birds, remained at similar concentrations in three birds, and increased between 30 and 60 min in two birds. When quail were handled for 30 min corticosterone continued to rise between 15 and 30 min in six of the birds, whilst corticosterone concentrations did not change markedly in another six birds and decreased in two birds (Fig. 5.1). Individual birds that had been handled for 30 min showed similar corticosterone patterns between 30 and 60 min compared with birds handled for 15 min, with corticosterone declining in eight birds, remaining relatively constant in two birds and increasing in four birds. Quail handled for 60 min showed a range of corticosterone responses. Corticosterone rose during the first 30 min in four birds, with little or no change between 15 and 30 min in seven birds and a decrease between 15 and 30 min in three birds. Corticosterone between 30 and 60 min fell in two birds, remained similar in five birds and increased in seven birds.

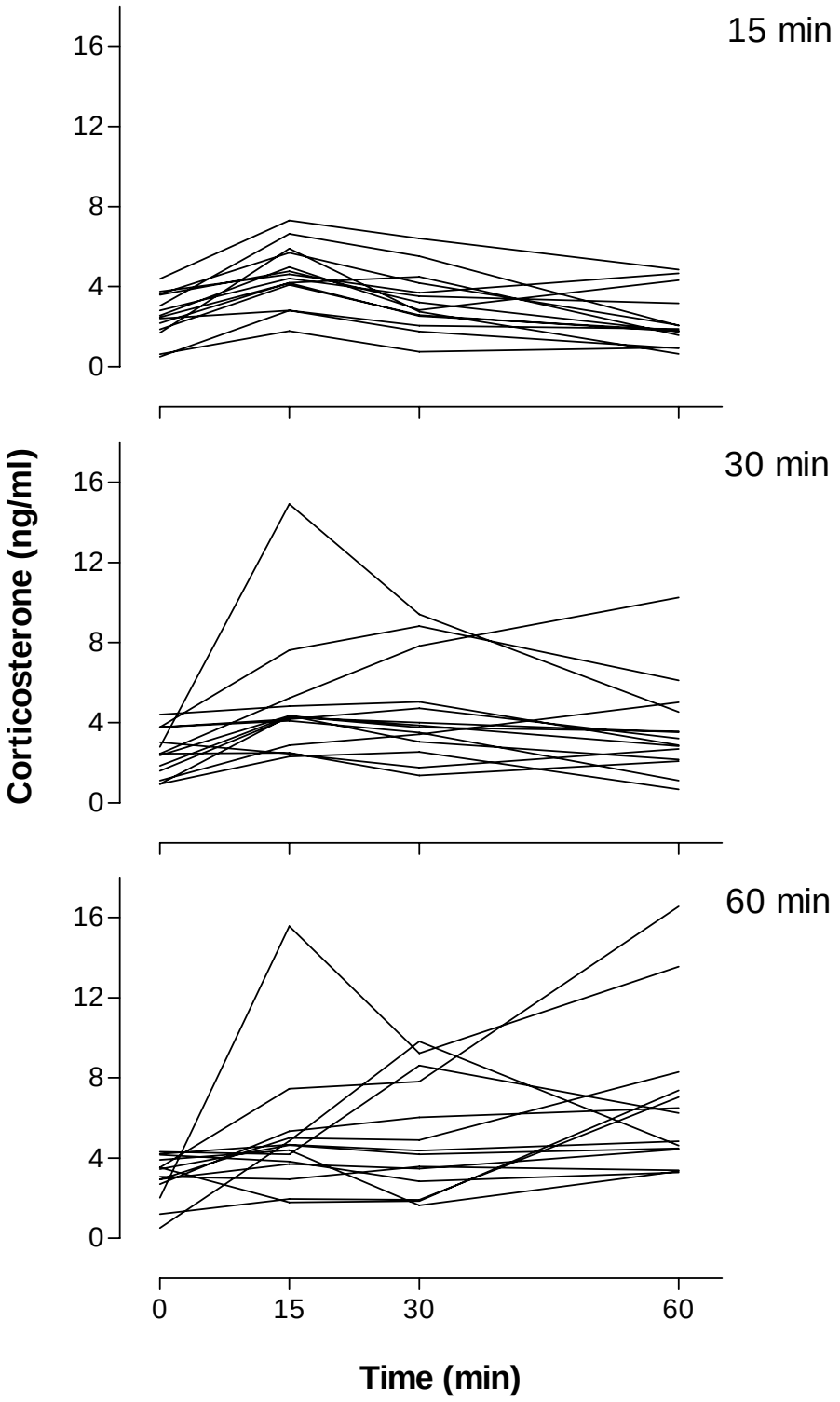


**Figure 5.1.** Individual plasma corticosterone responses to three emotional stressors in quail. 15 min, 15 min handling followed by 45 min confinement in a box; 30 min, 30 min handling followed by 30 min confinement in a box; 60 min, 60 min handling. N = 14 per group.

There were significant effects of time and handling time on mean corticosterone concentrations, but no significant effect of handling occasion (three way repeated measures ANOVA $F_{3,99} = 11.740$, $p < 0.001$; $F_{2,33} = 3.717$, $p = 0.035$; $F_{2,33} = 0.129$, $p = 0.880$; see Appendix Table 5.1 for further statistics). Mean corticosterone increased between 0 and 15 min in birds handled for a total of 15, 30 or 60 min ($F_{1,33} > 5.220$, $p < 0.030$; Fig. 5.2). Corticosterone decreased between 15 and 30 min and between 30 and 60 min in birds handled for 15 min ($F_{1,33} = 17.298$, $p < 0.001$ and $F_{1,33} = 5.836$, $p = 0.021$). Corticosterone concentrations in birds handled for 30 min did not change significantly between 15 and 30 min, or between 30 and 60 min ($F_{1,33} = 1.176$, $p = 0.286$ and $F_{1,33} = 2.131$, $p = 0.154$). Birds handled for 60 min had similar mean corticosterone concentrations at 15 and 30 min, whereas corticosterone tended to increase between 30 and 60 min ($F_{1,33} = 0.271$, $p = 0.606$ and $F_{1,33} = 3.234$, $p = 0.081$). There were no significant differences in corticosterone concentrations at 0, 15 and 30 min between quail handled for different periods ($F_{1,33} < 2.800$, $p > 0.100$), whereas at 60 min corticosterone was higher in birds handled for 60 min than for 15 or 30 min ($F_{1,33} = 19.045$, $p < 0.001$ and $F_{1,33} = 6.045$, $p = 0.019$). Mean corticosterone concentrations at 60 min were higher in birds handled for 30 min than in birds handled for 15 min, although this difference was marginally significant ($F_{1,33} = 3.976$, $p = 0.054$).

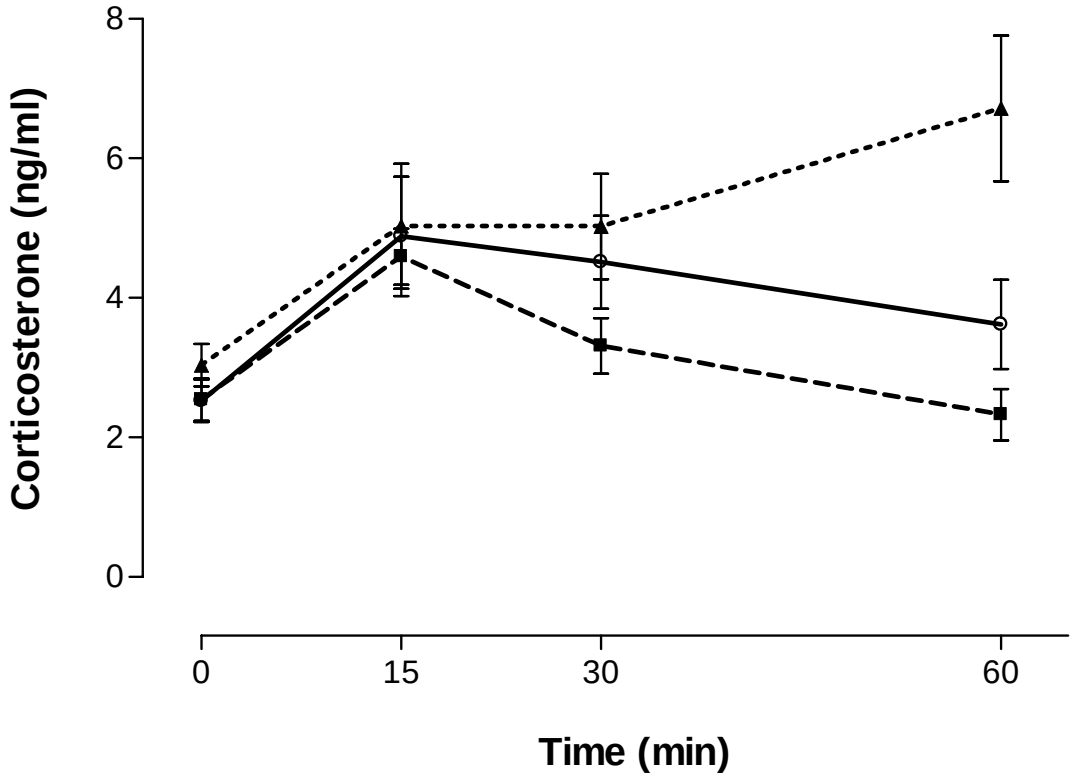


**Figure 5.2.** Plasma corticosterone responses to three emotional stressors in quail. 15 min handling followed by 45 min confinement in a box, - - - ■ - - -; 30 min handling followed by 30 min confinement in a box, —○—; 60 min handling, ····▲····. Data are presented as mean ± S.E.; N = 14 per group.

### 5.3.2 Effects of handling occasion on corticosterone concentrations

All quail were handled for 15 min on three occasions, so the effects of repeated handling on corticosterone concentrations at 0 and 15 min could be examined. Mean corticosterone in the first samples at 0 min gradually increased from 2.39 $\pm$ 0.28 to 2.98 $\pm$ 0.31 ng/ml over the three handling occasions (see Fig. 5.3), but there were no significant differences between handling occasions in corticosterone at 0 or 15 min ($F_{1,33} < 1.000$, $p > 0.340$ and $F_{1,33} < 1.000$, $p \geq 0.370$; see Appendix Table 5.1 for further statistics).

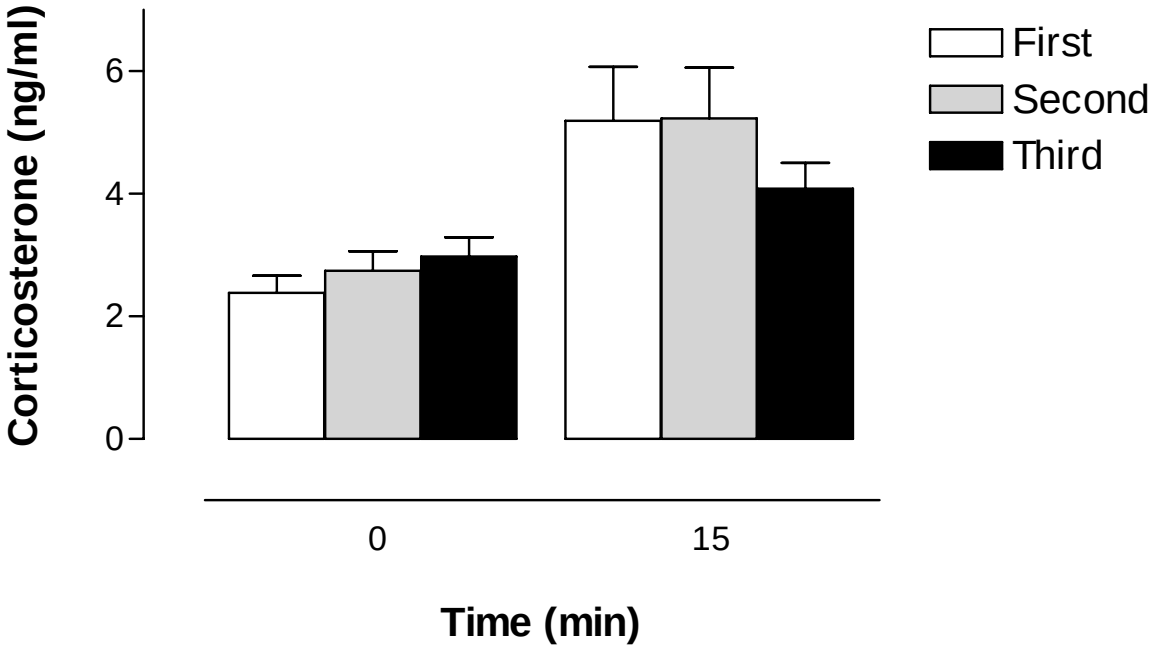


**Figure 5.3.** Plasma corticosterone concentrations at 0 and 15 min in quail handled for 15 min on three occasions (first, second, third). Data are presented as mean ± S.E.; N = 14 per group.

### 5.3.3 Repeatability of corticosterone concentrations

Corticosterone concentrations at 0 min were repeatable on the three sampling occasions (statistical repeatability $r = 0.492 \pm 0.157$, $p < 0.05$). Corticosterone at 15 min was not repeatable when all birds were considered together ($r = 0.030 \pm 0.161$, $p > 0.05$), but if two birds that had single outlying corticosterone concentrations >14 ng/ml are omitted then corticosterone concentrations were repeatable at 15 min ($r = 0.344 \pm 0.187$, $p < 0.05$).

### 5.3.4 Plasma corticosterone responses to handling and confinement in individual birds

There was a significant relationship between responses to 15 min handling followed by 45 min confinement, and responses to 60 min handling in quail (ranks of total area under the plasma corticosterone response curve; $r = 0.609$, $p = 0.021$; Fig. 5.4b). If all birds are included in the analyses, then there were no correlations between responses to 15 min handling followed by 45 min confinement, and 30 min handling followed by 30 min confinement, or between responses to 30 min handling followed by 30 min confinement, and 60 min handling (ranks of total area under the plasma corticosterone response curve; $r = 0.301$, $p = 0.296$; $r = 0.424$, $p = 0.131$; Fig. 5.4a and c). However, one bird had a spurious plasma corticosterone concentration of 14.9 ng/ml at 15 min on the second handling occasion, compared with 1.8 and 2.0 ng/ml at 15 min on the first and third handling occasions respectively. This bird was ranked 1$^{st}$, 14$^{th}$ and 2$^{nd}$ for total area under the plasma corticosterone response curve for the three stressors respectively. If this bird is removed from the analyses, then there were significant positive relationships between responses to 15 handling followed by 45 min confinement, and 30 min handling followed by 30 min confinement, and between responses to 30 min handling followed by 30 min confinement, and 60 min handling (ranks of total area under the plasma corticosterone response curve; $r = 0.626$, $p = 0.022$; $r = 0.731$, $p = 0.005$).

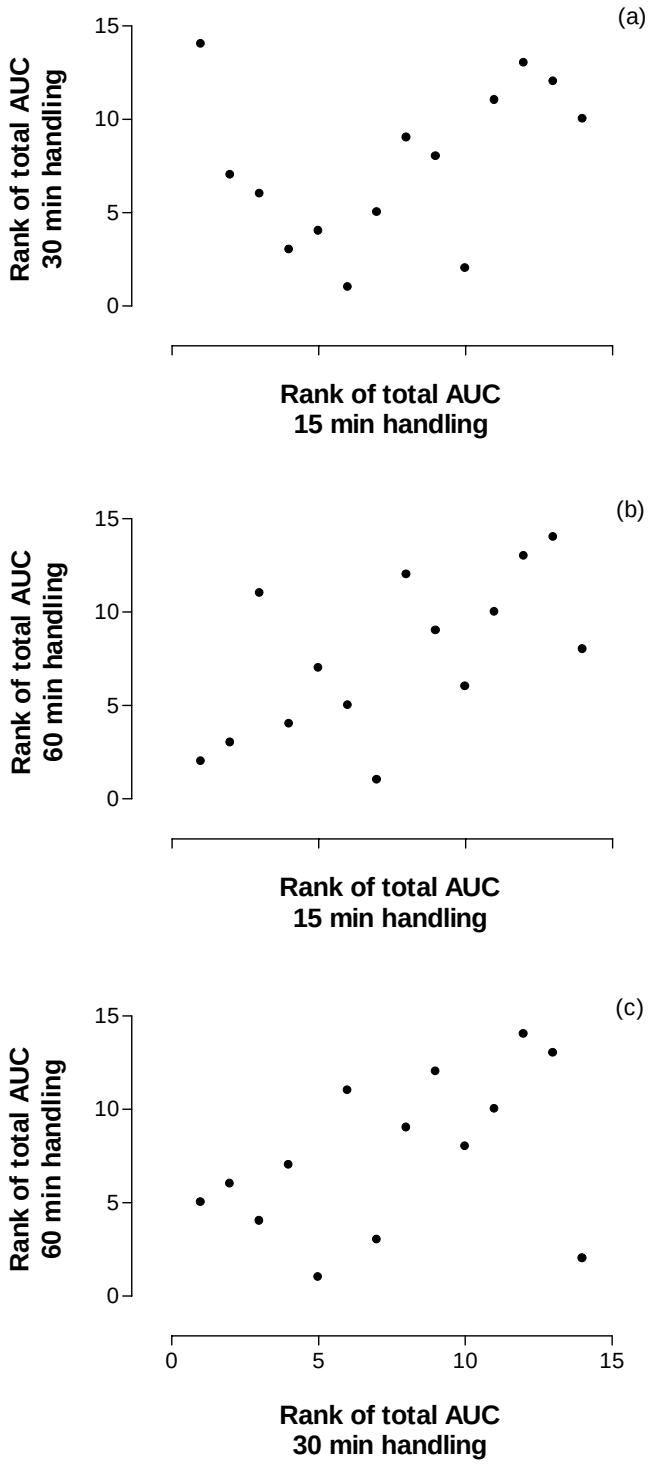


**Figure 5.4.** Correlations between ranks of total area under the plasma corticosterone response curve (AUC) in quail (a) handled for 15 min then confined in a box for 45 min or handled for 30 min then confined in a box for 30 min, (b) handled for 15 min then confined in a box for 45 min or handled for 60 min or (c) handled for 30 min then confined in a box for 30 min or handled for 60 min. Areas under the curve are in ng/ml.min. See section 5.3.4 for Spearman statistics. N = 14.

## 5.4 Discussion

This is the first study to measure plasma corticosterone responses of individual birds to three similar emotional stressors. There were significant positive relationships between the magnitudes of plasma corticosterone responses to 15, 30 or 60 min handling followed by 45, 30 or 0 min confinement respectively in individual Japanese quail. In general, quail with relatively low or high plasma corticosterone responses to 15 min handing also had relatively low or high responses to 30 or 60 min handling. These findings show for the first time the existence of consistent individual differences in the responses of birds to several similar emotional stressors.

The mean initial corticosterone concentration in quail was 2.7 ± 0.2 ng/ml for the 42 handling events, and was similar to that reported in a previous study of quail in our laboratory (Hull et al., 2007). Quail displayed a typical response to 15 min of handling followed by 45 min confinement, with an increase in mean corticosterone at 15 min, and a return to initial concentrations at 30 and 60 min. A mean corticosterone concentration at 15 min of 4.8 ± 0.4 ng/ml for the 42 handling events was consistent with a previous report in mixed-sex quail from the same stock (Hull et al., 2007). In the present study corticosterone did not increase significantly after 15 min in quail handled for 30 or 60 min. Plasma corticosterone responses to handling can be higher in domesticated chickens. Brown Hyline and white Leghorn laying hens for example, had mean corticosterone concentrations after handling for 15 min of approximately 8 and 12 ng/ml respectively (Littin and Cockrem, 2001; Fraisse and Cockrem, 2006). Responsiveness of the HPA axis in quail can also be determined by the measurement of plasma corticosterone responses to mechanical restraint in a crush-cage. Concentrations of corticosterone following restraint have been measured in lines of quail selected on the basis of low (low stress) or high (high stress) corticosterone responses to brief mechanical restraint itself, or short or long tonic immobility responses. Mean corticosterone concentrations following immobilisation were greater in quail of a high stress than a low stress line (Satterlee and Johnson, 1988), and greater in quail of a short tonic immobility response line than a long tonic immobility response line (Hazard et al., 2008). Mean corticosterone after 4-10 min of restraint is typically higher in divergent lines and unselected control lines of both populations, than in quail handled for up to 60 min in the present study (Satterlee and Johnson, 1988; Jones et al., 1994a; Jones et al.,

1994b; Jones and Satterlee, 1996; Odeh et al., 2003; Hazard et al., 2005; Hazard et al., 2008). The magnitude of the corticosterone response in quail therefore, appears to be dependent on the genetic history of the experimental birds and on the intensity of the stressor (repeated handling or continuous restraint).

Results of the present study show for the first time that plasma corticosterone responses of individual birds to similar emotional stressor are generally related. Handling for 15 min followed by confinement in a cardboard box for 45 min has been used in a study of corticosterone responses in Japanese quail (Hull et al., 2007), and responses to this stressor appear to reflect the responsiveness of the HPA axis of quail to 30 min handling followed by 30 min confinement, and to 60 min handling. Just one study has measured the responses of wild free-living birds to the same emotional stressor on three occasions (Cockrem et al., 2009), whilst other studies have measured corticosterone responses of birds on just one occasion. This novel study provides the first indication that measurement of corticosterone responses of birds to one emotional stressor may reflect the responsiveness of the HPA axis of birds to emotional stressors in general.

There were marked individual differences between birds in their corticosterone responses to the three emotional stressors used in the present study, with some quail showing relatively low responses to each stressor, and other quail showing relatively high responses. Whilst relatively low or high corticosterone responses may be advantageous to birds in constant or changing environments respectively, it has been suggested that there is no optimum corticosterone response for all conditions (Cockrem et al., 2009). Indeed, variation in the magnitudes of corticosterone responses in individuals within a species is likely to be advantageous in an evolutionary context. Consider a period of environmental change, for example, where individuals of a species of bird must respond successfully to a range of novel, and perhaps threatening stimuli in order to survive. If all individuals consistently expressed relatively low corticosterone responses to threatening events (i.e. emotional stressors) then the likelihood of a species persisting in a changing environment might be reduced. It is therefore adaptive for a species of bird to maintain individuals with the capacity to respond to a range of emotional stressors with consistently low or high corticosterone responses, and this capacity has been demonstrated for the first time in the present study. The current findings also have implications for the conservation of threatened species.

Identification of individual birds with consistently low corticosterone responses to emotional stressors may be advantageous in captive breeding programs, as these birds may be less susceptible to chronic stress. Chronic stress is a state in which plasma corticosterone concentrations can remain elevated above initial concentrations for days or even weeks, and plasma corticosterone concentrations can be negatively correlated with reproductive parameters in birds (Kitaysky et al., 2007; Schoech et al., 2009).

There was no significant effect of handling occasion on plasma corticosterone concentrations at 15 min in the present study, suggesting that quail did not become habituated to the handling stressor. Corticosterone responses to handling or capture followed by restraint have previously been measured on three or four occasions in chickens (Littin and Cockrem, 2001), great tits (Parus major; Cockrem and Silverin, 2002b) and Adelie penguins (Pygoscelis adeliae; Cockrem et al., 2009). Chickens had greater mean corticosterone concentrations after 15 min handling on the first occasion compared with subsequent occasions on which corticosterone concentrations were similar (Littin and Cockrem, 2001), and corticosterone concentrations at 30 min were similar on three occasions in great tits (Cockrem and Silverin, 2002b) and Adelie penguins (Cockrem et al., 2009). The present results from quail are consistent with these other reports of consistent corticosterone responses in birds subjected to handling or capture and restraint on more than one occasion. Indeed, a lack of habituation to an emotional stressor may be advantageous for birds in their natural environments. Just because a bird has experienced a particular stressor, for example, does not necessarily mean that subsequent exposure to the same stressor will be any less threatening, and consistently mounting robust corticosterone responses to stressors can promote physiological and behavioural changes that are needed for survival. A rapid return of plasma corticosterone to initial concentrations once exposure to a stressor ends, however, is essential to prevent the potentially deleterious consequences of glucocorticoid excess.

Quail in the present study were maintained in constant housing conditions and subjected to each handling stressor between 0900 and 1200 h. Concentrations of corticosterone in blood samples collected from quail immediately after removal from their cages were repeatable for the three handling occasions. Initial corticosterone concentrations in individual birds may vary however, depending on whether successive samples are

collected during the day or night, during different photoperiods, or during alternative life-history stages (Romero and Reed, 2008). Corticosterone concentrations in quail at 15 min were only repeatable if two birds that had single outlying corticosterone concentrations above 14 ng/ml were omitted. Maximum corticosterone concentrations during capture and restraint for 30 min were repeatable in female zebra finches (Taeniopygia guttata) between 16 days and three months of age (Wada et al., 2008).

In conclusion, corticosterone responses of Japanese quail were longer but not higher in birds handled for 30 min then confined for 30 min, and in birds handled for 60 min, compared with quail handled for 15 min then confined for 45 min. In general, the magnitudes of plasma corticosterone responses of individual quail to the three emotional stressors used in the present study were related. This is the first study in birds to demonstrate that the responsiveness of the HPA axis of birds to one emotional stressor may reflect the responsiveness of the HPA axis of birds to emotional stressors in general.

# 6. Plasma corticosterone responses to injection of insulin or lipopolysaccharide in Japanese quail

## 6.1 Introduction

The hypothalamo-pituitary-adrenal (HPA) axis is activated when animals respond to stimuli called stressors, with corticosterone the primary glucocorticoid secreted by the adrenal glands in birds (Selye, 1974; Carsia and Harvey, 2000). Stimuli that pose physical challenges to animals and initiate stress responses through disturbance of physical and chemical tissue parameters are called physical stressors, whilst stimuli that are perceived as a threat following comparison with innate predispositions or previous experience and initiate stress responses are called emotional stressors (Day et al., 1999). Physical stressors are also called physiological, homeostatic or systemic stressors, and emotional stressors may also be referred to as psychological, processive or neurogenic stressors (Reyes et al., 2003). Japanese quail (Coturnix coturnix japonica) have plasma corticosterone responses to emotional stressors such as social isolation (Mills et al., 1993), mechanical restraint (Jones and Satterlee, 1996), weighing or induction of tonic immobility (Jones et al., 2005), repeated handling (Hull et al., 2007), and presentation of a novel object in the home cage (Richard et al., 2008). No report in quail however, has discussed physical stressors per se, although treatment of quail with prostaglandin $E_2$ ($PGE_2$) did elevate plasma corticosterone concentrations (Satterlee et al., 1989).

Two potential physical stressors in birds are treatment with insulin and treatment with the endotoxin lipopolysaccharide (LPS). Insulin is a hormone released from pancreatic β-cells (Braun and Sweazea, 2008) and insulin can induce hypoglycaemia in chickens (Gallus domesticus; Akiba et al., 1999; Chida et al., 2000; Tokushima et al., 2003; Niezgoda et al., 2005), white-crowned sparrows (Zonotrichia leucophrys gambelii; Boswell et al., 1997) and mourning doves (Zenaidura macroura; Sweazea et al., 2006). Corticosterone is a glucocorticoid that can increase blood glucose concentrations in birds (Dong et al., 2007; Lin et al., 2007; Yuan et al., 2008), and there is one report in chickens of increased plasma corticosterone during an insulin-induced decrease in

plasma glucose (Niezgoda et al., 2005). Insulin-induced hypoglycaemia is often used to stimulate glucocorticoid responses in tests of HPA axis function in mammals (Suda et al., 1992; Sapolsky et al., 2000; Pacak and Palkovits, 2001). LPS forms part of the cell wall of Gram-negative bacteria, and infection with LPS invokes a series of behavioural, metabolic and endocrine changes known as the acute phase response (Koutsos and Klasing, 2001). Activation of the HPA axis typically occurs after treatment with LPS, and it is thought that cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) mediate this response (Beishuizen and Thijs, 2003). Corticosterone responses to injection of LPS have been shown in chickens (Johnson et al., 1993; Nakamura et al., 1998; Shini et al., 2008) and white-crowned sparrows (Owen-Ashley et al., 2006).

The present study was conducted to identify physical stressors in quail, with insulin and LPS treatments chosen as potential physical stressors. Whilst Japanese quail have been treated previously with insulin and LPS (Koutsos and Klasing, 2001; Macajova et al., 2003), effects of these treatments on corticosterone concentrations were not measured in these studies. Dose-response tests were therefore conducted, and plasma corticosterone was measured to determine if insulin and LPS can evoke corticosterone responses, and hence be classified as physical stressors in quail. Plasma glucose concentrations were also measured in samples collected during insulin dose-response tests to determine if concentrations decreased in quail following treatment with insulin. Three different doses of insulin and LPS were used, and blood samples were taken at three times after treatment, to determine suitable doses and blood sampling times to use in a study of plasma corticosterone responses to emotional and physical stressors in quail (see Chapter seven).

## 6.2 Materials and methods

### 6.2.1 Animals and husbandry

White female Japanese quail were purchased at five weeks of age from our usual supplier (Canter Valley Farm, Christchurch). Each bird was identified with a coloured, numbered leg band and housed in an individual cage measuring 35 x 40 x 24 cm (length x width x height). Quail were given four weeks to acclimatise to housing conditions

and were nine weeks of age when the experiment began. Please refer to section 2.2.1 for further details on animals and husbandry.

### 6.2.2 Experimental design

Twenty four quail were used in insulin dose-response tests and then left undisturbed for two weeks before use in LPS dose-response tests. Quail were randomly assigned to four groups of six birds on each occasion.

### 6.2.3 Blood sample collection

Please refer to section 2.2.5 for details on blood sample collection.

### 6.2.4 Injection preparation and administration

Solutions of human insulin (Sigma #I9278) and LPS from Escherichia coli (Sigma #L4005, serotype O55:B5) were prepared in 0.9 % sterile saline (Bomac). Injections were prepared at a volume of 200 µl the day before administration and stored at $4^0$C. Injections were delivered subcutaneously in the abdomen using a 1 ml syringe and a 27 g, ½″ needle.

### 6.2.5 Insulin and LPS dose-response tests

Insulin dose-response tests were conducted between 0900 and 1200 h. Birds were removed from their cages and an initial blood sample (-1.5 min) was taken. Birds then received an injection of 0.9 % saline (control), or 1, 2 or 4 IU/kg body weight of insulin (N = 6 per group) at 0 min, and were returned to their cages. Further blood samples were taken at 30, 60 and 150 min. LPS dose-response tests were conducted between 0800 and 1300 h. Birds were removed from their cages and an initial blood sample (-1.5 min) was taken. Birds then received an injection of 0.9 % saline (control), or 0.5, 1.0 or 2.0 mg/kg body weight of LPS (N = 6 per group) at 0 min, and were returned to their cages. Further blood samples were taken at 60, 120 and 240 min.

### 6.2.6 Corticosterone radioimmunoassay

Please refer to section 2.2.7 for details on corticosterone radioimmunoassay.

### 6.2.7 Plasma glucose

Concentrations of glucose were measured in undiluted plasma by enzymatic

colorimetric methods using Roche Diagnostics kits in a Hitachi 911 auto analyser. Glucose was only measured in samples for which there was sufficient plasma.

### 6.2.8 Statistics

Statistical analyses were performed using Systat (Systat Software). Plasma corticosterone concentrations were transformed to logarithms. Levene's tests to check for homogeneity of variances showed that parametric statistics could not be used. Instead, Kruskal-Wallis one way ANOVAs were used to compare corticosterone concentrations between groups for each time in the insulin and LPS dose-response tests, with pairwise comparisons between the control group and each insulin or LPS treatment group performed with Mann-Whitney U tests. Freidman's one way repeated measures ANOVAs were used to compare corticosterone concentrations between times for each group in insulin and LPS dose-response tests, with pairwise comparisons between times performed with Wilcoxon signed rank tests. Fifty three of the 96 samples collected during insulin dose-response tests had sufficient plasma for measurement of glucose concentrations, and there was sufficient plasma for measurement of glucose concentrations at all four times for just two of the 24 quail. No statistical analyses were performed on these data as N was $\leq 2$ for the control group at -1.5 min, the 1 IU/kg insulin group at -1.5 and 150 min, and the 4 IU/kg insulin group at 60 min. Data are presented as individual points or as mean ± standard error (S.E.).

## 6.3 Results

### 6.3.1 Insulin dose-response tests

#### 6.3.1.1 Plasma corticosterone

Initial plasma corticosterone concentrations at -1.5 min were <2 ng/ml in 22 of the 24 birds (see Fig. 6.1 for individual corticosterone responses). Corticosterone remained constant in control birds treated with saline, and showed little or no change between -1.5 and 30 min, and between 30 and 60 min, in most quail treated with insulin. There was a rise in plasma corticosterone from -1.5 to 30 min and from 30 to 60 min in one bird treated with 2 IU/kg of insulin. Corticosterone increased between 60 and 150 min in one of the six birds treated with either 1 or 2 IU/kg of insulin, and in three of the six birds treated with 4 IU/kg of insulin.

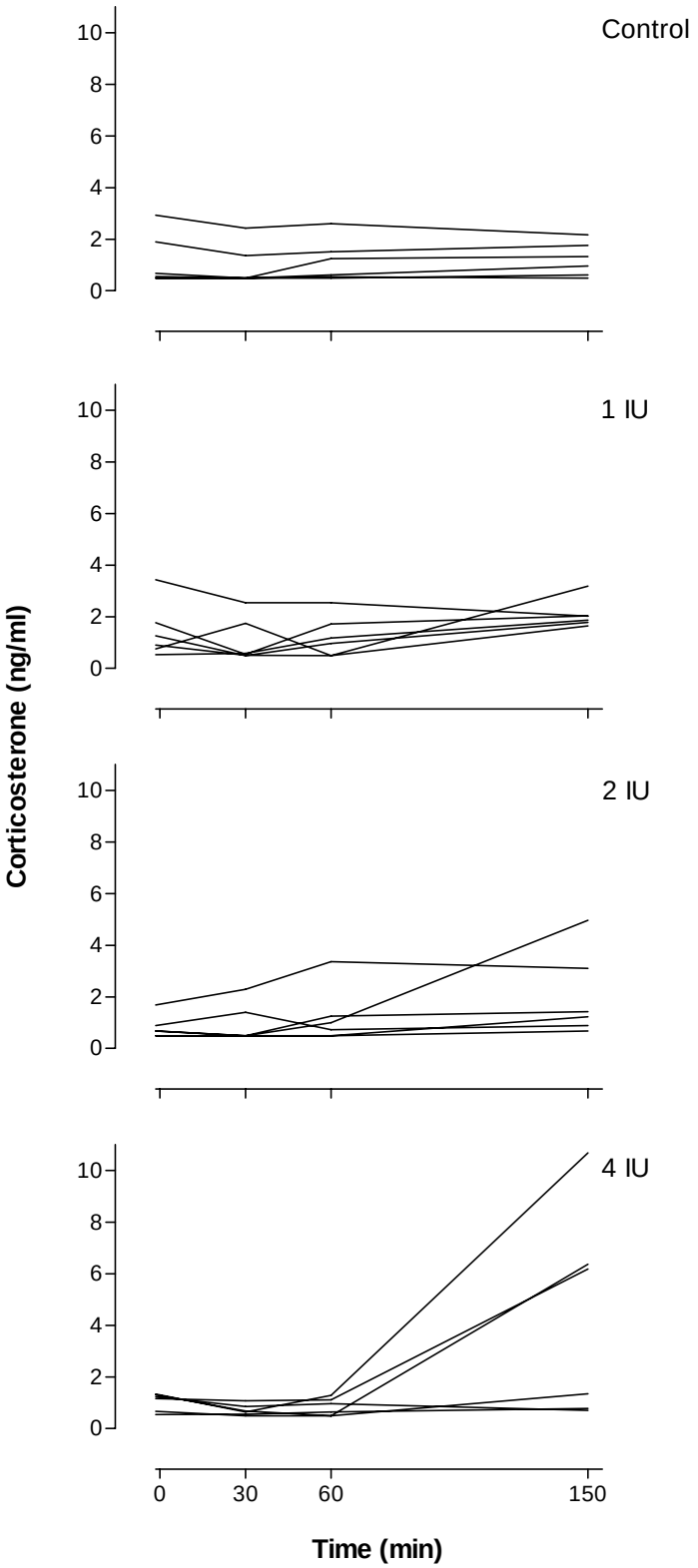


**Figure 6.1.** Individual plasma corticosterone responses in control quail and in quail treated with 1, 2 or 4 IU/kg of insulin. Initial blood samples were collected 1.5 min before injections were given at 0 min. N = 6 per group.

Mean plasma corticosterone concentrations did not differ between groups immediately before injection with saline or insulin (-1.5 min), or at any time after injection (Kruskal-Wallis one way ANOVA $K_3 \leq 4.013$, $p \geq 0.260$; Fig. 6.2, see Appendix Table 6.1 for further statistics). There were no differences in mean corticosterone between the control group and each of the insulin treatment groups at all times ($U \leq 22.000$, $p \geq 0.200$), although corticosterone in birds treated with 1 IU/kg of insulin was marginally higher than in controls at 150 min ($U = 6.000$, $p = 0.055$). Mean corticosterone concentrations in the control group and in the three insulin treatment groups ranged from 0.82 ± 1.82 to 1.45 ± 0.44 ng/ml at -1.5 min, 0.72 ± 0.09 to 1.07 ± 0.35 ng/ml at 30 min, 0.83 ± 0.14 to 1.23 ± 0.45 ng/ml at 60 min, and from 1.22 ± 0.27 to 4.35 ± 1.66 ng/ml at 150 min.

Mean corticosterone concentrations did not change with time in the control group or the two lowest insulin treatment groups (Friedman's one way repeated measures ANOVA $F_3 \leq 7.050$, $p \geq 0.070$; Fig. 6.2, see Appendix Table 6.2 for further statistics), whereas an effect of time was marginally significant in the highest insulin treatment group ($F_3 = 7.650$, $p = 0.054$). Mean corticosterone was significantly higher at 150 min than at -1.5 min in the group treated with 2 IU/kg of insulin (2.05 ± 0.68 vs 0.82 ± 0.18 ng/ml; $Z = 2.201$, $p = 0.028$), but mean corticosterone was not significantly elevated above initial concentrations at any time in the other treatment groups.

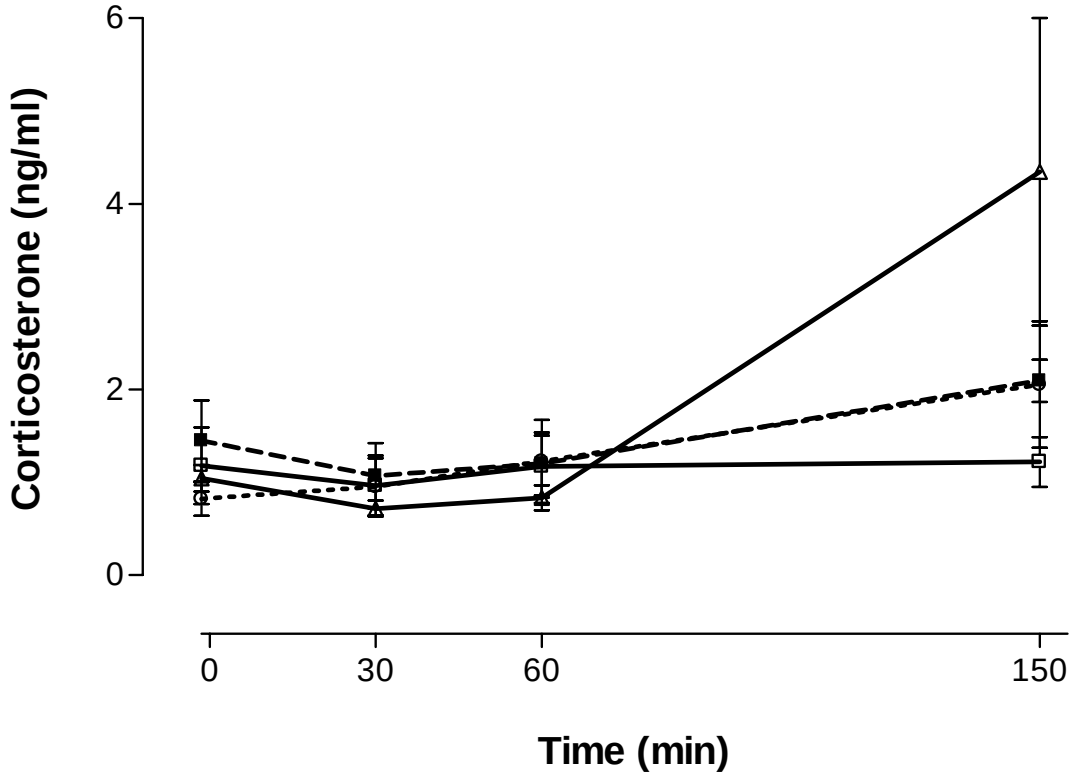


**Figure 6.2.** Plasma corticosterone responses in control quail and in quail treated with insulin. Control, —□—; 1 IU/kg insulin; - - - ■ - - -; 2 IU/kg insulin, ·····○·····; 4 IU/kg insulin, —Δ—. Initial blood samples were collected 1.5 min before injections were given at 0 min. Data are presented as mean ± S.E.; N = 6 per group.

#### 6.3.1.2 Plasma glucose

The three control birds with sufficient plasma for measurement of glucose at 150 min had plasma glucose concentrations of 17.60, 15.30 and 15.70 mmol/l, and plasma corticosterone concentrations at 150 min of 0.61, 0.97 and 2.17 ng/ml respectively. The bird in the 2 IU/kg insulin treatment group with a plasma corticosterone concentration at 150 min of 4.97 ng/ml had a plasma glucose concentration at 150 min of 5.00 mmol/l. The two birds in the 4 IU/kg insulin treatment group with plasma corticosterone concentrations at 150 min of 6.19 and 6.36 ng/ml had plasma glucose concentrations at 150 min of 4.40 and 5.10 mmol/l respectively. The bird in the 4 IU/kg insulin treatment group with a plasma corticosterone concentration at 150 min of 10.68 ng/ml had insufficient plasma for measurement of glucose concentration.

Mean plasma glucose concentrations remained relatively constant in control birds treated with saline, and ranged from 14.00 to 16.20 mmol/l at each time (see Fig. 6.3). Initial mean glucose concentrations at -1.5 min were >15.50 mmol/l in the three insulin treatment groups. Mean glucose then decreased between -1.5 and 30 min to 10.93, 9.70 and 7.74 mmol/l in the 1, 2 and 4 IU/kg insulin treatment groups respectively. Mean glucose increased steadily between 30 and 60 min and between 60 and 150 min in the lowest insulin treatment group, whereas mean glucose steadily decreased between these times in the two highest insulin treatment groups. Mean glucose concentrations at 150 min were 14.00, 8.02 and 5.58 mmol/l in the 1, 2 and 4 IU/kg insulin treatment groups respectively.

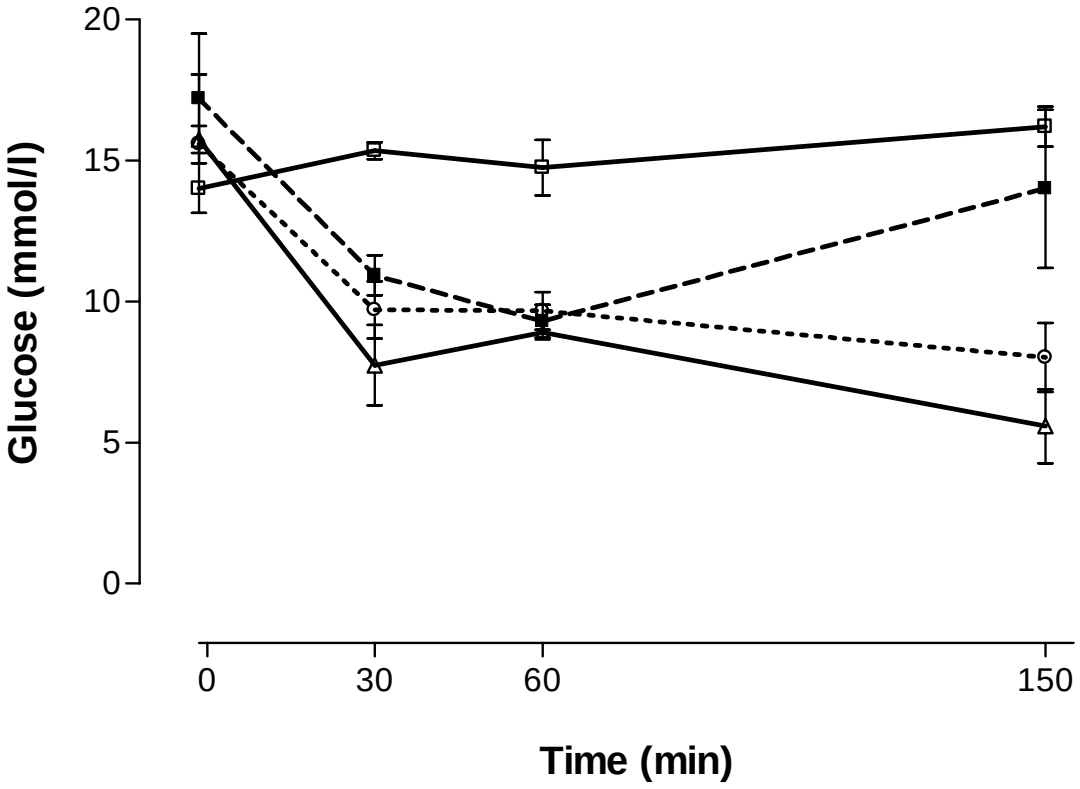


**Figure 6.3.** Plasma glucose responses in control quail and in quail treated with insulin. Control, —□—; 1 IU/kg insulin; - - - ■ - - -; 2 IU/kg insulin, ·····○·····; 4 IU/kg insulin, —Δ—. Initial blood samples were collected 1.5 min before injections were given at 0 min. Data are presented as mean ± S.E. Control: -1.5 min, N = 1; 30 min, N = 4; 60 min, N = 6; 150 min, N = 3; 1 IU/kg insulin: -1.5 min, N = 2; 30 min, N = 3; 60 min, N = 3; 150 min, N = 2; 2 IU/kg insulin: -1.5 min, N = 3; 30 min, N = 3; 60 min, N = 3; 150 min, N = 5; 4 IU/kg insulin: -1.5 min, N = 4; 30 min, N = 5; 60 min, N = 1; 150 min, N = 5.

### 6.3.2 LPS dose-response tests

Plasma corticosterone concentrations were <2 ng/ml in 21 of 24 birds at -1.5 min (see Fig. 6.4 for individual corticosterone responses). There were no changes in plasma corticosterone in control birds treated with saline, and corticosterone remained constant between -1.5 and 60 min in birds treated with 2 mg/kg of LPS. Corticosterone increased between 60 and 120 min in four of the six birds treated with 0.5 mg/kg LPS, and in five of the six birds treated with either 1.0 or 2.0 mg/kg LPS. Corticosterone then decreased or remained relatively constant from 120 to 240 min in 16 of 18 birds treated with LPS.

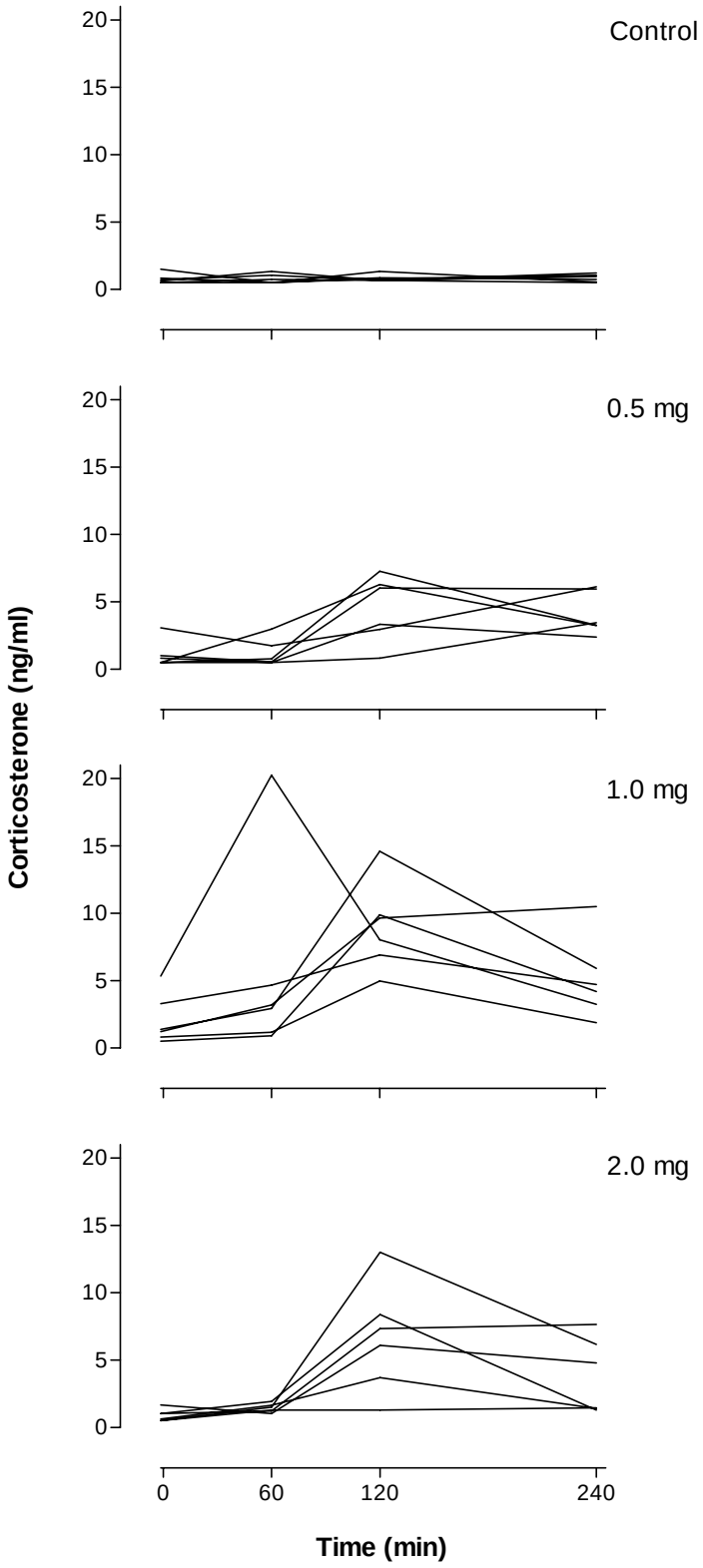


**Figure 6.4.** Individual plasma corticosterone responses in control quail and in quail treated with 0.5, 1.0 or 2.0 mg/kg of lipopolysaccharide (LPS). Initial blood samples were collected 1.5 min before injections were given at 0 min. N = 6 per group.

Mean corticosterone concentrations did not differ between groups immediately before injection with saline or LPS (Kruskal-Wallis one way ANOVA $K_3 = 3.259$, $p = 0.353$; Fig. 6.5, see Appendix Table 6.3 for further statistics), but differed between groups at all other times ($K_3 = 9.465$, $p = 0.024$; $K_3 = 14.940$, $p = 0.002$; $K_3 = 13.200$, $p = 0.004$). Mean corticosterone was greater than in the control group at 120 and 240 min in birds treated with 0.5 mg/kg of LPS ($U = 2.000$, $p = 0.010$; $U = 0.000$, $p = 0.004$), and at all times in birds treated with either 1.0 ($U = 3.000$, $p = 0.016$; $U = 0.000$, $p = 0.004$; $U = 0.000$, $p = 0.004$) or 2.0 mg/kg of LPS ($U = 3.500$, $p = 0.019$; $U = 1.000$, $p = 0.006$; $U = 0.000$, $p = 0.004$).

Mean corticosterone concentrations did not change with time in the control group (Friedman's one way repeated measures ANOVA $F_3 = 0.650$, $p = 0.885$, Fig. 6.5, see Appendix Table 6.4 for further statistics), but differed significantly between times in groups treated with LPS ($F_3 = 12.350$, $p = 0.006$; $F_3 = 11.800$, $p = 0.008$; $F_3 = 11.000$, $p = 0.012$). Corticosterone in birds treated with 0.5 mg/kg of LPS did not change between -1.5 and 60 min, increased to a maximum of 4.46 ± 1.01 ng/ml at 120 min, and remained relatively constant between 120 and 240 min (-1.5 vs 60 min, $F_3 = -0.405$, $p = 0.686$; 60 vs 120 min, $F_3 = 2.201$, $p = 0.028$; 120 vs 240 min, $F_3 = -0.524$, $p = 0.600$). Corticosterone in birds treated with 1.0 mg/kg of LPS increased from -1.5 to 60 min, reached a maximum of 9.01 ± 1.34 ng/ml at 120 min, then decreased from 120 to 240 min (-1.5 vs 60 min, $F_3 = 2.201$, $p = 0.028$; 60 vs 120 min, $F_3 = 0.943$, $p = 0.345$; 120 vs 240 min, $F_3 = -1.992$, $p = 0.046$). Corticosterone in birds treated with 2.0 mg/kg of LPS remained relatively constant from -1.5 to 60 min, increased to a maximum of 6.66 ± 1.65 ng/ml at 120 min, then decreased slightly but not significantly from 120 to 240 min (-1.5 vs 60 min, $F_3 = 1.782$, $p = 0.075$; 60 vs 120 min, $F_3 = 1.992$, $p = 0.046$; 120 vs 240 min, $F_3 = -1.572$, $p = 0.116$).

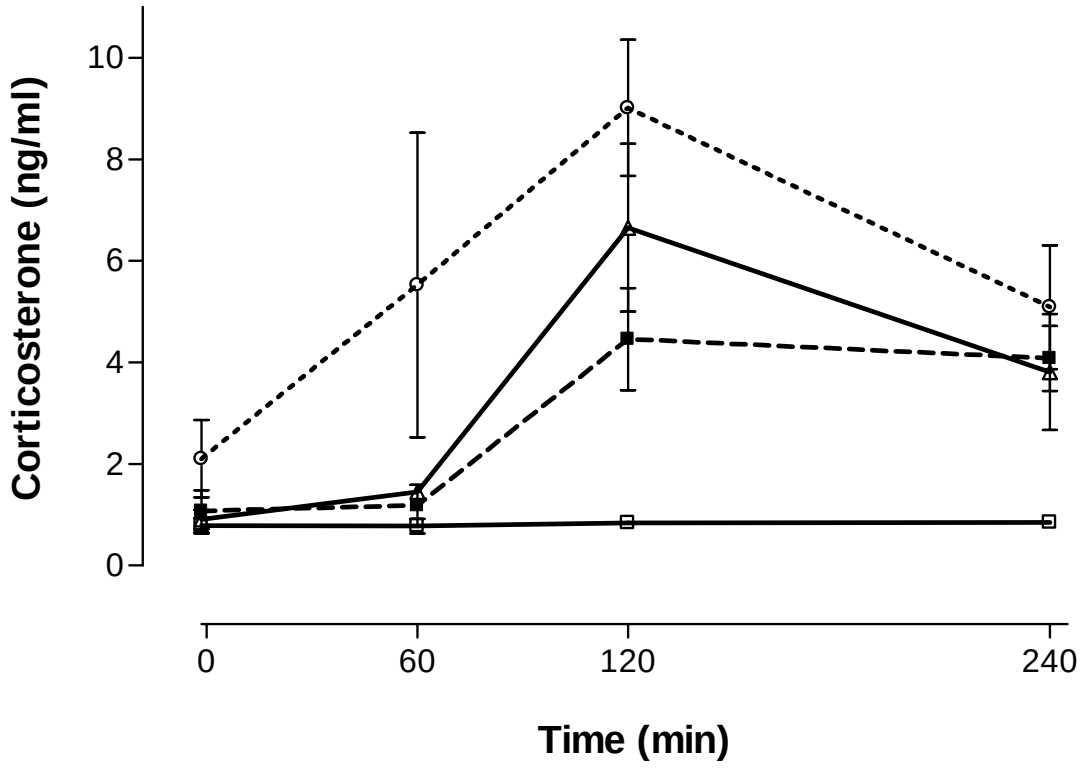


**Figure 6.5.** Plasma corticosterone responses in control quail and in quail treated with lipopolysaccharide (LPS). Control, —□—; 0.5 mg/kg LPS, - - - ■ - - -; 1.0 mg/kg LPS, ·····○·····; 2.0 mg/kg LPS, —Δ—. Initial blood samples were collected 1.5 min before injections were given at 0 min. Data are presented as mean ± S.E.; N = 6 per group.

## 6.4 Discussion

Treatment of Japanese quail with 4 IU/kg of insulin increased plasma corticosterone concentrations in three of the six birds, and treatment with 0.5, 1.0 or 2.0 mg/kg of LPS elevated corticosterone in at least five of the six birds receiving each dose. These findings show that treatment of quail with insulin or LPS can activate the HPA axis, and hence can be classified as physical stressors in quail.

This is the first report of physical stressors in Japanese quail. Corticotropin-releasing factor (CRF), arginine vasotocin (AVT), and adrenocorticotropic hormone (ACTH) are all components of the HPA axis, and treatment of quail with these hormones is not considered to represent treatment with a physical stressor. Also, although food and water deprivation can activate the HPA axis in quail (Scott et al., 1983), this treatment is not solely a physical stressor. Visual and metabolic stimuli associated with food and water deprivation can influence plasma corticosterone concentrations (Harvey et al., 1983; Harvey et al., 1985), so this stressor has both emotional and physical components.

Corticosterone and glucose concentrations have for the first time been measured together in plasma samples collected from Japanese quail following treatment with insulin. Insulin-induced hypoglycaemia can elevate plasma corticosterone concentrations in mammals (Robinson et al., 1992; Romero et al., 1993; Koenig and Cho, 2005) and in chickens. For example, chickens with a mean body weight of approximately 1 200 g were given intravenous injections of insulin at a dose of 4 IU/animal (Niezgoda et al., 2005). Plasma glucose decreased significantly from approximately 11 mmol/l at the time of injection to 7 mmol/l at 2 h, and was still lower than in controls at 6 h, whereas plasma corticosterone was significantly elevated at 2 h, reached a maximum concentration of around 9 ng/ml at 4 h, and was still higher than in controls at 6 h (Niezgoda et al., 2005). The results of the present study support the notion that elevated plasma corticosterone concentrations in quail treated with insulin resulted from hypoglycaemia. Whilst just 53 of the 96 samples collected during insulin dose-response tests had sufficient plasma for measurement of glucose concentrations, in many cases plasma glucose in quail treated with insulin was markedly lower than in untreated control quail. Plasma glucose concentrations were also noticeably reduced in treated quail that had markedly elevated plasma corticosterone concentrations.

Furthermore, mean glucose concentrations were markedly lower in all insulin treatment groups than in controls at 60 min, and lower in the two highest insulin treatment groups than in controls at 150 min. In general, however, plasma glucose concentrations in untreated Japanese quail appeared to be slightly higher than in untreated chickens (see Niezgoda et al., 2005).

The current findings show that treatment of quail with LPS can activate the HPA axis and increase the secretion of corticosterone, as has been shown in chickens. Intravenous treatment of male broilers with 1 mg/kg of LPS elevated plasma corticosterone to approximately 9 ng/ml after 2 h (Baert et al., 2005). Corticosterone concentrations in chickens treated with higher doses of LPS differed between studies. In eight week old male white Leghorns treated with 8 mg/kg of LPS, mean corticosterone was >100 ng/ml at 1 h, decreased to around 50 ng/ml at 6 h, and returned to similar concentrations to controls after 24 h (Gehad et al., 2002). Conversely, in brown Hyline chicks treated with 8 mg/kg of LPS, mean corticosterone concentrations were <8 ng/ml at 1 h, and around 6 ng/ml at 3 h, before returning to initial concentrations at 24 h (Shini et al., 2008). There is substantial evidence that activation of the HPA axis following treatment with the endotoxin LPS is mediated by cytokines (Turnbull and Rivier, 1999; Arkins et al., 2001; Beishuizen and Thijs, 2003). Studies in mammals show that different cytokines can act at more than one level of the HPA axis to increase glucocorticoid secretion. Indeed, the secretion of the cytokines IL-1, IL-6 and TNF-α occurred simultaneously with increased plasma corticosterone concentrations in chickens treated with LPS (Nakamura et al., 1998; Gehad et al., 2002; Ferdous et al., 2008).

This is the first report to demonstrate marked individual differences in the plasma corticosterone responses of birds to physical stressors. For example, three of the six quail treated with 4 IU/kg insulin had markedly elevated plasma corticosterone concentrations at 150 min, whilst plasma corticosterone in the other three quail in this group remained relatively constant after treatment with insulin. There was also considerable variation in the responses of individual birds to treatment with LPS, with quail showing relatively low or high corticosterone responses to treatment with all three doses. Whilst this is the first report of individual variation in corticosterone responses of birds to physical stressors, there is already considerable interest in the variation

between birds in their responses to emotional stressors. Studies that investigate individual variation in corticosterone responses typically measure responses of birds to standardised emotional stressors, such as capture followed by restraint in captive (Cockrem and Silverin, 2002b) or wild (Cockrem et al., 2009) free-living species, or handling followed by confinement in domesticated species (Littin and Cockrem, 2001). The functional significance of variation in corticosterone responses to emotional stressors has been examined (Blas et al., 2007; Cockrem, 2007; Williams, 2008; Breuner et al., 2008), but there are no data on how individual variation in corticosterone responses to physical stressors might relate to birds in an evolutionary context. Variation in corticosterone responses to emotional stressors may result from differences in the way a stressor is perceived, or from differences in the functioning of the HPA axis, and both genetics and previous experience may influence the response (Satterlee and Johnson, 1988; Cockrem, 2007). It is likely that these factors also account for some of the variation in corticosterone responses to physical stressors, although the perception of stimuli as threats, and hence as emotional stressors, will be absent when birds respond to physical stressors. Instead, factors such as the distribution and number of receptors in insulin target cells, and the concentrations of circulating cytokines may account for some of the variation in corticosterone responses of birds to the physical stressors used in the present study. To further examine the functional significance of individual variation in plasma corticosterone responses the first objective would clearly be to determine if responses of birds to emotional and physical stressors are related. It would then be apparent whether or not birds show consistent individual differences in plasma corticosterone response to stressors in general.

In conclusion, these findings in quail are consistent with reports of elevated plasma corticosterone concentrations in chickens following treatment with insulin or LPS. It is probable that decreased plasma glucose concentrations and increased secretion of cytokines were responsible for activating the HPA axis following treatment with insulin or LPS respectively. The corticosterone responses to different doses of insulin or LPS provide a basis for the choice of doses (4 IU/kg of insulin or 1.2 mg/kg of LPS) and sampling times (insulin, 60, 150 and 240 min; LPS, 60, 120 and 240 min) to be used in a study of plasma corticosterone responses to emotional and physical stressors in quail.

# 7. Plasma corticosterone responses to emotional and physical stressors in Japanese quail

## 7.1 Introduction

Animals respond to potentially harmful stimuli by adjusting their physiology and behaviour. If the hypothalamo-pituitary-adrenal (HPA) axis is activated, then the stimulus is called a stressor and the animal is said to be experiencing stress (Selye, 1974). Stimuli that pose physical challenges to animals and initiate stress responses through disturbance of physical and chemical tissue parameters are called physical stressors, whilst stimuli that are perceived as a threat following comparison with innate predispositions or previous experience and initiate stress responses are called emotional stressors (Day et al., 1999). Physical stressors are also called physiological, homeostatic or systemic stressors, and emotional stressors may also be referred to as psychological, processive or neurogenic stressors (Reyes et al., 2003). Corticosterone is the primary glucocorticoid secreted by the adrenal glands in birds during activation of the HPA axis (Carsia and Harvey, 2000), and corticosterone responses of birds to emotional stressors have been studied more extensively than responses to physical stressors. For example, the standardised artificial emotional stressors used to measure HPA axis activity are capture followed by restraint in free-living birds (Wingfield, 1994; Silverin, 1998), and mechanical restraint (Jones and Satterlee, 1996) or repeated handling followed by confinement in domesticated birds (Fraisse and Cockrem, 2006). Furthermore, lines of birds selected for low and high stress responses (e.g. chickens, Gallus domesticus, Gross and Siegel, 1985; Japanese quail, Coturnix coturnix japonica, Satterlee and Johnson, 1988; zebra finches, Taeniopygia guttata, Evans et al., 2006) have been selected on corticosterone responses to emotional rather than physical stressors. Corticosterone responses to standardised artificial emotional stressors are assumed to reflect the responsiveness of the HPA axis of birds to stressors in general, and hence to more natural stressors (Breuner et al., 2008), but there are no data on the relationships between corticosterone responses of individual birds to emotional and physical stressors.

There is increasing interest in the study of personality in animals, particularly in birds. Personalities have been defined as suites of correlated behaviours that are consistent across different situations and over time (Carere and Eens, 2005). Personalities vary along a continuum, but individuals can be classified as having either proactive or reactive personalities (Cockrem, 2007). Proactive and reactive personalities are thought to be associated with relatively low and high glucocorticoid responses to stressors respectively (Koolhaas et al., 1999; Cockrem, 2007). The glucocorticoid responses are responses to emotional stressors, but do animals assigned to personality groups on the basis of responses to emotional stressors also have similarly low or high glucocorticoid responses to physical stressors? Individual Japanese quail were subjected to three different types of novel emotional and physical stressors and plasma corticosterone concentrations were measured to address important questions about responsiveness of the avian HPA axis to stressors. The emotional stressor used was repeated handling followed by confinement and the two physical stressors used were treatment with insulin and treatment with lipopolysaccharide (LPS). These treatments were chosen as they can all activate the HPA axis in quail (Hull et al., 2007; see Chapter six) and chickens (Johnson et al., 1993; Nakamura et al., 1998; Niezgoda et al., 2005; Shini et al., 2008). Each bird was subjected to one emotional stressor and two physical stressors, so the relationships between the corticosterone response to an emotional stressor and responses to each physical stressor, and relationships between corticosterone responses to the two physical stressors could be examined.

It is proposed that birds with proactive personalities may also have lower levels of fearfulness than birds with reactive personalities (Cockrem, 2007). For example, low stress Japanese quail consistently show lower levels of fearfulness than high stress quail in a range of behaviour tests (Jones et al., 1992b; Satterlee et al., 1993; Jones et al., 1999). The novel object test has previously been used in studies of fearfulness in quail (Richard et al., 2008; Saint-Dizier et al., 2008), where birds that take longer to pass or peck a novel object are considered to have higher levels of fearfulness than birds that take shorter times (Miller et al., 2005). Behavioural responses to the presentation of a novel object in the home cage were therefore measured in the present study for comparison with plasma corticosterone responses to handling and confinement in individual birds.

## 7.2 Materials and methods

### 7.2.1 Animals and husbandry

White female Japanese quail were purchased at five weeks of age from our usual supplier (Canter Valley Farm, Christchurch). Each bird was identified with a coloured, numbered leg band and housed in an individual cage measuring 35 x 40 x 24 cm (length x width x height). Quail were given five weeks to acclimatise to housing conditions and were ten weeks of age when the experiment began. Please refer to section 2.2.1 for further details on animals and husbandry.

### 7.2.2 Experimental design

Behavioural responses to a novel object were recorded in 28 quail. The birds were subjected to the emotional stressor of handling followed by confinement two weeks later. Quail were then left undisturbed for three weeks before treatment with insulin as the first physical stressor, and three weeks after treatment quail were treated with LPS as the second physical stressor. It was not practical to expose different birds to handling, insulin or LPS on the same day, so all birds were exposed to the same stressor on each of the sampling days. This design was suitable to achieve the aim of the experiment which was to compare the relative rather than absolute responses of quail to the three stressors.

### 7.2.3 Blood sample collection

Please refer to section 2.2.5 for details on blood sample collection.

### 7.2.4 Injection preparation and administration

Please refer to section 6.2.4 for details on injection preparation and administration.

### 7.2.5 Behavioural responses to novel object

Behavioural responses to a novel object were measured in the bird's home cages between 0800 and 1300 h. Wire mesh inserted into cages 24 h before testing halved the floor area and decreased the ability of birds to move away from the novel object. Please refer to section 2.2.3.2 for further details on novel object tests.

### 7.2.6 Plasma corticosterone responses to handling and confinement

Plasma corticosterone responses to handling and confinement were measured as previously described in quail (Hull et al., 2007) and chickens (Littin and Cockrem, 2001; Fraisse and Cockrem, 2006). Handling and blood sample collection occurred between 0930 and 1200 h. Please refer to section 5.2.2 for further details on measuring plasma corticosterone responses to 15 min handling followed by 45 min confinement.

### 7.2.7 Plasma corticosterone responses to insulin or LPS

Insulin and LPS injection and blood sample collection occurred between 0800 and 1300 h. Birds were removed from their cages and an initial blood sample (-1.5 min) was taken. Birds then received an injection of 4 IU/kg body weight of insulin or 1.2 mg/kg body weight of LPS at 0 min, and were returned to their cages. Further blood samples were taken at 60, 150 and 240 min following injection with insulin, and at 60, 120 and 240 min following injection with LPS. The doses of insulin and LPS and the timing of blood sample collection were selected on the basis of results of dose-response tests (see Chapter six). There was no increase in plasma corticosterone 30, 60, 120, 150 or 240 min after injection with 0.9 % saline in control birds during dose-response tests.

### 7.2.8 Corticosterone radioimmunoassay

Please refer to section 2.2.7 for details on corticosterone radioimmunoassay.

### 7.2.9 Statistics

Statistical analyses were performed using Systat (Systat Software). Plasma corticosterone concentrations were transformed to logarithms. Levene's tests to check for homogeneity of variances showed that parametric statistics could not be used. Instead, Friedman's one way repeated measures ANOVAs were used to compare corticosterone concentrations between times in quail handled for 15 min and then confined for 45 min, and in quail treated with 4 IU/kg of insulin or 1.2 mg/kg of LPS, with pairwise comparisons between times performed with Wilcoxon signed rank tests. The total areas under plasma corticosterone response curves (AUCs) were determined in GraphPad Prism (GraphPad Software Inc) using the trapezoid rule (Cockrem and Silverin, 2002b). Total AUCs were calculated separately for plasma corticosterone responses to handling for 15 min followed by 45 min confinement, and for treatment with 4 IU/kg of insulin or 1.2 mg/kg of LPS. Birds were then ranked on their total

AUCs for each treatment, where low and high ranks indicate relatively low and high total AUCs respectively. Data are presented as individual points or as mean ± standard error (S.E.).

### 7.2.10 Relationships between plasma corticosterone responses to emotional and physical stressors

Relationships between corticosterone variables (ranks of total area under the plasma corticosterone response curve; ranks of maximum plasma corticosterone concentration) calculated for plasma corticosterone responses to 15 min handling followed by 45 min confinement, and treatment with 4 IU/kg of insulin or 1.2 mg/kg of LPS were determined with Spearman correlations.

### 7.2.11 Fear score ranks

Please refer to section 2.2.10 for further details on the calculation of fear score ranks.

### 7.2.12 Relationships between corticosterone and behaviour

Relationships between corticosterone variables (ranks of total area under the plasma corticosterone response curve during handling and confinement; ranks of maximum plasma corticosterone concentration during handling and confinement) and behaviour variables (fear score ranks; ranks for each of the three behaviour variables) were determined with Spearman correlations.

## 7.3 Results

### 7.3.1 Plasma corticosterone responses to handling and confinement

Plasma corticosterone concentrations in individual quail immediately after removal from cages (0 min) were <2 ng/ml. Corticosterone remained relatively constant in five of the 28 birds (see Fig. 7.1 for individual corticosterone responses). Of the other 23 birds, corticosterone increased in 21 and remained at similar concentrations in two, between 0 and 15 min. Between 15 and 30 min and between 30 and 60 min, corticosterone declined in 15 quail, declined then increased in two quail, remained relatively constant then increased in three quail or increased then declined in three quail. Corticosterone concentrations ranged from 0.50 to 9.75 ng/ml at 15 min.

Mean plasma corticosterone concentrations in quail were significantly affected by handling for 15 min (Friedman's one way repeated measures ANOVA $F_3 = 32.604$, $p < 0.001$; Fig. 7.2, see Appendix Table 7.1 for further statistics). Initial (0 min) mean corticosterone concentrations were 1.00 ± 0.08 ng/ml. Mean corticosterone then increased to 3.51 ± 0.43 ng/ml at 15 min, before declining to 2.06 ± 0.30 ng/ml at 30 min (0 vs 15 min, $Z = 4.349$, $p < 0.001$; 15 vs 30 min, $Z = -3.363$, $p < 0.001$). Mean corticosterone then returned towards initial concentrations at 60 min (1.68 ± 0.30 ng/ml; 0 vs 60 min, $Z = 1.574$, $p = 0.115$).

### 7.3.2 Plasma corticosterone responses to insulin

Individual plasma corticosterone concentrations were <2 ng/ml immediately before (-1.5 min) injection with 4 IU/kg of insulin. Corticosterone concentrations after treatment with insulin were similar to initial concentrations in nine birds (see Fig. 7.1 for individual corticosterone responses). Of the other 19 birds, corticosterone remained relatively constant in 13 and increased in six, between -1.5 and 60 min. Between 60 and 150 min and between 150 and 240 min, corticosterone continued to increase then declined in four of the six birds, increased in one bird, and declined in one bird. In the 13 quail with relatively constant corticosterone concentrations from -1.5 to 60 min, corticosterone between 60 and 150 min and between 150 and 240 min increased then declined in nine quail, increased in two quail, and increased then remained at similar concentrations in two quail. Corticosterone concentrations at 150 min varied from 0.50 to 13.39 ng/ml.

Treatment of quail with 4 IU/kg of insulin significantly affected mean plasma corticosterone concentrations (Friedman's one way repeated measures ANOVA $F_3 = 41.839$, $p < 0.001$; Fig. 7.2, see Appendix Table 7.1 for further statistics). Mean corticosterone concentrations at -1.5 min were 0.93 ± 0.09 ng/ml. Mean corticosterone then increased to 1.75 ± 0.34 ng/ml at 60 min, and continued to increase to a maximum concentration of 3.41 ± 0.52 ng/ml at 150 min (-1.5 vs 60 min, $Z = 3.417$, $p = 0.001$; 60 vs 150 min, $Z = 3.940$, $p < 0.001$). Corticosterone declined between 150 and 240 min, but concentrations at 240 min were still significantly elevated above initial concentrations (2.01 ± 0.23 ng/ml; 150 vs 240 min, $Z = -2.095$, $p = 0.036$; -1.5 vs 240 min, $Z = 4.517$, $p < 0.001$).

### 7.3.3 Plasma corticosterone responses to LPS

Initial (-1.5 min) plasma corticosterone concentrations were <1.7 ng/ml in individual quail before injection with 1.2 mg/kg of LPS. Twenty seven of the 28 quail showed a marked increase in plasma corticosterone following injection with LPS (see Fig. 7.1 for individual corticosterone responses). Corticosterone remained relatively constant in 12 of the 27 birds and increased in the other 15 birds between -1.5 and 60 min. Between 60 and 120 min and between 120 and 240 min, corticosterone continued to increase then declined in six of the 15 birds, remained at similar concentrations then increased in two birds, declined then increased in six birds, and declined in one bird. In the 12 quail with relatively constant corticosterone concentrations between -1.5 and 60 min, corticosterone between 60 and 120 min and between 120 and 240 min, increased then declined in three quail, increased in three quail, increased then remained at similar concentrations in three quail, and remained relatively constant then increased in three quail. Corticosterone concentrations varied from 1.28 to 13.20 ng/ml at 240 min.

Mean plasma corticosterone concentrations in quail increased significantly after treatment with 1.2 mg/kg LPS (Friedman's one way repeated measures ANOVA $F_3$ = 47.625, $p < 0.001$; Fig. 7.2, see Appendix Table 7.1 for further statistics). Initial (-1.5 min) mean plasma corticosterone concentrations were low at 0.90 ± 0.07 ng/ml, and increased markedly at 60 min (3.53 ± 0.61 ng/ml; $Z = 4.440$, $p < 0.001$). Corticosterone continued to increase between 60 and 120 min, and between 120 and 240 min (4.88 ± 0.70 ng/ml at 120 min; 60 vs 120 min, $Z = 1.571$, $p = 0.116$; 5.23 ± 0.46 ng/ml at 240 min; 120 vs 240 min, $Z = 0.672$, $p = 0.502$). Corticosterone was significantly elevated above initial concentrations at 240 min (-1.5 vs 240 min, $Z = 4.623$, $p < 0.001$).

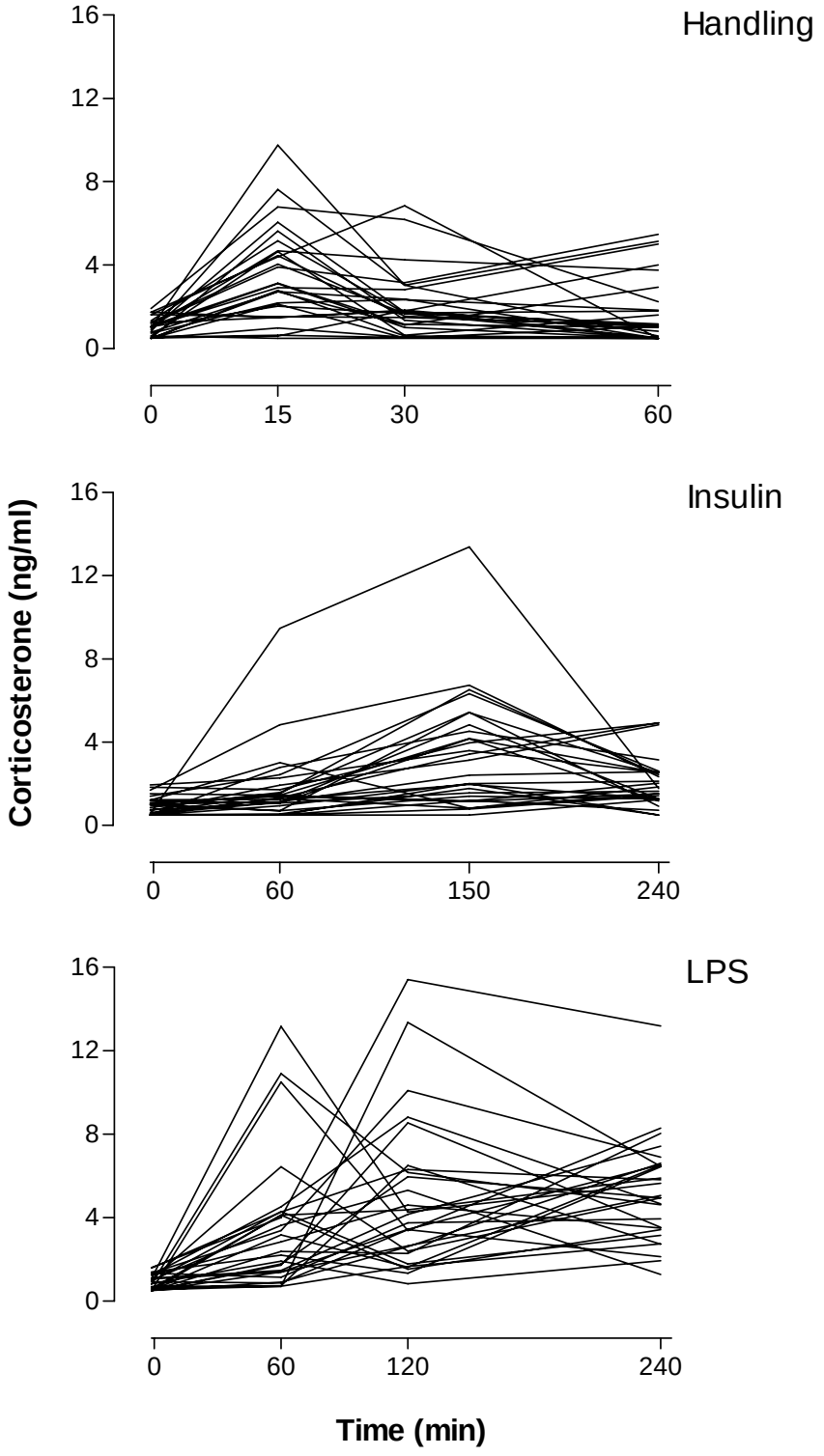


**Figure 7.1.** Individual plasma corticosterone responses in quail handled for 15 min and then confined for 45 min, and in quail treated with 4 IU/kg of insulin or 1.2 mg/kg of lipopolysaccharide (LPS). Initial blood samples were taken 1.5 min before injections were given at 0 min. N = 28.

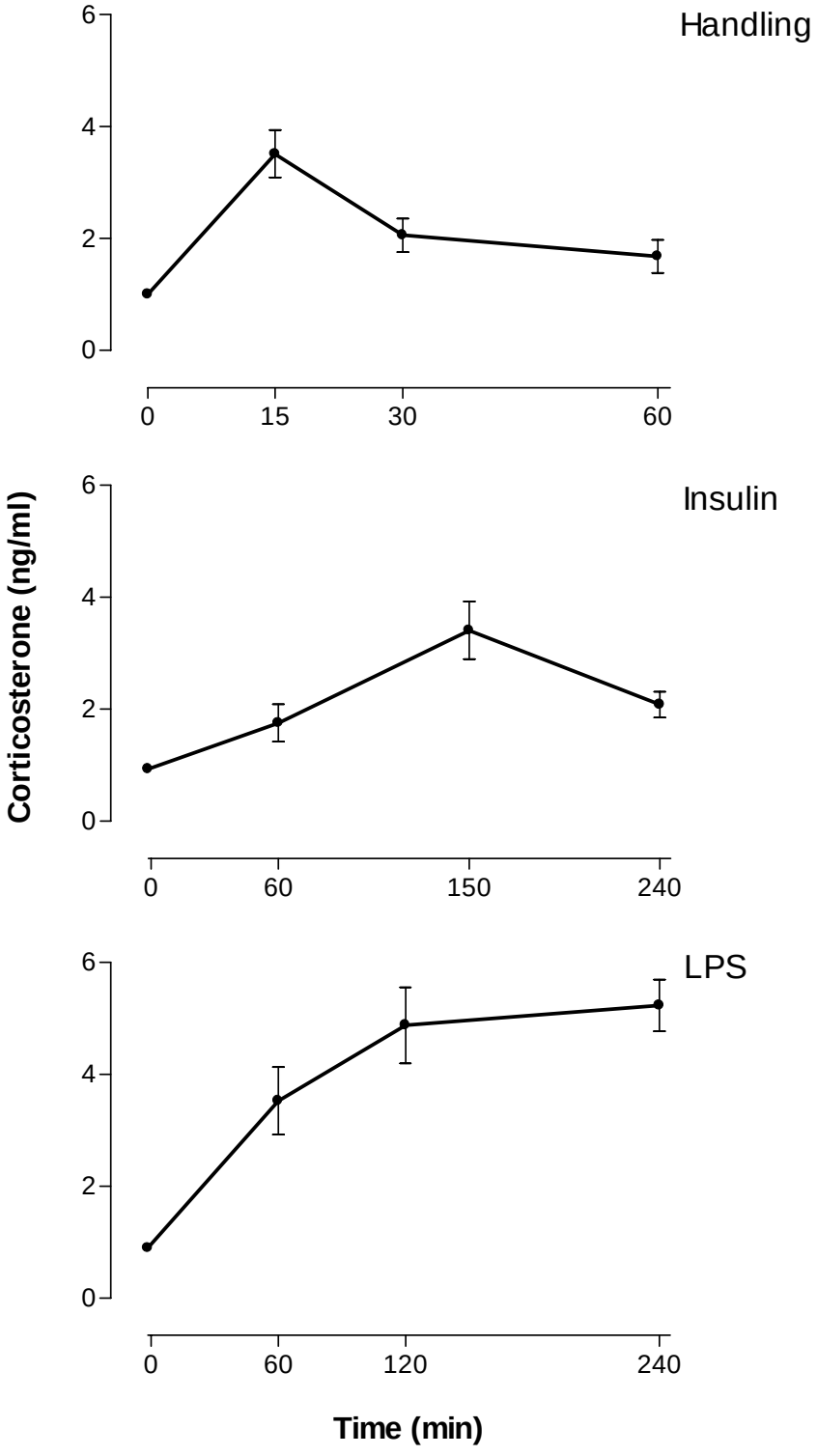


**Figure 7.2.** Plasma corticosterone responses in quail handled for 15 min and then confined for 45 min, and in quail treated with 4 IU/kg of insulin or 1.2 mg/kg of lipopolysaccharide (LPS). Initial blood samples were taken 1.5 min before injections were given at 0 min. Data are presented as mean ± S.E. N = 28.

### 7.3.4 Plasma corticosterone responses to emotional and physical stressors in individual birds

Plasma corticosterone responses to emotional and physical stressors are shown for four quail in Fig. 7.3 and for three quail in Fig. 7.4 (see Appendix Fig. 7.1 for plasma corticosterone responses to the three stressors in all 28 birds). Approximately one quarter of the quail showed similar responses to each of the four combinations illustrated in Fig. 7.3, suggesting there was no clear relationship between corticosterone responses to emotional and physical stressors within individual birds. Corticosterone responses to the two physical stressors generally resembled the responses shown in Fig. 7.4 (a) or Fig. 7.4 (b) in 22 of the 28 birds. In other words, if a bird had a relatively low corticosterone response to one physical stressor, it generally had a relatively low response to the other physical stressor, and if a bird had a relatively high corticosterone response to one physical stressor, it generally had a relatively high response to the other physical stressor.

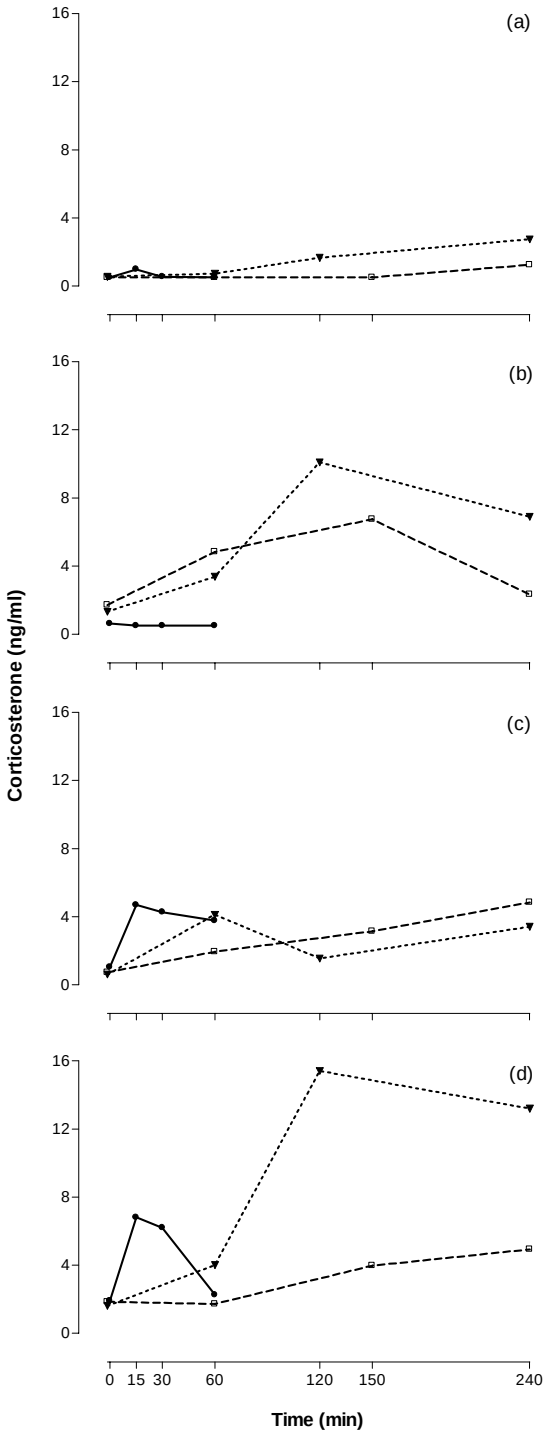


**Figure 7.3.** Plasma corticosterone responses to emotional and physical stressors in four quail. Relatively (a) low responses to emotional and physical stressors (b) low response to emotional stressor and high response to at least one physical stressor (c) high response to emotional stressor and low responses to physical stressors (d) high response to emotional stressor and high response to at least one physical stressor. Handling for 15 min followed by confinement for 45 min, —●—; treatment with 4 IU/kg insulin, - - - □ - - -; treatment with 1.2 mg/kg LPS, ·····▼·····.

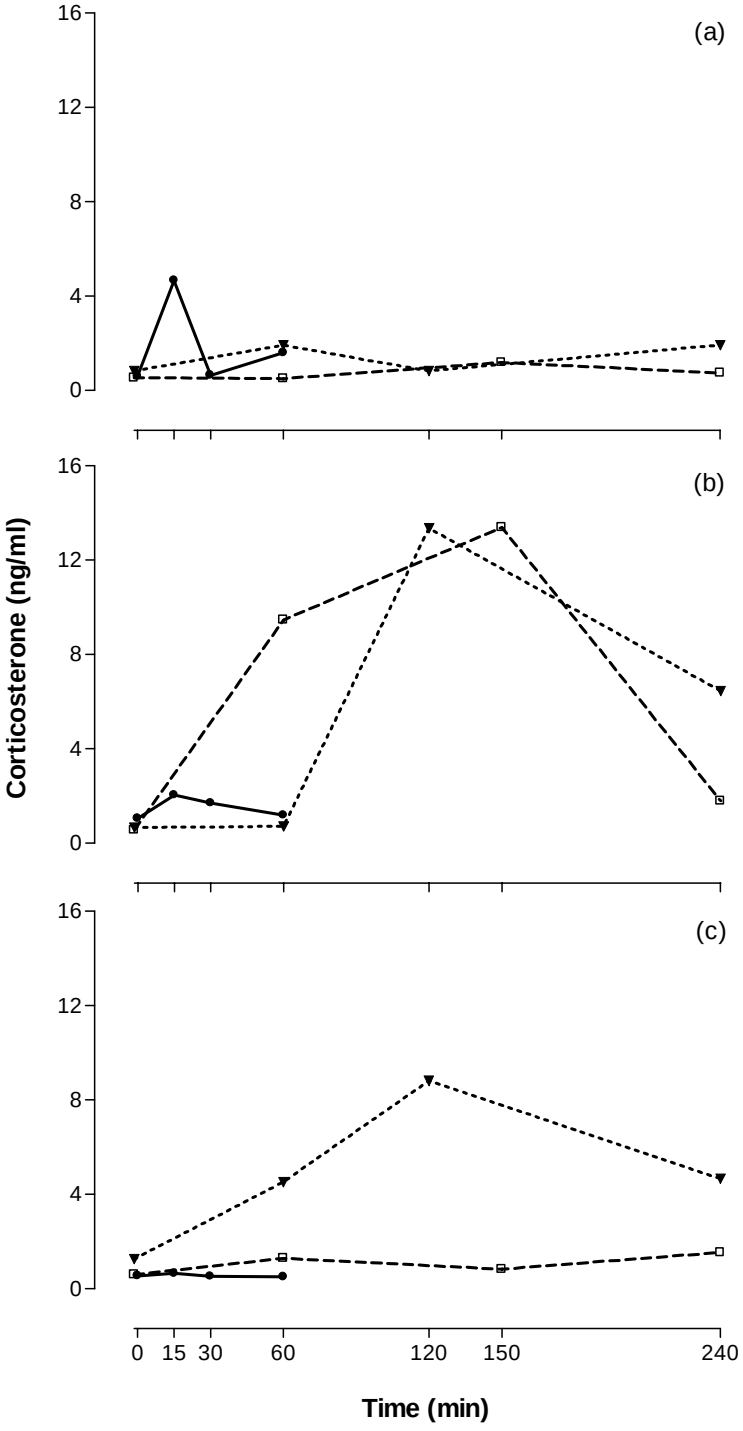


**Figure 7.4.** Plasma corticosterone responses to emotional and physical stressors in three quail. Relatively (a) low responses to two different physical stressors (b) high responses to two different physical stressors (c) low response to one physical stressor and a high response to a second physical stressor. Handling for 15 min followed by confinement for 45 min, —●—; treatment with 4 IU/kg insulin, - - - □ - - -; treatment with 1.2 mg/kg LPS, ····▼····.

### 7.3.5 Relationships between plasma corticosterone responses to emotional and physical stressors

There were no significant relationships between responses to emotional and physical stressors in the calculated variables (ranks of total area under the plasma corticosterone response curve; handling vs insulin, $r = 0.306$, $p = 0.113$; handling vs LPS, $r = 0.057$, $p = 0.773$; ranks of maximum plasma corticosterone concentration; handling vs insulin, $r = 0.174$, $p = 0.376$; handling vs LPS, $r = -0.060$, $p = 0.761$; Fig. 7.5 and 7.6). There were however, positive correlations between the two physical stressors for both corticosterone variables. There were significant relationships between the two stressors for ranks of total areas under the plasma corticosterone response curve, and for ranks of maximum plasma corticosterone concentration following treatment with insulin or LPS ($r = 0.636$, $p < 0.001$; $r = 0.387$, $p = 0.042$; Fig. 7.5 and Fig. 7.6). The three birds with the lowest ranks of total area under the corticosterone response curve after treatment with insulin (i.e. ranks 1, 2 and 3) were ranked 2$^{nd}$, 3$^{rd}$ and 1$^{st}$ respectively for total area under the corticosterone response curve after treatment with LPS (bird # 3, 6 and 13 respectively in Appendix Fig. 7.1). Furthermore, the three birds with the highest ranks of total area under the corticosterone response curve after treatment with insulin (i.e. ranks 26, 27 and 28) were ranked 24$^{th}$, 26$^{th}$ and 27$^{th}$ respectively for total area under the corticosterone response curve after treatment with LPS (bird # 19, 1, and 10 respectively in Appendix Fig. 7.1).

### 7.3.6 Relationships between corticosterone and behaviour

Relationships between the two corticosterone variables and each of the four behaviour variables were not significant ($p \geq 0.190$).

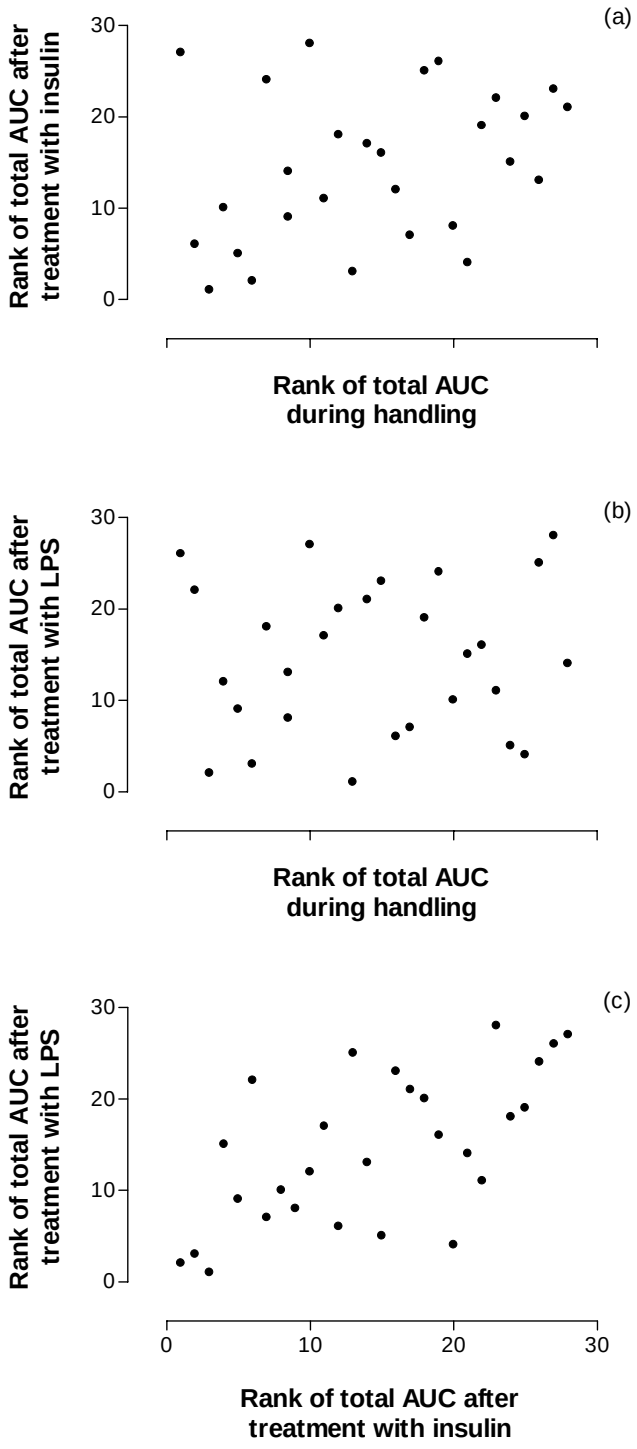


**Figure 7.5.** Correlations between ranks of total area under the plasma corticosterone response curve (AUC) in quail (a) handled for 15 min then confined for 45 min, or treated with 4 IU/kg of insulin, (b) handled for 15 min then confined for 45 min, or treated with 1.2 mg/kg of lipopolysaccharide (LPS) or (c) treated with 4 IU/kg of insulin or 1.2 mg/kg of LPS. Areas under the curve are in ng/ml.min. See section 7.3.5 for Spearman statistics. N = 28.

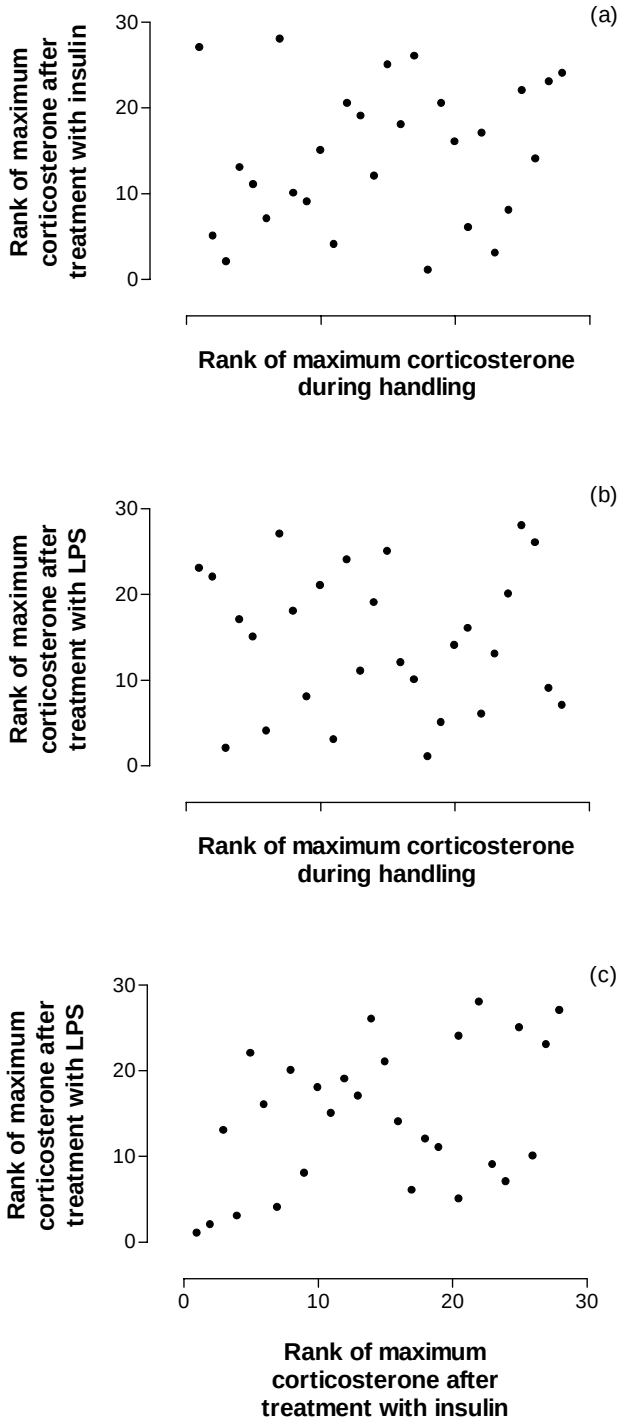


**Figure 7.6.** Correlations between ranks of maximum plasma corticosterone concentrations in quail (a) handled for 15 min then confined for 45 min, or treated with 4 IU/kg of insulin, (b) handled for 15 min then confined for 45 min, or treated with 1.2 mg/kg of lipopolysaccharide (LPS) or (c) treated with 4 IU/kg of insulin or 1.2 mg/kg of LPS. Corticosterone concentrations are in ng/ml. See section 7.3.5 for Spearman statistics. N = 28.

## 7.4 Discussion

This study in Japanese quail is the first in any species of bird to describe plasma corticosterone responses to emotional and physical stressors in individual birds. Each bird was subjected to an emotional stressor of handling for 15 min followed by 45 min of confinement, and the two physical stressors of treatment with 4 IU/kg of insulin and treatment with 1.2 mg/kg of lipopolysaccharide (LPS). Mean corticosterone concentrations were significantly elevated following all three stressors. There were no relationships between corticosterone responses of individual birds to emotional and physical stressors, or between responses of birds to handling and fear behaviour in novel object tests. There was, however, a significant correlation between corticosterone responses of birds to the two physical stressors. These findings suggest that the magnitude of the corticosterone response to standardised emotional stressors in birds may not reflect the responsiveness of the HPA axis to stressors in general.

Mean initial corticosterone concentrations in quail were ≤1 ng/ml on all occasions, so HPA axis activity in undisturbed birds was similar immediately before handling or treatment with insulin or LPS. Changes in mean corticosterone concentrations across time were different for each of the three stressors, as expected. The mean corticosterone response to 15 min handling followed by 45 min confinement in quail was typical of the response reported elsewhere (Hull et al., 2007; see Chapter five). The mean corticosterone concentration 150 min after treatment with 4 IU/kg of insulin was 3.4 ± 0.5 ng/ml, and was similar to the mean concentration in quail following treatment with 4 IU/kg of insulin in another study (see Chapter 6). In a separate study, mean plasma corticosterone concentrations in quail 120 min after treatment with 0.5, 1.0 or 2.0 mg/kg of LPS were 4.5 ± 1.0, 9.0 ± 1.3 and 6.7 ± 1.7 ng/ml respectively, and it appears that the effects of LPS on plasma corticosterone in quail are not dose-dependent (see Chapter six). Quail have not been treated with 1.2 mg/kg LPS before, although a mean plasma corticosterone concentration of 4.9 ± 0.70 ng/ml 120 min after treatment with 1.2 mg/kg of LPS in the present study is slightly lower than what might of been expected. There are marked individual differences between birds in their corticosterone responses to treatment with LPS (see Chapter six; Fig. 7.1), and it is likely that individual variation resulted in different mean plasma corticosterone concentrations between studies. Maximum mean corticosterone concentrations in quail given subcutaneous injections of

4 IU/kg insulin or 1.2 mg/kg LPS were 3-5 ng/ml less than in chickens treated with lower doses of insulin or LPS. For example, in chickens given intravenous injections of approximately 3.4 IU/kg mean body weight of insulin or 1 mg/kg of LPS, mean corticosterone concentrations increased from <2 ng/ml to around 9 ng/ml at 4 h and 2 h respectively (Baert et al., 2005; Niezgoda et al., 2005). Differences in the route of administration, species differences, or the source of insulin or LPS, could explain the more pronounced effects of insulin and LPS on HPA axis activity in chickens compared with quail.

There were no correlations between the responses of individual quail to emotional and physical stressors in the present study, as measured by ranks of total area under plasma corticosterone response curves, and by ranks of maximum corticosterone concentrations. If a bird had a relatively low corticosterone response to 15 min handling, for example, the same bird did not necessarily show a low response to treatment with insulin or LPS. Whilst mean corticosterone responses of free-living species can vary with time of year or breeding stage (Romero, 2002; Adams et al., 2005), migratory status (Romero et al., 1997) and body condition (Heath and Dufty, 1998), the responses of individual birds are generally consistent when subjected to the same emotional stressor on three or four occasions, as demonstrated in chickens (Littin and Cockrem, 2001), and in free-living species either held in captivity (great tits, Parus major; Cockrem and Silverin, 2002b), or captured in the wild (Adelie penguins, Pygoscelis adeliae; Cockrem et al., 2009). The current data, however, show for the first time in birds that magnitudes of corticosterone responses to emotional stressors may not be related to corticosterone responses of birds to physical stressors.

The present findings have implications for the study of avian personalities. For example, low and high stress Japanese quail have been selected on the magnitude of corticosterone responses to an emotional stressor (mechanical restraint; Satterlee and Johnson, 1988), and have subsequently been proposed to have proactive and reactive personalities respectively (Cockrem, 2007). Similarly, zebra finches selected on low or high corticosterone responses to manual restraint differ in their risk-taking and exploratory behaviour, and are also considered to have proactive and reactive personalities (Martins et al., 2007). Corticosterone responses to physical stressors have not been measured in divergent lines of Japanese quail or zebra finches, however, and

the results of the present study suggest that birds assigned to personality groups on the basis of corticosterone responses to emotional stressors may not have similarly low or high responses to physical stressors.

There is considerable evidence from studies in mammals to suggest that different neurocircuitry is involved in generating responses to emotional and physical stressors (Herman and Cullinan, 1997; Day et al., 1999; Dayas et al., 2001; Herman et al., 2003; Reyes et al., 2003). A study of rats (Rattus norvegicus), for example, found that emotional stressors, such as restraint or noise, activated different regions of the amygdala and different medullary noradrenergic cell groups to physical stressors, such as immune challenge or haemorrhage (Dayas et al., 2001). The paraventricular nucleus (PVN) is a region of the hypothalamus that secretes corticotropin-releasing hormone during stress responses (Lopez et al., 1999), and it has also been reported that exposure to an emotional stressor (30 min restraint) and to a physical stressor (treatment with LPS) recruited few genes in common in the hypothalamic PVN of rats (Reyes et al., 2003). A difference in the processing of emotional and physical stressors in the brain might explain the discrepancies in responses of individual quail to the two classes of stressor in the present study. In support of this notion, there was a significant correlation in the responses of individual quail to the two physical stressors, as measured by ranks of total area under plasma corticosterone response curves, and by ranks of maximum corticosterone concentrations. If a bird had a relatively low corticosterone response to treatment with insulin, for example, the same bird generally had a low response to treatment with LPS. It is likely that hypoglycaemia was responsible for activating the HPA axis in quail following treatment with insulin, as insulin-induced hypoglycaemia can elevate plasma corticosterone concentrations in mammals (Robinson et al., 1992; Romero et al., 1993; Koenig and Cho, 2005) and in chickens (Niezgoda et al., 2005). In contrast, activity of the HPA axis is thought to be mediated by cytokines, such as interleukin-1 and -6, and tumour necrosis factor-α, following treatment with LPS (Beishuizen and Thijs, 2003). Thus, differences in processing are likely to contribute to differences in corticosterone responses to physical stressors in birds.

Handling and confinement is an analogous stressor to capture and restraint which is a standardised emotional stressor widely used to measure HPA axis activity in free-living

species of birds. Measuring corticosterone responses to standardised emotional stressors provides a useful method of comparing HPA axis activity between individuals, populations and species, and studies in free-living birds unequivocally measure the responses of individuals to standardised stressors with no consideration of how birds might respond to physical stressors. Indeed, magnitudes of corticosterone responses to standardised emotional stressors are simply assumed to reflect the responsiveness of the HPA axis of birds to stressors in general (Breuner et al., 2008). The findings of the present study do not support this notion as plasma corticosterone responses to emotional stressors were not related to the responses of birds to physical stressors. The current results, however, do not discount the possibility that standardised stressors reflect the responsiveness of the HPA axis of birds to emotional stressors in general, and hence, to more natural emotional stressors. Indeed, there is evidence from studies in wild free-living species of birds that biologically relevant data can be collected from studies of plasma corticosterone responses to standardised emotional stressors, even though these are artificial situations for birds. For example, magnitudes of plasma corticosterone responses to capture followed by restraint were negatively correlated with survival in European white storks (Ciconia ciconia; Blas et al., 2007), and with food abundance in the previous month in common murres (Uria aalge; Kitaysky et al., 2007). Thus, measuring corticosterone responses of birds to standardised emotional stressors can provide important information about stress responses of birds in their natural environments.

In the present study, there was no relationship between the magnitude of plasma corticosterone responses to an emotional stressor and fear behaviour in quail. In other words, if a bird had a relatively high corticosterone response to handling and confinement, the same bird did not necessarily show high levels of fearfulness in the novel object test. There is considerable experimental evidence to suggest that there is a positive relationship between HPA axis activity and fearfulness in low and high stress quail that are selected on the basis of their plasma corticosterone responses to mechanical restraint (Jones, 1996). Results of the present study, however, suggest that the strength of the relationship between fearfulness and HPA axis activity in unselected populations of quail is lower than is predicted from studies in selected populations of birds (see Chapter eight for further discussion).

In conclusion, it has been shown for the first time in birds that the magnitude of the corticosterone response to an emotional stressor is not related to the size of responses to physical stressors. In contrast, the responses of birds to different types of physical stressors were related. Differences in the neurocircuitry involved in generating responses to emotional and physical stressors may contribute to differences in corticosterone responses of birds to the two classes of stressor. These findings suggest that birds assigned to personality groups on the basis of relatively low or high corticosterone responses to emotional stressors may not have similarly low or high responses to physical stressors.

# 8. General discussion

The current study in Japanese quail (Coturnix coturnix japonica) provided unique opportunities to address novel questions relating to corticosterone, fear behaviour and plasma corticosterone responses to stressors in birds. The effects of corticosterone in laying quail were examined both during and after a 21 day treatment period. Corticosterone had no effect on the fearfulness of birds in tonic immobility, novel object and open field tests, suggesting that elevated plasma corticosterone is not responsible for the stressor-induced increases in fearfulness reported in other populations of quail. Fear score ranks for individual birds in three tests of fear were not correlated, and there was no relationship between corticosterone responses of birds to an emotional stressor and fear behaviour in novel object tests. These findings suggest that studying avian personalities in unselected populations of quail may be more difficult than in selected populations of quail. Body weight gain and reproductive function were affected for up to 22 days after corticosterone treatment ended in some quail, demonstrating that the effects of chronically elevated plasma corticosterone concentrations in birds may persist after corticosterone treatment ends. Food deprivation for 24 h was not a stressor for quail so the effects of elevated plasma corticosterone concentrations on the responses of quail to subsequent stressors could not be determined. Plasma corticosterone responses of quail were measured to examine if responses to similar emotional stressors or to different types of emotional and physical stressors are related in individual birds. In general, quail with relatively low or high plasma corticosterone responses to 15 min handing also had relatively low or high responses to 30 or 60 min handling, suggesting that plasma corticosterone responses of birds to one emotional stressor may reflect the responsiveness of the hypothalamo-pituitary-adrenal (HPA) axis of birds to emotional stressors in general. Treatment with insulin and treatment with LPS were shown to be physical stressors in quail, and a subsequent study found that there were no relationships between corticosterone responses of individual birds to emotional and physical stressors. There was, however, a significant correlation between corticosterone responses of birds to two physical stressors. These findings suggest that the magnitude of the corticosterone response to standardised emotional stressors in birds may not reflect the responsiveness of the HPA axis to stressors in general.

Corticosterone secretion by the adrenal glands increases during activation of the HPA axis (Carsia and Harvey, 2000), and treatment of birds with corticosterone simulates the rise in plasma corticosterone concentrations that occur during stress. Birds treated with corticosterone, however, do not experience stress per se; rather they experience the result of stress (i.e. an increase in plasma corticosterone concentrations). Increased plasma corticosterone following exogenous corticosterone treatment could potentially have different effects in birds from stressor-induced activation of the HPA axis. In the present study for example, there was little evidence that treatment of quail with corticosterone increased fearfulness in tonic immobility, novel object or open field tests of fear behaviour. Quail subjected to the emotional stressor of mechanical restraint 0 or 55 min prior to tonic immobility or open field testing however, showed higher levels of fearfulness than control birds left undisturbed before behaviour testing (Satterlee et al., 1993; Satterlee and Marin, 2006). Birds experience fear when they respond to emotional stressors (LeDoux, 1994), and it is suggested in the present study that stimulation of the amygdala during threatening situations like restraint, rather than an elevation in plasma corticosterone, is responsible for the stressor-induced increases in fearfulness reported in other populations of quail. Knowledge about how birds respond to stimuli in their natural environments can be gained from studies in Japanese quail. Exposure to an emotional stressor (i.e. a potential predator) in the wild, for example, can stimulate the amygdala and produce a heightened state of fear in birds. A heightened state of fear is likely to be associated with the expression of avoidance behaviours, flight or escape behaviours, or immobility postures, such as crouching or freezing (Jones, 1996), and such behaviours are likely to be adaptive in an ecological context. A bird displaying such behaviours, for example, is more likely to escape predation resulting in increased fitness (survival and reproduction). When birds respond to physical stressors (i.e. immune challenge), however, they are responding to an actual physical challenge rather than a threat, and brain regions involved in the perception of threats will not be stimulated in the same way as when birds experience emotional stressors. Results of the present study suggest that birds with increased corticosterone secretion in response to physical stressors will not show a corresponding increase in fearfulness. Indeed, there is no obvious adaptive advantage for birds to exhibit increased fearfulness when responding to physical stressors such as an immune challenge or a decrease in plasma glucose.

In the present study, the effects of chronic stress in birds were investigated by treating quail with corticosterone in their drinking water for 21 days. It was found that plasma corticosterone concentrations remained markedly elevated in quail for up to eight days after treatment ended, and reproductive function and body weight of quail were affected for up to 22 days after treatment ended. These findings are particularly interesting, and suggest that birds may continue to be affected by elevated plasma corticosterone concentrations after exposure to a stressor ends. Whilst the effects of elevated plasma corticosterone on some physiological and behavioural parameters in birds have been extensively studied, less is known about how elevated plasma corticosterone concentrations might affect the responsiveness of the HPA axis of birds to subsequent stressors. Given the adaptive value of the acute glucocorticoid stress response, it is important to determine how HPA axis responsiveness may be affected by chronic stress. Treatment of quail with corticosterone in the present study allowed the effects of elevated plasma corticosterone on responses of birds to a potential stressor to be investigated. Whilst the effects of elevated plasma corticosterone on responses of birds to artificial stressors have been examined (Hull et al., 2007; Busch et al., 2008b; Muller et al., 2009), fasting was chosen as a potential stressor in the present study because it is a more natural stressor and because 24 h of food deprivation elevated plasma corticosterone concentrations in immature (Harvey et al., 1983; Geris et al., 1999) and adult (Scanes et al., 1980) chickens. Plasma corticosterone concentrations in quail, however, were generally unaffected after 24 h of fasting in the present study. Whilst questions about the effects of elevated plasma corticosterone concentrations on responses of birds to natural stressors such as fasting remain unanswered, studies in free-living birds suggest that chronic elevations in plasma corticosterone can affect responses to artificial stressors. For example, American stonechats (Setophaga ruticilla) in poorer habitats had higher plasma corticosterone concentrations than individuals in better quality habitats, and plasma corticosterone responses to capture followed by restraint were markedly lower in birds with higher initial corticosterone concentrations than in birds with lower initial corticosterone concentrations (Marra and Holberton, 1998). Furthermore, white-crowned sparrows (Zonotrichia leucophrys gambelii), Eurasian kestrels (Falco tinnunculus) and barn owls (Tyto alba) treated with corticosterone had lower plasma corticosterone responses to capture followed by restraint than untreated control birds (Busch et al., 2008b; Muller et al., 2009). It is suggested that elevated plasma corticosterone concentrations might reduce HPA activity

by altering the way in which birds perceive stressors, or by providing negative feedback on the HPA axis (Busch et al., 2008b). Indeed, reducing the responsiveness of the HPA axis to acute stressors at a time when plasma corticosterone concentrations are already elevated may help reduce the effects of glucocorticoid excess. The present study has shown that plasma corticosterone responses of individual birds to emotional and physical stressors can differ, so further research could examine if treatment with corticosterone influences the responses of birds to each class of stressor, and also to more natural stressors.

Results of the present study show that plasma corticosterone concentrations can remain markedly elevated in quail for the duration of an acute stressor lasting 60 min, and that plasma corticosterone can remain elevated for a period of days after chronic corticosterone treatment has ended. Together, these results suggest that if birds experience a series of acute stressors, or if birds are constantly exposed to a stressor, then a state of chronic stress could develop, and the effects of elevated plasma corticosterone concentrations could potentially continue for several days or even weeks after exposure to a stressor ends. Whilst animals have evolved stress responses to help them cope with natural changes in their environments, such as changes in predation pressure or food abundance, it is likely that in the foreseeable future animals will increasingly be exposed to stressors that are anthropogenic in origin. A combination of climate change and human disturbance, also referred to as global change, has the potential to cause catastrophic effects in animals in nearly every habitat on earth (Wingfield, 2008). Although the fitness consequences of global change in birds are relatively unknown, recent studies suggest that environmental disturbances of anthropogenic origin have the potential to cause chronic stress in birds. For example, faecal corticosterone concentrations in male spotted owls (Strix occidentalis caurina) were higher in birds that had home ranges closer to intensively logged forest areas compared to birds that occupied home ranges in less disturbed areas of the forest (Wasser et al., 1997), and more recently it was shown that Arctic-breeding glaucous gulls (Larus hyperboreus) with higher plasma concentrations of persistent organic pollutants (POPs) had higher plasma corticosterone concentrations than gulls with lower plasma POP concentrations (Verboven et al., 2010). Furthermore, magnitudes of plasma corticosterone responses to capture followed by restraint were negatively associated with plasma POP concentrations in male gulls (Verboven et al., 2010). The

study of Verboven et al., (2010) suggests that pollution of Arctic environments by humans may be reducing the capacity of birds to respond successfully to acute stressors, which may increase in frequency with the Arctic's changing climatic conditions. It is also likely that birds exposed to stressors of anthropogenic origin will have reduced fitness as it is generally found that free-living birds with relatively low plasma corticosterone concentrations have higher annual reproductive success than birds with higher corticosterone concentrations (Bonier et al., 2009; Busch and Hayward, 2009). It has recently been shown that the effects of slight, chronic elevations in plasma corticosterone concentrations on immune function and feather length in American kestrel (Falco sparverius) nestlings presented almost a week after corticosterone treatment had ended (Butler et al., 2010). Coupled with the results of the present study, there is new evidence that chronic elevations in plasma corticosterone concentrations in birds that result from global change may continue for several days or even weeks after exposure to a stressor ends. It is likely that such elevations in plasma corticosterone will have detrimental effects on reproductive function and body condition and eventually lead to a reduction in fitness in some species of birds.

There is variability within and between studies in the effects of corticosterone treatment on physiology and behaviour in birds. Within studies for example, treatment of chickens (Gallus domesticus) with high but not low doses of corticosterone increased plasma glucose concentrations and abdominal fat content compared with untreated birds (Davison et al., 1983; Hayashi et al., 1994), and treatment of white-crowned sparrows with low but not high doses of corticosterone increased locomotor activity compared with untreated controls (Breuner et al., 1998; Breuner and Wingfield, 2000). Furthermore, treatment of chickens with corticosterone increased food intake in some studies (Nasir et al., 1999; El-Lethey et al., 2001), but not others (Davison et al., 1983; Williams et al., 1985; Kafri et al., 1988). In the present study in quail, variables measured in tonic immobility tests were unaffected by corticosterone treatment, whereas in two studies in chickens, durations of tonic immobility were significantly longer in birds treated with corticosterone than in untreated controls (Jones et al., 1988; El-Lethey et al., 2001). There were also variable effects of corticosterone treatment on food intake in quail. For example, there was no clear effect of corticosterone on food intake in the second week of treatment, whereas food intake was higher in birds treated with corticosterone than in untreated controls in the 3 h following 24 h of fasting. In

contrast, food intake was lower in the three highest treatment groups than in controls in the week after corticosterone treatment ended. Furthermore, quail in the highest treatment group had significantly lower egg production compared with controls in the third week after corticosterone treatment ended, whereas quail in the three lowest treatment groups had similar egg production to controls in the third week after treatment ended. Differences within or between studies in the effects of corticosterone in birds could depend on the method and duration of treatment, dose of corticosterone, concentrations of corticosterone in the plasma, and on the species, strain or sex of experimental birds. Global conclusions about the actions of corticosterone in birds, therefore, should not be made on the basis of the findings of one study.

Mean corticosterone was <7 ng/ml in quail handled repeatedly for up to 60 min in the present study. In two different populations of domesticated quail, mean corticosterone was <7 ng/ml following restraint in a cloth bag for 30 min (Hayward et al., 2006), and <20 ng/ml following 60 min of continuous mechanical restraint (Hazard et al., 2008). In contrast, mean corticosterone 30 or 60 min after capture and restraint in a cloth bag or box is usually markedly higher in a diverse range of free-living birds. Mean corticosterone can be greater than 40 ng/ml after 30 min restraint in Adelie penguins (Pygoscelis adeliae; Cockrem et al., 2006), white-crowned sparrows (Romero et al., 1997) or bar-tailed godwits (Limosa lapponica; Landys-Ciannelli et al., 2002), and greater than 80 ng/ml after 60 min restraint in grey-faced petrels (Pterodroma macroptera gouldi; Adams et al., 2005), Smith's longspur's (Calcarius pictus; Meddle et al., 2003) or willow warblers (Phylloscopus trochilus; Silverin et al., 1997). Domesticated birds such as Japanese quail and chickens live in cages and aviaries and have been selected over many generations for parameters such as increased growth rate or egg production (Al-Nasser et al., 2007), and it is likely that selective breeding during domestication is responsible for a reduction in the magnitude of corticosterone responses in domesticated animals compared to free-living animals. Indeed, previous studies in ducks (Anas platyrhynchos; Martin, 1978), Norway rats (Rattus norvegicus; Naumenko et al., 1989), silver foxes (Vulpes vulpes; Trut, 1999) and guinea pigs (Cavia aperea f. porcellus; Kunzl et al., 2003) support the notion that HPA axis responsiveness is less pronounced in domesticated populations, compared to their wild counterparts. Whilst it is suggested that relatively low corticosterone responses in domesticated quail are likely a consequence of artificial selection for increased docility, there are no data

on corticosterone responses in free-living quail. Free-living populations of Japanese quail occur in Eastern Asia (Chang et al., 2005), and although measuring plasma corticosterone responses to handling followed by confinement in these birds is a practical challenge, it would be valuable to know if responses are higher in free-living compared with domesticated quail.

There are marked differences between strains of chickens in plasma corticosterone responses, and white Leghorn hens have higher corticosterone responses to handling than brown Hyline hens (Fraisse and Cockrem, 2006). The magnitudes of corticosterone responses to the same stressor, however, have not been compared between different strains of domesticated quail. Quail are an important species for meat and egg production (Hinshaw et al., 1969; Baumgartner, 1994), and studies in low and high stress quail report that low stress birds show a range of desirable production traits, such as reduced osteoporosis and developmental instability (Satterlee and Roberts, 1990; Satterlee et al., 2000), and earlier sexual maturity in both sexes (Marin et al., 2002). Identification of strains of domesticated quail that have relatively low corticosterone responses to stressors could lead to improvements in production traits and welfare in quail.

The Japanese quail used in the present study can be considered "unselected" quail, in the sense that they have not been artificially selected on the basis of low (low stress) or high (high stress) plasma corticosterone responses to an emotional stressor, or selected for short or long tonic immobility responses, like other selected populations of quail that have been extensively researched (Mills and Faure, 1991; Jones, 1996). The consistency of responses of quail in three behaviour tests of fear were examined, and this was the first study to investigate relationships between fearfulness and HPA axis activity in an unselected population of quail. There were no correlations between fear score ranks assigned to individual birds in tonic immobility, novel object and open field tests, or between fear score ranks assigned to birds in novel object tests and magnitudes of plasma corticosterone responses to an emotional stressor. The present findings suggest that fearfulness is not an easily identifiable personality trait in an unselected population of quail, and that the strength of the relationship between fearfulness and HPA axis activity in unselected quail is lower than is predicted from studies in selected populations of birds. The majority of research on avian personalities has been

conducted in artificially selected lines of slow and fast exploring great tits (Parus major) that are held in captivity, and consistent differences in the behavioural responses of birds in the two lines have been identified (Groothuis and Carere, 2005). Proactive great tits from a fast exploring line also demonstrate lower HPA axis activity during social challenges compared to reactive great tits from a slow exploring line (Carere et al., 2003). In support of the existence of avian personalities in artificially selected populations of birds, proactive Japanese quail from a low stress line demonstrate lower levels of fearfulness in a range of behaviour tests compared to reactive quail from a high stress line (Jones et al., 1992a; Jones et al., 1992b; Jones, 1996; Kembro et al., 2008), and zebra finches (Taeniopygia guttata) selected on low or high plasma corticosterone responses to restraint differ in their risk-taking and exploratory behaviour, and are also considered to have proactive and reactive personalities respectively (Martins et al., 2007). Whilst the unselected quail used in the present study did not display consistent individual differences in personality traits, wild populations of birds that are not subjected to artificial selection can display consistent individual differences in personality traits. For example, wild great tits had consistent behaviours when subjected to open field tests on two occasions (Dingemanse et al., 2002); proactive wild great tits that explored novel environments faster than reactive great tits showed a greater intensity in alarm calling toward a human intruder (Hollander et al., 2008); and wild juvenile starlings (Sturnus vulgaris) had consistent individual differences in tests of exploratory behaviour (Minderman et al., 2009). Given that personality types relate to foraging, exploration and aggression, it is likely that avian personalities are adaptive and are maintained by natural selection (Dingemanse et al., 2002). The maintenance of avian personalities in the wild relies on the processes of natural selection acting on whole suites of traits, and results in individuals showing correlated behaviours across a range of environmental situations (Dingemanse and Reale, 2005). It is possible that the processes of artificial selection act in a similar way to natural selection (Fuller et al., 2005), acting on whole suites of traits, which results in marked differences in personality types between selected lines of birds, and correlations between traits within individuals. Taken together, the results of the present study suggest that whilst Japanese quail of low and high stress selection lines are suitable subjects for the study of fearfulness as an avian personality trait, fearfulness as a personality trait is more difficult to identify in unselected populations of quail.

The current thesis describes the most comprehensive study of plasma corticosterone responses to emotional and physical stressors in individuals of any species of bird. Relationships between plasma corticosterone responses to three similar emotional stressors, two different physical stressors, and three different emotional and physical stressors were examined in individual Japanese quail. Indeed, studies of HPA axis activity in birds have overwhelmingly focused on mean responses of groups of birds to standardised emotional stressors. The current study, therefore, provides important new information on whether or not birds display consistent individual differences in their corticosterone responses to threatening events, to physical challenges, or to both. The current data will be discussed in terms of the mechanistic and functional factors that relate to plasma corticosterone responses to emotional and physical stressors in birds.

Plasma corticosterone responses to three similar emotional stressors were generally related in individual Japanese quail. It was also shown that plasma corticosterone responses to two different physical stressors were generally related in individual birds. In other words, if a bird had a relatively low plasma corticosterone response to 15 min handling followed by 45 min confinement, the same bird generally had a relatively low response to 30 min handling followed by 30 min confinement, and to 60 min handling, and if a bird had a relatively high plasma corticosterone response to treatment with insulin, the same bird generally had a relatively high response to treatment with lipopolysaccharide (LPS). Although it is acknowledged that the consistency or repeatability of individual variation in acute glucocorticoid stress responses should be examined to determine if individual differences reflect actual physiological phenotypes or result from sampling error (Williams, 2008; Wingfield et al., 2008), no previous study has investigated if birds respond consistently to different classes of stressor. Indeed, the current results show for the first time the existence of consistent individual differences in the magnitudes of plasma corticosterone responses of birds to emotional stressors and to physical stressors. Given that repeatable individual variation within populations provides the raw material on which the processes of natural selection act (Garland and Carter, 1994; Wingfield et al., 2008), the current experiments in Japanese quail provide important new information about the evolution of stress responses in free-living birds. Indeed, there is recent interest in how the acute glucocorticoid stress response relates to fitness in birds, and demonstration of consistent phenotypic differences in plasma corticosterone responses to stressors strengthens the evidence that

the processes of natural selection act on HPA axis activity (Breuner et al., 2008). Evidence that a phenotypic trait is selected for also relies on the existence of correlations between a given trait and fitness of individuals in natural environments (Garland and Carter, 1994). Just three studies, however, have examined the relationship between plasma corticosterone responses to emotional stressors and fitness measures in wild birds, whilst no study has investigated the relationship between corticosterone responses to physical stressors and fitness. Magnitudes of plasma corticosterone responses to capture and restraint were negatively correlated with survival in European white storks (Ciconia ciconia; Blas et al., 2007), and with the likelihood of returning to breed the following year in male song sparrows (Melospiza melodia; MacDougall-Shackleton et al., 2009), whilst magnitudes of corticosterone responses to capture and restraint were positively correlated with the likelihood of American redstarts (Setophaga ruticilla) returning to their non-breeding territory the following winter, although this relationship depended on habitat type (Angelier et al., 2009). Whilst the results of these studies appear contradictory, they support the notion that there is no optimal corticosterone response for all environments (see Cockrem, 2005; Cockrem et al., 2009), and that individuals with relatively low or high corticosterone responses may do better depending on the current environmental conditions. The present study provides the first indication that measuring plasma corticosterone responses of birds on one occasion generally reflects the phenotypic response of that individual to a particular class of stressor, and further studies are clearly required to determine how consistent individual differences in plasma corticosterone responses to emotional stressors and to physical stressors relate to fitness in wild birds.

There was no relationship between magnitudes of plasma corticosterone responses to emotional and physical stressors in individual birds. For example, some quail showed relatively low corticosterone responses to handling and confinement but high responses to treatment with insulin or LPS, and some birds with relatively high corticosterone responses to handling and confinement had relatively low responses to treatment with insulin or LPS. Although there is empirical evidence from studies in mammals to suggest that the neural circuitry involved in the generation of stress responses differs for emotional and physical stressors (Herman and Cullinan, 1997; Day et al., 1999; Dayas et al., 2001; Herman et al., 2003; Reyes et al., 2003), there is no previous evidence from studies in birds to suggest that differences in central processing translate into variation

in magnitudes of corticosterone responses to different classes of stressor. In addition to differences in central processing, other factors could contribute to the disparity in responses of individual birds to emotional and physical stressors. For example, whilst the present study has shown that individuals can display consistently low or high corticosterone responses to emotional stressors and to physical stressors, there is evidence that animals can also demonstrate phenotypic plasticity, where, for example, the physiological responses of individuals can change as a result of environmental cues (Dufty et al., 2002). Indeed, there is recent interest in how maternal effects can regulate the developmental environment within avian eggs to alter plasma corticosterone responses in offspring. Hayward and Wingfield (2004) treated female quail with corticosterone and found higher corticosterone concentrations in the yolk of eggs from treated females than in eggs from untreated controls, and offspring from treated birds had higher plasma corticosterone responses to restraint than offspring from control birds. Furthermore, wood duck (Aix sponsa) ducklings that hatched from eggs that were artificially incubated at a temperature at the low end of the natural range had higher plasma corticosterone responses to restraint compared to ducklings that hatched from eggs incubated at temperatures at the mid to high end of the natural range (DuRant et al., 2010). These studies show that the developmental environment can alter corticosterone responses of birds to emotional stressors, suggesting that there is some plasticity in this phenotypic trait. Environmental effects could alter functioning of the avian HPA axis, in which case responses to both emotional and physical stressors would be affected. However, it is likely that environmental effects can change the way in which individuals perceive stressors, which would alter magnitudes of responses to emotional stressors more so than responses to physical stressors. Differences in neural processing combined with varying effects of the developmental environmental, therefore, are two factors that could result in disparity in magnitudes of plasma corticosterone responses of individual birds to emotional and physical stressors.

The results of the present study show for the first time in birds that although responses to emotional and physical stressors share a common physiological pathway in that they both involve activation of the HPA axis, birds may possess at least two quite distinct stress responses i.e. a stress response that helps individuals to cope with threatening events and a stress response that helps individuals to cope with actual physical challenges. The current study was conducted in domesticated Japanese quail, and it is

likely that magnitudes of corticosterone responses to emotional stressors are also unrelated to magnitudes of corticosterone responses to physical stressors in individuals of free-living species of birds. Whilst plasma corticosterone responses of individual quail to the two classes of stressor were unrelated, quail demonstrated consistent individual differences in their plasma corticosterone responses to a particular class of stressor (i.e. emotional or physical). Repeatable individual variation within populations provides the raw material on which the processes of natural selection act (Garland and Carter, 1994; Wingfield et al., 2008), and if correlations exist in free-living species of birds between some measures of fitness and magnitudes of corticosterone responses to emotional stressors, and between other measures of fitness and magnitudes of plasma corticosterone responses to physical stressors, then it is possible that different selection pressures act on responses of birds to the two classes of stressor (see Garland and Carter, 1994). In order to examine possible relationships between fitness and corticosterone responses to physical stressors in free-living birds, however, researchers will need to look past the standard procedure of measuring the responses of birds to the emotional stressor of capture and restraint. Indeed, capture and restraint is perhaps only used so widely as a standardised stressor in free-living birds because of its simplicity and relative ease with which it can be applied to individuals from different populations and species (see Wingfield et al., 1994; Silverin, 1998). Plasma corticosterone responses to physical stressors have not been measured in wild free-living species of birds, and there are some practical challenges involved with measuring the responses of birds to physical stressors in the field. Characterising corticosterone responses of wild free-living birds to treatment with insulin or LPS, for example, requires each bird to be captured so that an initial blood sample can be taken, and for the bird to be treated with insulin or LPS. The bird would then be released and then recaptured within hours for a second blood sample, which might be easier in birds that occupy smaller territories. Another issue to address is what physical stressor would be most suitable for examination of HPA axis activity in wild free-living species of birds. Insulin-induced hypoglycaemia is often used to stimulate glucocorticoid responses in tests of HPA axis function in mammals (Suda et al., 1992; Sapolsky et al., 2000; Pacak and Palkovits, 2001), and a fall in plasma glucose concentrations represents a natural physical challenge for animals. It is suggested therefore, that treatment with insulin is a suitable physical stressor to use in studies of corticosterone responses in free-living species of birds. Whilst there are some practical issues associated with characterising

corticosterone responses of birds to physical stressors in the field, the present study has provided the impetus and the framework on which studies of corticosterone responses to physical stressors in wild free-living birds can be based. Indeed, this area of research will benefit from further studies of corticosterone responses of birds to physical stressors, and of possible relationships between corticosterone responses to physical stressors and the fitness of birds in their natural environments.

This study of corticosterone, fear behaviour and plasma corticosterone responses to stressors in Japanese quail has provided important new information on stress and the effects of stress in birds. As birds are exposed to increasing numbers of stressors of anthropogenic origin, particularly though climate change and human disturbance, knowledge about the effects of stress in birds will be crucial in ensuring the continued existence of countless species of birds, and of other groups of animals. Very important findings of the present study were that whilst quail displayed consistent individual differences in their plasma corticosterone responses to emotional stressors and to physical stressors, magnitudes of corticosterone responses to both classes of stressor were unrelated in individual birds. Studies of avian stress endocrinology are based on the assumption that corticosterone responses to standardised artificial emotional stressors reflect the responsiveness of the HPA axis of birds to stressors in general. The present study in Japanese quail, however, provides the first indication that this assumption is incorrect, and has highlighted that birds may possess at least two quite distinct stress responses to help them cope with changes in their environments.

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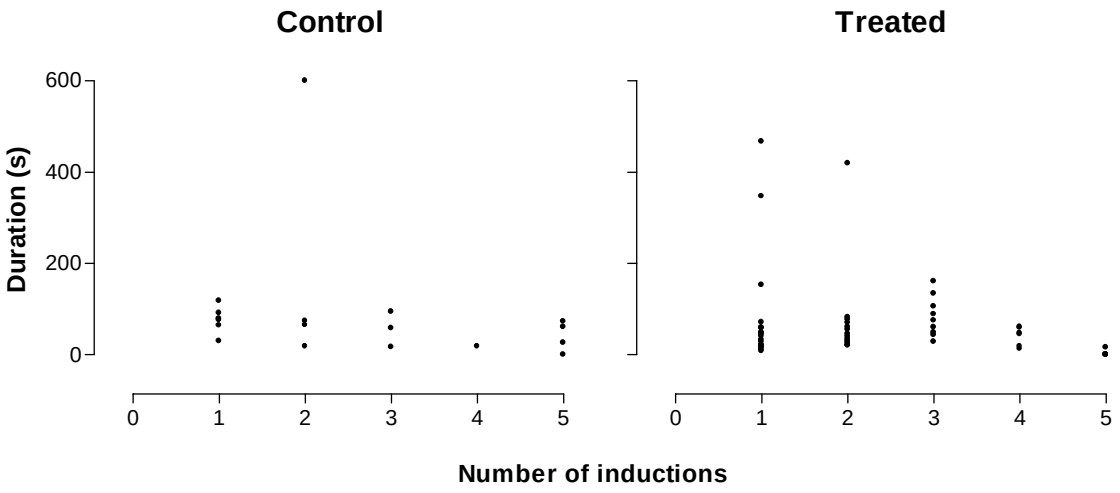
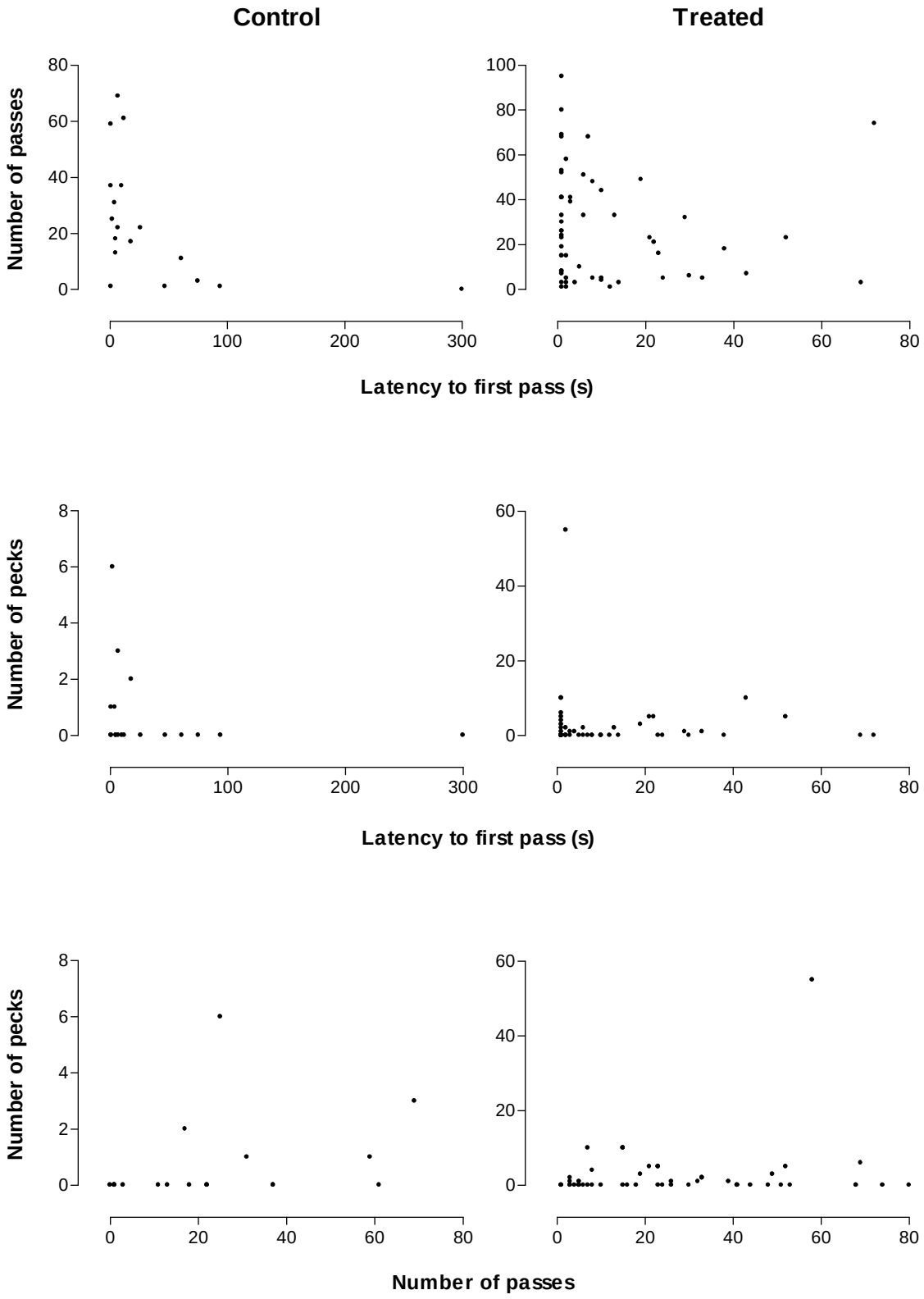
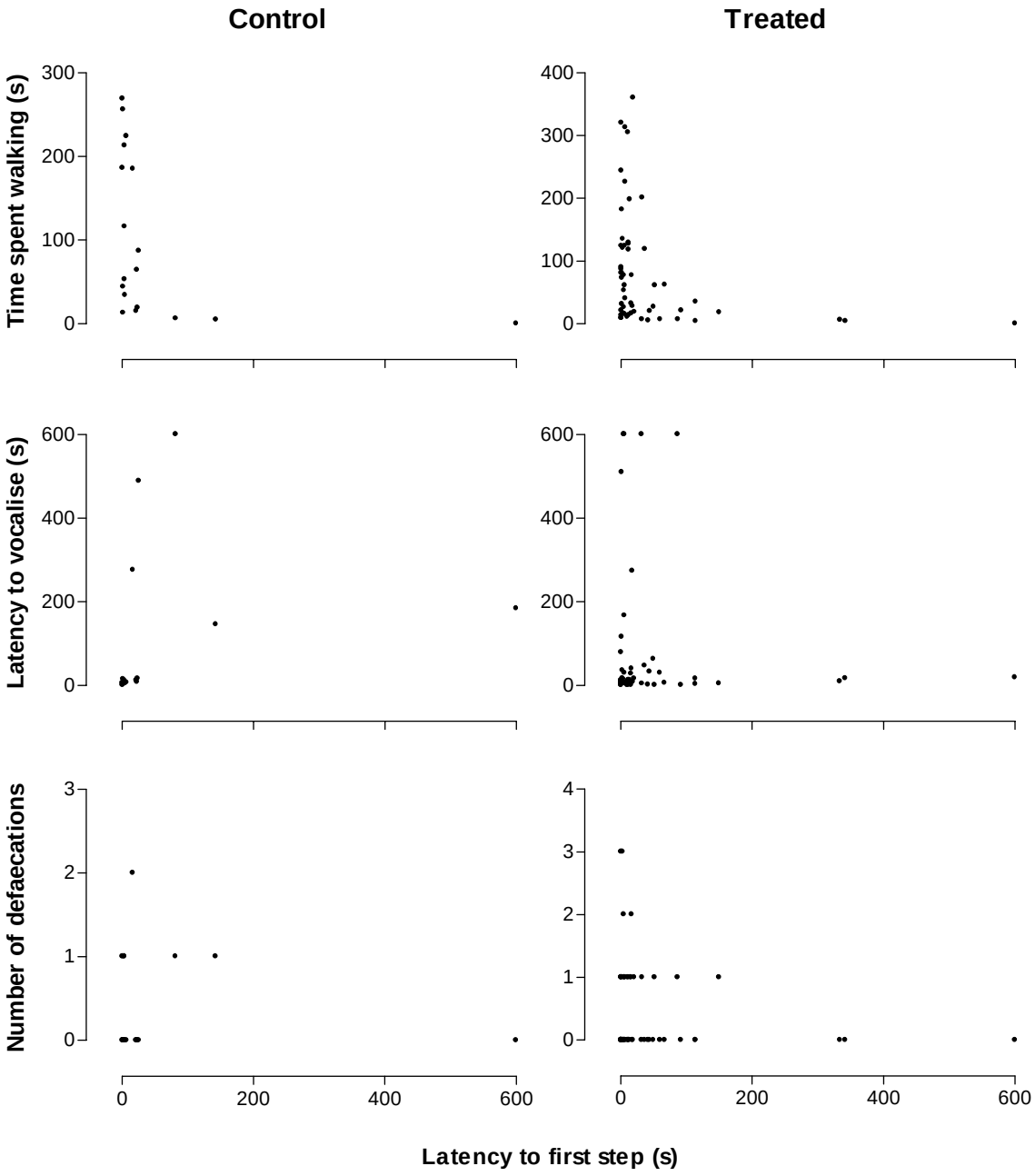
# Appendix figures and tables



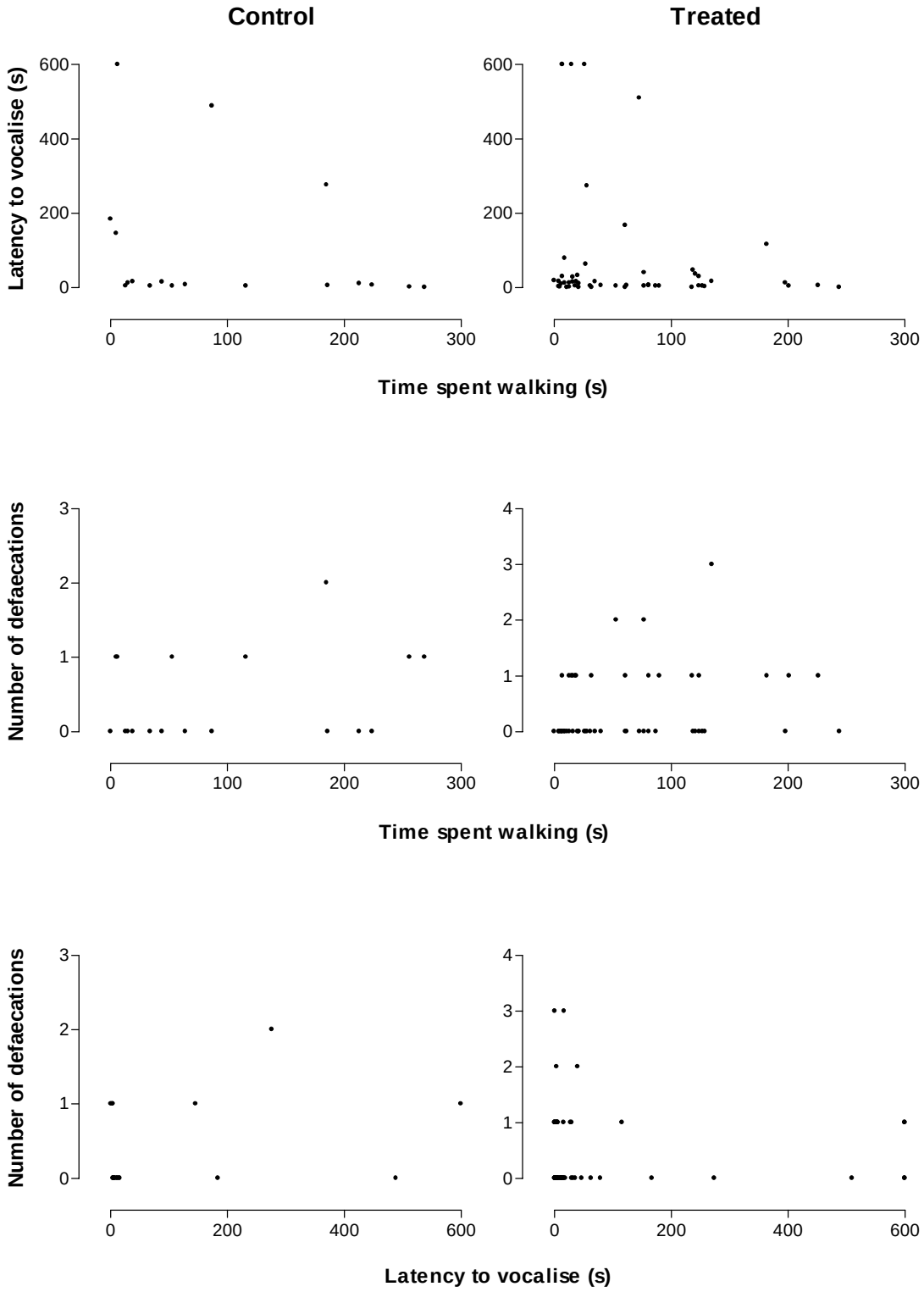

**Appendix Figure 2.1.** Relationships between the two behaviour variables of the first tonic immobility test in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. See Table 2.1 and 2.2 for Pearson statistics.



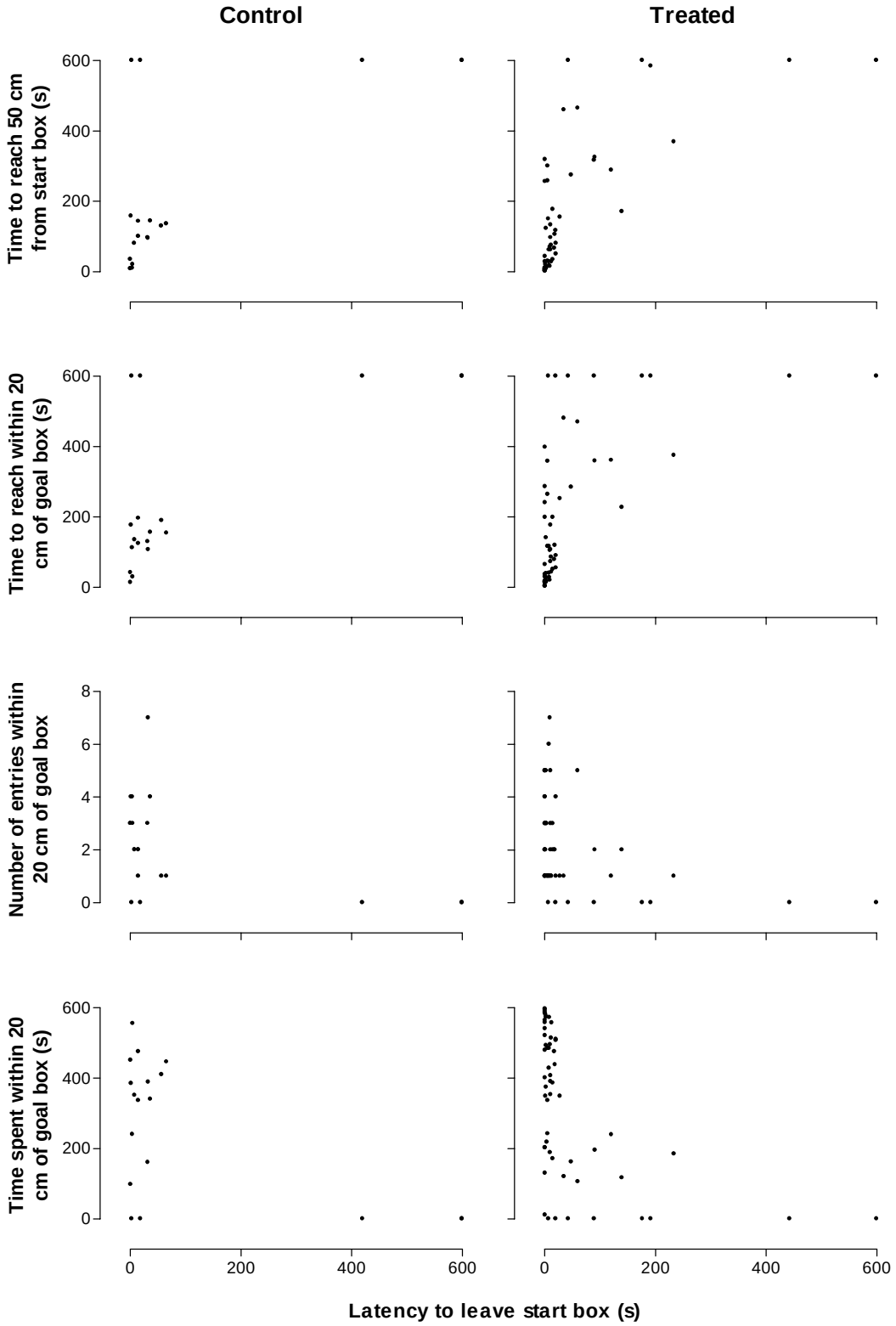

**Appendix Figure 2.2.** Relationships between the three behaviour variables of the novel object test in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. See Table 2.1 and 2.2 for Pearson statistics.



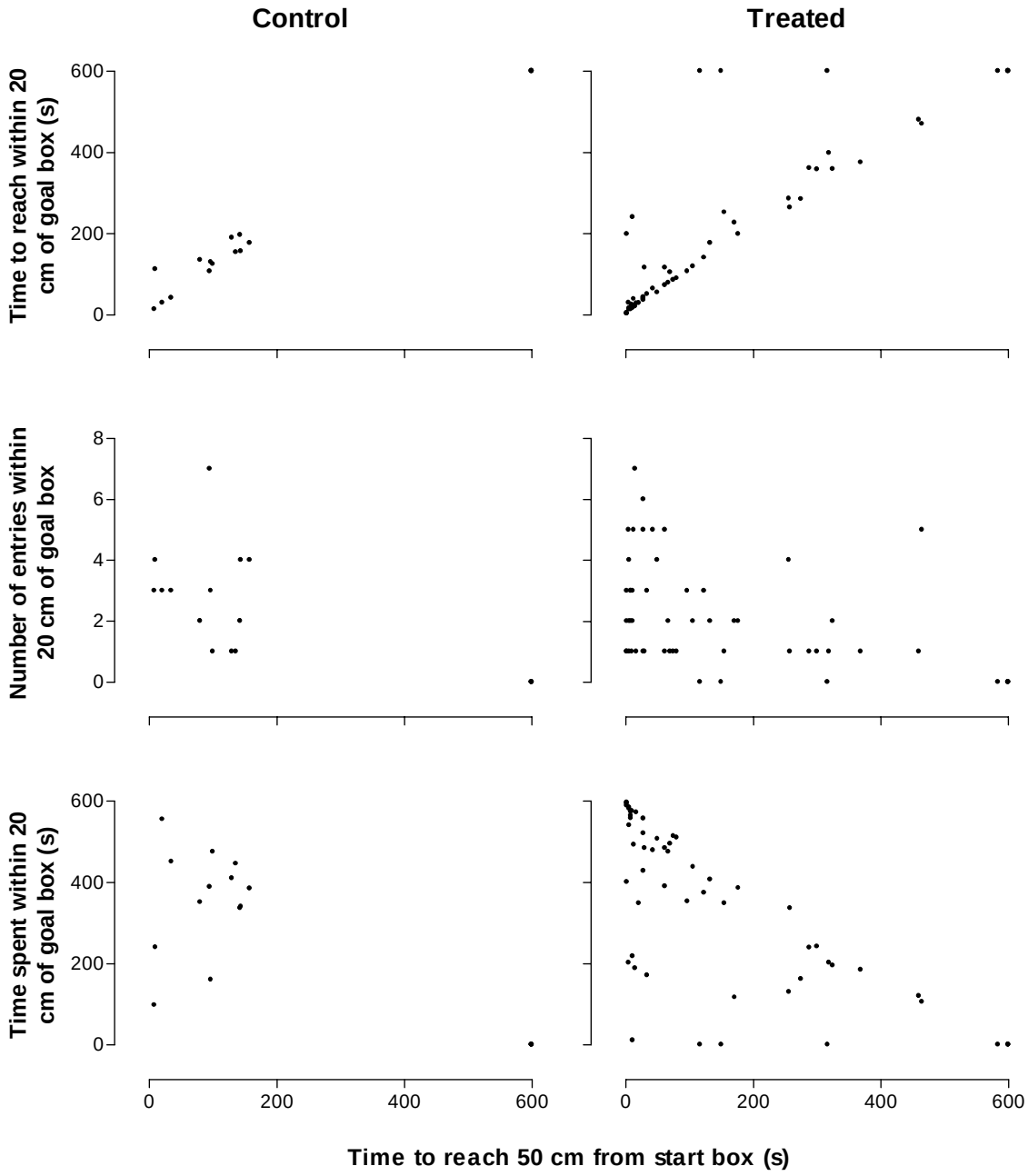

**Appendix Figure 2.3.** Relationships between latency to first step and the other three behaviour variables of the open field test in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. See Table 2.1 and 2.2 for Pearson statistics.



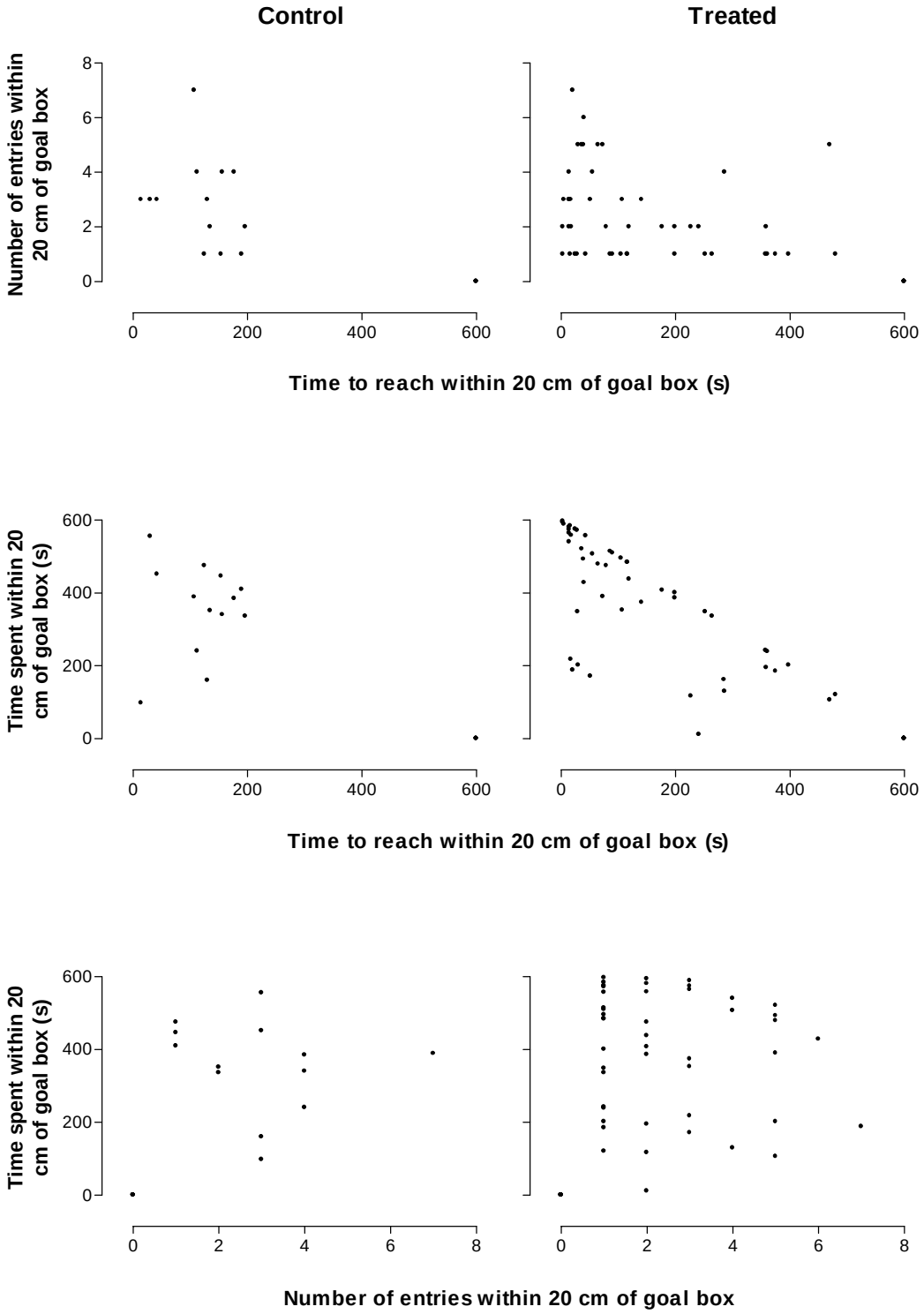

**Appendix Figure 2.4.** Relationships of time spent walking with latency to vocalise and the number of defaecations and the latency to vocalise with the number of defaecations during the open field test in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. See Table 2.1 and 2.2 for Pearson statistics.



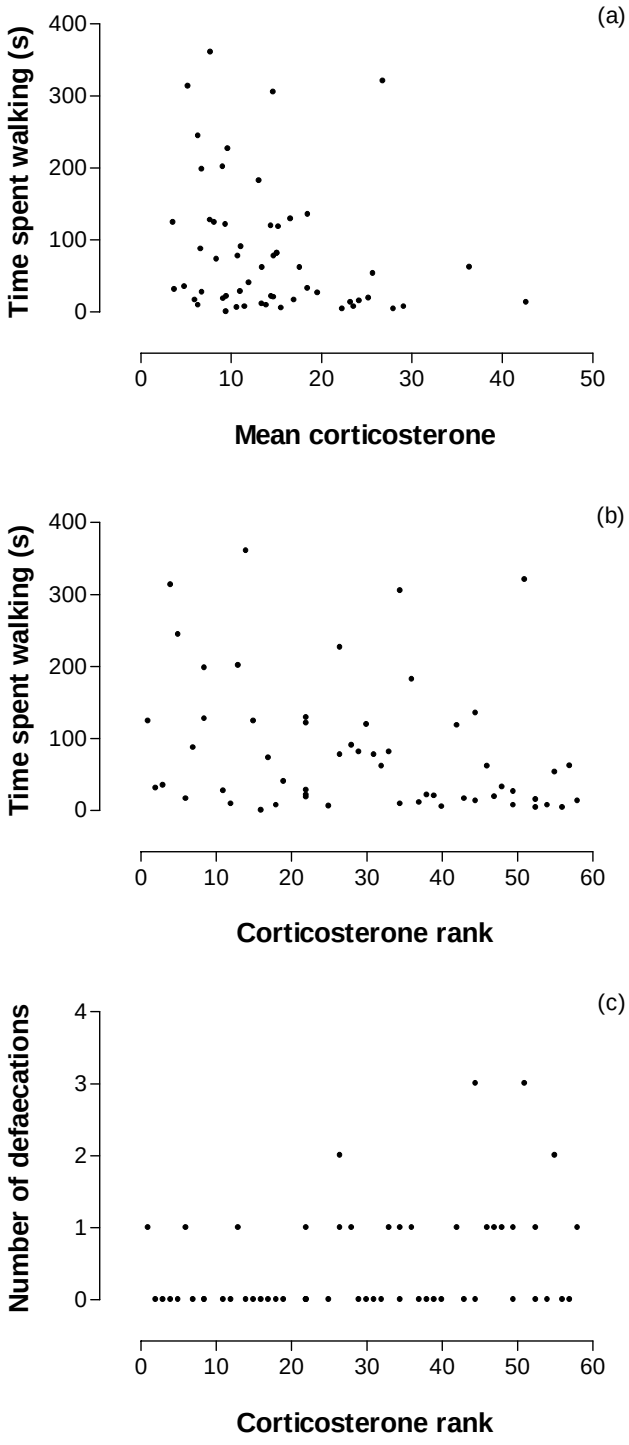

**Appendix Figure 2.5.** Relationships between the latency to leave the start box and the other four behaviour variables of the runway test in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. See Table 2.3 for Pearson statistics.



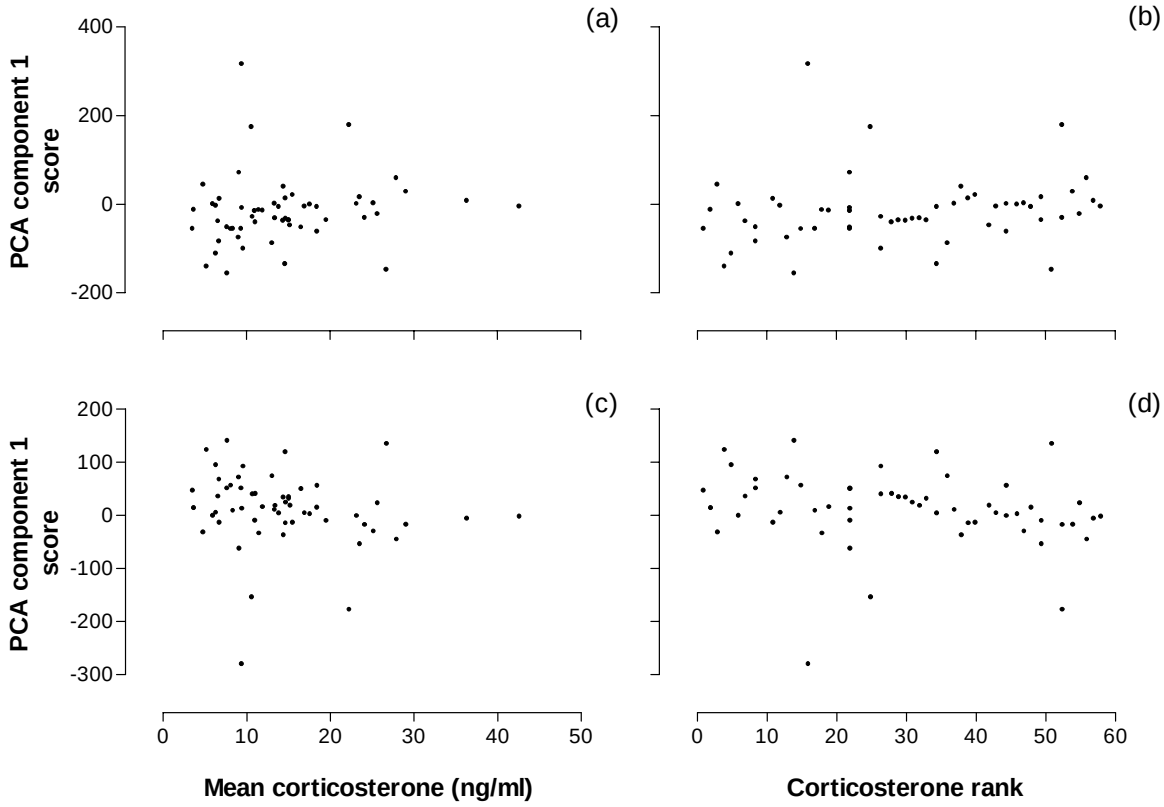

**Appendix Figure 2.6.** Relationships of time to reach 50 cm from start box with time to reach within 20 cm of goal box, the number of entries within 20 cm of goal box and the time spent within 20 cm of goal box during the runway test in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. See Table 2.3 for Pearson statistics.



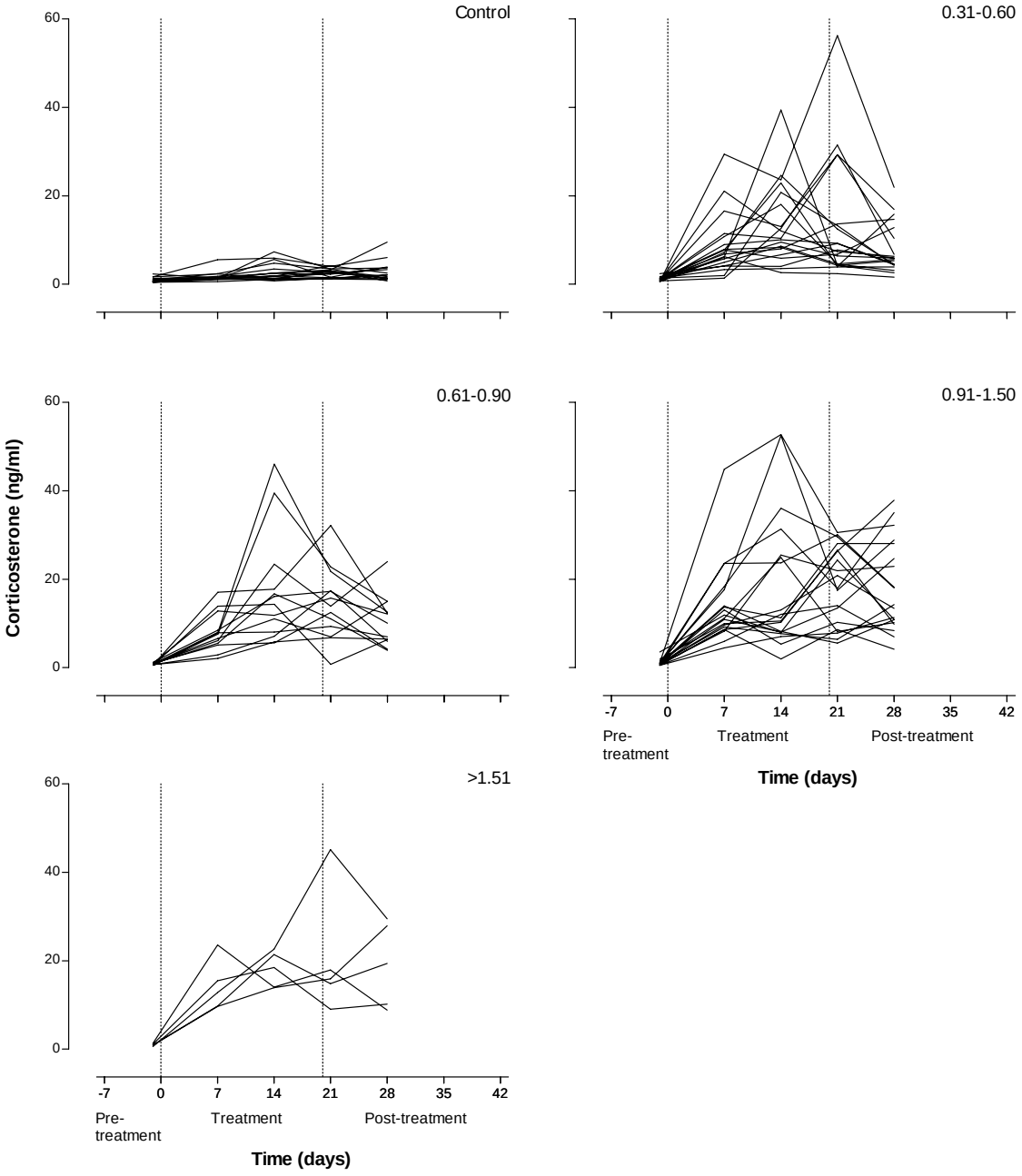

**Appendix Figure 2.7.** Relationships of time to reach within 20 cm of goal box with number of entries within 20 cm of goal box and time spent within 20 cm of goal box and number of entries within 20 cm of goal box with time spent within 20 cm of goal box during the runway test in control quail and in quail treated with corticosterone. Control, N = 18; treated, N = 56. See Table 2.3 for Pearson statistics.



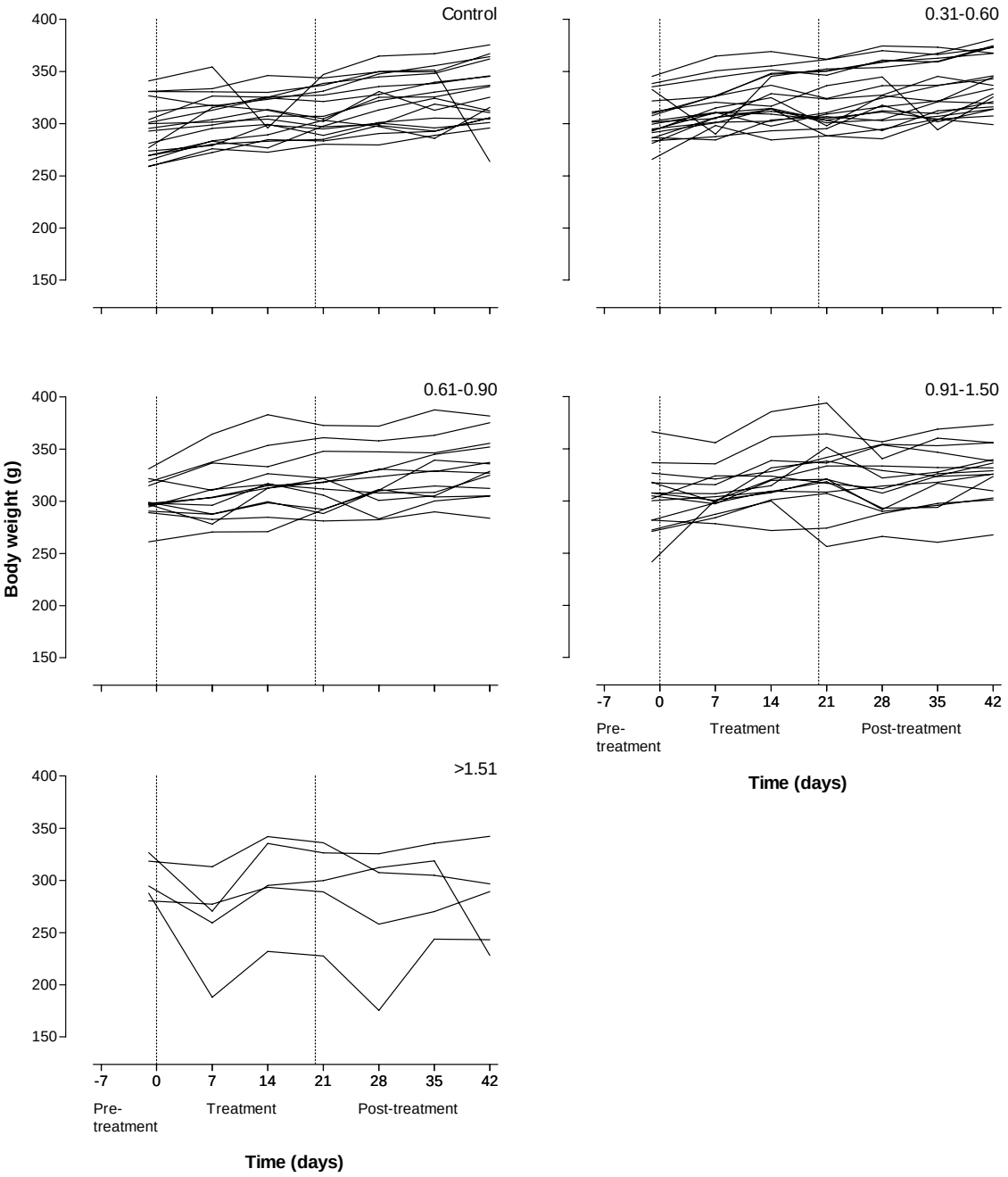

**Appendix Figure 2.8.** Significant relationships of variables from the four behaviour tests with corticosterone for treated birds. Mean plasma corticosterone concentration (ng/ml) over days 7, 14 and 21 vs (a) time spent walking in the open field test; overall corticosterone rank for days 7, 14 and 21 vs (b) time spent walking in the open field test and (c) number of defaecations in the open field test. N = 56. See Appendix Table 2.7 for Spearman statistics.



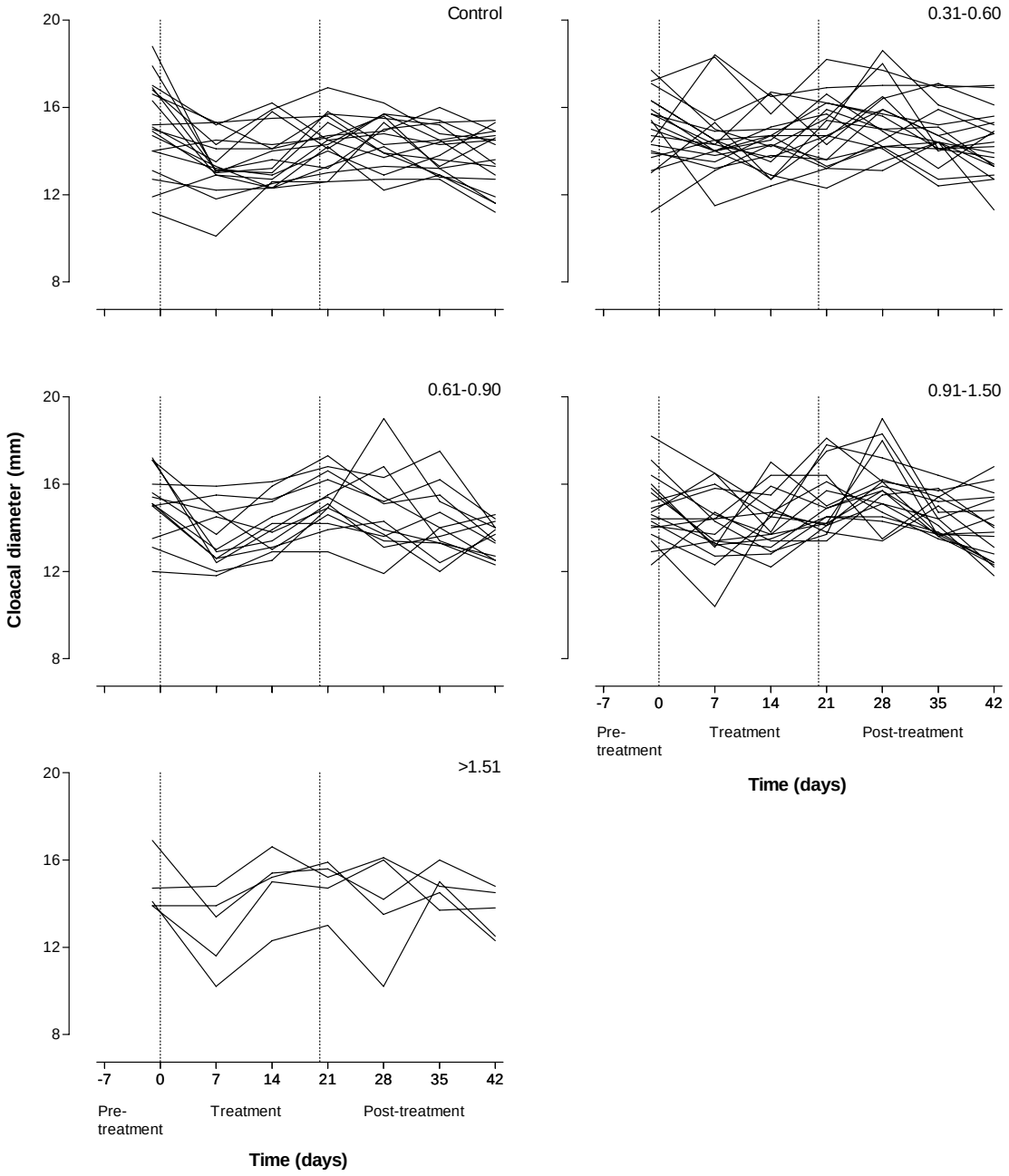

**Appendix Figure 2.9.** Significant relationships of variables from the four behaviour tests with corticosterone for treated birds. Mean plasma corticosterone concentration (ng/ml) over days 7, 14 and 21 vs (a) PCA component 1 score for the open field test and (c) PCA component 1 score for the three fear behaviour tests combined; overall corticosterone rank for days 7, 14 and 21 vs (b) PCA component 1 score for the open field test and (d) PCA component 1 score for the three fear behaviour tests combined. N = 56. See Appendix Table 2.8 for Spearman statistics.



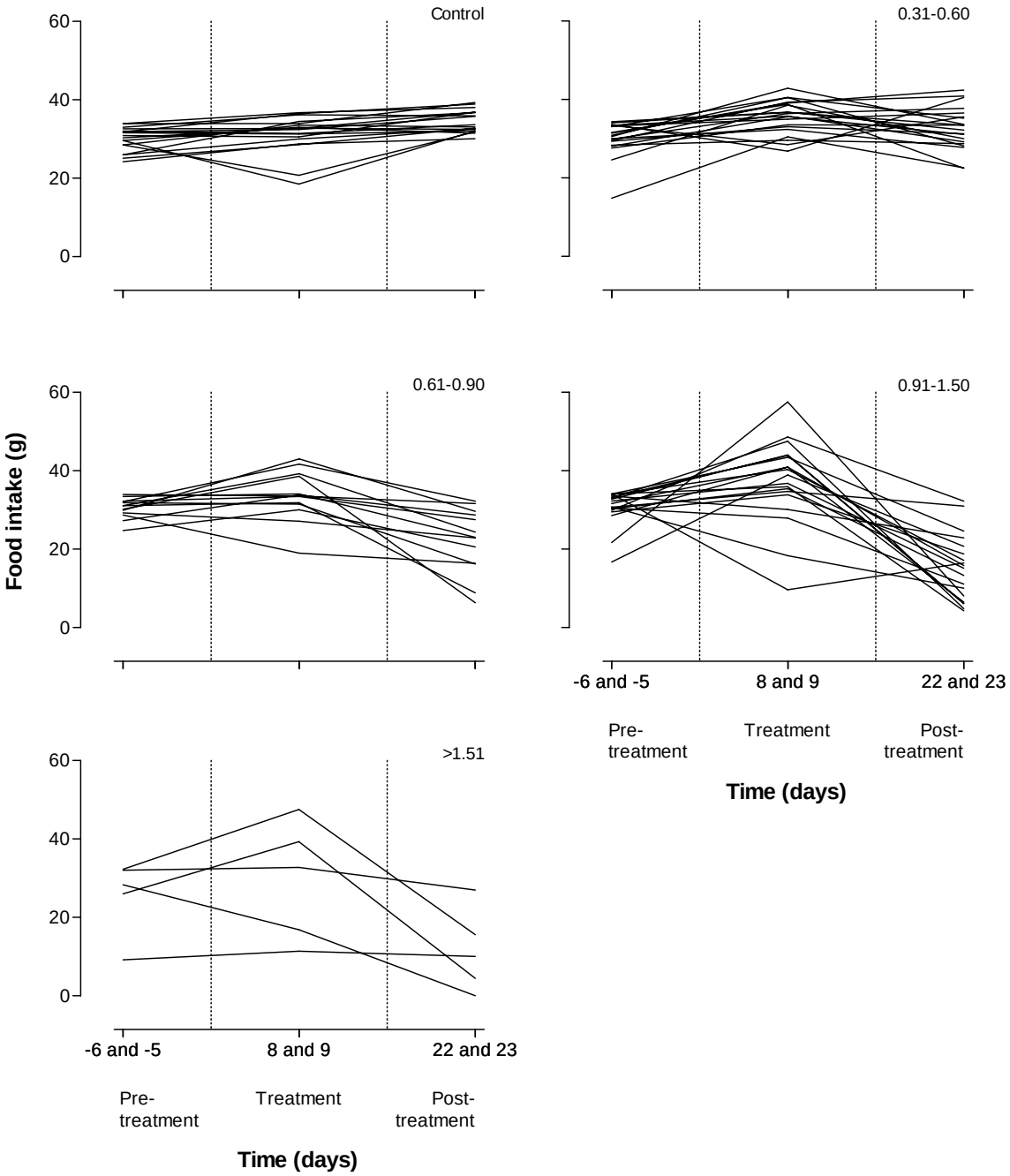

**Appendix Figure 3.1.** Individual plasma corticosterone concentrations in control quail and in quail treated with corticosterone. Control, N = 17; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. Initial samples were collected one day before corticosterone treatment began, and vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment.



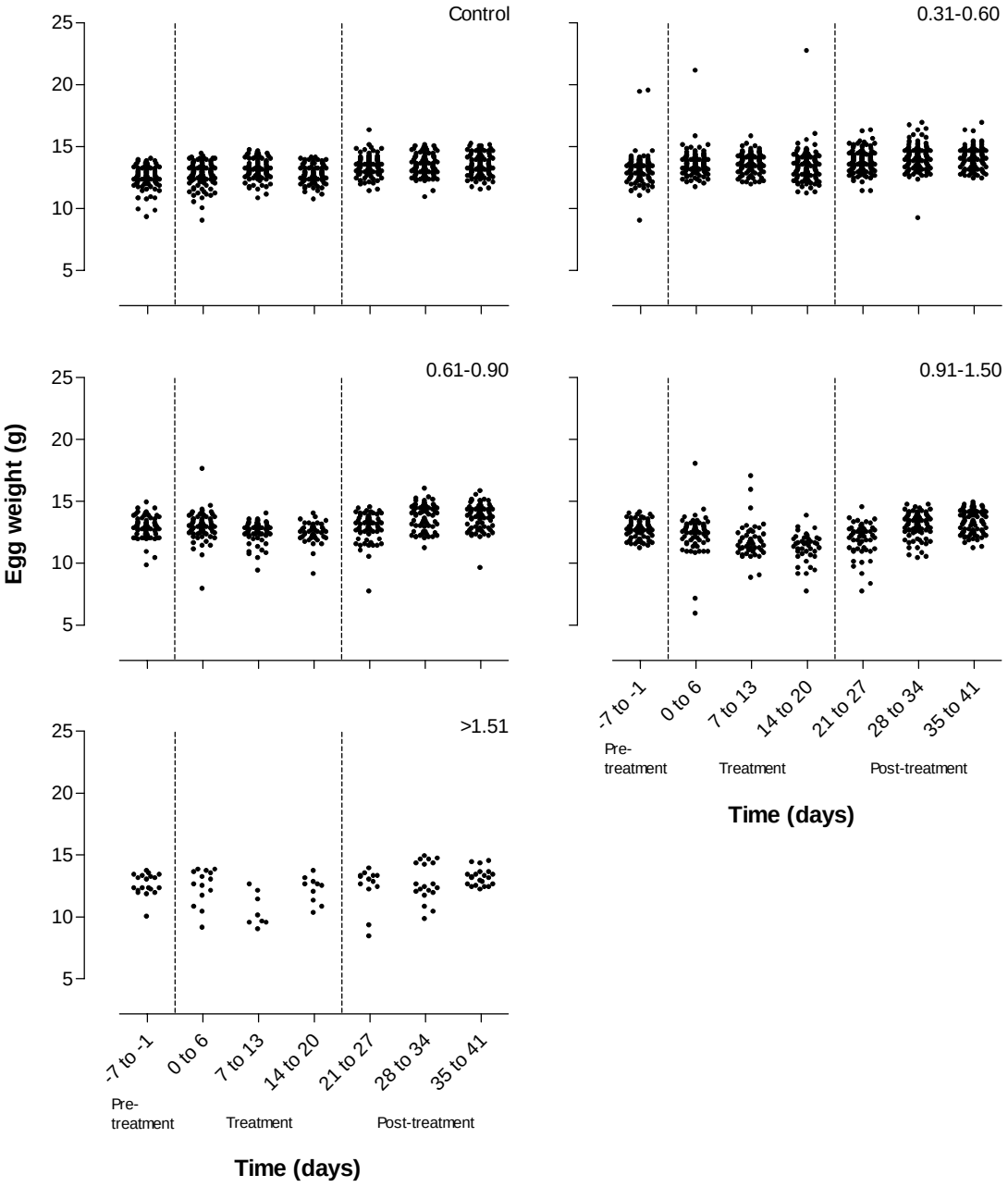

**Appendix Figure 3.2.** Individual body weights in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. Initial measurements were taken one day before corticosterone treatment began, and vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment.



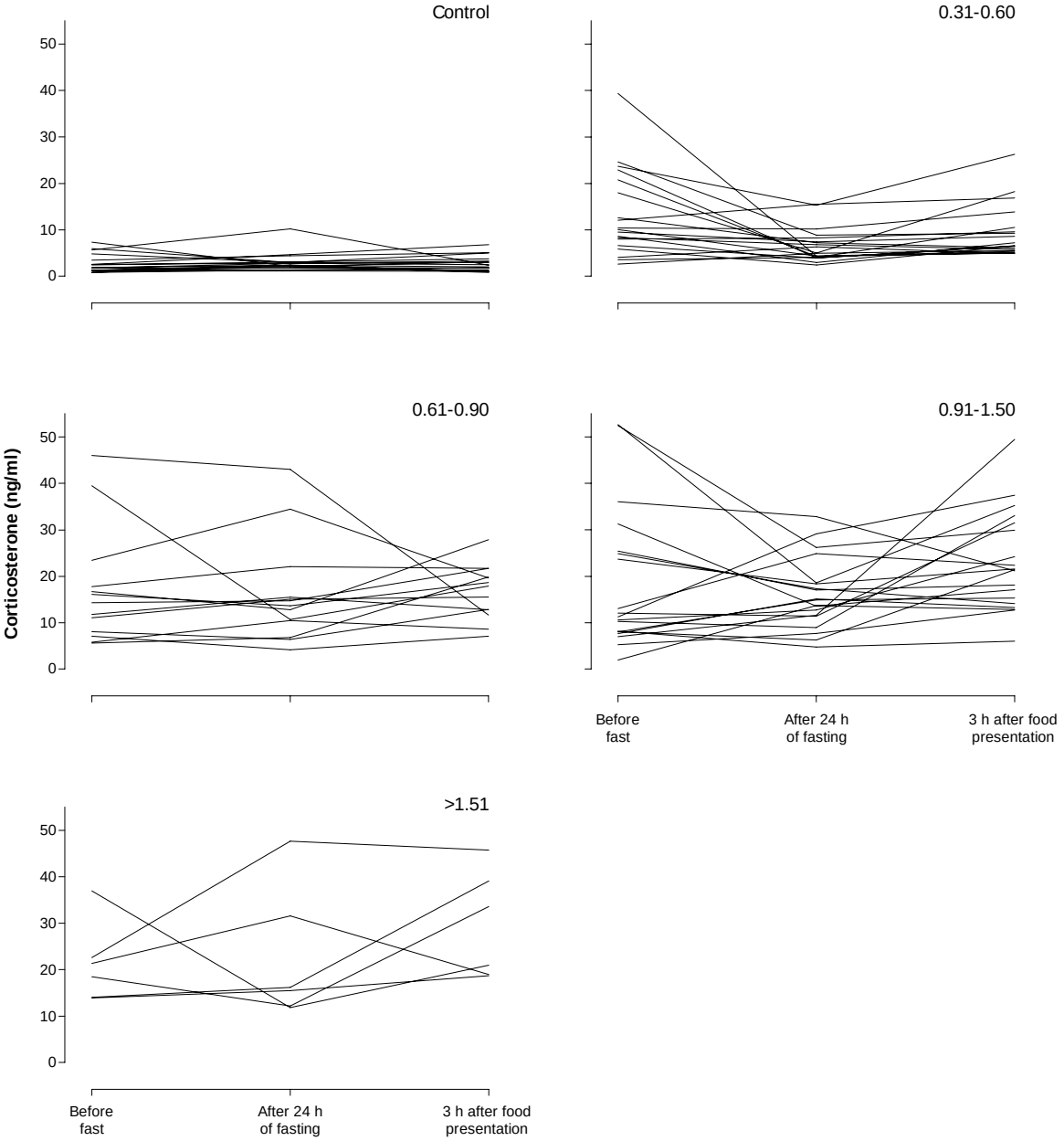

**Appendix Figure 3.3.** Individual cloacal diameters in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. Initial measurements were taken one day before corticosterone treatment began, and vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment.



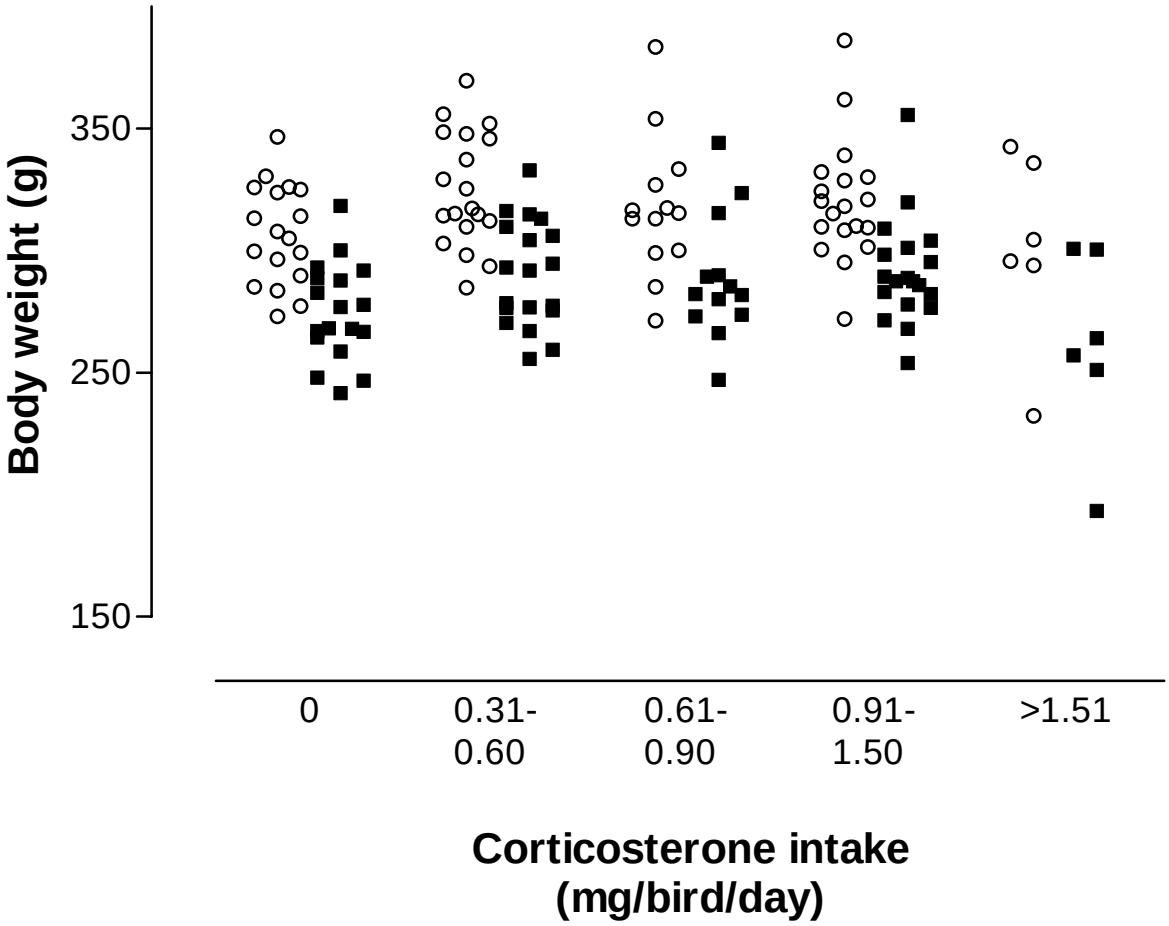

**Appendix Figure 3.4.** Individual food intakes over 24 h in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. Vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment.



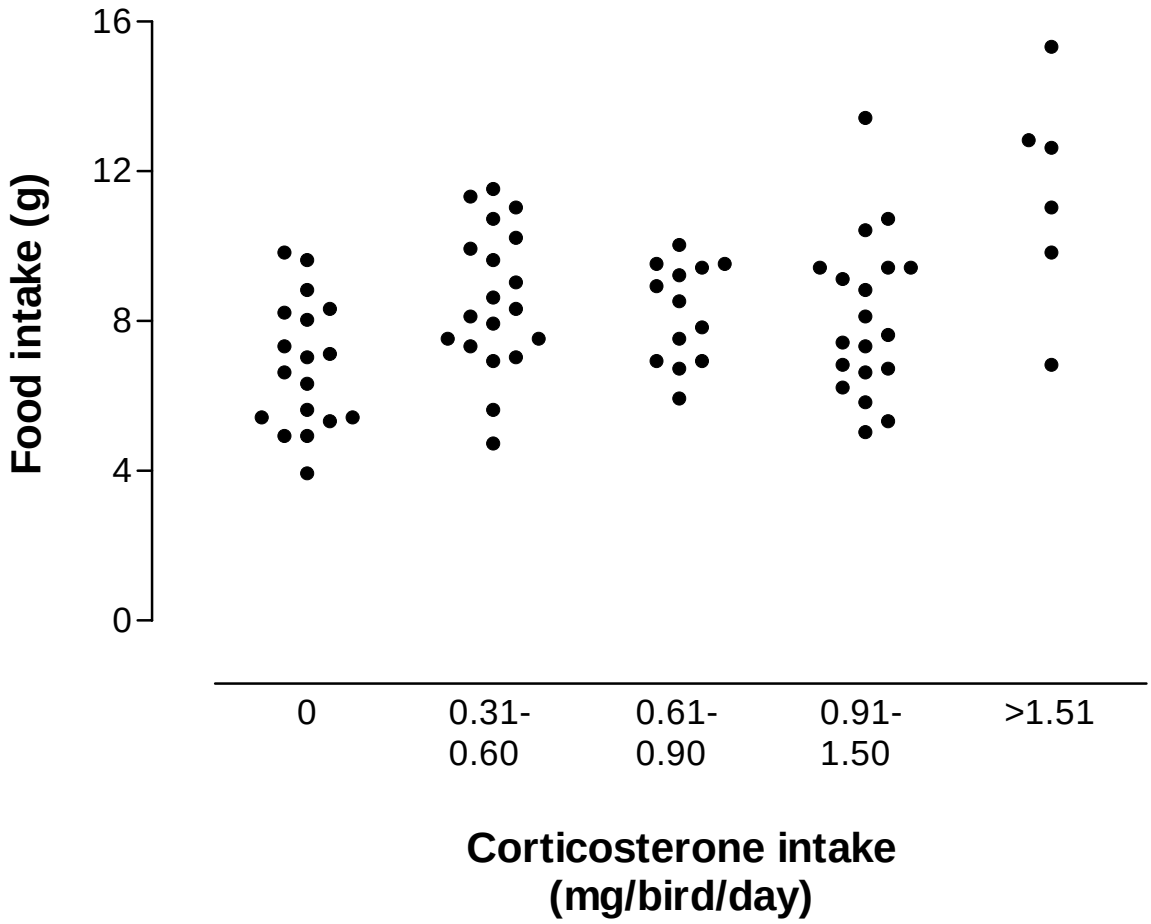

**Appendix Figure 3.5.** Individual egg weights in control quail and in quail treated with corticosterone. Control, N = 17; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 11; 0.91-1.50 mg corticosterone/bird/day, N = 12; >1.51 mg corticosterone/bird/day, N = 3. Vertical dotted lines denote the beginning (day 0) and end (day 20) of corticosterone treatment.



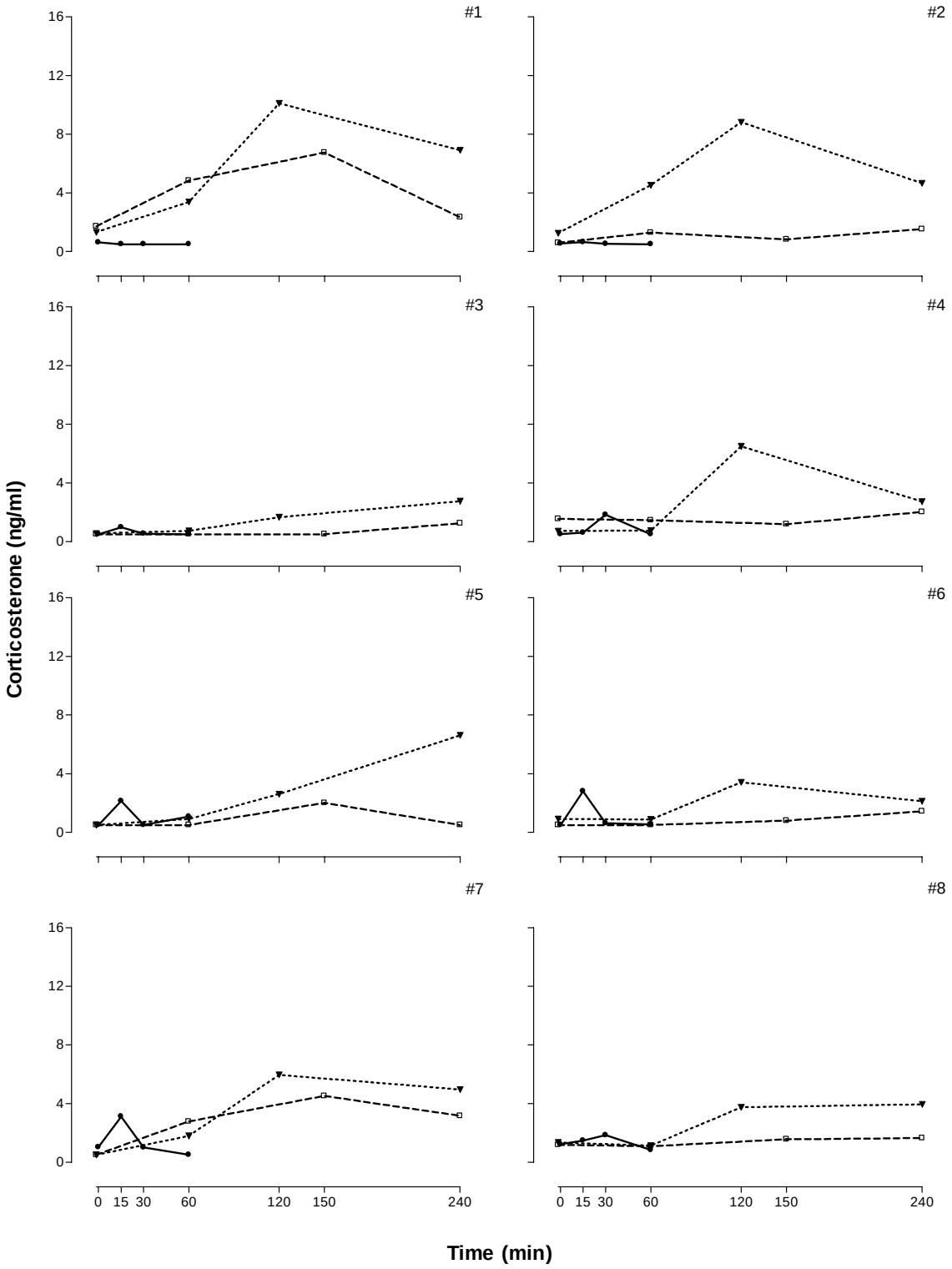

**Appendix Figure 4.1.** Individual plasma corticosterone concentrations before and after 24 h of fasting and 3 h after food presentation in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6.



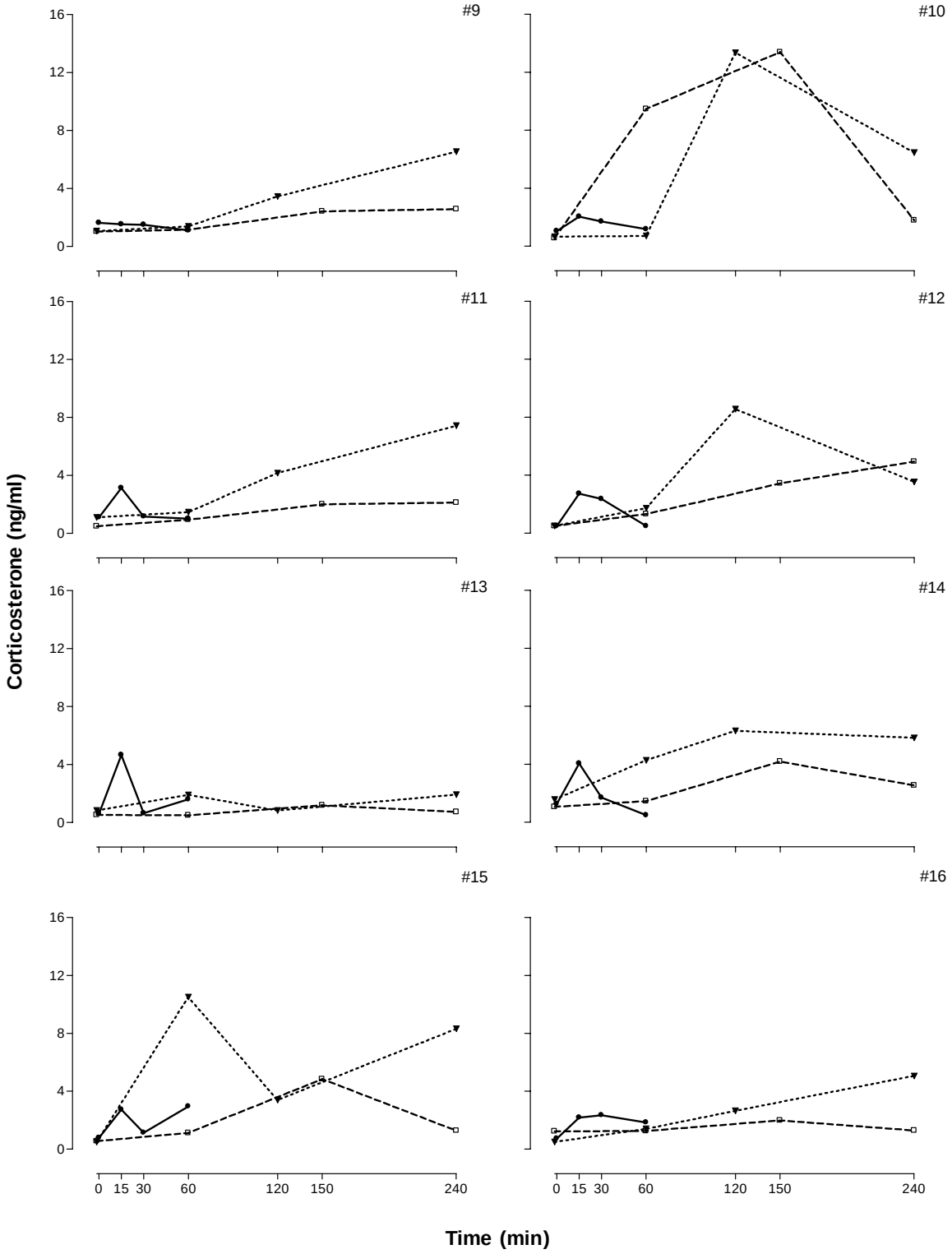

**Appendix Figure 4.2.** Individual body weights before (open circles) and after (closed squares) 24 h of fasting in control quail and in quail treated with corticosterone. Control (0), N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6.



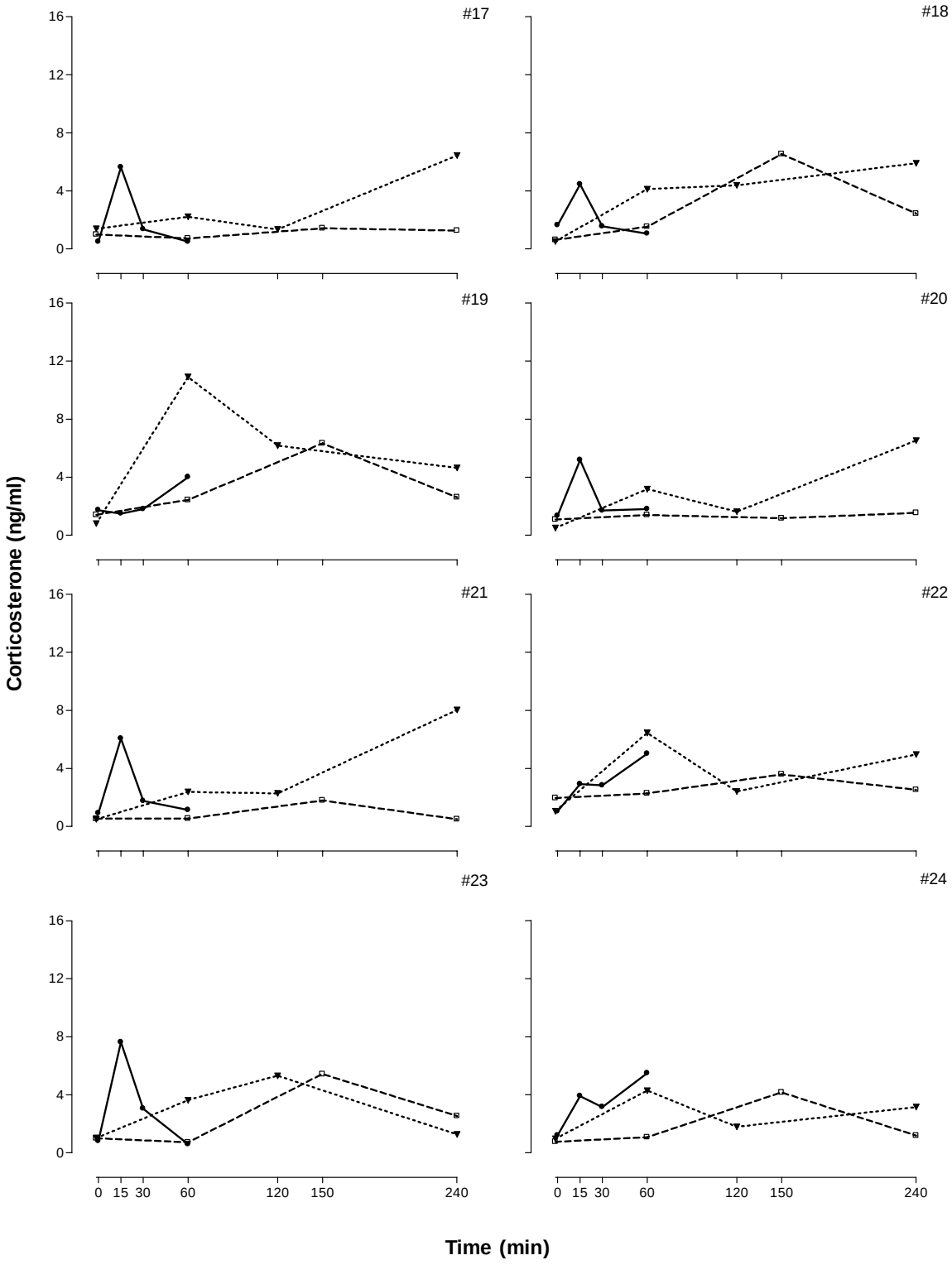

**Appendix Figure 4.3.** Individual food intakes in the three hours after 24 h of fasting in control quail and in quail treated with corticosterone. Control (0), N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6.



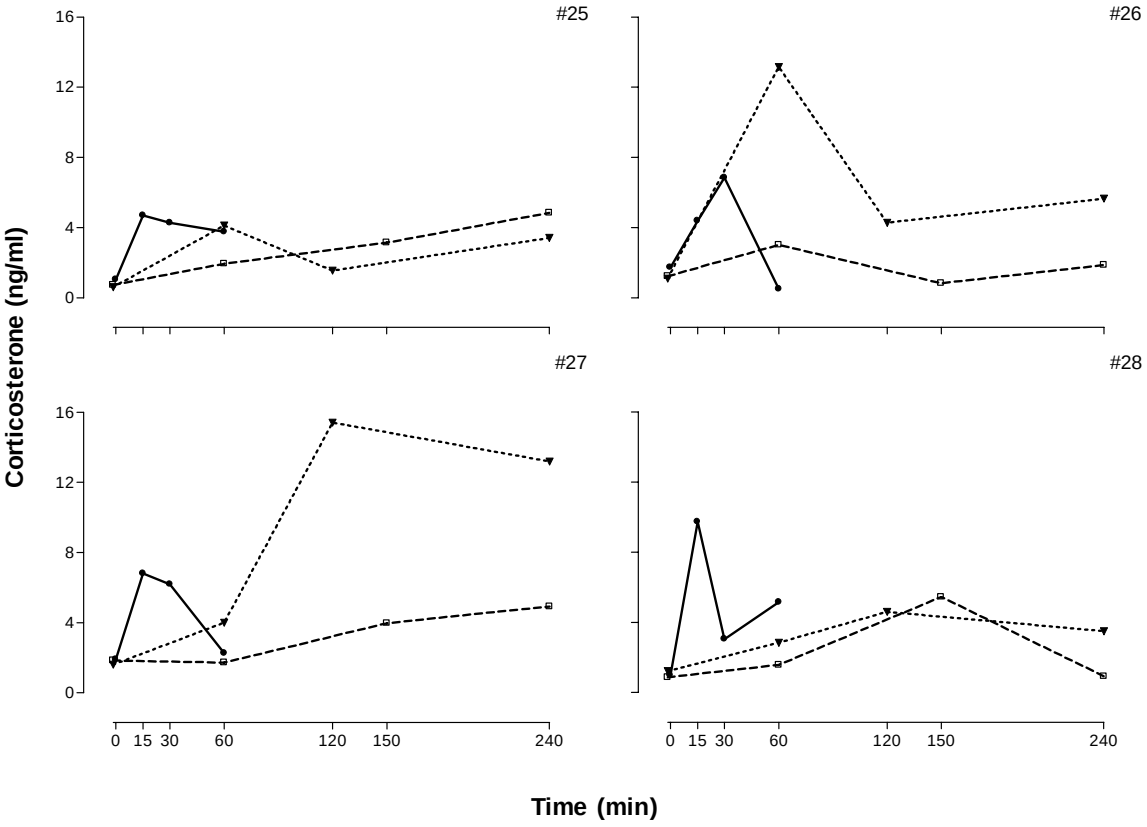

**Appendix Figure 7.1.** Individual plasma corticosterone responses in quail handled for 15 min, or treated with 4 IU/kg of insulin or 1.2 mg/kg of lipopolysaccharide (LPS). Birds are arranged in order of lowest to highest values for total area under the corticosterone response curve during handling. Handling, —●—; insulin, - - - □ - - -; LPS, ·····▼·····. N = 28.




**Appendix Figure 7.1 continued.** Individual plasma corticosterone responses in quail handled for 15 min, or treated with 4 IU/kg of insulin or 1.2 mg/kg of lipopolysaccharide (LPS). Birds are arranged in order of lowest to highest values for total area under the corticosterone response curve during handling. Handling, —●—; insulin, - - - □ - - -; LPS, ·····▼·····. N = 28.




**Appendix Figure 7.1 continued.** Individual plasma corticosterone responses in quail handled for 15 min, or treated with 4 IU/kg of insulin or 1.2 mg/kg of lipopolysaccharide (LPS). Birds are arranged in order of lowest to highest values for total area under the corticosterone response curve during handling. Handling, —●—; insulin, - - - □ - - -; LPS, ·····▼·····. N = 28.




**Appendix Figure 7.1 continued.** Individual plasma corticosterone responses in quail handled for 15 min, or treated with 4 IU/kg of insulin or 1.2 mg/kg of lipopolysaccharide (LPS). Birds are arranged in order of lowest to highest values for total area under the corticosterone response curve during handling. Handling, —●—; insulin, - - - □ - - -; LPS, ·····▼·····. N = 28.

**Appendix Table 2.1.** Kruskal-Wallis one way ANOVA analyses of variables from four tests of behaviour in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| Tonic immobility | | | |
| Number of inductions | 6.101 | 4 | 0.192 |
| Duration | 3.566 | 4 | 0.468 |
| Novel object | | | |
| Latency to first pass | 6.231 | 4 | 0.183 |
| Number of passes | 2.568 | 4 | 0.633 |
| Number of pecks | 1.500 | 4 | 0.827 |
| Open field | | | |
| Latency to first step | 1.357 | 4 | 0.852 |
| Time spent walking | 2.147 | 4 | 0.709 |
| Latency to vocalise | 3.370 | 4 | 0.498 |
| Number of defaecations | 15.014 | 4 | 0.005* |
| Runway | | | |
| Latency to leave start box | 5.145 | 4 | 0.273 |
| Time to reach 50 cm from start box | 4.101 | 4 | 0.393 |
| Time to reach within 20 cm of goal box | 2.470 | 4 | 0.650 |
| Number of entries within 20 cm of goal box | 3.586 | 4 | 0.465 |
| Time spent within 20 cm of goal box | 4.496 | 4 | 0.343 |

**Appendix Table 2.2.** Kruskal-Wallis one way ANOVA analyses of PCA component scores and fear score ranks from four tests of behaviour in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| Tonic immobility | | | |
| PCA component 1 score | 2.972 | 4 | 0.563 |
| Fear score rank | 2.600 | 4 | 0.627 |
| Novel object | | | |
| PCA component 1 score | 1.374 | 4 | 0.849 |
| Fear score rank | 3.377 | 4 | 0.497 |
| Open field | | | |
| PCA component 1 score | 3.741 | 4 | 0.442 |
| PCA component 2 score | 0.454 | 4 | 0.978 |
| Fear score rank | 3.418 | 4 | 0.491 |
| Runway | | | |
| PCA component 1 score | 4.227 | 4 | 0.376 |

**Appendix Table 2.3.** Kruskal-Wallis one way ANOVA analyses of combined PCA component scores from three tests of fear behaviour in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| PCA component scores for tonic immobility, novel object and open field tests combined | | | |
| Component 1 | 1.159 | 4 | 0.885 |
| Component 2 | 0.094 | 4 | 0.999 |
| Component 3 | 7.205 | 4 | 0.125 |
| Component 4 | 0.683 | 4 | 0.953 |

**Appendix Table 2.4.** Mann-Whitney comparisons of control quail with each group of quail treated with corticosterone for all variables from four tests of behaviour. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | 0.31-0.60 | | 0.61-0.90 | | 0.91-1.50 | | >1.51 | |
|---|---|---|---|---|---|---|---|---|
| | U | p | U | p | U | p | U | p |
| Tonic immobility | | | | | | | | |
| Number of inductions | 155.0 | 0.617 | 108.0 | 0.712 | 203.5 | 0.299 | 65.0 | 0.119 |
| Duration | 220.0 | 0.132 | 139.0 | 0.378 | 221.5 | 0.125 | 57.0 | 0.371 |
| Novel object | | | | | | | | |
| Latency to first pass | 246.5 | 0.019* | 140.0 | 0.354 | 224.0 | 0.104 | 57.0 | 0.368 |
| Number of passes | 165.5 | 0.867 | 92.0 | 0.316 | 159.5 | 0.726 | 29.0 | 0.232 |
| Number of pecks | 138.5 | 0.247 | 110.0 | 0.726 | 153.5 | 0.522 | 39.0 | 0.582 |
| Open field | | | | | | | | |
| Latency to first step | 177.5 | 0.843 | 104.0 | 0.602 | 154.5 | 0.615 | 49.5 | 0.736 |
| Time spent walking | 176.0 | 0.879 | 129.0 | 0.631 | 198.5 | 0.403 | 39.0 | 0.655 |
| Latency to vocalise | 163.5 | 0.819 | 107.5 | 0.703 | 206.5 | 0.280 | 58.0 | 0.331 |
| Number of defaecations | 212.0 | 0.107 | 137.0 | 0.326 | 150.0 | 0.471 | 16.0 | 0.016* |
| Runway | | | | | | | | |
| Latency to leave start box | 181.0 | 0.761 | 159.0 | 0.091 | 219.5 | 0.138 | 53.5 | 0.526 |
| Time to reach 50 cm from start box | 204.5 | 0.308 | 157.5 | 0.104 | 227.5 | 0.085 | 48.0 | 0.821 |
| Time to reach within 20 cm of goal box | 204.0 | 0.314 | 129.5 | 0.614 | 227.5 | 0.085 | 47.5 | 0.851 |
| Number of entries within 20 cm of goal box | 133.0 | 0.242 | 118.5 | 0.951 | 170.5 | 0.988 | 56.0 | 0.401 |
| Time spent within 20 cm of goal box | 137.0 | 0.300 | 100.5 | 0.505 | 103.0 | 0.038* | 33.5 | 0.387 |

**Appendix Table 2.5.** Mann-Whitney comparisons of control quail with each group of quail treated with corticosterone for PCA component scores and fear score ranks from four tests of behaviour. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | 0.31-0.60 | | 0.61-0.90 | | 0.91-1.50 | | >1.51 | |
|---|---|---|---|---|---|---|---|---|
| | U | p | U | p | U | p | U | p |
| Tonic immobility | | | | | | | | |
| PCA component 1 score | 124.0 | 0.153 | 95.5 | 0.389 | 124.5 | 0.157 | 34.0 | 0.412 |
| Fear score rank | 213.0 | 0.202 | 130.0 | 0.603 | 179.0 | 0.808 | 46.0 | 0.941 |
| Novel object | | | | | | | | |
| PCA component 1 score | 146.5 | 0.457 | 95.0 | 0.378 | 154.0 | 0.605 | 31.0 | 0.297 |
| Fear score rank | 224.0 | 0.107 | 145.5 | 0.254 | 213.0 | 0.196 | 57.0 | 0.371 |
| Open field | | | | | | | | |
| PCA component 1 score | 181.0 | 0.761 | 133.0 | 0.522 | 210.0 | 0.236 | 35.0 | 0.456 |
| PCA component 2 score | 167.0 | 0.903 | 104.0 | 0.603 | 177.0 | 0.855 | 47.0 | 0.881 |
| Fear score rank | 163.5 | 0.820 | 99.0 | 0.471 | 174.5 | 0.915 | 61.0 | 0.233 |
| Runway | | | | | | | | |
| PCA component 1 score | 213.0 | 0.202 | 148.0 | 0.215 | 238.0 | 0.042* | 48.0 | 0.832 |

**Appendix Table 2.6.** Mann-Whitney comparisons of control quail with each group of quail treated with corticosterone for combined PCA component scores from three tests of fear behaviour. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | 0.31-0.60 | | 0.61-0.90 | | 0.91-1.50 | | >1.51 | |
|---|---|---|---|---|---|---|---|---|
| | U | p | U | p | U | p | U | p |
| PCA component scores for tonic immobility, novel object and open field tests combined | | | | | | | | |
| Component 1 | 174.0 | 0.927 | 132.0 | 0.548 | 187.0 | 0.429 | 43.0 | 0.881 |
| Component 2 | 167.0 | 0.903 | 123.0 | 0.810 | 176.0 | 0.879 | 49.0 | 0.766 |
| Component 3 | 208.0 | 0.261 | 127.0 | 0.689 | 228.0 | 0.083 | 31.0 | 0.297 |
| Component 4 | 150.0 | 0.523 | 99.0 | 0.471 | 169.0 | 0.952 | 39.0 | 0.655 |

**Appendix Table 2.7.** Spearman correlation coefficients between corticosterone and behaviour variables. Mean values of plasma corticosterone concentration (ng/ml) over days 7, 14 and 21 were calculated for each bird. Corticosterone concentrations on each day were also ranked within control and treated birds, then used to calculate an overall corticosterone rank for each bird for the three days. Correlations with behaviour variables were calculated for mean corticosterone concentrations and for corticosterone ranks. Control birds, N = 17; treated birds, N = 56. *Indicates a significant result ($p < 0.05$).

| | Control | | | | Treated | | | |
|---|---|---|---|---|---|---|---|---|
| | Corticosterone | | Corticosterone rank | | Corticosterone | | Corticosterone rank | |
| | r | p | r | p | r | p | r | p |
| Tonic immobility | | | | | | | | |
| Number of inductions | 0.109 | 0.677 | 0.219 | 0.398 | -0.015 | 0.913 | -0.085 | 0.535 |
| Duration | -0.232 | 0.371 | -0.279 | 0.279 | -0.163 | 0.231 | -0.164 | 0.227 |
| Novel object | | | | | | | | |
| Latency to first pass | -0.064 | 0.808 | -0.040 | 0.879 | 0.087 | 0.526 | 0.114 | 0.401 |
| Number of passes | -0.072 | 0.782 | -0.104 | 0.692 | -0.015 | 0.915 | 0.003 | 0.980 |
| Number of pecks | -0.014 | 0.958 | 0.047 | 0.857 | -0.011 | 0.935 | 0.018 | 0.897 |
| Open field | | | | | | | | |
| Latency to first step | 0.059 | 0.822 | 0.100 | 0.702 | 0.127 | 0.352 | 0.131 | 0.337 |
| Time spent walking | -0.093 | 0.722 | -0.150 | 0.567 | -0.308 | 0.021* | -0.347 | 0.009* |
| Latency to vocalise | -0.241 | 0.352 | -0.132 | 0.615 | -0.079 | 0.564 | -0.060 | 0.663 |
| Number of defaecations | -0.310 | 0.225 | -0.323 | 0.206 | 0.263 | 0.051 | 0.304 | 0.023* |
| Runway | | | | | | | | |
| Latency to leave start box | -0.012 | 0.963 | 0.046 | 0.861 | 0.175 | 0.197 | 0.179 | 0.187 |
| Time to reach 50 cm from start box | -0.122 | 0.641 | -0.194 | 0.456 | 0.190 | 0.160 | 0.206 | 0.127 |
| Time to reach within 20 cm of goal box | -0.149 | 0.568 | -0.139 | 0.594 | 0.113 | 0.406 | 0.140 | 0.302 |
| Number of entries within 20 cm of goal box | 0.208 | 0.422 | 0.199 | 0.444 | -0.146 | 0.283 | -0.214 | 0.113 |
| Time spent within 20 cm of goal box | 0.191 | 0.462 | 0.021 | 0.936 | -0.119 | 0.381 | -0.124 | 0.362 |

**Appendix Table 2.8.** Spearman correlation coefficients between corticosterone variables and PCA component scores for individual and combined behaviour tests and between corticosterone variables and fear score ranks. Mean values of plasma corticosterone concentration (ng/ml) over days 7, 14 and 21 were calculated for each bird. Corticosterone concentrations on each day were also ranked within control and treated birds, then used to calculate an overall corticosterone rank for each bird for the three days. Correlations with PCA component scores and fear score ranks were calculated for mean corticosterone concentrations and for corticosterone ranks. Control birds, N = 17; treated birds, N = 56. *Indicates a significant result ($p < 0.05$).

| | Control | | | | Treated | | | |
|---|---|---|---|---|---|---|---|---|
| | Corticosterone | | Corticosterone rank | | Corticosterone | | Corticosterone rank | |
| | r | p | r | p | r | p | r | p |
| PCA component scores for individual behaviour tests | | | | | | | | |
| Tonic immobility component 1 | 0.232 | 0.371 | 0.286 | 0.267 | 0.156 | 0.251 | 0.153 | 0.262 |
| Novel object component 1 | 0.037 | 0.889 | -0.007 | 0.978 | -0.005 | 0.972 | 0.014 | 0.917 |
| Open field component 1 | 0.088 | 0.736 | 0.163 | 0.532 | 0.269 | 0.045* | 0.308 | 0.021* |
| Open field component 2 | -0.373 | 0.141 | -0.438 | 0.079 | -0.122 | 0.370 | -0.149 | 0.274 |
| Runway component 1 | -0.088 | 0.736 | -0.004 | 0.989 | 0.166 | 0.223 | 0.188 | 0.165 |
| PCA component scores for tonic immobility, novel object and open field tests combined | | | | | | | | |
| Component 1 | 0.363 | 0.152 | 0.359 | 0.157 | -0.304 | 0.023* | -0.332 | 0.012* |
| Component 2 | -0.338 | 0.184 | -0.428 | 0.087 | -0.078 | 0.566 | -0.060 | 0.659 |
| Component 3 | 0.152 | 0.560 | 0.022 | 0.933 | -0.117 | 0.390 | -0.164 | 0.228 |
| Component 4 | 0.186 | 0.474 | 0.324 | 0.205 | 0.152 | 0.263 | 0.173 | 0.202 |
| Fear score rank | | | | | | | | |
| Tonic immobility | -0.210 | 0.419 | -0.309 | 0.227 | -0.087 | 0.525 | -0.049 | 0.723 |
| Novel object | 0.022 | 0.933 | 0.021 | 0.935 | 0.092 | 0.500 | 0.086 | 0.530 |
| Open field | 0.018 | 0.944 | 0.059 | 0.822 | 0.088 | 0.517 | 0.096 | 0.483 |

**Appendix Table 3.1.** Statistical analyses of plasma corticosterone concentrations in control quail and in quail treated with corticosterone. Control, N = 17; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| Effect | F | df | p |
|---|---|---|---|
| Group | 40.327 | 4,69 | <0.001* |
| Time | 209.217 | 4,276 | <0.001* |
| Interaction of group and time | 8.319 | 16,276 | <0.001* |
| | | | |
| Comparisons between times for each group | | | |
| Day -1[a] vs day 7[b] | | | |
| control | 10.070 | 1,69 | 0.002* |
| 0.31-0.60 | 148.694 | 1,69 | <0.001* |
| 0.61-0.90 | 130.555 | 1,69 | <0.001* |
| 0.91-1.50 | 276.653 | 1,69 | <0.001* |
| >1.51 | 77.224 | 1,69 | <0.001* |
| | | | |
| Day -1[a] vs day 14[c] | | | |
| control | 22.769 | 1,69 | <0.001* |
| 0.31-0.60 | 164.498 | 1,69 | <0.001* |
| 0.61-0.90 | 163.898 | 1,69 | <0.001* |
| 0.91-1.50 | 214.156 | 1,69 | <0.001* |
| >1.51 | 67.489 | 1,69 | <0.001* |
| | | | |
| Day -1[a] vs day 21[d] | | | |
| control | 26.485 | 1,69 | <0.001* |
| 0.31-0.60 | 146.719 | 1,69 | <0.001* |
| 0.61-0.90 | 146.854 | 1,69 | <0.001* |
| 0.91-1.50 | 251.660 | 1,69 | <0.001* |
| >1.51 | 70.042 | 1,69 | <0.001* |
| | | | |
| Day 14[c] vs day 21[d] | | | |
| control | 0.081 | 1,69 | 0.777 |
| 0.31-0.60 | 0.925 | 1,69 | 0.340 |
| 0.61-0.90 | 0.869 | 1,69 | 0.355 |
| 0.91-1.50 | 0.911 | 1,69 | 0.343 |
| >1.51 | 0.000 | 1,69 | 0.998 |
| | | | |
| Day 21[d] vs day 28[e] | | | |
| control | 0.146 | 1,69 | 0.704 |
| 0.31-0.60 | 5.189 | 1,69 | 0.026* |
| 0.61-0.90 | 1.372 | 1,69 | 0.246 |
| 0.91-1.50 | 0.035 | 1,69 | 0.851 |
| >1.51 | 0.015 | 1,69 | 0.904 |

Table continued on next page

**Appendix Table 3.1 continued.** Statistical analyses of plasma corticosterone concentrations in control quail and in quail treated with corticosterone. Control, N = 17; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Comparisons between control and treatment groups | | | |
| Day -1[a] | | | |
| control vs 0.31-0.60 | 1.793 | 1,69 | 0.185 |
| control vs 0.61-0.90 | 0.439 | 1,69 | 0.510 |
| control vs 0.91-1.50 | 0.024 | 1,69 | 0.878 |
| control vs >1.51 | 0.042 | 1,69 | 0.837 |
| Day 7[b] | | | |
| control vs 0.31-0.60 | 62.208 | 1,69 | <0.001* |
| control vs 0.61-0.90 | 51.235 | 1,69 | <0.001* |
| control vs 0.91-1.50 | 116.432 | 1,69 | <0.001* |
| control vs >1.51 | 55.016 | 1,69 | <0.001* |
| Day 14[c] | | | |
| control vs 0.31-0.60 | 44.317 | 1,69 | <0.001* |
| control vs 0.61-0.90 | 48.814 | 1,69 | <0.001* |
| control vs 0.91-1.50 | 57.230 | 1,69 | <0.001* |
| control vs >1.51 | 32.845 | 1,69 | <0.001* |
| Day 21[d] | | | |
| control vs 0.31-0.60 | 33.546 | 1,69 | <0.001* |
| control vs 0.61-0.90 | 37.153 | 1,69 | <0.001* |
| control vs 0.91-1.50 | 67.067 | 1,69 | <0.001* |
| control vs >1.51 | 32.097 | 1,69 | <0.001* |
| Day 28[e] | | | |
| control vs 0.31-0.60 | 25.548 | 1,69 | <0.001* |
| control vs 0.61-0.90 | 36.966 | 1,69 | <0.001* |
| control vs 0.91-1.50 | 85.585 | 1,69 | <0.001* |
| control vs >1.51 | 40.582 | 1,69 | <0.001* |

Note: The first three rows show the results of two way repeated measures ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons between times for each group and between groups at each time. [a] Day before corticosterone treatment began; [b] 7th day of corticosterone treatment; [c] 14th day of corticosterone treatment; [d] day after corticosterone treatment ended; [e] 8 days after corticosterone treatment ended.

**Appendix Table 3.2.** Statistical analyses of body weights in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| Effect | F | df | p |
|---|---|---|---|
| Group | 2.309 | 4,69 | 0.066 |
| Time | 27.278 | 6,414 | <0.001* |
| Interaction of group and time | 3.671 | 24,414 | <0.001* |
| | | | |
| Comparisons between times for each group | | | |
| Day -1[a] vs day 21[d] | | | |
| control | 15.910 | 1,69 | <0.001* |
| 0.31-0.60 | 17.561 | 1,69 | <0.001* |
| 0.61-0.90 | 12.015 | 1,69 | 0.001* |
| 0.91-1.50 | 19.396 | 1,69 | <0.001* |
| >1.51 | 0.519 | 1,69 | 0.474 |
| | | | |
| Day 21[d] vs day 42[e] | | | |
| control | 12.125 | 1,69 | 0.001* |
| 0.31-0.60 | 18.767 | 1,69 | <0.001* |
| 0.61-0.90 | 7.661 | 1,69 | 0.007* |
| 0.91-1.50 | 1.128 | 1,69 | 0.292 |
| >1.51 | 5.453 | 1,69 | 0.067 |
| | | | |
| Comparisons between control and treatment groups | | | |
| Day -1[a] | | | |
| control vs 0.31-0.60 | 1.775 | 1,69 | 0.187 |
| control vs 0.61-0.90 | 0.649 | 1,69 | 0.423 |
| control vs 0.91-1.50 | 1.473 | 1,69 | 0.229 |
| control vs >1.51 | 0.415 | 1,69 | 0.522 |
| | | | |
| Day 7[b] | | | |
| control vs 0.31-0.60 | 1.437 | 1,69 | 0.235 |
| control vs 0.61-0.90 | 0.009 | 1,69 | 0.926 |
| control vs 0.91-1.50 | 0.006 | 1,69 | 0.939 |
| control vs >1.51 | 11.619 | 1,69 | 0.001* |
| | | | |
| Day 14[c] | | | |
| control vs 0.31-0.60 | 4.185 | 1,69 | 0.045* |
| control vs 0.61-0.90 | 1.329 | 1,69 | 0.253 |
| control vs 0.91-1.50 | 2.470 | 1,69 | 0.121 |
| control vs >1.51 | 0.261 | 1,69 | 0.611 |

Table continued on next page

**Appendix Table 3.2 continued.** Statistical analyses of body weights in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Comparisons between control and treatment groups | | | |
| Day 21[d] | | | |
| control vs 0.31-0.60 | 1.257 | 1,69 | 0.266 |
| control vs 0.61-0.90 | 0.517 | 1,69 | 0.475 |
| control vs 0.91-1.50 | 1.473 | 1,69 | 0.229 |
| control vs >1.51 | 1.093 | 1,69 | 0.300 |
| Day 42[e] | | | |
| control vs 0.31-0.60 | 1.978 | 1,69 | 0.164 |
| control vs 0.61-0.90 | 0.371 | 1,69 | 0.545 |
| control vs 0.91-1.50 | 0.002 | 1,69 | 0.962 |
| control vs >1.51 | 10.326 | 1,69 | 0.002* |

Note: The first three rows show the results of two way repeated measures ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons between times for each group and between groups at each time. [a] Day before corticosterone treatment began; [b] 7th day of corticosterone treatment; [c] 14th day of corticosterone treatment; [d] day after corticosterone treatment ended; [e] 22 days after corticosterone treatment ended.

**Appendix Table 3.3.** Statistical analyses of cloacal diameters in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| Effect | F | df | p |
|---|---|---|---|
| Group | 1.392 | 4,69 | 0.246 |
| Time | 13.654 | 6,414 | <0.001* |
| Interaction of group and time | 1.352 | 24,414 | 0.125 |
| | | | |
| Comparisons between times for each group | | | |
| Day -1[a] vs day 21[d] | | | |
| control | 3.741 | 1,69 | 0.057 |
| 0.31-0.60 | 0.012 | 1,69 | 0.912 |
| 0.61-0.90 | 0.036 | 1,69 | 0.850 |
| 0.91-1.50 | 0.603 | 1,69 | 0.440 |
| >1.51 | 0.063 | 1,69 | 0.803 |
| | | | |
| Day 21[d] vs day 42[e] | | | |
| control | 3.964 | 1,69 | 0.050* |
| 0.31-0.60 | 3.111 | 1,69 | 0.082 |
| 0.61-0.90 | 15.291 | 1,69 | <0.001* |
| 0.91-1.50 | 10.185 | 1,69 | 0.002* |
| >1.51 | 3.260 | 1,69 | 0.075 |
| | | | |
| Comparisons between control and treatment groups | | | |
| Day -1[a] | | | |
| control vs 0.31-0.60 | 0.050 | 1,69 | 0.824 |
| control vs 0.61-0.90 | 0.007 | 1,69 | 0.932 |
| control vs 0.91-1.50 | 0.199 | 1,69 | 0.657 |
| control vs >1.51 | 0.240 | 1,69 | 0.626 |
| | | | |
| Day 7[b] | | | |
| control vs 0.31-0.60 | 5.628 | 1,69 | 0.020* |
| control vs 0.61-0.90 | 0.115 | 1,69 | 0.735 |
| control vs 0.91-1.50 | 1.775 | 1,69 | 0.187 |
| control vs >1.51 | 0.631 | 1,69 | 0.430 |
| | | | |
| Day 14[c] | | | |
| control vs 0.31-0.60 | 2.700 | 1,69 | 0.105 |
| control vs 0.61-0.90 | 0.709 | 1,69 | 0.403 |
| control vs 0.91-1.50 | 1.602 | 1,69 | 0.210 |
| control vs >1.51 | 3.103 | 1,69 | 0.083 |

Table continued on next page

**Appendix Table 3.3 continued.** Statistical analyses of cloacal diameters in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Comparisons between control and treatment groups | | | |
| Day 21[d] | | | |
| control vs 0.31-0.60 | 1.698 | 1,69 | 0.197 |
| control vs 0.61-0.90 | 3.136 | 1,69 | 0.081 |
| control vs 0.91-1.50 | 2.985 | 1,69 | 0.088 |
| control vs >1.51 | 0.529 | 1,69 | 0.469 |
| Day 42[e] | | | |
| control vs 0.31-0.60 | 2.527 | 1,69 | 0.116 |
| control vs 0.61-0.90 | 0.061 | 1,69 | 0.806 |
| control vs 0.91-1.50 | 0.520 | 1,69 | 0.473 |
| control vs >1.51 | 0.005 | 1,69 | 0.944 |

Note: The first three rows show the results of two way repeated measures ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons between times for each group and between groups at each time. [a] Day before corticosterone treatment began; [b] 7th day of corticosterone treatment; [c] 14th day of corticosterone treatment; [d] day after corticosterone treatment ended; [e] 22 days after corticosterone treatment ended.

**Appendix Table 3.4.** Statistical analyses of food intakes over 24 h in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| Day -6 to day -5[a] | 3.506 | 4 | 0.477 |
| Day 8 to day 9[b] | 10.900 | 4 | 0.028* |
| Day 22 to day 23[c] | 48.226 | 4 | <0.001* |
| | **U** | | **p** |
| Comparisons between control and treatment groups | | | |
| Day -6 to day -5[a] | | | |
| Control vs 0.31-0.60 | 164.000 | | 0.640 |
| Control vs 0.61-0.90 | 108.500 | | 0.733 |
| Control vs 0.91-1.50 | 128.500 | | 0.196 |
| Control vs >1.51 | 55.500 | | 0.433 |
| Day 8 to day 9[b] | | | |
| Control vs 0.31-0.60 | 91.000 | | 0.009* |
| Control vs 0.61-0.90 | 87.000 | | 0.230 |
| Control vs 0.91-1.50 | 78.500 | | 0.005* |
| Control vs >1.51 | 43.500 | | 0.911 |
| Day 22 to day 23[c] | | | |
| Control vs 0.31-0.60 | 224.500 | | 0.193 |
| Control vs 0.61-0.90 | 227.000 | | <0.001* |
| Control vs 0.91-1.50 | 336.000 | | <0.001* |
| Control vs >1.51 | 90.000 | | <0.001* |

Note: The first three rows show the results of Kruskal-Wallis one way ANOVA for the effects of group on food intake at each time. The remaining rows show the results of Mann-Whitney comparisons of the control group with each treatment group at each time. [a] Week before corticosterone treatment began; [b] 2nd week of corticosterone treatment; [c] week after corticosterone treatment ended.

**Appendix Table 3.5.** Statistical analyses of food intakes over 24 h in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Control | 19.694 | 2 | <0.001* |
| 0.31-0.60 | 14.400 | 2 | 0.001* |
| 0.61-0.90 | 15.846 | 2 | <0.001* |
| 0.91-1.50 | 22.211 | 2 | <0.001* |
| >1.51 | 6.400 | 2 | 0.041* |
| | **Z** | | **p** |
| Comparisons between times for each group | | | |
| Control | | | |
| Day -6 to day -5[a] vs day 8 to day 9[b] | 1.633 | | 0.102 |
| Day -6 to day -5[a] vs day 22 to day 23[c] | 3.724 | | <0.001* |
| Day 8 to day 9[b] vs day 22 to day 23[c] | 3.158 | | 0.002* |
| 0.31-0.60 | | | |
| Day -6 to day -5[a] vs day 8 to day 9[b] | 3.211 | | 0.001* |
| Day -6 to day -5[a] vs day 22 to day 23[c] | 2.035 | | 0.042* |
| Day 8 to day 9[b] vs day 22 to day 23[c] | -0.941 | | 0.052 |
| 0.61-0.90 | | | |
| Day -6 to day -5[a] vs day 8 to day 9[b] | 1.712 | | 0.087 |
| Day -6 to day -5[a] vs day 22 to day 23[c] | -2.900 | | 0.004* |
| Day 8 to day 9[b] vs day 22 to day 23[c] | -3.180 | | 0.001* |
| 0.91-1.50 | | | |
| Day -6 to day -5[a] vs day 8 to day 9[b] | 2.334 | | 0.020* |
| Day -6 to day -5[a] vs day 22 to day 23[c] | -3.582 | | <0.001* |
| Day 8 to day 9[b] vs day 22 to day 23[c] | -3.743 | | <0.001* |
| >1.51 | | | |
| Day -6 to day -5[a] vs day 8 to day 9[b] | 1.214 | | 0.255 |
| Day -6 to day -5[a] vs day 22 to day 23[c] | -1.753 | | 0.080 |
| Day 8 to day 9[b] vs day 22 to day 23[c] | -2.023 | | 0.043* |

Note: The first five rows show the results of Friedman's one way repeated measures ANOVA for the effects of time on food intake in each group. The remaining rows show the results of Wilcoxon comparisons between times in each group. [a] Week before corticosterone treatment began; [b] 2nd week of corticosterone treatment; [c] week after corticosterone treatment ended.

**Appendix Table 3.6.** Statistical analyses of egg production (calculated for each week as the mean percentage of birds in the group that laid an egg each day that week) in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| Day -7 to day -1[a] | 6.369 | 4 | 0.173 |
| Day 0 to day 6[b] | 28.318 | 4 | <0.001* |
| Day 7 to day 13[c] | 142.800 | 4 | <0.001* |
| Day 14 to day 20[d] | 82.233 | 4 | <0.001* |
| Day 21 to day 27[e] | 111.123 | 4 | <0.001* |
| Day 28 to day 34[f] | 21.957 | 4 | <0.001* |
| Day 35 to day 41[g] | 12.605 | 4 | 0.013* |
| | **U** | | **p** |
| Comparisons between control and treatment groups | | | |
| Day -7 to day -1[a] | | | |
| Control vs 0.31-0.60 | 7952.000 | | 0.024* |
| Control vs 0.61-0.90 | 5414.500 | | 0.289 |
| Control vs 0.91-1.50 | 7623.000 | | 0.412 |
| Control vs >1.51 | 1956.500 | | 0.121 |
| Day 14 to day 20[d] | | | |
| Control vs 0.31-0.60 | 8232.000 | | 0.178 |
| Control vs 0.61-0.90 | 7455.000 | | <0.001* |
| Control vs 0.91-1.50 | 10710.000 | | <0.001* |
| Control vs >1.51 | 3192.000 | | <0.001* |
| Day 35 to day 41[g] | | | |
| Control vs 0.31-0.60 | 9023.000 | | 0.456 |
| Control vs 0.61-0.90 | 5855.000 | | 0.528 |
| Control vs 0.91-1.50 | 7749.000 | | 0.356 |
| Control vs >1.51 | 2523.500 | | 0.007* |

Note: The first seven rows show the results of Kruskal-Wallis one way ANOVA for the effects of group on egg production at each time. The remaining rows show the results of Mann-Whitney comparisons of the control group with each treatment group. [a] Week before corticosterone treatment began; [b] 1st week of corticosterone treatment; [c] 2nd week of corticosterone treatment; [d] 3rd week of corticosterone treatment; [e] week after corticosterone ended; [f] 2nd week after corticosterone treatment ended; [g] 3rd week after corticosterone treatment ended.

**Appendix Table 3.7.** Statistical analyses of egg production (calculated for each week as the mean percentage of birds in the group that laid an egg each day that week) in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 18; >1.51 mg corticosterone/bird/day, N = 5. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Control | 13.369 | 6 | 0.038* |
| 0.31-0.60 | 3.289 | 6 | 0.772 |
| 0.61-0.90 | 33.832 | 6 | <0.001* |
| 0.91-1.50 | 97.167 | 6 | <0.001* |
| >1.51 | 35.614 | 6 | <0.001* |
| | **Z** | | **p** |
| Comparisons between times for each group | | | |
| Control | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -0.729 | | 0.466 |
| Day -7 to day -1[a] vs day 35 to day 41[c] | -3.402 | | 0.001* |
| Day 14 to day 20[b] vs day 35 to day 41[c] | -4.131 | | <0.001* |
| 0.31-0.60 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -1.715 | | 0.086 |
| Day -7 to day -1[a] vs day 35 to day 41[c] | -0.600 | | 0.549 |
| Day 14 to day 20[b] vs day 35 to day 41[c] | -2.414 | | 0.016* |
| 0.61-0.90 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -5.427 | | <0.001* |
| Day -7 to day -1[a] vs day 35 to day 41[c] | -1.897 | | 0.058 |
| Day 14 to day 20[b] vs day 35 to day 41[c] | -6.193 | | <0.001* |
| 0.91-1.50 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -6.454 | | <0.001* |
| Day -7 to day -1[a] vs day 35 to day 41[c] | -3.411 | | 0.001* |
| Day 14 to day 20[b] vs day 35 to day 41[c] | -8.137 | | <0.001* |
| >1.51 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -4.379 | | <0.001* |
| Day -7 to day -1[a] vs day 35 to day 41[c] | -1.414 | | 0.157 |
| Day 14 to day 20[b] vs day 35 to day 41[c] | -4.123 | | <0.001* |

Note: The first five rows show the results of Friedman's one way repeated measures ANOVA for the effects of time on egg production in each group. The remaining rows show the results of Wilcoxon comparisons between times in each group. [a] Week before corticosterone treatment began; [b] 3rd week of corticosterone treatment; [c] 3rd week after corticosterone treatment ended.

**Appendix Table 3.8.** Statistical analyses of egg weights (calculated for each week as the mean weight of all eggs laid in each group, using only data from birds that laid at least one egg every week) in control quail and in quail treated with corticosterone. Control, N = 17; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 11; 0.91-1.50 mg corticosterone/bird/day, N = 12; >1.51 mg corticosterone/bird/day, N = 3. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| Day -7 to day -1[a] | 45.450 | 4 | <0.001* |
| Day 0 to day 6[b] | 78.311 | 4 | <0.001* |
| Day 7 to day 13[c] | 142.586 | 4 | <0.001* |
| Day 14 to day 20[d] | 100.193 | 4 | <0.001* |
| Day 21 to day 27[e] | 103.542 | 4 | <0.001* |
| Day 28 to day 34[f] | 66.278 | 4 | <0.001* |
| Day 35 to day 41[g] | 61.899 | 4 | <0.001* |
| | **U** | | **p** |
| Comparisons between control and treatment groups | | | |
| Day -7 to day -1[a] | | | |
| Control vs 0.31-0.60 | 3393.000 | | <0.001* |
| Control vs 0.61-0.90 | 2492.000 | | 0.001* |
| Control vs 0.91-1.50 | 3527.000 | | 0.182 |
| Control vs >1.51 | 874.000 | | 0.538 |
| Day 14 to day 20[d] | | | |
| Control vs 0.31-0.60 | 3234.500 | | <0.001* |
| Control vs 0.61-0.90 | 2307.000 | | 0.055 |
| Control vs 0.91-1.50 | 3227.000 | | <0.001* |
| Control vs >1.51 | 587.500 | | 0.131 |
| Day 35 to day 41[g] | | | |
| Control vs 0.31-0.60 | 3833.000 | | <0.001* |
| Control vs 0.61-0.90 | 3427.000 | | 0.030* |
| Control vs 0.91-1.50 | 4794.000 | | 0.873 |
| Control vs >1.51 | 1384.000 | | 0.250 |

Note: The first seven rows show the results of Kruskal-Wallis one way ANOVA for the effects of group on egg weight at each time. The remaining rows show the results of Mann-Whitney comparisons of the control group with each treatment group at each time. [a] Week before corticosterone treatment began; [b] 1st week of corticosterone treatment; [c] 2nd week of corticosterone treatment; [d] 3rd week of corticosterone treatment; [e] week after corticosterone ended; [f] 2nd week after corticosterone treatment ended; [g] 3rd week after corticosterone treatment ended.

**Appendix Table 3.9.** Statistical analyses of egg weights (calculated for each week as the mean weight of all eggs laid in each group, using only data from birds that laid at least one egg every week) in control quail and in quail treated with corticosterone. Control, N = 17; 0.31-0.60 mg corticosterone/bird/day, N = 20; 0.61-0.90 mg corticosterone/bird/day, N = 11; 0.91-1.50 mg corticosterone/bird/day, N = 12; >1.51 mg corticosterone/bird/day, N = 3. *Indicates a significant result ($p < 0.05$); † insufficient data for statistical analysis.

| | F | df | p |
|---|---|---|---|
| Control | 122.128 | 6 | <0.001* |
| 0.31-0.60 | 193.410 | 6 | <0.001* |
| 0.61-0.90 | 48.321 | 6 | <0.001* |
| 0.91-1.50 | 45.468 | 6 | <0.001* |
| >1.51 | † | | |
| | **Z** | | **p** |
| Comparisons between times for each group | | | |
| Control | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | 3.770 | | <0.001* |
| Day -7 to day -1[a] vs day 35 to day 41[c] | 7.608 | | <0.001* |
| Day 14 to day 20[b] vs day 35 to day 41[c] | 5.535 | | <0.001* |
| 0.31-0.60 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | 2.645 | | 0.008* |
| Day -7 to day -1[a] vs day 35 to day 41[c] | 8.097 | | <0.001* |
| Day 14 to day 20[b] vs day 35 to day 41[c] | 7.746 | | <0.001* |
| 0.61-0.90 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -2.749 | | 0.006* |
| Day -7 to day -1[a] vs day 35 to day 41[c] | 5.095 | | <0.001* |
| Day 14 to day 20[b] vs day 35 to day 41[c] | 5.371 | | <0.001* |
| 0.91-1.50 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -4.730 | | <0.001* |
| Day -7 to day -1[a] vs day 35 to day 41[c] | 4.913 | | <0.001* |
| Day 14 to day 20[b] vs day 35 to day 41[c] | 5.387 | | <0.001* |
| >1.51 | | | |
| Day -7 to day -1[a] vs day 14 to day 20[b] | -0.654 | | 0.513 |
| Day -7 to day -1[a] vs day 35 to day 41[c] | 1.895 | | 0.058 |
| Day 14 to day 20[b] vs day 35 to day 41[c] | 2.599 | | 0.009* |

Note: The first five rows show the results of Friedman's one way repeated measures ANOVA for the effects of time on egg weight in each group. The remaining rows show the results of Wilcoxon comparisons between times in each group. [a] Week before corticosterone treatment began; [b] 3rd week of corticosterone treatment; [c] 3rd week after corticosterone treatment ended.

**Appendix Table 4.1.** Statistical analyses of plasma corticosterone concentrations before and after 24 h of fasting and three hours after food presentation in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6. *Indicates a significant result ($p < 0.05$).

| Effect | F | df | p |
|---|---|---|---|
| Group | 54.325 | 4,70 | <0.001* |
| Time | 3.525 | 2,140 | 0.032 |
| Interaction of group and time | 2.980 | 8,140 | 0.004* |
| | | | |
| Comparisons between times for each group | | | |
| Before fast vs after 24 h of fasting | | | |
| control | 1.370 | 1,70 | 0.246 |
| 0.31-0.60 | 14.996 | 1,70 | <0.001* |
| 0.61-0.90 | 0.059 | 1,70 | 0.808 |
| 0.91-1.50 | 0.189 | 1,70 | 0.665 |
| >1.51 | 0.011 | 1,70 | 0.916 |
| | | | |
| After 24 of fasting vs 3 h after food presentation | | | |
| control | 1.410 | 1,70 | 0.239 |
| 0.31-0.60 | 7.559 | 1,70 | 0.008* |
| 0.61-0.90 | 1.126 | 1,70 | 0.292 |
| 0.91-1.50 | 8.780 | 1,70 | 0.004* |
| >1.51 | 2.576 | 1,70 | 0.113 |
| | | | |
| Comparisons between control and treatment groups | | | |
| Before fast | | | |
| control vs 0.31-0.60 | 46.052 | 1,70 | <0.001* |
| control vs 0.61-0.90 | 51.854 | 1,70 | <0.001* |
| control vs 0.91-1.50 | 61.191 | 1,70 | <0.001* |
| control vs >1.51 | 44.080 | 1,70 | <0.001* |
| | | | |
| After 24 h of fasting | | | |
| control vs 0.31-0.60 | 22.275 | 1,70 | <0.001* |
| control vs 0.61-0.90 | 71.263 | 1,70 | <0.001* |
| control vs 0.91-1.50 | 95.418 | 1,70 | <0.001* |
| control vs >1.51 | 64.311 | 1,70 | <0.001* |
| | | | |
| 3 h after food presentation | | | |
| control vs 0.31-0.60 | 63.147 | 1,70 | <0.001* |
| control vs 0.61-0.90 | 113.142 | 1,70 | <0.001* |
| control vs 0.91-1.50 | 181.328 | 1,70 | <0.001* |
| control vs >1.51 | 112.754 | 1,70 | <0.001* |

Note: The first three rows show the results of two way repeated measures ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons between times for each group and between groups at each time.

**Appendix Table 4.2.** Statistical analyses of body weights before and after 24 h of fasting in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6. *Indicates a significant result ($p < 0.05$).

| Effect | F | df | p |
|---|---|---|---|
| Group | 2.329 | 4,70 | 0.064 |
| Time | 1365.456 | 1,70 | <0.001* |
| Interaction of group and time | 4.120 | 4,70 | 0.005* |
| | | | |
| Comparisons between times for each group | | | |
| Before fast vs after 24 h of fasting | | | |
| control | 376.311 | 1,70 | <0.001* |
| 0.31-0.60 | 470.182 | 1,70 | <0.001* |
| 0.61-0.90 | 223.794 | 1,70 | <0.001* |
| 0.91-1.50 | 323.597 | 1,70 | <0.001* |
| >1.51 | 193.307 | 1,70 | <0.001* |
| | | | |
| Comparisons between control and treatment groups | | | |
| Before fast | | | |
| control vs 0.31-0.60 | 4.701 | 1,70 | 0.034* |
| control vs 0.61-0.90 | 1.360 | 1,70 | 0.247 |
| control vs 0.91-1.50 | 2.552 | 1,70 | 0.115 |
| control vs >1.51 | 0.245 | 1,70 | 0.622 |
| | | | |
| After 24 of fasting | | | |
| control vs 0.31-0.60 | 3.890 | 1,70 | 0.053 |
| control vs 0.61-0.90 | 2.550 | 1,70 | 0.115 |
| control vs 0.91-1.50 | 4.483 | 1,70 | 0.038* |
| control vs >1.51 | 1.492 | 1,70 | 0.226 |

Note: The first three rows show the results of two way repeated measures ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons between times for each group and between groups at each time.

**Appendix Table 4.3.** Statistical analyses of food intakes in the three hours after 24 h of fasting in control quail and in quail treated with corticosterone. Control, N = 18; 0.31-0.60 mg corticosterone/bird/day, N = 19; 0.61-0.90 mg corticosterone/bird/day, N = 13; 0.91-1.50 mg corticosterone/bird/day, N = 19; >1.51 mg corticosterone/bird/day, N = 6. *Indicates a significant result ($p < 0.05$).

| Effect | F | df | p |
|---|---|---|---|
| Group | 6.642 | 4,70 | <0.001* |
| | | | |
| Comparisons between control and treatment groups | | | |
| control vs 0.31-0.60 | 7.682 | 1,70 | 0.007* |
| control vs 0.61-0.90 | 4.023 | 1,70 | 0.049* |
| control vs 0.91-1.50 | 4.033 | 1,70 | 0.048* |
| control vs >1.51 | 25.425 | 1,70 | <0.001* |
| | | | |
| Comparisons between treatment groups | | | |
| 0.31-0.60 vs 0.61-0.90 | 0.255 | 1,70 | 0.615 |
| 0.31-0.60 vs 0.91-1.50 | 0.599 | 1,70 | 0.442 |
| 0.31-0.60 vs >1.51 | 9.791 | 1,70 | 0.003* |
| | | | |
| 0.61-0.90 vs 0.91-1.50 | 0.037 | 1,70 | 0.847 |
| 0.61-0.90 vs >1.51 | 11.135 | 1,70 | 0.001* |
| | | | |
| 0.91-1.50 vs >1.51 | 13.434 | 1,70 | <0.001* |

Note: The first row shows the results of a one way ANOVA for the main effect. The remaining rows show the results of post hoc comparisons of food intake between the control group and each treatment group, and comparisons between treatment groups.

**Appendix Table 5.1.** Statistical analyses of plasma corticosterone concentrations in quail handled for 15, 30 or 60 min and then confined for 45, 30 or 0 min respectively. N = 14. *Indicates a significant result ($p < 0.05$).

| Effect | F | df | p |
|---|---|---|---|
| Time | 11.740 | 3,99 | <0.001* |
| Handling time | 3.717 | 2,33 | 0.035* |
| Handling occasion | 0.129 | 2,33 | 0.880 |
| Interaction of time and handling time | 3.274 | 6,99 | 0.006* |
| Interaction of handling time and handling occasion | 2.678 | 4,33 | 0.049* |
| Interaction of time and handling occasion | 0.855 | 6,99 | 0.531 |
| | | | |
| Comparisons between times for each handling time | | | |
| 0 min vs 15 min | | | |
| 15 min handling | 21.128 | 1,33 | <0.001* |
| 30 min handling | 13.778 | 1,33 | 0.001* |
| 60 min handling | 5.224 | 1,33 | 0.029* |
| | | | |
| 15 min vs 30 min | | | |
| 15 min handling | 17.298 | 1,33 | <0.001* |
| 30 min handling | 1.176 | 1,33 | 0.286 |
| 60 min handling | 0.271 | 1,33 | 0.606 |
| | | | |
| 30 min vs 60 min | | | |
| 15 min handling | 5.836 | 1,33 | 0.021* |
| 30 min handling | 2.131 | 1,33 | 0.154 |
| 60 min handling | 3.234 | 1,33 | 0.081 |
| | | | |
| 0 min vs 60 min | | | |
| 15 min handling | 0.062 | 1,33 | 0.805 |
| 30 min handling | 1.442 | 1,33 | 0.238 |
| 60 min handling | 7.665 | 1,33 | 0.009* |

Table continued on next page

Note: The first six rows show the results of a three way repeated measures ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons between times for each handling time, comparisons between handling times for each time and comparisons between handling occasions at 0 and 15 min.

**Appendix Table 5.1 continued.** Statistical analyses of plasma corticosterone concentrations in quail handled for 15, 30 or 60 min and then confined for 45, 30 or 0 min respectively. N = 14. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Comparisons between handling times for each time | | | |
| 0 min | | | |
| 15 min vs 30 min handling | 0.462 | 1,33 | 0.502 |
| 15 min vs 60 min handling | 2.353 | 1,33 | 0.135 |
| 30 min vs 60 min handling | 0.783 | 1,33 | 0.383 |
| 15 min | | | |
| 15 min vs 30 min handling | 0.003 | 1,33 | 0.960 |
| 15 min vs 60 min handling | 0.011 | 1,33 | 0.918 |
| 30 min vs 60 min handling | 0.003 | 1,33 | 0.956 |
| 30 min | | | |
| 15 min vs 30 min handling | 2.103 | 1,33 | 0.156 |
| 15 min vs 60 min handling | 2.753 | 1,33 | 0.107 |
| 30 min vs 60 min handling | 0.075 | 1,33 | 0.786 |
| 60 min | | | |
| 15 min vs 30 min handling | 3.976 | 1,33 | 0.054 |
| 15 min vs 60 min handling | 19.045 | 1,33 | <0.001* |
| 30 min vs 60 min handling | 6.045 | 1,33 | 0.019* |
| Comparisons between handling occasions for two times | | | |
| 0 min | | | |
| First vs second handling occasion | 0.902 | 1,33 | 0.349 |
| First vs third handling occasion | 0.325 | 1,33 | 0.572 |
| Second vs third handling occasion | 0.164 | 1,33 | 0.688 |
| 15 min | | | |
| First vs second handling occasion | 0.027 | 1,33 | 0.872 |
| First vs third handling occasion | 0.610 | 1,33 | 0.440 |
| Second vs third handling occasion | 0.826 | 1,33 | 0.370 |

**Appendix Table 6.1.** Statistical analyses of plasma corticosterone concentrations in control quail and in quail treated with 1, 2 or 4 IU/kg of insulin. N = 6 per group. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| -1.5 min | 2.435 | 3 | 0.487 |
| 30 min | 1.754 | 3 | 0.625 |
| 60 min | 0.486 | 3 | 0.922 |
| 150 min | 4.013 | 3 | 0.260 |
| | U | | p |
| Comparisons between control and insulin treatment groups | | | |
| -1.5 min | | | |
| control vs 1 IU | 12.000 | | 0.336 |
| control vs 2 IU | 19.000 | | 0.870 |
| control vs 4 IU | 15.000 | | 0.630 |
| 30 min | | | |
| control vs 1 IU | 11.000 | | 0.245 |
| control vs 2 IU | 18.000 | | 1.000 |
| control vs 4 IU | 14.000 | | 0.507 |
| 60 min | | | |
| control vs 1 IU | 19.500 | | 0.810 |
| control vs 2 IU | 19.500 | | 0.809 |
| control vs 4 IU | 22.000 | | 0.519 |
| 150 min | | | |
| control vs 1 IU | 6.000 | | 0.055 |
| control vs 2 IU | 13.000 | | 0.423 |
| control vs 4 IU | 10.000 | | 0.200 |

Note: The first four rows show the results of Kruskal-Wallis one way ANOVA for the effects of group on corticosterone concentration at each time. The remaining rows show the results of Mann-Whitney comparisons of the control group with each insulin treatment group at each time. Injections were delivered at 0 min.

**Appendix Table 6.2.** Statistical analyses of plasma corticosterone concentrations in control quail and in quail treated with 1, 2 or 4 IU/kg of insulin. N = 6 per group. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Control | 4.850 | 3 | 0.183 |
| 1 IU | 6.150 | 3 | 0.105 |
| 2 IU | 7.050 | 3 | 0.070 |
| 4 IU | 7.650 | 3 | 0.054 |
| | **Z** | | **p** |
| Comparisons between times for each group | | | |
| Control | | | |
| -1.5 min vs 30 min | -1.826 | | 0.068 |
| 30 min vs 60 min | 2.023 | | 0.043* |
| 60 min vs 150 min | 0.734 | | 0.463 |
| -1.5 min vs 60 min | -0.314 | | 0.753 |
| -1.5 min vs 150 min | 0.135 | | 0.893 |
| 1 IU | | | |
| -1.5 min vs 30 min | -0.943 | | 0.345 |
| 30 min vs 60 min | 0.405 | | 0.686 |
| 60 min vs 150 min | 1.782 | | 0.075 |
| -1.5 min vs 60 min | -1.153 | | 0.249 |
| -1.5 min vs 150 min | 1.153 | | 0.249 |
| 2 IU | | | |
| -1.5 min vs 30 min | 0.736 | | 0.461 |
| 30 min vs 60 min | 1.095 | | 0.273 |
| 60 min vs 150 min | 1.367 | | 0.172 |
| -1.5 min vs 60 min | 1.214 | | 0.225 |
| -1.5 min vs 150 min | 2.201 | | 0.028* |
| 4 IU | | | |
| -1.5 min vs 30 min | -1.992 | | 0.046* |
| 30 min vs 60 min | 0.944 | | 0.345 |
| 60 min vs 150 min | 1.782 | | 0.075 |
| -1.5 min vs 60 min | -1.572 | | 0.116 |
| -1.5 min vs 150 min | 1.572 | | 0.116 |

Note: The first four rows show the results of Friedman's one way repeated measures ANOVA for the effects of time on plasma corticosterone concentration in each group. The remaining rows show the results of Wilcoxon comparisons of times in each group. Injections were delivered at 0 min.

**Appendix Table 6.3.** Statistical analyses of plasma corticosterone concentrations in control quail and in quail treated with 0.5, 1.0 or 2.0 mg/kg of lipopolysaccharide (LPS). N = 6 per group. *Indicates a significant result ($p < 0.05$).

| | K | df | p |
|---|---|---|---|
| -1.5 min | 3.259 | 3 | 0.353 |
| 60 min | 9.465 | 3 | 0.024* |
| 120 min | 14.940 | 3 | 0.002* |
| 240 min | 13.200 | 3 | 0.004* |
| | **U** | | **p** |
| Comparisons between control and LPS treatment groups | | | |
| -1.5 min | | | |
| control vs 0.5 mg | 17.000 | | 0.871 |
| control vs 1.0 mg | 9.000 | | 0.147 |
| control vs 2.0 mg | 15.000 | | 0.625 |
| 60 min | | | |
| control vs 0.5 mg | 14.000 | | 0.507 |
| control vs 1.0 mg | 3.000 | | 0.016* |
| control vs 2.0 mg | 3.500 | | 0.019* |
| 120 min | | | |
| control vs 0.5 mg | 2.000 | | 0.010* |
| control vs 1.0 mg | 0.000 | | 0.004* |
| control vs 2.0 mg | 1.000 | | 0.006* |
| 240 min | | | |
| control vs 0.5 mg | 0.000 | | 0.004* |
| control vs 1.0 mg | 0.000 | | 0.004* |
| control vs 2.0 mg | 0.000 | | 0.004* |

Note: The first four rows show the results of Kruskal-Wallis one way ANOVA for the effects of group on corticosterone concentration at each time. The remaining rows show the results of Mann-Whitney comparisons of the control group with each LPS treatment group at each time. Injections were delivered at 0 min.

**Appendix Table 6.4.** Statistical analyses of plasma corticosterone concentrations in control quail and in quail treated with 0.5, 1.0 or 2.0 mg/kg of lipopolysaccharide (LPS). N = 6 per group. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Control | 0.650 | 3 | 0.885 |
| 0.5 mg | 12.350 | 3 | 0.006* |
| 1.0 mg | 11.800 | 3 | 0.008* |
| 2.0 mg | 11.000 | 3 | 0.012* |
| | **Z** | | **p** |
| Comparisons between times for each group | | | |
| Control | | | |
| -1.5 min vs 60 min | -0.135 | | 0.893 |
| 60 min vs 120 min | 0.105 | | 0.917 |
| 120 min vs 240 min | 0.314 | | 0.753 |
| -1.5 min vs 120 min | 0.736 | | 0.462 |
| -1.5 min vs 240 min | 0.736 | | 0.462 |
| 0.5 mg | | | |
| -1.5 min vs 60 min | -0.405 | | 0.686 |
| 60 min vs 120 min | 2.201 | | 0.028* |
| 120 min vs 240 min | -0.524 | | 0.600 |
| -1.5 min vs 120 min | 1.782 | | 0.075 |
| -1.5 min vs 240 min | 2.201 | | 0.028* |
| 1.0 mg | | | |
| -1.5 min vs 60 min | 2.201 | | 0.028* |
| 60 min vs 120 min | 0.943 | | 0.345 |
| 120 min vs 240 min | -1.992 | | 0.046* |
| -1.5 min vs 120 min | 2.201 | | 0.028* |
| -1.5 min vs 240 min | 1.572 | | 0.116 |
| 2.0 mg | | | |
| -1.5 min vs 60 min | 1.782 | | 0.075 |
| 60 min vs 120 min | 1.992 | | 0.046* |
| 120 min vs 240 min | -1.572 | | 0.116 |
| -1.5 min vs 120 min | 2.201 | | 0.028* |
| -1.5 min vs 240 min | 2.201 | | 0.028* |

Note: The first four rows show the results of Friedman's one way repeated measures ANOVA for the effects of time on plasma corticosterone concentration in each group. The remaining rows show the results of Wilcoxon comparisons of times in each group. Injections were delivered at 0 min.

**Appendix Table 7.1.** Statistical analyses of plasma corticosterone concentrations in quail handled for 15 min, or treated with 4 IU/kg of insulin or 1.2 mg/kg of lipopolysaccharide (LPS). N = 28. *Indicates a significant result ($p < 0.05$).

| | F | df | p |
|---|---|---|---|
| Handling | 32.604 | 3 | <0.001* |
| Insulin | 41.839 | 3 | <0.001* |
| LPS | 47.625 | 3 | <0.001* |
| | **Z** | | **p** |
| Comparisons between times for each treatment | | | |
| Handling | | | |
| 0 min vs 15 min | 4.349 | | <0.001* |
| 15 min vs 30 min | -3.363 | | 0.001* |
| 30 min vs 60 min | -1.177 | | 0.239 |
| 0 min vs 60 min | 1.574 | | 0.115 |
| Insulin | | | |
| -1.5 min vs 60 min | 3.417 | | 0.001* |
| 60 min vs 150 min | 3.940 | | <0.001* |
| 150 min vs 240 min | -2.095 | | 0.036* |
| -1.5 min vs 240 min | 4.517 | | <0.001* |
| LPS | | | |
| -1.5 min vs 60 min | 4.440 | | <0.001* |
| 60 min vs 120 min | 1.571 | | 0.116 |
| 120 min vs 240 min | 0.672 | | 0.502 |
| -1.5 min vs 240 min | 4.623 | | <0.001* |

Note: The first three rows show the results of Friedman's one way repeated measures ANOVAs for the effects of time on corticosterone concentration. The remaining rows show the results of Wilcoxon comparisons of times for each treatment. Insulin and LPS injections were delivered at 0 min.