Corticosterone responses to handling and effects of corticosterone injections in the Japanese quail (*Coturnix coturnix japonica*)

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ABSTRACT

These studies examined the effects of corticosterone on the reproductive system, investigated the relationship between plasma and faecal corticosterone levels and defined corticosterones response to handling in the Japanese quail (*Coturnix coturnix japonica*). Six days of daily corticosterone injections decreased the area of the cloacal protuberance in both seven week old and six month old male quail. However, plasma testosterone levels 24 hours after an injection were only decreased in the six month old birds. There was a strong correlation between basal plasma and faecal corticosterone concentrations in the six month old birds.

The effects of corticosterone during the 24 hours after an injection were then examined before and after six days of corticosterone injections in male quail. Corticosterone injections decreased plasma testosterone levels three-fold for 6-12 hours both after a single corticosterone injection and after six days of treatment. However, there were no changes in plasma luteinising hormone levels during the 24 hours after an injection. This result is consistent with corticosterone acting directly on the testes to decrease testosterone release. The rate of corticosterone removal from the blood after an injection increased after six days of corticosterone injections.

Handling female Japanese quail for 15 minutes resulted in increased plasma corticosterone levels for less than 30 minutes. Mean corticosterone response curves were almost identical when the same birds were handled on three occasions. Although corticosterone response curves were similar during the early afternoon and during the night, basal corticosterone levels and the area under the corticosterone response curves were lower at night. Plasma corticosterone levels 0 and 15 minutes after the initiation of handling were more than twice as high in birds with large gonads than birds with small gonads.

This study provides the first information in birds of a decrease in plasma testosterone levels within three hours of a corticosterone injection, independent of changes in plasma LH levels. It is also the first study in a domestic species to show larger corticosterone responses in female birds with large gonads than in birds with small gonads.

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1 General Introduction

Corticosterone is the predominant glucocorticoid released in response to various stressors in birds (Carsia and Harvey, 2000). Corticosterone has many effects in birds, influencing metabolism, body composition, behaviour and the reproductive system. Although much work has been carried out on the effects of glucocorticoids on mammals (usually rodents), relatively few studies have been conducted in birds.

In this study, corticosterone was administered to male Japanese quail (*Coturnix coturnix japonica*) to examine its effects on reproductive hormones (luteinising hormone (LH) and testosterone) and on secondary sexual characteristics (cloacal gland area and cloacal foam production). The relationship between plasma and faecal levels of corticosterone was also examined. Finally, the corticosterone responses of Japanese quail to handling were defined to give an indication of how these vary within birds, and between birds in similar and different situations.

Japanese quail were used in this study as they are a commonly studied domestic bird, are easy to house and maintain, and are readily available from a nearby quail farm.

1.1 Stressors, stress and the stress response

The term "stress" is widely used, with various definitions. Therefore, it is important to define the terms stressor, stress and the stress response as they are used in this study.

A stressor is defined as any factor that has the potential to disrupt the physiological equilibrium (homeostasis) of an animal. Stressors can be environmental (e.g. overcrowding, extremes in temperature), physiological (injury or disease), nutritional (malnutrition, starvation, or decreased food availability), or psychological (fear, anxiety).

A stress response is an animal's reaction to a stressor. It includes both a short-term neural response (i.e. activation of the sympathetic nervous system), and the release of hormones from the adrenal medulla and cortex. Catecholamines (adrenaline and noradrenaline) and glucocorticoids (cortisol or corticosterone) are the predominant hormones released during a stress response. Their function is to restore the physiological balance of the animal either by promoting behaviours that will remove of the animal from the stressor, or by adaptation to the new situation. As corticosterone is the predominant glucocorticoid released in birds, the corticosterone response provides a convenient measure of the response of a bird to a stressor.

An animal is said to be stressed (or experiencing stress) when a stress response has been initiated in response to one or more stressors.

1.2 The hypothalamo-pituitary-adrenal axis

The hypothalamo-pituitary-adrenal (HPA) axis maintains basal levels of glucocorticoids in the plasma and increases glucocorticoid levels in response to a stressor. It is well established that corticotropin-releasing factor (CRF) is a predominant secretagog of adrenocorticotropic hormone (ACTH) in both mammals (reviewed in Chrousos, 1998) and birds (Mikami and Yamada, 1984). CRF is released from nerve terminals at the median eminence of the hypothalamus, and enters the hypothalamo-hypophysial portal system. From here it is carried to the anterior pituitary, where it induces the release of ACTH from specific pituitary cells (corticotrophs). CRF is not the only hormone able to induce the release of ACTH, however. Arginine vasopressin (AVP) has been found to induce the release of ACTH in both mammals (Konakchieva *et al.*, 1997; Ma *et al.*, 1997; Chrousos, 1998) and birds (Gilles *et al.*, 1982; Westerhof *et al.*, 1992), as does arginine vasotocin (AVT) (Westerhof *et al.*, 1992). Serotonin is another ACTH secretagog at least in mammals (reviewed in Dinan, 1996; Lefebvre *et al.*, 1998).

ACTH released from the pituitary gland in birds is carried in the systemic blood stream to it's target tissue, the adrenal gland, where it stimulates release of corticosterone (Schmeling and Nockels, 1978; Freeman and Manning, 1979; Davison *et al.,* 1980; Davison *et al.,* 1985; Beuving and Vonder, 1986), aldosterone (Radke *et al.,* 1984; Radke *et al.,* 1985) and the catecholamines (Zachariasen and Newcomer, 1971). In birds, plasma ACTH levels reach a peak 5-10 minutes after the initiation of a stress response (Kovacs and Peczely, 1991), followed by peak levels of plasma corticosterone 10-20 minutes after initiation of the response (Freeman and Flack, 1980; Beuving and Vonder, 1986; Kovacs and Peczely, 1991; Astheimer *et al.,* 1994; Astheimer *et al.,* 1995; Schwabl, 1995).

1.3 Circadian rhythms in hypothalamo-pituitary-adrenal axis activity

There is a circadian rhythm of the HPA axis that is closely controlled and usually results in higher glucocorticoid levels at the time of increasing activity in an animal. Basal activation of this axis appears to be controlled by a diurnal rhythm, mediated via the suprachiasmatic nucleus in both mammals (Kwat *et al.,* 1992) and birds (King and Follett, 1997). In birds that are active during the day, this rhythm usually results in increasing plasma corticosterone levels at night with a peak during the late night (Boissin and Assenmacher, 1970; Joseph and Meier, 1973; Beuving and Vonder, 1977; Etches, 1979; Wilson and Cunningham, 1981; Breuner *et al.,* 1999). However, some studies have shown peaks in basal plasma corticosterone levels during the early night (Dusseau and Meier, 1971; Kovacs and Peczely, 1983); or even mid-morning (Johnson, 1981; Proudman, 1991). It appears that the amplitude of daily variation in basal corticosterone levels is partially dependent on the photoperiod the birds experience. Boissin and Assenmacher (1970) found that Japanese quail held on short days (6L:18D) had similar trough levels of corticosterone to birds held on a longer photoperiod (12L:12D), but peak basal corticosterone levels were twice as high. Under both photoperiods, however, basal corticosterone levels peaked just before lights-on. Although basal corticosterone levels show a distinct circadian rhythm, even peak basal levels are relatively low compared to

the increases in plasma corticosterone due to egg laying or the stress response (Beuving , 1980).

In mammals, when glucocorticoid levels are lowest during the circadian rhythm there is little, if any, release of CRF from the hypothalamus (Dallman *et al.,* 1994). During this 'trough' of secretion, glucocorticoid release is likely to be due solely to constitutive ACTH release from the pituitary, independent of CRF stimulation. At the peak of the daily rhythm, the majority of ACTH released is induced by CRF. It is not known whether the same situation occurs in birds.

1.4 Measurement of the stress response

Plasma levels of hormones released during the stress response can be measured in order to define and compare stress responses. Plasma levels of ACTH, adrenaline, noradrenaline, and glucocorticoids all increase during the stress response, and therefore measurements of these hormones could be used to define a stress response. Plasma corticosterone levels are most commonly used to measure a stress response in birds as corticosterone has a longer half-life in plasma than the other hormones and is released during all types of stress. Also, and the magnitude and duration of a corticosterone response is dependent on the severity of a stressor (Harvey *et al.,* 1984).

Plasma corticosterone levels increase rapidly In response to a stressor, and have usually increased significantly within one or two minutes of the initiation of a stress response (Beuving and Vonder, 1978; Harvey *et al.,* 1980; Dawson and Howe, 1983; Scatterlee and Johnson, 1988). The duration of elevated corticosterone levels in the plasma varies between species of bird. Plasma corticosterone levels in some species such as chickens (Freeman and Flack, 1980) and ducks (Harvey *et al.,* 1980) returned to basal levels within 60 minutes after the initiation of a corticosterone response. However, in free-living species corticosterone levels are usually still greatly elevated one hour after a stressor is applied (Wingfield *et al.,* 1982; Astheimer *et al.,* 1994; Smith *et al.,* 1994; Schwabl, 1995; Dufty and Beltoff, 1997). Corticosterone responses also

vary within a species depending on the bird's age (Schmeling and Nockells, 1978; Schwabl, 1995; Romero *et al.,* 1998c) and sex (Jones *et al.,* 1994; Astheimer *et al.,* 1995; Schwabl, 1995) and on its reproductive status (Wingfield *et al.,* 1982; Astheimer *et al.,* 1994; Astheimer *et al.,* 1995; Romero *et al.,* 1997; Romero *et al.,* 1998a, b, c). Moreover, corticosterone responses can be modified in birds that are repeatedly (or chronically) stressed (Astheimer *et al.,* 1995).

In order to study the stress response in a species of bird the corticosterone response must be defined using a consistent method. The most commonly used method is the 'capture stress protocol' (e.g. Wingfield *et al.,* 1982; Dawson and Howe, 1983; Beuving and Vonder, 1986; Astheimer *et al.,* 1995; Romero *et al.,* 1997). In this procedure, a bird is captured (if free-living) or picked up (if kept in captivity), and a blood sample collected as rapidly as possible to determine basal corticosterone levels. A series of blood samples are then collected over a period of time (usually one hour) during which the bird is kept in a small box or cloth bag. The changes in plasma corticosterone levels over this sampling period define the corticosterone response to handling in that bird. Peak plasma corticosterone levels, the duration of elevated corticosterone levels, and the areas under the corticosterone versus time curves can all be used to compare birds of different sex, age, reproductive states, or environmental situation (i.e. comparisons between free-living and captive birds, or captive birds kept in different situations).

1.5 Actions of glucocorticoids in birds

Corticosterone's main role in the stress response is help the bird adapt to a stressor and return to a physiological balance. Corticosterone acts in a variety of ways to achieve this.

One of corticosterone's effects is to increase locomotor activity and foraging behaviour (Heath, 1997; Beltoff and Dufty, 1998; Breuner *et al.,* 1998). This is

thought to be an adaptive mechanism that would help a bird to remove itself from, or adapt to, the stressors of overcrowding or lack of food availability. This effect of corticosterone may also be involved in the dispersal of young birds after fledging (Heath, 1997; Beltoff and Dufty, 1998). Unlike many of the other effects of corticosterone, changes in locomotion occur rapidly, within 15 minutes of corticosterone administration (Breuner *et al.,* 1998).

Corticosterone also has significant effects on metabolism. Long-term elevation of plasma corticosterone levels increases fat deposits and decreases muscle weight (Davison *et al.,* 1983; Simon, 1984; Hayashi *et al.,* 1994; Covasa and Forbes, 1995). This is often accompanied by increases in food intake (Gross *et al.,* 1980; Bray, 1993; Covasa and Forbes, 1995) and plasma glucose levels (Peebles *et al.,* 1997). The metabolic alterations that result from these changes are beneficial in the short-term, as the bird has more rapidly available energy. However, with prolonged stress this mechanism can result in considerable muscle wastage.

Corticosterone can also have an inhibitory effect on reproduction in birds, and glucocorticoids are thought to be the most important mediators of the effects of stress on reproduction in both mammals and birds. This role of corticosterone can have obvious advantages, as animals that are chronically stressed due to the environment (inclement weather, lack of food) or social situation (overcrowding) are unlikely to be able to successfully raise young. Inhibition of reproduction could occur at the level of the hypothalamus (inhibition of GnRH release), at the level of the pituitary (inhibition of LH or FSH release), or at the level of the gonads (inhibition of oestradiol or testosterone release). The inhibition of reproduction by stressors is a complicated process, and most of the previous work in this area has been carried out on mammals. However, various studies in birds have shown decreases in plasma testosterone levels with stressors such as decreased food availability (Wilson *et al.,* 1979), stormy weather (Wingfield *et al.,* 1983) and handling (Wilson *et al.,* 1979; Wingfield *et al.,* 1982). A number of studies have also been carried out in birds on the

effects of administration of either ACTH (Deviche *et al.,* 1980a; Chaturvedi and Suresh, 1990) or corticosterone (Deviche *et al.,* 1982; Wingfield and Silverin, 1986; Petitte and Etches, 1988; Petitte and Etches, 1989) on the reproductive systems of various avian species. In these studies, administration of corticosterone has generally been seen to decrease plasma levels of the reproductive hormones. Corticosterone has been reported to decrease GnRH release *in vivo* (Petitte and Etches, 1989), and decrease the response of pituitary cells to GnRH *in vitro* (Connolly and Callard, 1987). It is not known whether corticosterone also acts at the level of the gonads in birds, or at which level corticosterone predominantly acts *in vivo.* It is also not known whether corticosterone can have acute effects on the reproductive system, or whether long-term elevation of plasma corticosterone is necessary for an effect to be seen.

In mammals, CRF and ACTH appear to have inhibitory effects on the reproductive system independent of glucocorticoids (Rivier and Plotsky, 1986; Calogero *et al.,* 1996; Magiakou *et al.,* 1997; Phogat *et al.,* 1997), and glucocorticoids can inhibit reproduction at the level of the hypothalamus (Li, 1993), the pituitary (Rivier and Rivest, 1991) and the gonads (Hayashi and Moberg, 1990; Rivier and Rivest, 1991). It is thought that in most mammals corticosterone acts predominantly at the levels of the hypothalamus and pituitary, and has only minor effects directly on the gonads (Tilbrook *et al.,* 2000). However, there is evidence in rats that physiological levels of corticosterone have a direct, inhibitory action on testosterone synthesis in Leydig cells (Gao *et al.,* 1996). The HPA axis can also be activated without appearing to affect the reproductive axis at all (Rivest and Rivier, 1995), and different types of stress have different affects. For example, although chronic stress can result in a decrease in reproductive hormone levels, acute stress has been seen to cause a transitory increase in LH (Briski and Sylvester, 1988).

The presence of other hormones may also be important in mediating the effects of stress on reproduction. Adrenaline and noradrenaline, which are

neurotransmitters as well as being released in response to some stressors, have been seen to increase GnRH release in mammals (Scott *et al.,* 1992; Uemura *et al.,* 1997), and birds (Millam *et al.,* 1984).

1.6 Glucocorticoid metabolism

Hormones are rapidly metabolised and excreted after they are secreted into the systemic blood stream. This process is important as it results in fine controls on plasma hormone levels. The half-life of radioactive corticosterone in plasma has been reported as 7.5 minutes in the plasma of the duck (*Anas platyrhynchos,* Holmes *et al.,* 1974), 22 minutes in eight week old male broiler chickens (Birrenkott and Wiggins, 1984) and 9.8 minutes (Kovacs and Peczely, 1983) or 10.2 minutes (Kovacs *et al.,* 1983) in male Japanese quail.

The liver is the predominant site of metabolism of most hormones in birds, including corticosterone (Holmes and Slikker, 1976). However, it is not the only site, and there is evidence that a significant proportion of corticosterone metabolism also occurs in the intestine of both mammals (Vylitova *et al.,* 1998) and birds (Holmes and Slikker, 1976; Vylitova *et al.,* 1998). Metabolism of corticosterone involves its transformation into various derivatives, which are thought to have limited biological activity, and conjugation predominantly to ester sulfates (Helton and Holmes, 1973), which is thought to increase the rate of excretion of the metabolites (Bentley, 1998).

Unaltered corticosterone in the blood of birds first passes through either the liver or the kidneys. A large proportion is metabolised In the liver, being changed into derivatives or conjugated to ester sulfates. Most of the corticosterone (and derivatives) returns to the blood stream via the hepatic circulation, while the rest is incorporated into bile and transported to the intestine through the bile duct. In the intestine there is the potential for further metabolism of the corticosterone into derivatives (e.g. 11-dehydro-corticosterone) by enzymes in mucosal cells as the corticosterone is reabsorbed (Vylitova *et al.,* 1998), or cleavage by microbes in the intestine

(Holmes and Slikker, 1976). A proportion of modified and unmodified corticosterone is re-absorbed into the blood stream, while the remainder is excreted in the faeces.

Corticosterone and its derivatives in the blood will also pass through the kidneys, where the hormone and metabolites are filtered and excreted in the urine (Helton and Holmes, 1973). The majority of corticosterone is excreted via the kidneys rather than the intestine (Holmes and Slikker, 1976), but in birds urine is excreted with the faeces, so measurements of faecal corticosterone levels include corticosterone from both urine and faeces.

The process of corticosterone metabolism and excretion described above results in the great majority of corticosterone being excreted in a modified form either as a derivative or a conjugate. Holmes and Slikker (1976), found that only 1% of injected corticosterone was excreted unmodified in ducks.

1.7 Relationship between faecal and plasma corticosterone

As corticosterone in birds is excreted in the faeces, faecal corticosterone levels can, in theory, be used as estimates of plasma levels of the hormones. The use of faecal hormone levels as a non-invasive measure of plasma hormones has been validated for the sex steroids (testosterone, progesterone and oestradiol) in a number of mammalian (reviewed in Schwarzenberger *et al.*, 1996) and avian (e.g. Bishop and Hall, 1991; Cockrem and Rounce, 1994) species, allowing non-invasive measurement of hormone levels in different seasons and situations in both captive and free-living animals. The use of faecal hormone measurements instead of plasma measurements has several advantages, the most important being that it is not necessary to capture the animal to be tested. This is of most benefit when the animal being studied is large and difficult to restrain and sample, or for endangered and protected species from which capture and sampling may not be allowed. An additional advantage of faecal measurements of glucocorticoids is that the stress

response is not initiated by collection of the sample, as sample collection is non-invasive and the animal does not need to be handled.

Validation of faecal hormone measurements includes comparison of plasma and faecal levels of the hormone to ensure that measured faecal levels reflect plasma levels of the hormone. Validation of measurement of faecal corticosterone levels is more difficult than validation of faecal sex steroid measurements. Plasma levels of the sex steroids tend to remain relatively constant over several hours and reproductively mature animals will generally have high levels of these hormones whereas reproductively immature animals will have low levels. However, plasma corticosterone levels increase rapidly in response to a stressor and remain elevated for a relatively short time (usually less than one hour - depending on the species) before returning to basal levels. It also takes time for hormones from the plasma to be excreted in the faeces, dependent both on the time needed for the hormone to be transferred to the faeces and on the frequency of defecation. The time needed for corticosterone from the plasma to be excreted in the faeces is around six hours in a number of rodent species (Harper and Austad, 2000), but is only about one hour in the duck (Holmes and Slikker, 1976), and two hours in the northern spotted owl (*Strix occidentalis caurina,* Wasser *et al.,* 1997).

Comparison of basal corticosterone levels in the plasma and faeces can also be compared as part of the validation of faecal corticosterone measures. As basal glucocorticoid levels are relatively constant, correlation of basal plasma and faecal glucocorticoids should not be as difficult.

Due to the difficulties inherent in correlating plasma and faecal glucocorticoid levels, many researchers have measured faecal glucocorticoids without comparing faecal and plasma glucocorticoid levels. Faecal corticosterone levels have been measured in a small number of bird species such as rock ptarmigans (*Lagopus mutus*, Kikuchi *et al.,* 1996), greylag geese (*Anser anser*, Kotrschal *et al.,* 1998) and northern spotted owls (Wasser *et al.,* 1997). Similar

mammalian studies have been carried out in rodents (Harper and Austad, 2000); ring-tailed lemurs (*Lemur catta*, Cavigelli, 1999), muriquis (*Brachyteles arachnoides*, Strier *et al.,* 1999), cheetahs (*Acinonyx jubatus*, Jurke *et al.,* 1997), spotted hyenas (*Crocuta crocuta*, Goymann *et al.,* 1999), domestic cats (*Felix domesticus*; Graham and Brown, 1996), chimpanzees (*Pan troglodytes*, Whitten *et al.,* 1998), Barbary macaques (*Macaca sylvanus*, Wallner *et al.,* 1999) and African wild dogs (*Lycaon pictus*, Monfort *et al.,* 1998). In a number of these studies faecal glucocorticoid measurements were not validated in any way (Kikuchi *et al.,* 1996; Kotrschal *et al.,* 1998; Strier *et al.,* 1999; Wallner *et al.*, 1999). In several other studies, faecal measures were partially validated by collecting faeces before and after a stressor was applied (Jurke *et al.,* 1997; Whitten *et al.,* 1998; Goymann *et al.,* 1999) or ACTH was administered (Wasser *et al.,* 1997; Monfort *et al.,* 1998). In only three studies (Graham and Brown, 1996; Wasser *et al.,* 1997; Harper and Austad, 2000) were plasma and faecal levels of glucocorticoids compared after plasma glucocorticoid levels were raised by injection of ACTH or handling stress, and in only one study were basal plasma and faecal corticosterone levels correlated (Cavigelli, 1999).

1.8 Outline of thesis

This thesis consists of five chapters: a general introduction, three chapters of experimental studies, and a general discussion. The overall aim of the thesis was to define the corticosterone responses to handling in the Japanese quail and to examine the affects of corticosterone on the reproductive system in male quail. The first two experimental chapters (chapters 2 and 3) describe studies of the effects of corticosterone administration by daily injections on the reproductive system of male Japanese quail and the relationship between faecal and plasma corticosterone levels. The third experimental chapter (chapter 4) describes corticosterone responses to handling in female Japanese quail in various situations. In the general discussion the significance on the results and areas for further work are examined.

2 Effects of daily corticosterone injections

2.1 Abstract

Reproductively mature male Japanese quail (*Coturnix coturnix japonica*) of two different ages were injected daily with oil or corticosterone in oil for six days. In the first experiment, six month old quail were injected with oil or 3, 6, or 12 mg/kg corticosterone per day. Blood samples were collected 24 hours after the previous injection on days 0, 2,4 and 6. Twenty-four hour faecal samples were collected daily over the treatment period. In the second experiment, seven week old quail were injected with oil or 0.075, 0.15, 0.3, 0.6 or 1.2 mg corticosterone per day, and plasma samples were collected as described above. The area of the cloacal gland protuberance was measured throughout the treatment period in both experiments. Plasma and faecal corticosterone concentrations were compared in oil injected birds from the first experiment and a strong correlation between the two was found. In six month old birds, plasma testosterone levels decreased in all of the corticosterone treatment groups, but there were no changes with treatment in the seven week old birds. However, the area of the cloacal protuberance decreased with corticosterone treatment in both experiments. The results from these experiments suggest that faecal corticosterone levels can give an indication of plasma levels when corticosterone is at basal levels. Also, administration of corticosterone by injections has an inhibitory effect on the reproductive axis in the male Japanese quail, the degree of which appears to be more pronounced in older compared to younger birds.

2.2 Introduction

Corticosterone can have a number of effects on the body composition of birds, as well as having an inhibitory effect on reproduction. A preliminary experiment was carried out to determine the effects of daily corticosterone injections on Japanese quail, and to examine whether corticosterone could inhibit the reproductive systems of birds when administered by this method. The relationship between plasma and faecal corticosterone was also examined. The results of this preliminary study were then used to design a second experiment in which a range of corticosterone doses were administered to determine the lowest dose of corticosterone that would inhibit the reproductive system of the male quail.

Administration of corticosterone can have a number of metabolic effects on birds. Corticosterone has been reported to decrease body weight (Gross *et al.,* 1980; Bartov, 1982; Davison *et al.,* 1983; Davison *et al.,* 1985; Bray, 1993; Hayashi *et al.,* 1994; Astheimer *et al.,* 2000) or increase body weight (Petitte and Etches, 1989), decrease muscle mass (Hayashi *et al.,* 1994; Covasa and Forbes, 1995; Astheimer *et al.,* 2000), increase fat deposits (Bartov, 1982; Davison *et al.,* 1983; Simon, 1984; Buyse *et al.,* 1987; Bray, 1993; Covasa and Forbes, 1995) and increase liver weight (Gross *et al.,* 1980; Bartov, 1982; Davison *et al.,* 1983; Simon, 1984; Davison *et al.,* 1985; Williams *et al.,* 1985; Buyse *et al.,* 1987). In these studies corticosterone was administered either by daily injections, corticosterone implants, or the addition of corticosterone to the food. Most of these studies were carried out for prolonged periods (10 - 30 days of treatment) on young birds that were still growing (2 - 4 weeks old). Only three of these studies (Williams *et al.,* 1985; Petitte and Etches, 1989; Astheimer *et al.,* 2000) used birds over six weeks of age. Therefore, there is little information on the metabolic effects of corticosterone treatment on birds that are no longer growing.

Corticosterone is also known to have a inhibitory effect on the reproductive systems of female birds. Corticosterone treatment has been reported to decrease plasma LH (Etches *et al.,* 1984; Petitte and Etches, 1988; Petitte and Etches, 1989) and oestradiol levels (Petitte and Etches, 1989), ovary or oviduct weights (Williams *et al.,* 1985; Petitte and Etches, 1989) and egg laying (Etches *et al.,* 1984; John *et al.,* 1987). In most of these studies corticosterone was administered using osmotic pumps (Etches *et al.,* 1984; Williams *et al.*, 1985; John *et al.*, 1987; Petitte and Etches, 1988; Petitte and Etches, 1989) or other implants (Davison *et al.*, 1985) which result in constantly elevated corticosterone levels in the plasma.

The effects of corticosterone treatment on the reproductive systems of male birds is less clear. Deviche *et al.* (1982) found that corticosterone injections can increase the cloacal gland area and testis weight of Japanese quail held on short days, while decreasing the cloacal gland area of birds held on long days. Chaturvedi and Suresh (1990) similarly found that corticosterone only affected testis weight in the male redheaded bunting (*Emberiza bruniceps*) when the testes were either growing (preparatory phase) or regressing (regressive phase). Changes in plasma LH levels with corticosterone injections have been reported (Deviche *et al.*, 1979), but other studies reported no changes in plasma LH levels with ACTH or corticosterone treatment, even when plasma testosterone levels (Deviche *et al.*, 1980b) or cloacal gland area (Deviche *et al.*, 1982) decreased. It is also possible for corticosterone to be elevated (e.g. by silastic implant) without decreasing either plasma LH or testosterone (Astheimer *et al.,* 2000). There are clearly many unanswered questions about the actions of corticosterone on the reproductive systems of male birds. The level of the reproductive axis most affected by corticosterone is not known, nor is the time-scale of corticosterone's effects. These questions can be examined using corticosterone injections, but it must first be established that administration of corticosterone by injections does have an inhibitory effect on the reproductive system of the study bird.

Development of a non-invasive measure of corticosterone in birds would be beneficial, as the handling involved in collection blood samples is known to increase plasma corticosterone levels and blood sampling of birds is not always practical. Measurements of faecal corticosterone levels may provide a measure of the adrenal activity of a bird without the need for blood samples to be collected. Although a number of studies have examined faecal corticosterone or cortisol levels in mammals (Monfort *et al.,* 1998; Whitten *et al.,* 1998; Goymann *et al.,* 1999; Wallner *et al.,* 1999) and birds (Kikuchi *et al.,* 1996; Wasser *et al.,* 1997; Kotrschal *et al.,* 1998), a complete validation of faecal corticosterone as a measure of plasma corticosterone has not yet been carried out in any species.

The primary aim of the current study was to examine the effects of daily corticosterone injections on the Japanese quail, and to gain experience with the procedures of handling and injecting birds, collecting blood samples and carrying out radioimmunoassays.

The specific questions that were addressed are as follows:

1) What effects do daily corticosterone injections have on body weight and composition in the male quail?

2) What effects do daily corticosterone injections have on the reproductive systems of male quail?

3) What is the relationship between plasma and faecal corticosterone when corticosterone is both at basal levels and elevated by corticosterone injections?

4) What is the lowest dose of corticosterone able to inhibit the reproductive system of male quail?

2.3 Materials and methods

2.3.1 Animals

Male Japanese quail were obtained from Rangitikei Game Birds Ltd (Bulls). They were transported to Massey University and held in individual cages in a light and temperature controlled room in the Veterinary Science Building. They were provided with food (quail pellets, Feed Processing Unit, Massey University) and water *ad libitum,* and held under a long day photoperiod (16h light: 8h dark; lights on from 0800 - 0000) at an ambient temperature of 20°C.

2.3.2 Experimental design

Quail in both studies were injected daily for six days with corticosterone or with oil and killed 24 hours after the last injection. These studies were approved by the Massey University Animal Ethics Committee.

2.3.2.1 Six month old birds

A preliminary experiment was first conducted to examine the effects of corticosterone injections in a small number of male Japanese quail. Twelve male quail were obtained at three weeks of age and held under long days. When they were six months old they were divided into four treatment groups. The birds were then injected daily, starting at 9 am, with either oil or 3, 6 or 12 mg/kg corticosterone solution. The quail were arranged so that there was an empty cage between adjacent birds to facilitate the collection of faeces.

2.3.2.2 Seven week old birds

The results of the preliminary experiment were used to design a second experiment to define the lowest dose of corticosterone needed to inhibit the reproductive system of male Japanese quail. Forty-eight male quail were obtained at six weeks of age. They were divided into six treatment groups when they were seven weeks old; so that the average body weight of each group was similar. The birds were injected daily with either oil or 0.075, 0.15,

0.3, 0.6 or 1.2 mg of corticosterone (approximately 0, 0.375, 0.75, 1.5, 3 and 6 mg/kg corticosterone).

2.3.3 Hormone administration

Hormone solutions were prepared on the day before treatment started and stored at 4°C. The same solutions were used throughout the treatment period. Control birds received an injection of benzyl alcohol in corn oil, with the concentration of alcohol being the mean concentration of benzyl alcohol used in the hormone solutions (34.5 µl alcohol/ml oil). The volume of each injection was 200 µl.

2.3.3.1 Six month old birds

One hormone solution was prepared for each bird. A stock corticosterone suspension was first made by adding 200 mg of corticosterone (Sigma) to 800 µl benzyl alcohol (GPR, BDH) in a 10 ml glass bottle, and mixing with a magnetic stirrer (Chiltern Scientific). Once the corticosterone was fully dissolved, 7 200 µl of corn oil was added to make a stock solution of 25 mg/ml. The corticosterone came out of solution when the corn oil was added and did not redissolve. Stirred aliquots of the stock suspension were transferred into polystyrene tubes (75 x 12 mm) and diluted with corn oil so that each treated bird received the appropriate daily dose of corticosterone (3, 6 or 12 mg/kg) according to the individual's body weight.

Every morning, starting at 9 am, each bird was injected with its individual solution. Injection solutions were vortexed vigorously before the solution was drawn into the syringe to ensure that a uniform suspension of corticosterone was administered. Injections were administered subcutaneously into the torso of the bird, under the wing. The injection site was varied between the left and right side of the bird in order to minimise skin irritation.

2.3.3.2 Seven week old birds

The birds were weighed three days before the start of the corticosterone treatment. The mean weight of all birds was 202.55 ± 2.88 g. The average weight in each group ranged between 199.26 ± 8.75 g and 204.61 ± 6.16 g. One solution of corticosterone in oil was prepared for each group, rather than preparing individual solutions for each bird according to its exact weight. The concentration of corticosterone in each solution was calculated using 200 g as an average body weight. This approach was taken so that all birds in a single group would be injected with the same total amount of corticosterone, despite small differences in individual body weight. A 25 mg/ml solution of corticosterone in oil was first made by dissolving 250 mg of corticosterone in 1 ml benzyl alcohol then adding 9 ml of corn oil as described for six month old birds. Aliquots of the stock solution were further diluted with corn oil to make up the doses of corticosterone for each treatment group.

Birds were injected daily, starting at 9 am. Oil treated (control) birds were injected first each day, then the other groups were injected in order of increasing corticosterone dose. Groups were injected in this order to decrease the likelihood of cross-contamination between injection solutions and blood samples. Injection solutions were stirred (magnetic stirrer, Chiltern Scientific) for at least 5 minutes before use to resuspend the corticosterone. Birds were injected subcutaneously in the abdominal area. The exact injection site was varied from day to day to minimise skin irritation.

2.3.4 Data collection

2.3.4.1 Six month old birds

2.3.4.1.1 Cloacal gland area and foam production

The cloacal gland of male Japanese quail is an androgen-dependent gland (Balthazart *et al.*, 1979; Balthazart *et al.*, 1980; Massa *et al.*, 1980) that produces a white foam. The area of the cloacal gland and the amount of foam

produced were recorded as indicators of plasma androgen levels. Cloacal gland area was determined by measurement of the width and height of the cloacal protuberance using vernier callipers (± 0.05 mm). Measurements were made before the daily injections on days 0, 2 and 4 of the study. All birds had large cloacal protuberances and were secreting cloacal foam at the beginning of the experiment.

The presence of cloacal foam under the cages was recorded each day after the daily injections were completed. The quantity of foam present was scored as a normal amount, small amount, or none. The amount of foam considered 'normal' was determined from observations of the average amount of foam produced over 24 hours in birds before treatment.

2.3.4.1.2 Condition index

Body weight was measured (Mettler P1200 scales ± 0.05 g) every second day during the experiment. At the end of the experiment, the length of the tarsometatarsus was measured using vernier callipers (± 0.05 mm) as an indication of body size. A condition index was calculated for each bird at the beginning and end of the experiment by dividing body weight (g) by the cube of the leg length (cm^3).

2.3.4.1.3 Blood, faecal and tissue samples

Blood samples were collected immediately before the first of the daily injections and after two and four days of injections. Blood samples (up to 200 µl) were collected by puncture of the brachial vein and collection of the blood into heparinised capillary tubes. All samples were collected within three minutes from the time the bird was removed from its cage, except for one sample, which took four minutes. Blood was expelled from the capillary tubes into a heparinised 1 ml polystyrene test-tube (BDH), and kept on ice until centrifugation. Terminal blood samples were collected by decapitation (after

stunning) on day 6. These samples were collected into heparinised 10 ml polypropylene centrifuge tubes. Samples collected by venipuncture were centrifuged at 2 000 g for 15 minutes (Beckman GS-6R refrigerated centrifuge), whereas terminal samples were centrifuged at 1 900 g for 15 minutes (Beckman TJ-6). Plasma was removed with a glass Hamilton syringe, stored in 1 ml polypropylene titre tubes and frozen at -20°C until assay.

All faeces produced by each bird over 24 hours were collected daily and combined into one sample for each day. Twenty-four hour faecal samples were also collected for five days preceding the treatment in order to determine basal faecal corticosterone levels. Faeces were collected on a plastic sheet, which was placed on top of a flat aluminium tray under each cage. The plastic sheet was changed each morning after the daily injections were completed. Before the sheet was changed, the bottom of each cage was checked, and any faeces that had not fallen through onto the plastic sheet were pushed through. The plastic sheet was then removed, and replaced with a new sheet. Pellets of quail food that had fallen on to the plastic were removed and all the faecal material was transferred to a pre-weighed 35 ml polypropylene screw-top container. Some of the samples collected were very liquid. It was difficult to remove all the food from these samples, so small amounts of food were included in these samples (<5% of total dry weight). The faecal samples were weighed, and the weight of the container subtracted to give the wet weight of each sample. Samples were then frozen at -20°C until assay.

The birds were killed on day 6 and the testes, liver and pectoral muscle were removed and weighed (Mettler Toledo AB104-S +/- 0.0005 g). Finally, the skin around the neck (from the base of the skull to the shoulder) was plucked, and the skin with all associated fat was removed and weighed as an indication of total body fat.

2.3.4.2 Seven week old birds

2.3.4.2.1 Weight and cloacal gland area

Body weight and the cloacal gland area of quail were measured three days before treatment started and on days 0, 2, 4 and 6 of treatment. All birds had large cloacal glands, and were secreting cloacal foam at the beginning of the experiment.

2.3.4.2.2 Blood and tissue samples

Blood samples were collected immediately before the daily injections on days 0, 2 and 4. Blood samples were collected by puncture of the brachial vein with a 27 gauge needle and collection of approximately 200 µl of blood into a heparinised 1 ml syringe. All samples were collected within three minutes from the time each bird was removed from its cage. Terminal blood samples were collected by decapitation (after stunning) on day 6, approximately 24 hours after the previous injection. All blood samples were treated as described for six month old birds.

Testes were removed and paired testis weights were measured (Mettler Toledo AB104-S scales ± 0.0005 g) after collection of the terminal blood samples.

2.3.5 Hormone Assays

Corticosterone and testosterone in plasma samples, and corticosterone in faecal samples were measured by radioimmunoassay.

2.3.5.1 Hormone extraction from faeces

Faecal samples were thawed and freeze-dried for three days (Cuddon 0610 Freeze Drier). Samples were then ground to a powder using a grinding machine (RETCH 2M100; 0.25 sieve) and 0.05 g of powder (± 0.005 g) was transferred into a glass screw top tube (13 x 100 mm). 2.5 ml of 90% ethanol (Analar, BDH) was added using a Merck bottle-top dispenser and the tube was vortexed briefly. Tubes were brought to a rolling boil in an 80°C water bath, and boiled

for approximately 20 minutes. After boiling, 90% ethanol was added to return the volume of ethanol in each tube to 2.5 ml. The tubes were capped and samples were centrifuged for 20 minutes at 1 900 g (Beckman TJ-6 centrifuge). The supernatant was pipetted into a second glass screw-top test-tube. A further 1.25 ml of 90% ethanol was added to the pellet, which was vortexed for 30 seconds, and centrifuged for 15 minutes at 1 900 g (Beckman TJ-6 centrifuge). The supernatant was added to that from the previous spin, and the pellet was discarded. The ethanol extracts were dried under a stream of air in a heating block at 50°C, then allowed to cool before being reconstituted in 1 ml of phosphate-buffered saline with gelatine (PBSG; 0.1M, pH 7.0) and left overnight at 4°C. The next day, samples were vortexed for 30 seconds, shaken for one hour at room temperature, revortexed, and left overnight again at 4°C. After the second night of refrigeration, samples were vortexed, the extract was transferred into a 1.5 ml polypropylene eppendorf tube and was centrifuged for 10 minutes at 14 000 g (IEC Micromax ventilated microcentrifuge OM3590). The supernatant was then transferred into a 1 ml polypropylene titre tube, and frozen at -20°C. The PBSG extracts were defrosted on the day of assay, diluted by 1:40 in PBSG, and 100 µl aliquots transferred into polystyrene assay tubes (75 x 12 mm).

The recovery of corticosterone following extraction was measured by adding 100 µl of tritiated corticosterone (^{3}H corticosterone Amersham; 5 000 cpm) to each sample before boiling, and to two scintillation vials (polypropylene, 5 ml) for total counts. After extraction, a 100 µl aliquot of the reconstituted extract containing label was used to determine the percentage recovery of each sample. The mean recovery was 98.9 ± 0.5%. The level of corticosterone measured in each sample was adjusted by the recovery for that sample.

2.3.5.2 Extraction from plasma

Each plasma sample was thawed and spun at 14 000 g for five minutes (IEC Micromax ventilated microcentrifuge OM3590) in a 1.5 ml Eppendorf tube to separate lipid from the plasma. Corticosterone and testosterone were both extracted at the same time from each sample. A 40 µl aliquot of clear plasma from below the lipid layer was transferred to a glass screw-top extraction tube, (13 mm x 100 mm) and 2 ml of distilled dichloromethane (Analar, BDH) was added using a Merck bottle-top dispenser. The plasma and dichloromethane were vortexed together for 10 seconds, centrifuged at 1 900 g (Beckman TJ-6 centrifuge) for five minutes to ensure none of the plasma was left on the sides of the tube and shaken for one hour on an orbital shaker (Chiltern Scientific SS70). Samples were then centrifuged at 1 900 g for 10 minutes to separate the organic and aqueous phases. A 1 600 µl aliquot from the organic phase was removed from each tube, placed into an open-top glass tube (Kimak, 13 x 100 mm) and dried under a stream of air in a heating block at 37°C. The extract was reconstituted in 900 µl PBSG, vortexed three times at five minute intervals, shaken for one hour at room temperature and left overnight at 4°C. Four 100 µl aliquots of this extract were transferred into polystyrene assay tubes (75 x 12 mm), and frozen at -20°C.

The recovery of testosterone during the extraction process was measured by adding 5 µl of tritiated testosterone solution (1 500 cpm) to three or four plasma samples in each extraction. Duplicates of 100 µl of reconstituted extract and 5 µl triplicates of non-extracted label were used to calculate the percentage recovery for the sample. Corticosterone extraction efficiencies were similarly determined by adding tritiated corticosterone to between two and six samples from each extraction. The percentage recoveries were calculated for each extraction (Table 2.1) and applied to all samples from that extraction. The mean percentage recovery for testosterone in quail plasma was 100.30 ± 1.93 % (n=14), and the mean percentage recovery for corticosterone in quail plasma was 101.01±1.72 % (n=48).

Table 2.1: Extraction efficiency table for testosterone and corticosterone extracted from quail plasma.

Extraction efficiencies for testosterone				
Extraction #	Mean.	SE	%CV	n
1	101.95	1.28	2.51	4
2	94.57	0.81	1.72	4
3	101.76	1.11	2.45	3
4	102.92	5.01	10.89	3
Mean	100.30			
SE	1.93			

Extraction efficiencies for corticosterone				
Extraction #	Mean.	SE	%CV	n
1	104.86	4.48	8.54	4
2	96.99	2.70	5.56	4
3	97.16	1.47	3.03	4
4	101.42	2.07	4.09	4
5	93.76	3.45	7.36	4
6	90.92	3.55	7.82	4
7	98.68	6.86	12.47	2
8	103.85	1.84	3.54	4
9	103.61	3.33	5.57	3
10	113.40	1.14	1.74	3
11	105.03	0.76	3.63	6
12	102.41	2.75	5.49	6
Mean	101.01			
SE	1.72			

Note: Extraction Efficiencies from all chapters are contained in this table.

2.3.5.3 Radioimmunoassay of LH

Plasma LH was assayed in the Department of Biology, Waseda University, Tokyo, Japan by Dr. Kikuchi, using their validated radioimmunoassay for LH in Japanese quail (Hattori and Wakabayashi, 1979; Kikuchi and Ishii, 1989).

2.3.5.4 Radioimmunoassay of testosterone

Testosterone levels in extracted quail plasma were measured by radioimmunoassay, using a modification of the method described by Wingfield *et al.* (1997). Samples were assayed in duplicate. Samples from six month old birds were assayed in a single assay, whereas samples from seven week old birds were randomly distributed between two assays.

100 µl of reconstituted plasma extract was incubated with 100 µl of antibody (Endocrine Sciences CA, US; testosterone antiserum T3-125) and 100 µl of tritiated testosterone (^{3}H-testosterone TRK.406 Amersham, UK ;5 000 cpm) overnight at 4°C. 500 µl of dextran-coated charcoal (2.5 g/l charcoal (Sigma), 0.25 g/l dextran (Dextran T70, Amersham Pharmacia) in PBSG) was added to each sample using an Eppendorf multi-pipetter and was incubated with the sample for 15 minutes at 4°C to separate bound and free testosterone. Samples were then centrifuged at 2 000 g for 15 minutes at 4°C (Beckman GS-6R refrigerated centrifuge), and the supernatant poured off into a 5 ml polypropylene scintillation vial. A Merck bottle-top dispenser was used to add 3 ml of scintillant ((5 g/l PPO (2,3-diphenyl-oxazole, Sigma), 0.3 g/l dimethyl POPOP (1,4-bis-[methyl-5-phenyl-2-oxazoly]-benzene, Sigma) in toluene (Mobil)) to each vial, then the samples were shaken for one hour on an orbital shaker. The samples were left at room temperature for one hour, then were counted in a Wallac 1409-411 liquid scintillation counter for five minutes each.

The sensitivity of the radioimmunoassay for testosterone was determined as the hormone concentration at the mean minus two standard deviations from the percentage bound for the zero hormone tubes. The sensitivity was 12.37 pg/ml on the standard curve, which was equivalent to a testosterone concentration in

quail plasma of 0.36 ng/ml (n=10 assays). Serial dilutions of extracted quail plasma in assay buffer (PBSG) were parallel to the testosterone standard curve (n=3). Recovery of testosterone added to quail plasma was 97.4 ± 1.7%, 99.1 ± 8.1% and 102.0 ± 2.7% for three different samples.

Solutions of testosterone in PBSG at concentrations that gave approximately 20, 50 and 80% binding on the standard curve were used as high, medium and low quality controls in every assay. The mean concentrations of these standards were 561.08 ± 22.6, 113.09 ± 4.79 and 36.23 ± 4.22 pg/ml respectively. Intra-assay coefficients of variation were determined by conducting an assay with ten duplicates of each quality control. The intra-assay coefficients of variation for testosterone were 3.2%, 7.2%, and 18.0% for high, medium and low quality controls respectively. Inter-assay coefficients of variation were calculated from duplicates of the quality controls included at the beginning and end of each assay. The inter-assay coefficients of variation for five assays were 9.01%, 10.70% and 14.35% for high, medium and low quality controls respectively.

The cross-reactivity of the testosterone antibody with other steroids was reported by Endocrine Sciences as follows: dihydrotestosterone (20%), corticosterone (<0.01%), oestradiol (0.14%), Δ-1-testosterone (52%), 4-androsten-3β-17β-diol (3%), 5α-androstan-3β-17β-diol (1.8%), Δ-4-androstenedione (0.5%) and others (<0.5%).

2.3.5.5 Radioimmunoassay of corticosterone

Corticosterone levels were measured in extracted quail plasma and extracted faeces by radioimmunoassay, using the same procedure as described for testosterone, using corticosterone antibody (Dr R. J. Etches, University of Guelpeg, Ontario, Canada; 1:18 000 final dilution) and label (tritiated corticosterone, approximately 5 000 cpm; Amersham, UK).

The sensitivity of the radioimmunoassay for corticosterone was determined as the hormone concentration at the mean minus two standard deviations from the

percentage bound for the zero hormone tubes. The sensitivity was 30.35 pg/ml on the standard curve, which was equivalent to a corticosterone concentration in quail plasma of 0.73 ng/ml (n=12 assays). Serial dilutions of extracted quail plasma in assay buffer (PBSG) were parallel to the testosterone standard curve (n=3). Recovery of corticosterone added to quail plasma was 93.9 ± 4.9%, 98.6 ± 7.3% and 88.9 ± 5.5% for 3 different samples.

Solutions of corticosterone in PBSG at concentrations that gave approximately 20, 50 and 80% binding on the standard curve were used as high, medium and low quality controls in every assay. The mean concentrations of these standards were 1414.56 ± 54.25, 309.55 ± 13.18 and 60.49 ± 9.96 pg/ml respectively. Intra-assay coefficients of variation were determined by conducting an assay with ten duplicates of each quality control. The intra-assay coefficients of variation for corticosterone were 13.2%, 14.8% and 13.43% for high, medium and low quality controls respectively. Inter-assay coefficients of variation were calculated from duplicates of the quality controls included at the beginning and end of each assay. The inter-assay coefficients of variation for ten assays were 11.9%, 16.2% and 19.5% for high, medium and low quality controls respectively.

The cross-reactivity of the corticosterone antibody with other steroids was reported by Etches (1976) as follows: deoxycorticosterone (27.9%), cortisol, (6.9%), progesterone (37.6%), 11β-hydroxyprogesterone (21.3%) and oestradiol, testosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, aldosterone and pregnenalone (<5%). Although this antibody was found to cross-react strongly with progesterone and 11β-hydroxyprogesterone, these hormones are poorly extracted in dichloromethane, so corticosterone is the predominant hormone measured by this antibody.

All the samples from the six month old birds were assayed twice, as some time 0 samples appeared to have abnormally high plasma corticosterone levels (>50 ng/ml). Similar results were seen in the second assay, so a number of samples were re-extracted and assayed a third time. This group contained some

samples with abnormally high plasma corticosterone, as well as samples that contained low corticosterone concentrations. Again, the results were similar to those originally seen and it was concluded that the high plasma corticosterone levels were not caused by the extraction or assay procedures.

2.3.6 Calculation of the area under the curve

Both the raw data and the area under the curves were used to compare plasma corticosterone and testosterone values in different treatment groups. The area under the curve was calculated for each bird with data points at each sampling time (days 0, 2, 4 and 6) by the trapezoid rule using GraphPad Prism version 3 (1999; GraphPad Software Inc.). All areas were calculated as the area between the curve and zero on the y-axis.

2.3.7 Statistics

Graphs were prepared and correlations were performed using GraphPad Prism version 3 (1999; GraphPad Software Inc.). Statistical analyses were performed using Systat version 5.0 and Systat version 8.0 (Systat Inc., Illinois). Normally distributed data with homogeneous variances (as determined by Bartlett's test) were analysed using one- or two-way ANOVA's, or t-tests (as defined in the results). Data was transformed by $\log_{10}$ if necessary to increase homogeneity for parametric analysis. Where parametric tests were not able to be performed, Kruskal-Wallis or Friedman's non-parametric analyses were performed. Non-parametric analysis were performed on raw data.

Statistical analyses were not performed on data from the experiment on six month old birds because of the small sample sizes in this preliminary experiment.

2.4 Results

2.4.1 Six month old birds

Three of the 12 birds died due to unknown causes during the six days of treatment. The birds were one oil injected bird, one bird treated with 6 mg/kg and one bird treated with 12 mg/kg corticosterone. All other birds remained in good health throughout the treatment period. Data from birds that did not survive for the entire treatment period was included in individual graphs but excluded from graphs of mean data.

2.4.1.1 Plasma corticosterone

A number of plasma samples from this study appeared to be contaminated with corticosterone from the injection solutions. Some time 0 samples (taken before the first corticosterone injection), and other samples from oil injected birds had levels of corticosterone (> 50 ng/ml) many times higher than any levels previously reported for Japanese quail. This contamination occurred after the blood samples were collected from the birds, as many of the samples with high plasma corticosterone were collected before the bird's first injection. Contamination could have resulted from contamination of the blood sample containers or the syringes used to draw blood, or could have been due to corticosterone aerosols produced by the vortexing of injection solutions. All corticosterone data from corticosterone treated birds was therefore excluded, as it was impossible to determine whether the high plasma corticosterone levels in samples on days 2, 4 and 6 were due to the treatment or to contamination. However, plasma corticosterone results from oil injected birds that were within the expected range (<20 ng/ml) were not excluded and were used to compare basal corticosterone levels in plasma and faeces.

2.4.1.2 Faecal corticosterone

Faecal corticosterone levels were expressed as ng corticosterone per g of dry weight of sample. The total amount of corticosterone excreted over 24 hours was also calculated from the corticosterone concentration and the total weight of each sample. This measure of total corticosterone excretion over 24 hours (in ng) was independent of variations in dry weight between samples.

Faecal corticosterone levels remained low and relatively constant in all oil injected birds throughout the treatment period (Fig. 2.1). All corticosterone injected birds within each group showed similar patterns of faecal corticosterone levels over the treatment period, except one bird from the 12 mg/kg group, whose faecal corticosterone levels increased with treatment but remained considerably lower than those of other birds in the same group. Mean faecal corticosterone levels increased with corticosterone injections (Fig. 2.2). Faecal corticosterone levels and total daily excretion of corticosterone rose to a peak in corticosterone treated birds during the 24 hours after the first injection (day 1). Levels decreased during the second day, but remained elevated above basal levels. Faecal corticosterone then remained at similar levels for the remainder of the treatment period. The number of birds in each group was too small, however, to determine whether these changes were significant.

There was a dose-dependent effect of corticosterone treatment on the area under the faecal corticosterone excretion curves (Fig. 2.3, linear regression, r^2=0.7907, p=0.0013), however, there was no significant relationship between corticosterone dose and area under the faecal corticosterone concentration curves (linear regression, r^2=0.3732, p=0.0805).

The percentage of corticosterone injected that was excreted in the faeces was calculated for each 24 hour period (Table 2.2). The percentage excreted was highest on day 1 for all groups, and progressively decreased over the treatment period. The percentage excretion on day 6 was less than half that seen on day 0 in each group.

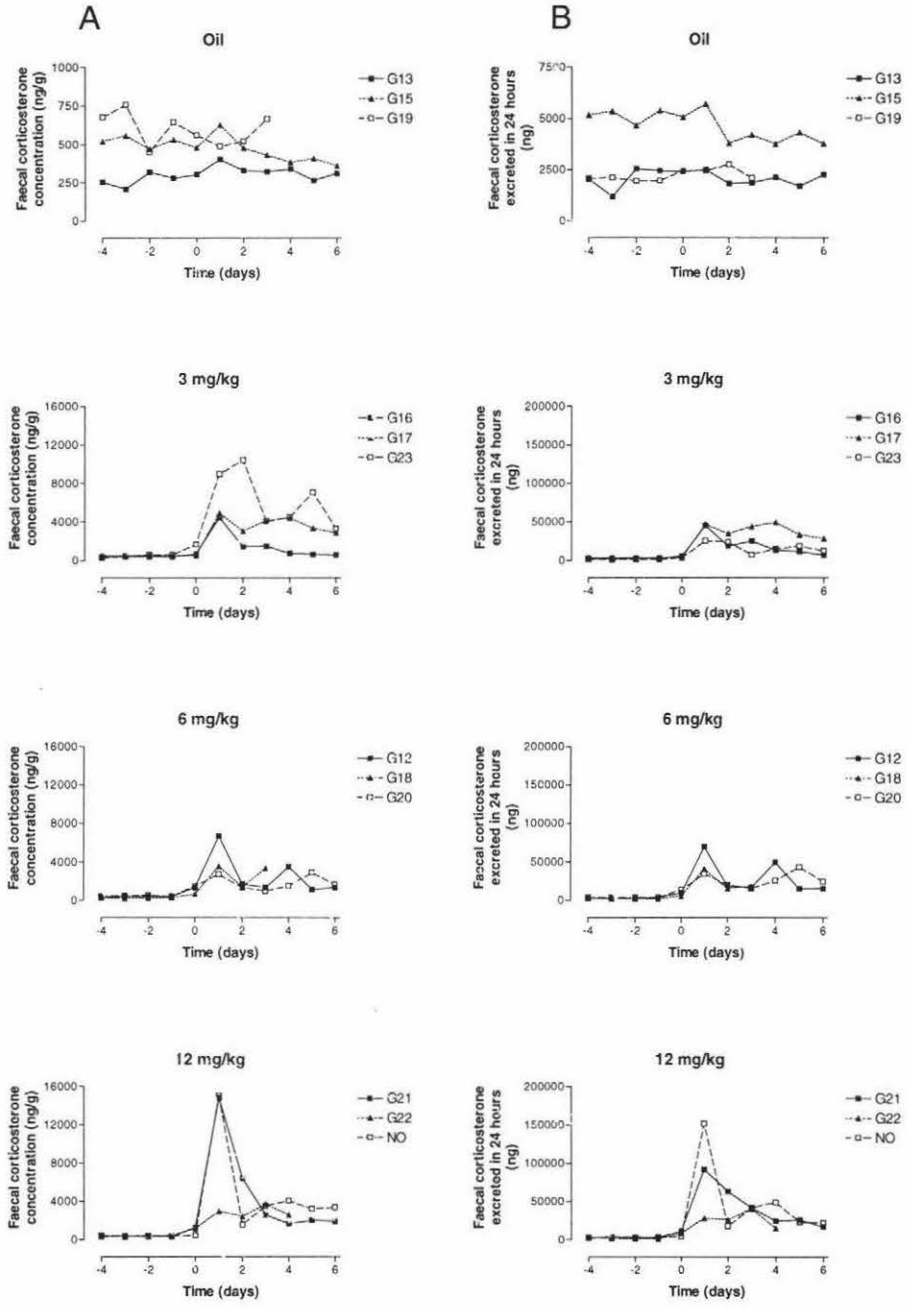


Fig. 2.1: Individual faecal corticosterone levels on days 0 - 6. Each sample consisted of all faeces from a single bird collected over the previous 24 hours. A) faecal corticosterone concentration (ng/g) B) faecal corticosterone excretion over 24 hours (ng). (Note different scales for oil injected and corticosterone treated birds).

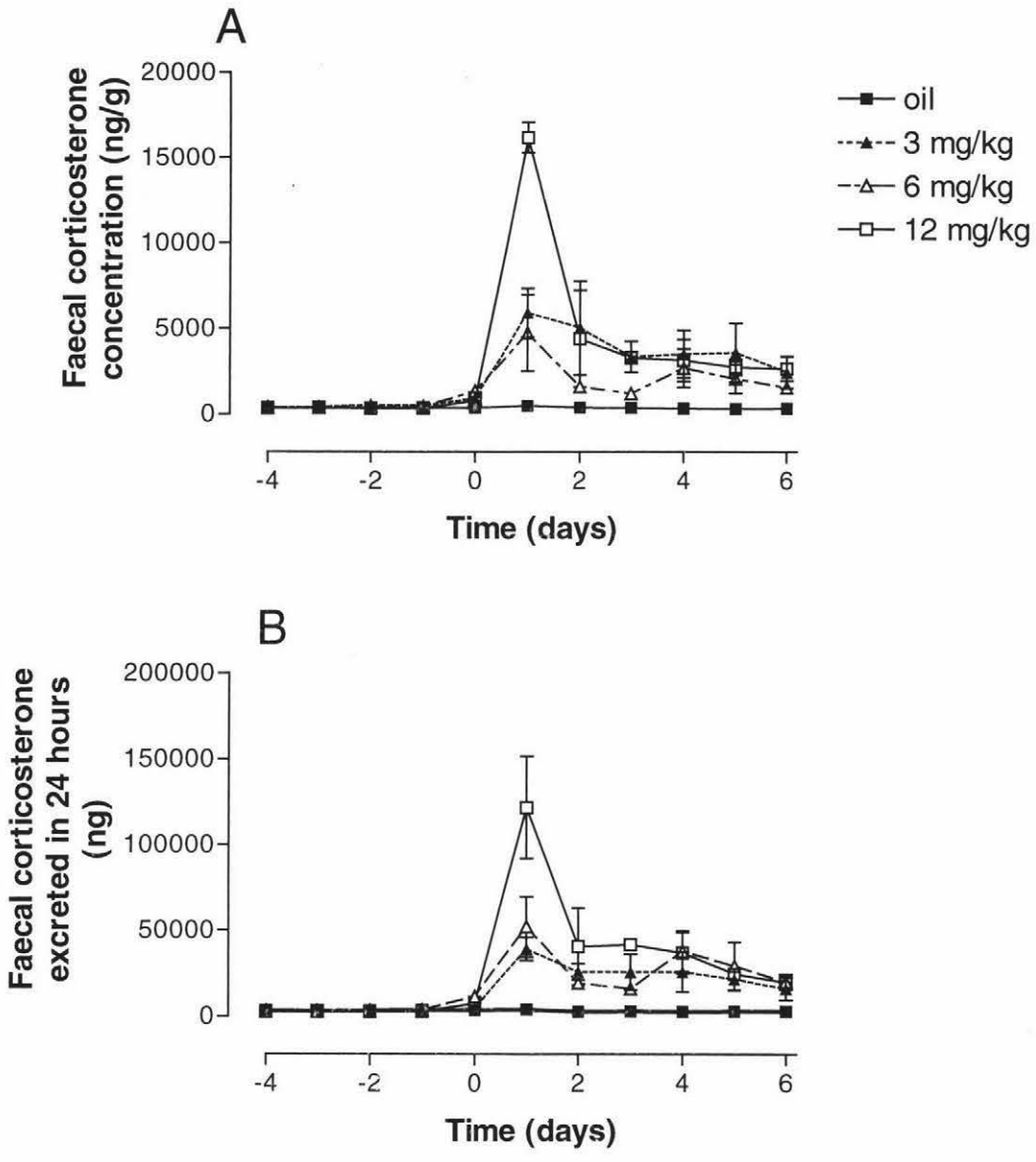


Fig. 2.2: Mean faecal corticosterone levels on days 0 - 6. Each sample consisted of all faeces from a single bird collected over the previous 24 hours (mean ± SE; oil injected, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2). A) faecal corticosterone concentration (ng/g) B) faecal corticosterone excretion over 24 hours (ng).

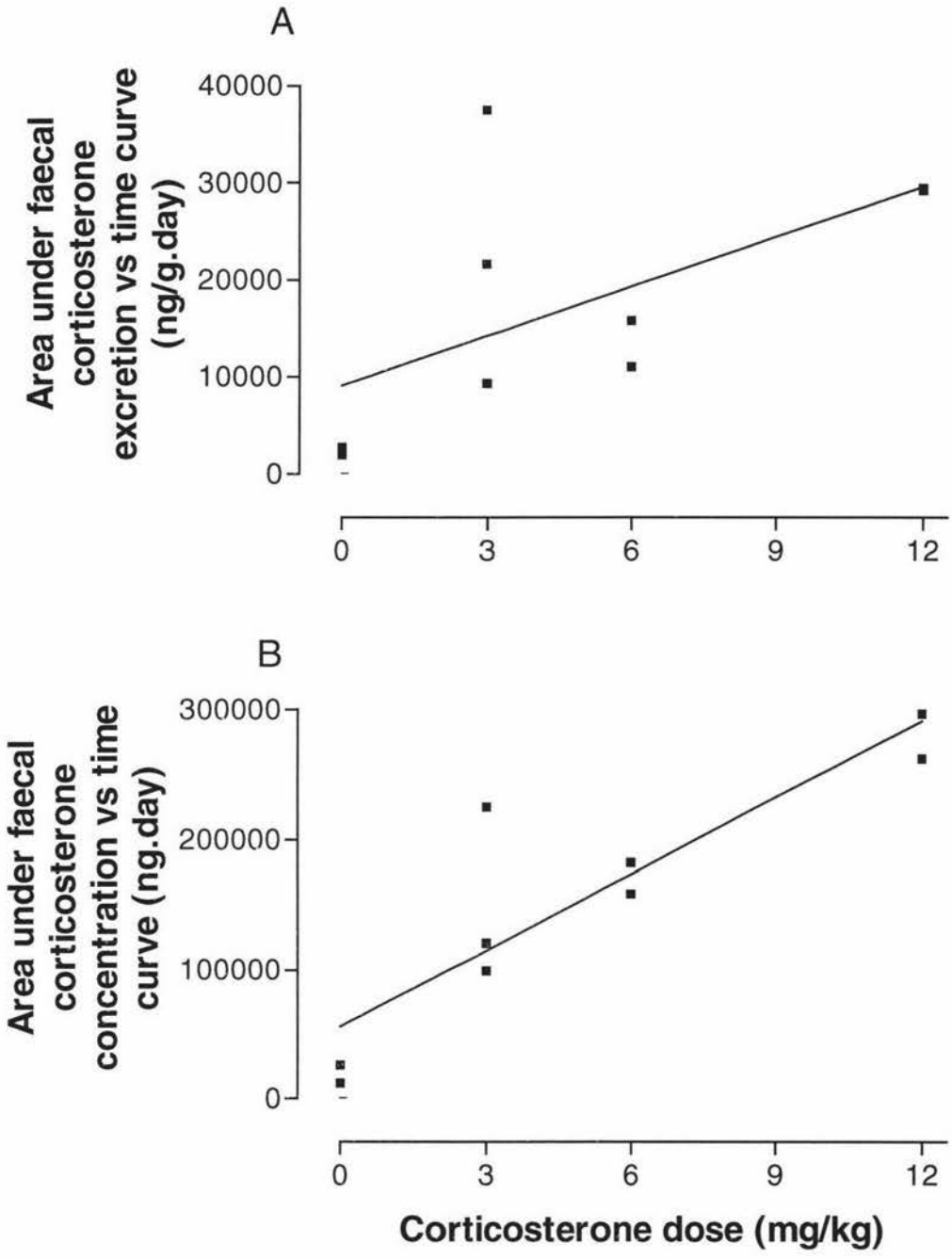


Fig. 2.3: Areas under faecal corticosterone versus time curves over the treatment period. A) faecal corticosterone concentration (ng/g) B) faecal corticosterone excretion over 24 hours (ng).

Table 2.2: Percentage of injected corticosterone that was excreted into the faeces of corticosterone injected birds during each 24 hour period over the six days of treatment.

		Day of treatment					
Group		1	2	3	4	5	6
3 mg/kg	Mean %	4.79	3.22	3.2	3.29	2.7	2.04
	SE	0.88	0.74	1.43	1.62	0.93	0.91
	n	3	3	3	3	3	3
6 mg/kg	Mean %	3.38	1.23	1.03	2.45	1.82	1.26
	SE	1.3	0.02	0.1	0.89	0.79	0.22
	n	2	2	2	2	2	2
12 mg/kg	Mean %	3.86	1.24	1.31	1.17	0.77	0.62
	SE	1.1	0.65	0.05	0.43	0.02	0.1
	n	2	2	2	2	2	2

2.4.1.3 Relationship between plasma and faecal corticosterone

There was a very clear linear relationship between plasma and faecal corticosterone concentrations in oil injected birds (Fig. 2.4, linear regression, r^2=0.7991 p=0.0066, n=7), but not between plasma corticosterone and total faecal corticosterone excreted (linear regression, r^2=0.03632, p=0.6823, n=7).

2.4.1.4 Plasma LH and testosterone

There was no clear relationship between dose of corticosterone and plasma LH levels (Fig. 2.5). The samples sizes were small, but it appears that corticosterone treatment did not markedly affect plasma LH levels 24 hours after an injection. Areas under the curves were not calculated as only four birds had complete LH curves over the six days of treatment.

Plasma testosterone levels remained relatively constant in oil injected birds throughout the treatment period (Fig. 2.6), whereas levels decreased to similar levels in all groups of birds treated with corticosterone. Plasma testosterone levels in oil injected and corticosterone treated birds were similar on day 0 (range = 3.29 -7.58 ng/ml; mean = 4.92 ± 0.56 ng/ml). However, plasma testosterone levels in the corticosterone treated groups had decreased to 0.92 ± 0.18 ng/ml (range = 0.36 -1.49 ng/ml) by day 4, and remained at similar levels on day 6 (mean =0.71 ± 0.13 ng/ml; range = 0.36 -1.33 ng/ml), whereas levels in oil injected birds remained at 5.78 ± 1.63 ng/ml on day 6. This decrease in plasma testosterone levels in corticosterone treated birds resulted in smaller areas under the testosterone curves versus time curves for corticosterone treated compared to oil injected birds (Fig. 2.7). The areas under the testosterone curves were similar for each corticosterone treatment group.

There were inverse relationships between plasma testosterone levels and faecal corticosterone concentration and excretion (Fig. 2.8, Non-linear regression r^2 = 0.382, n=43 and r^2 = 0.403, n=43 respectively) when data from all groups and days of treatment was combined.

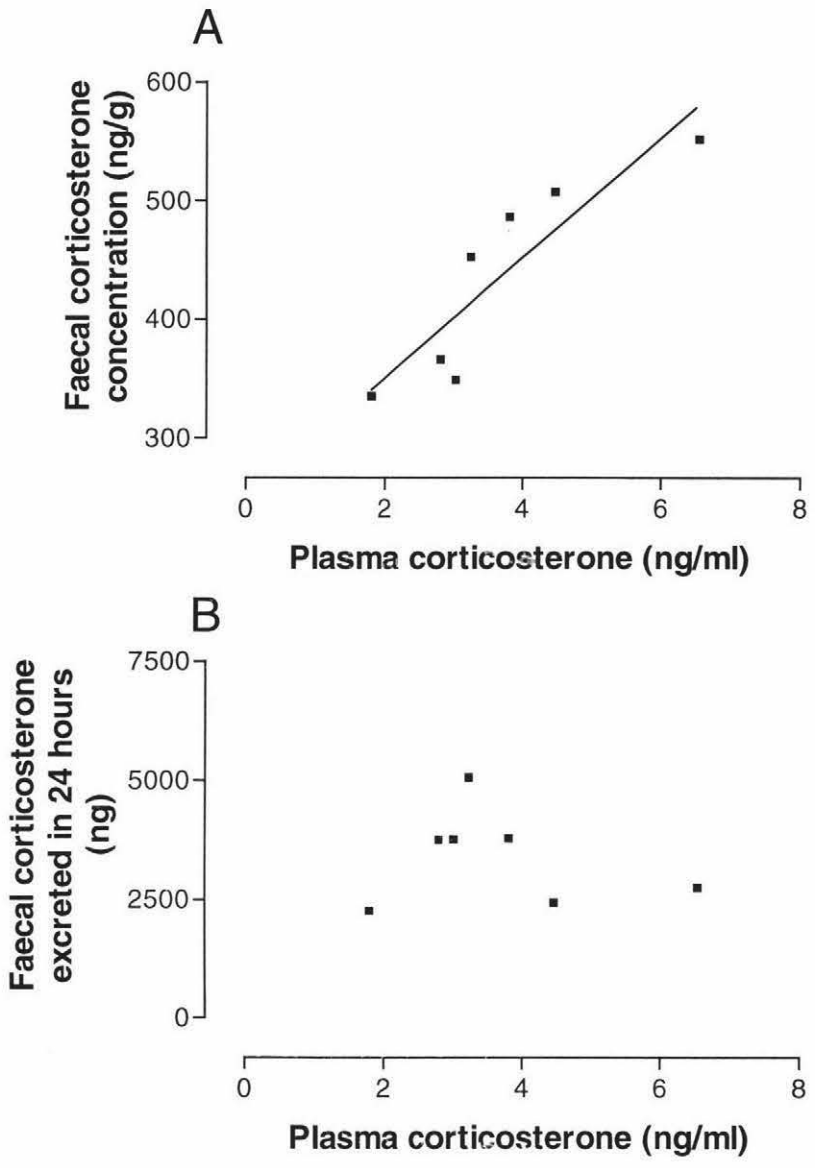


Fig. 2.4: Correlation between faecal and plasma corticosterone levels in oil injected birds during the treatment period. A) plasma corticosterone concentration versus faecal corticosterone concentration (ng/g) B) plasma corticosterone concentration versus faecal corticosterone excretion over 24 hours (ng).

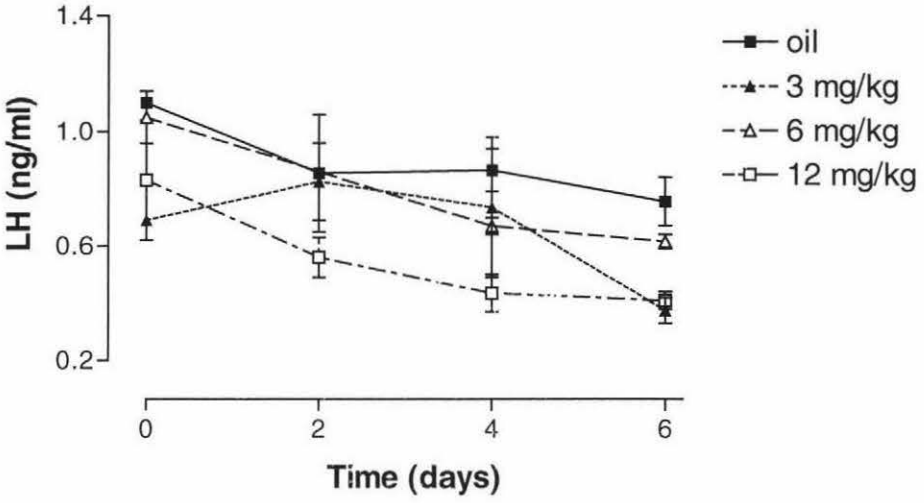


Fig. 2.5: Mean plasma LH levels on days 0, 2, 4 and 6. Each sample was collected approximately 24 hours after the previous injection (mean ± SE; n=2 for each treatment group).

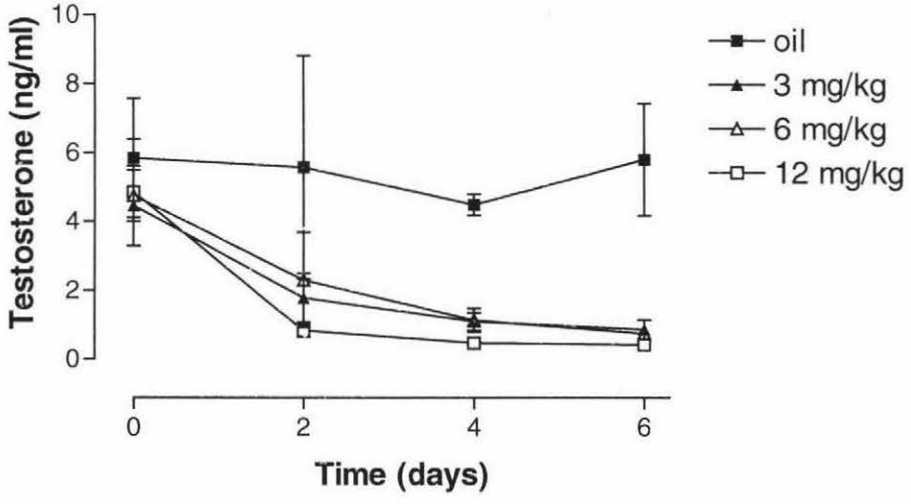


Fig. 2.6: Mean plasma testosterone levels on days 0, 2, 4 and 6. Each sample was collected approximately 24 hours after the previous injection (mean ± SE; oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2).

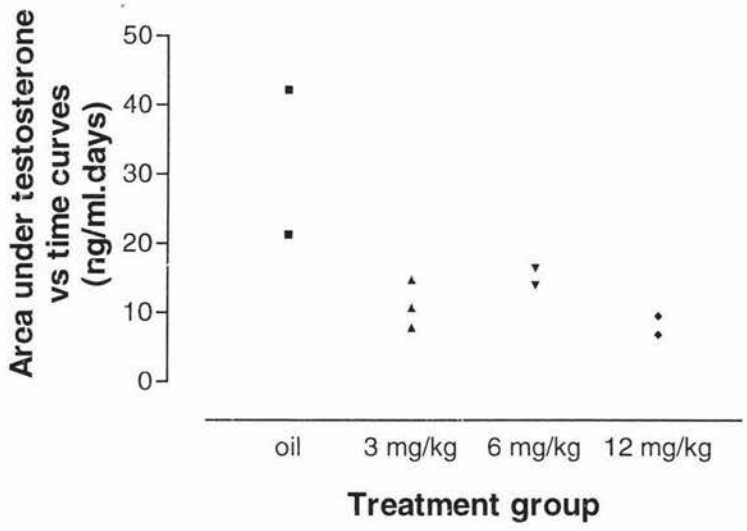


Fig. 2.7: Individual areas under plasma testosterone versus time curves over the treatment period.

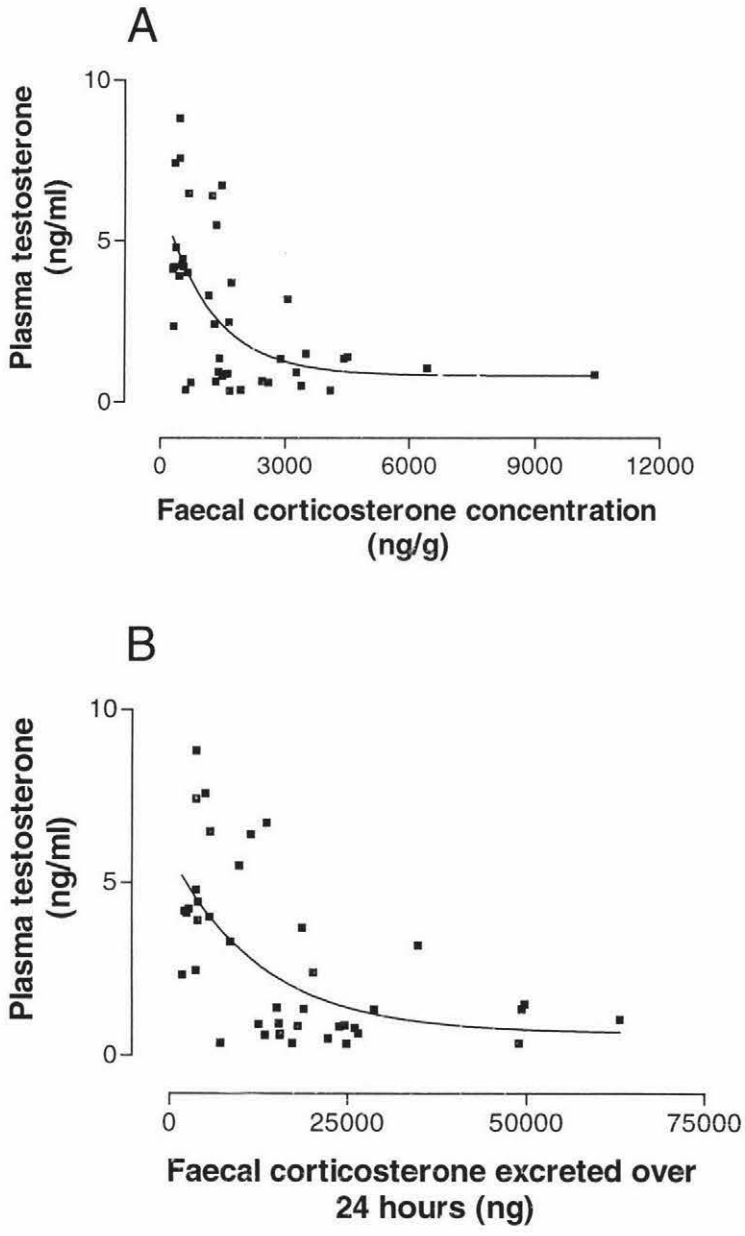


Fig. 2.8: Correlation between plasma testosterone and faecal corticosterone levels during the treatment period. A) plasma testosterone concentration versus faecal corticosterone concentration (ng/g).
B) plasma testosterone concentration versus faecal corticosterone excretion over 24 hours (ng).

2.4.1.5 Faecal composition

Each faecal sample was weighed after collection and again after being freeze-dried. The percentage of water in each faecal sample was calculated from the wet and dry weights. There was considerable variation between birds in the wet weights and dry weights of the 24 hour faecal samples. Some birds produced small, dry faeces whereas others produced larger, wetter faeces. The wet weights and dry weights of faeces produced by oil injected birds remained relatively constant over the treatment period (Fig. 2.9A). Faecal wet weights and percentage water both increased with corticosterone injections, the increase being most pronounced in the 12 mg/kg group (Fig. 2.9A and C). Dry faecal weights increased markedly in 6 mg/kg birds, but only transiently in the 12 mg/kg group (Fig 2.9B).

2.4.1.6 Body measurements

2.4.1.6.1 Body weight and condition index

Mean body weights of quail varied between groups at the start of the experiment. The relative body weight of each bird was therefore calculated, with a value of 0 g assigned to day 0. There was no consistent pattern in changes in relative body weight with treatment (Fig. 2.10A), and no differences in condition index before and after the treatment period (Fig. 2.10B). Relative body weights of birds in the 6 mg/kg group increased slightly with treatment and relative weights of birds in the 12 mg/kg group decreased slightly, but these changes were not large enough to affect the condition index of the birds.

2.4.1.6.2 Muscle to fat ratio

Pectoral muscle and neck skin were removed and weighed at the end of six days of treatment. Mean pectoral muscle weights were lower in 12 mg/kg treated birds than in oil injected birds, but no trends were evident in the neck skin weight (Fig. 2.11). When the muscle to fat ratio was calculated, there was a trend towards a decrease in the ratio with higher doses of corticosterone.

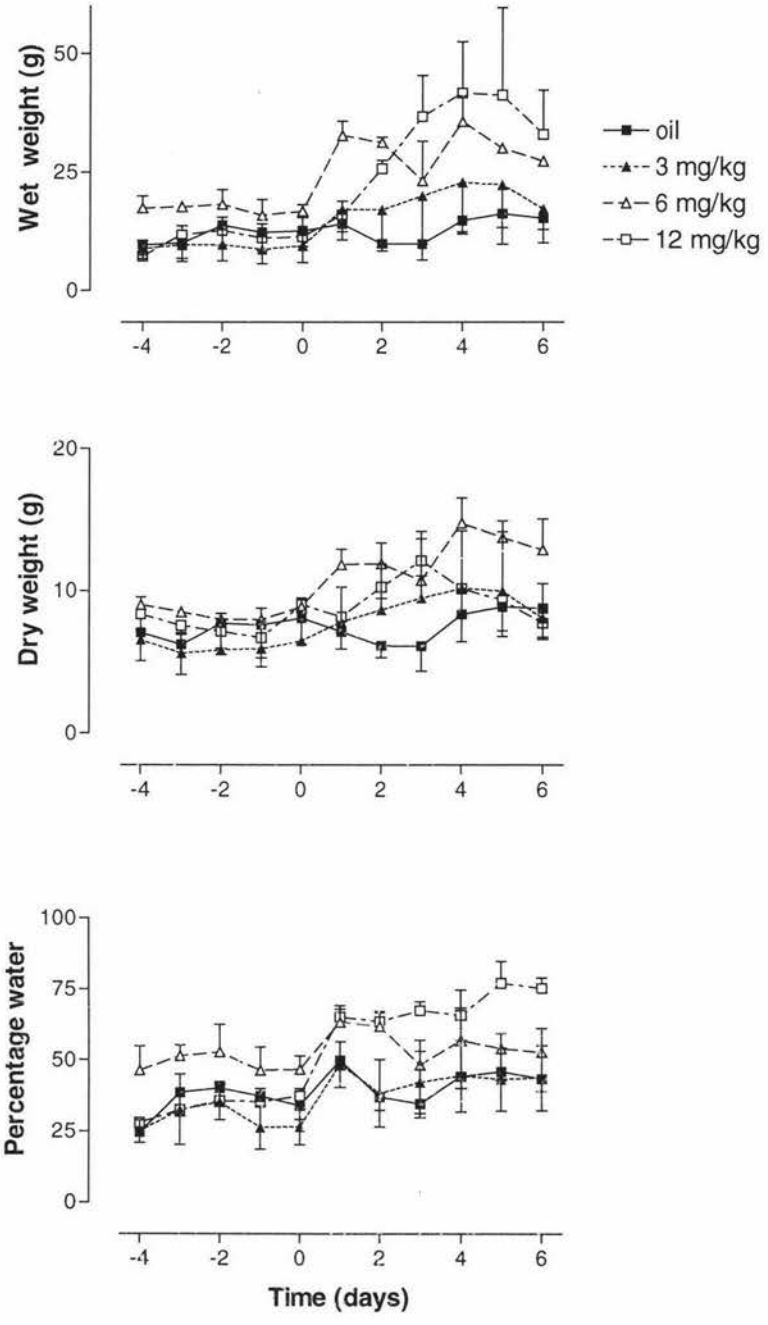


Fig. 2.9: Composition of faecal samples on days 0 - 6. Each sample was collected approximately 24 hours after the last injection (mean ± SE; oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2).

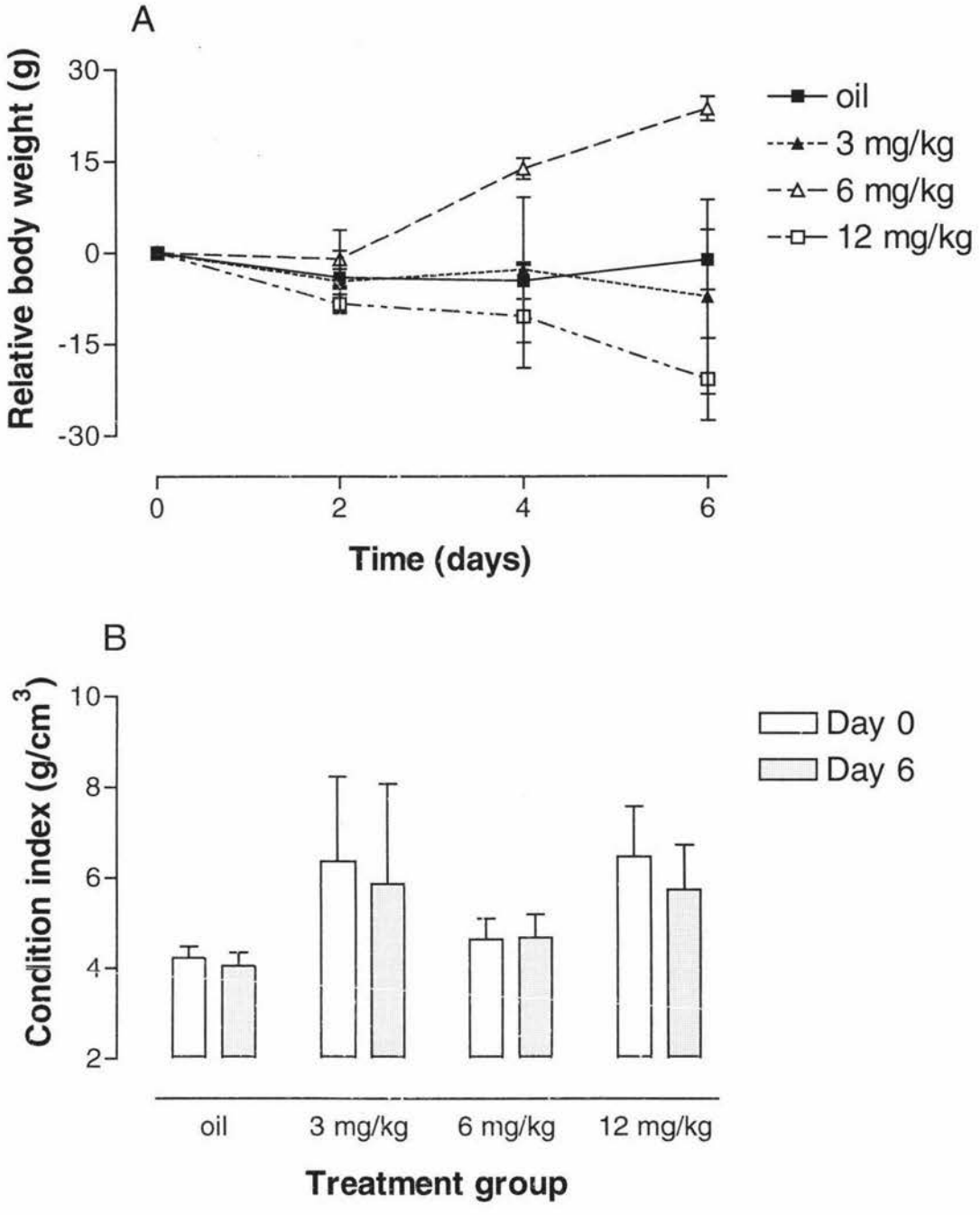


Fig. 2.10: Relative body weights on days 0, 2, 4 and 6 and condition index on days 0 and 6 (mean ± SE; oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2). A) Body weights relative to weight on day 0.
B) Condition index (body weight divided by the cube of the tarsus length) on days 0 and 6.

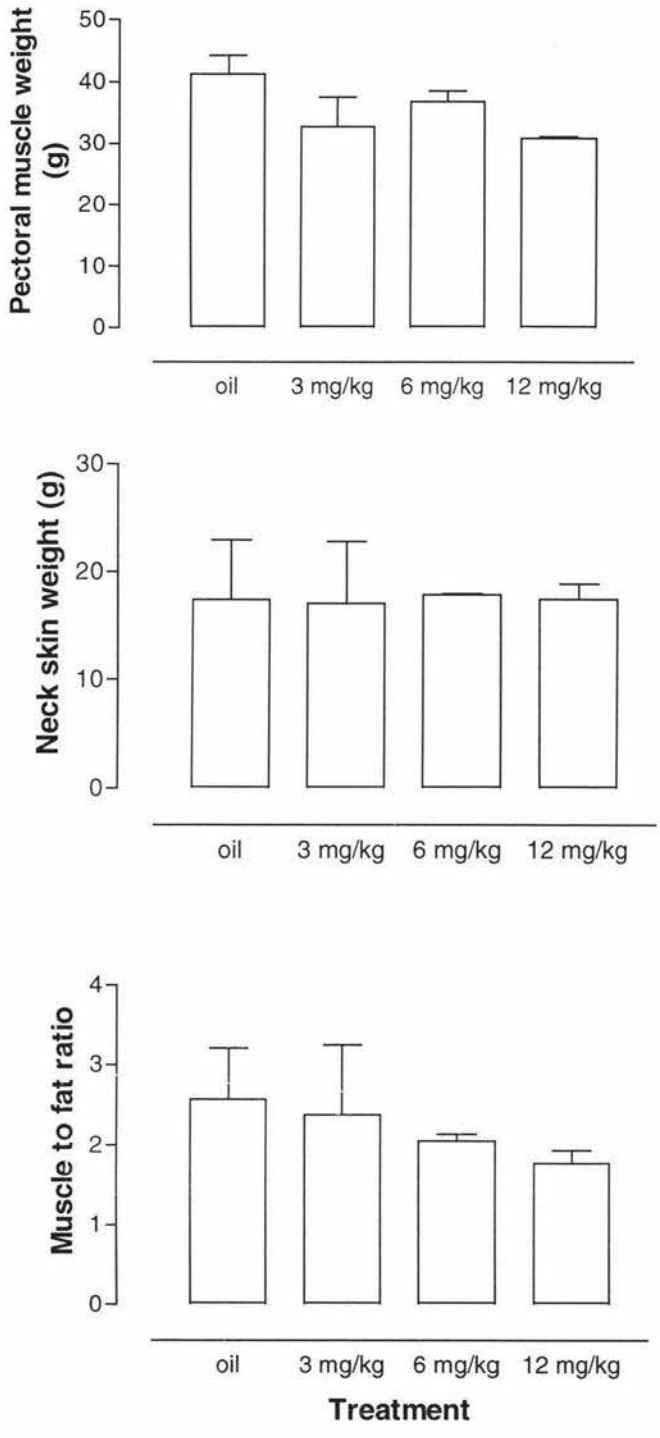


Fig. 2.11: Pectoral muscle weight, neck skin weight and ratio of pectoral muscle to neck skin weight on day 6 (mean ± SE, oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2).

2.4.1.6.3 Organ weights

Liver weights were expressed as a percentage of body weight (Fig. 2.12). The mean liver weights of all corticosterone treated groups were similar, and were more than double those of oil injected birds.

Paired testes weights were expressed both in grams and as a percentage of body weight (Fig. 2.13). Paired testes weights were less in the 12 mg/kg birds than in the other groups. Because of the small sample sizes, though, it is not clear whether or not this is an effect of the corticosterone treatment.

2.4.1.6.4 Secondary sexual characteristics

Cloacal gland areas were expressed as a percentage of the area of the gland on day 0 for each bird. Relative cloacal gland areas decreased markedly at the same rate in all treatment groups (Fig. 2.14) while remaining constant in the oil injected birds.

Oil injected birds continued to produce normal amounts of cloacal foam throughout the treatment period (Fig. 2.15). Birds treated with corticosterone, however, produced progressively less foam over the six days of injections. Foam production had stopped altogether by day 6 in all quail treated with 6 or 12 mg/kg corticosterone, and in two of the 3 birds treated with 3 mg/kg corticosterone. The amount of cloacal foam produced was inversely related to plasma testosterone levels (Fig. 2.16, one way ANOVA $F_{2,39}=27.862$, $p<0.001$). Birds producing normal amounts of foam (rated 2) had significantly higher plasma testosterone levels than those producing small amounts (rated 1) or no foam (rated 0) (Bonferroni $p=0.001$, $p<0.001$ for 2 versus 1 and 2 versus 0 respectively). However, there was no significant difference in testosterone levels between birds producing small amounts and those producing no foam (Bonferroni $p=0.773$).

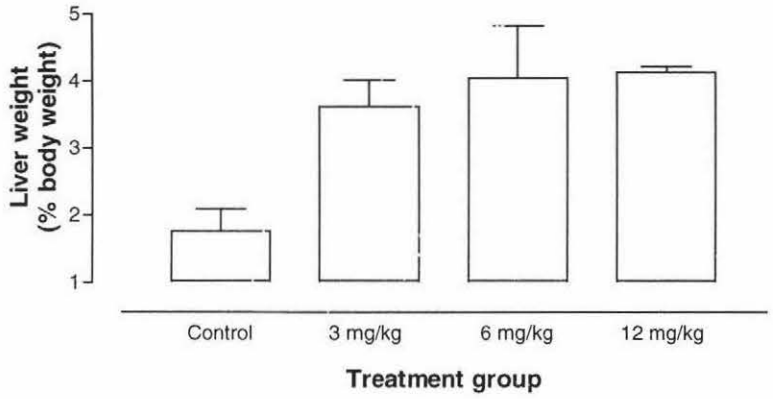


Fig. 2.12: Liver weight as a percentage of body weight on day 6 (mean ± SE; oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2).

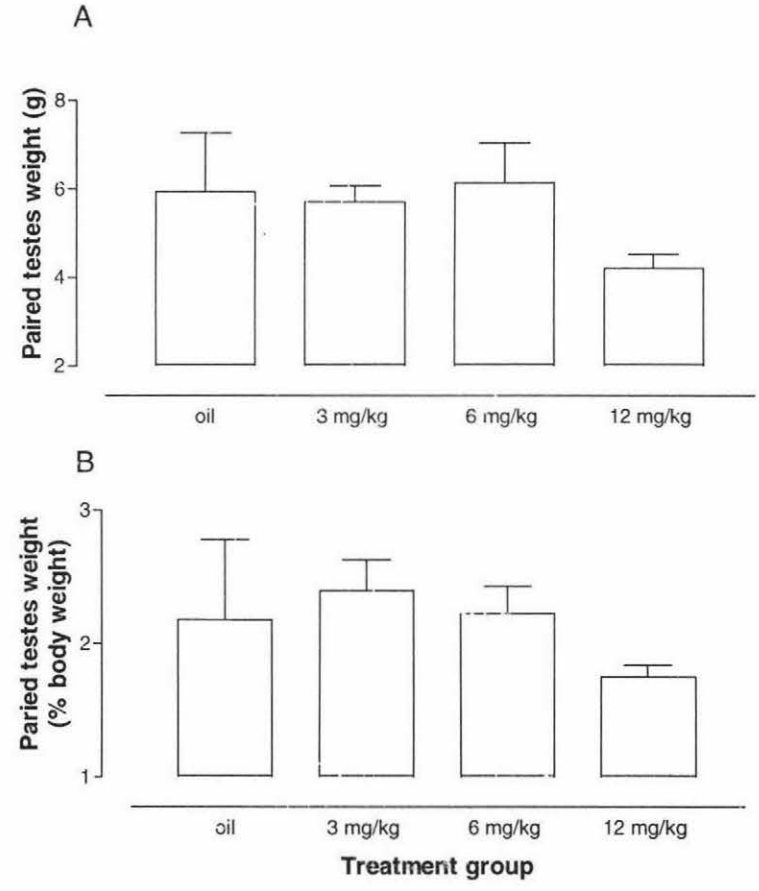


Fig. 2.13: Paired testes weights on day 6. (mean ± SE; oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2). A) weight in grams B) percentage body weight.

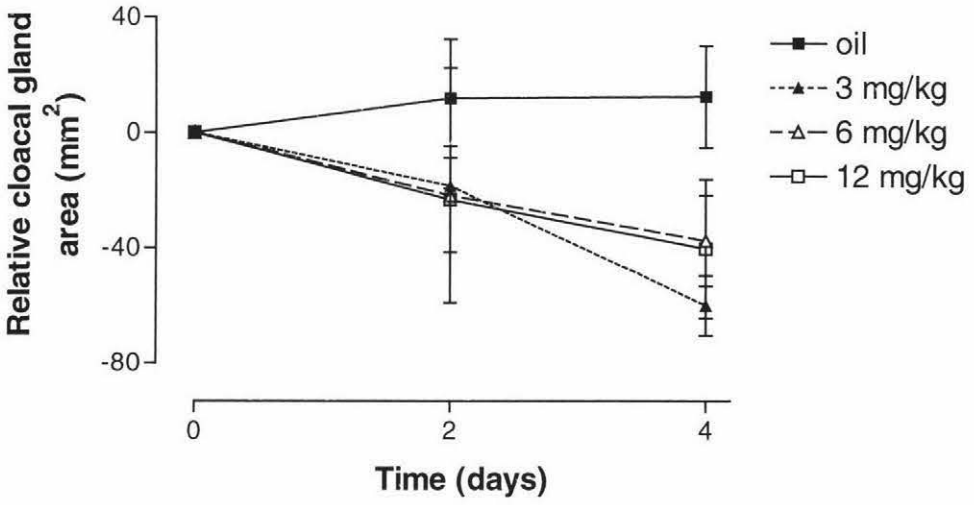


Fig. 2.14: Cloacal gland area on days 0, 2 and 4 relative to area on day 0 (mean ± SE; oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=3).

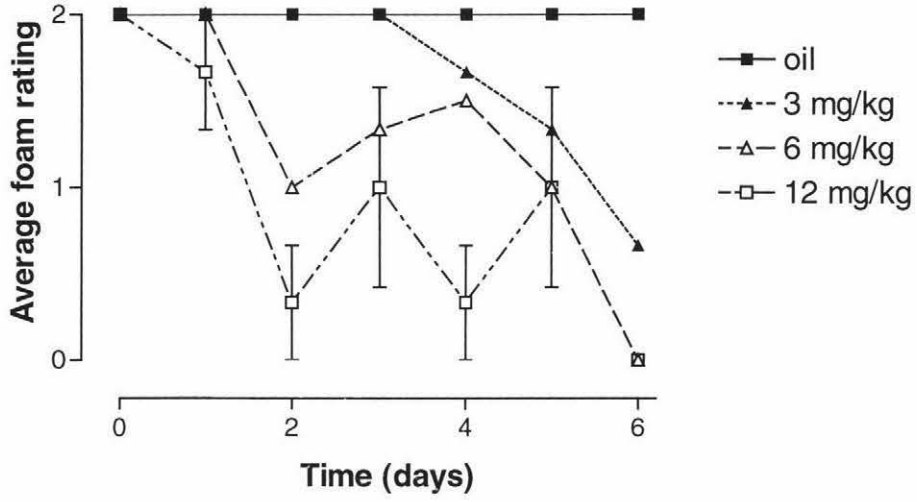


Fig. 2.15: Cloacal foam production over 24 hours on days 0, 2, 4 and 6. 2 = normal amount of foam produced; 1 = small amount of foam produced; 0 = no foam produced (mean ± SE; oil, n=2; 3 mg/kg, n=3; 6 mg/kg, n=2; 12 mg/kg, n=2).

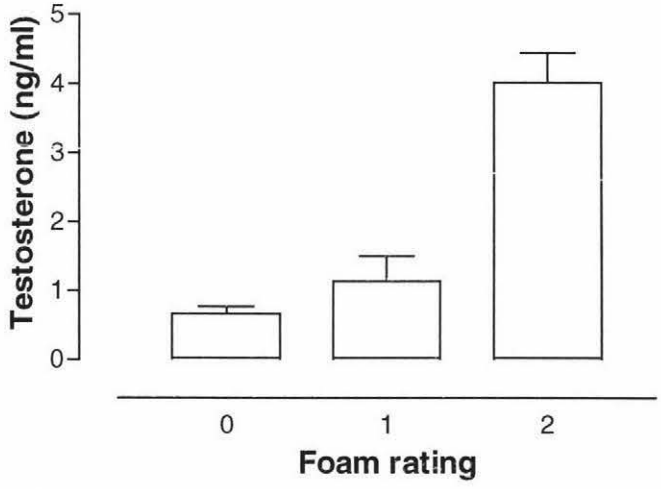


Fig. 2.16: Plasma testosterone levels of quail producing different amounts of cloacal foam over the treatment period (mean ± SE; 0 (no foam), n=10; 1 (small amounts of foam), n=5; 2 (normal amounts of foam), n=27).

2.4.2 Seven week old birds

2.4.2.1 Plasma corticosterone

Plasma corticosterone levels varied over the six days of treatment, but did not differ between treatments (Fig. 2.17; see Table 2.3 for statistics). Plasma corticosterone levels decreased between days 0 and 6 only in oil injected birds and birds treated with 0.075 and 0.3 mg corticosterone. However, plasma corticosterone levels did not differ between oil injected birds and any of the groups treated with corticosterone on day 0 or day 6 (Table 2.3). There were also no differences between groups in the area under the corticosterone versus time curves (Fig. 2.18, one way ANOVA $F_{5,34}=0.902$, $p=0.491$).

2.4.2.2 Plasma LH and testosterone

Plasma LH levels were measured on days 0 and 6 in oil injected birds and birds treated with 0.6 and 1.2 mg corticosterone (Fig. 2.19). Plasma LH levels were significantly higher on day 6 than day 0 in birds treated with 1.2 mg corticosterone (see Table 2.4 for statistics). There was no difference in plasma LH levels between treatment groups on either day.

Plasma testosterone levels varied over the six days of treatment in oil injected birds and those treated with 0.075, 0.15 and 1.2 mg corticosterone (Fig. 2.20, see Table 2.5 for statistics). Plasma testosterone levels were greater on day 6 than on day 0 in birds treated with 0.075, 0.15, and 1.2 mg corticosterone. (Table 2.5). However, there were no differences between treatment groups on any day (Kruskal-Wallis; $t=9.788$, $p=0.081$; $t=7.906$, $p=0.161$; $t=8.031$, $p=0.155$; $t=6.371$, $p=0.272$ for days 0, 2, 4 and 6 respectively). There were no differences between groups in the areas under testosterone versus time curves (Fig. 2.21, one way ANOVA $F_{5,40}=1.357$, $p=0.261$) over the six days of treatment.

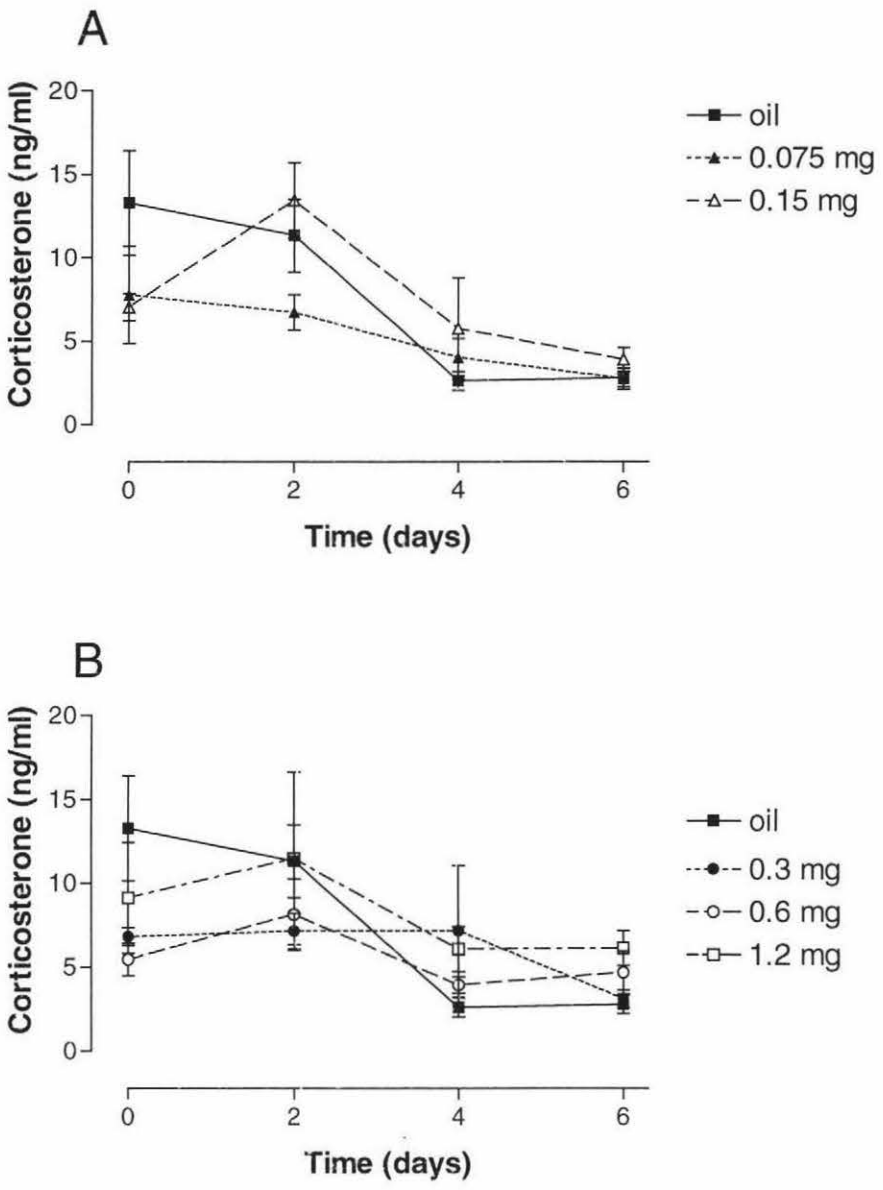


Fig. 2.17: Mean plasma corticosterone levels on days 0, 2, 4 and 6. Each sample was collected approximately 24 hours after the last injection (mean ± SE; n=8). A) groups treated with oil or 0.075 or 0.15 mg corticosterone B) groups treated with oil or 0.3, 0.6 or 1.2 mg corticosterone.

Table 2.3: Summary of two-way repeat measures ANOVA for plasma corticosterone levels on days 0, 2, 4 and 6.

	Plasma corticosterone		
Effect	F	DF	P
Day	27.529	3,108	<0.001 **
Treatment	0.848	5,36	0.525
Interaction	1.695	15,108	0.062
Comparisons between days			
Day 0 versus 6			
oil	13.843	1,36	0.001 **
0.075 mg	6.766	1,36	0.013 *
0.15 mg	3.411	1,36	0.073
0.3 mg	6.305	1,36	0.017 *
0.6 mg	2.721	1,36	0.108
1.2 mg	0.834	1,36	0.367
Comparisons between treatments			
Day 0			
oil versus 0.075 mg	3.078	1,36	0.088
oil versus 0.15 mg	1.426	1,36	0.240
oil versus 0.3 mg	1.645	1,36	0.208
oil versus 0.6 mg	1.889	1,36	0.178
oil versus 1.2 mg	0.865	1,36	0.359
Day 6			
oil versus 0.075 mg	0.436	1,36	0.513
oil versus 0.15 mg	0.612	1,36	0.439
oil versus 0.3 mg	0.046	1,36	0.831
oil versus 0.6 mg	0.907	1,36	0.347
oil versus 1.2 mg	3.480	1,36	0.070

Note: The first three rows show the overall effects of day (days 0, 2, 4 and 6) and treatment and the interaction between the two. The following rows show post hoc comparisons between days and between treatment groups. Significant differences ($p<0.05$) are marked with asterisks, and highly significant differences ($p<0.01$) are marked with double asterisks.

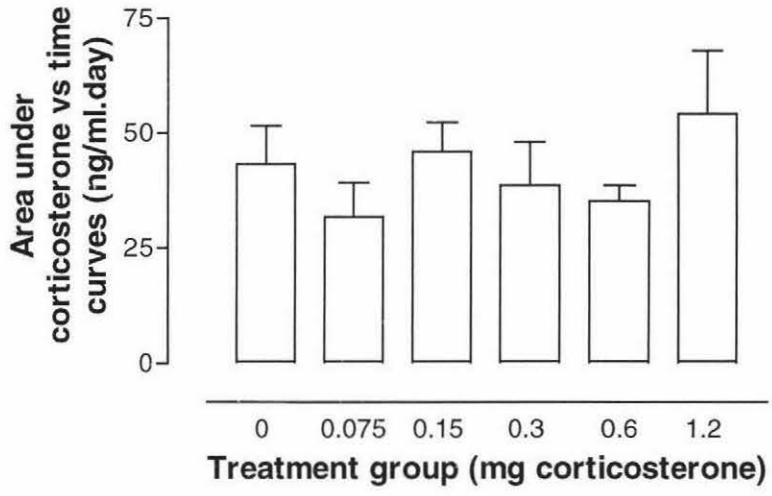


Fig. 2.18: Areas under the plasma corticosterone versus time curves over the treatment period (mean ± SE; n=8 per group).

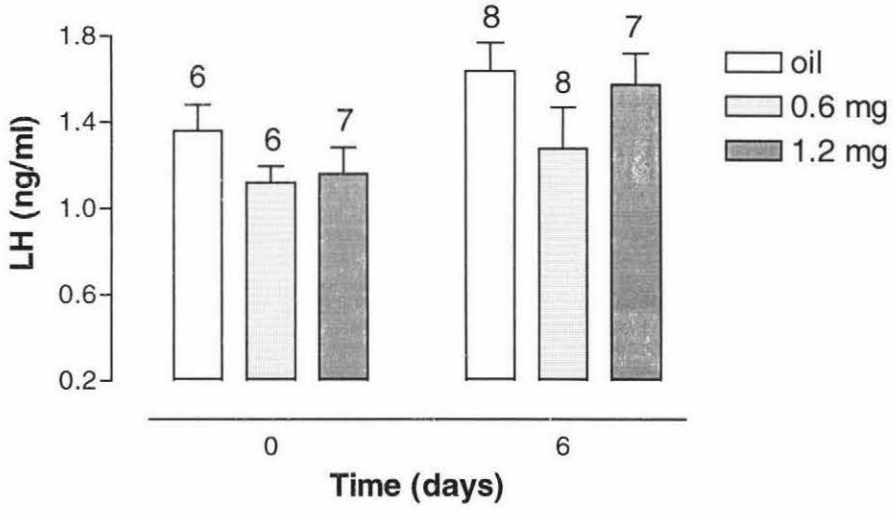


Fig. 2.19: Mean plasma LH levels on days 0 and 6 for birds treated with daily injections of or oil or 0.6 or 1.2 mg corticosterone. Each sample was collected approximately 24 hours after the previous injection (mean ± SE; sample sizes shown on graph).

Table 2.4: Summary of two-way repeat measures ANOVA for plasma LH levels on days 0 and 6.

		Plasma LH		
Effect		F	DF	P
Day		6.285	1,15	0.024 *
Treatment		1.132	2,15	0.349
Interaction		0.771	2,15	0.480
Comparisons between days				
Day 0 versus 6				
Oil		1.058	1,15	0.320
0.6 mg		0.734	1,15	0.405
1.2 mg		6.036	1,15	0.027 *
Comparisons between treatments				
Day 0				
	oil vs 0.6	1.737	1,15	0.207
	oil vs 1.2	1.076	1,15	0.316
Day 6				
	oil vs 0.6	1.189	1,15	0.293
	oil vs 1.2	0.072	1,15	0.793

Note: The first three rows show the overall effects of day (days 0 and 6) and treatment (0, 0.6 and 1.2 mg corticosterone) and the interaction between the two. The following rows show post hoc comparisons between days 0 and 6 and between treatment groups. Significant differences ($p<0.05$) are marked with asterisks, and highly significant differences ($p<0.01$) are marked with double asterisks.

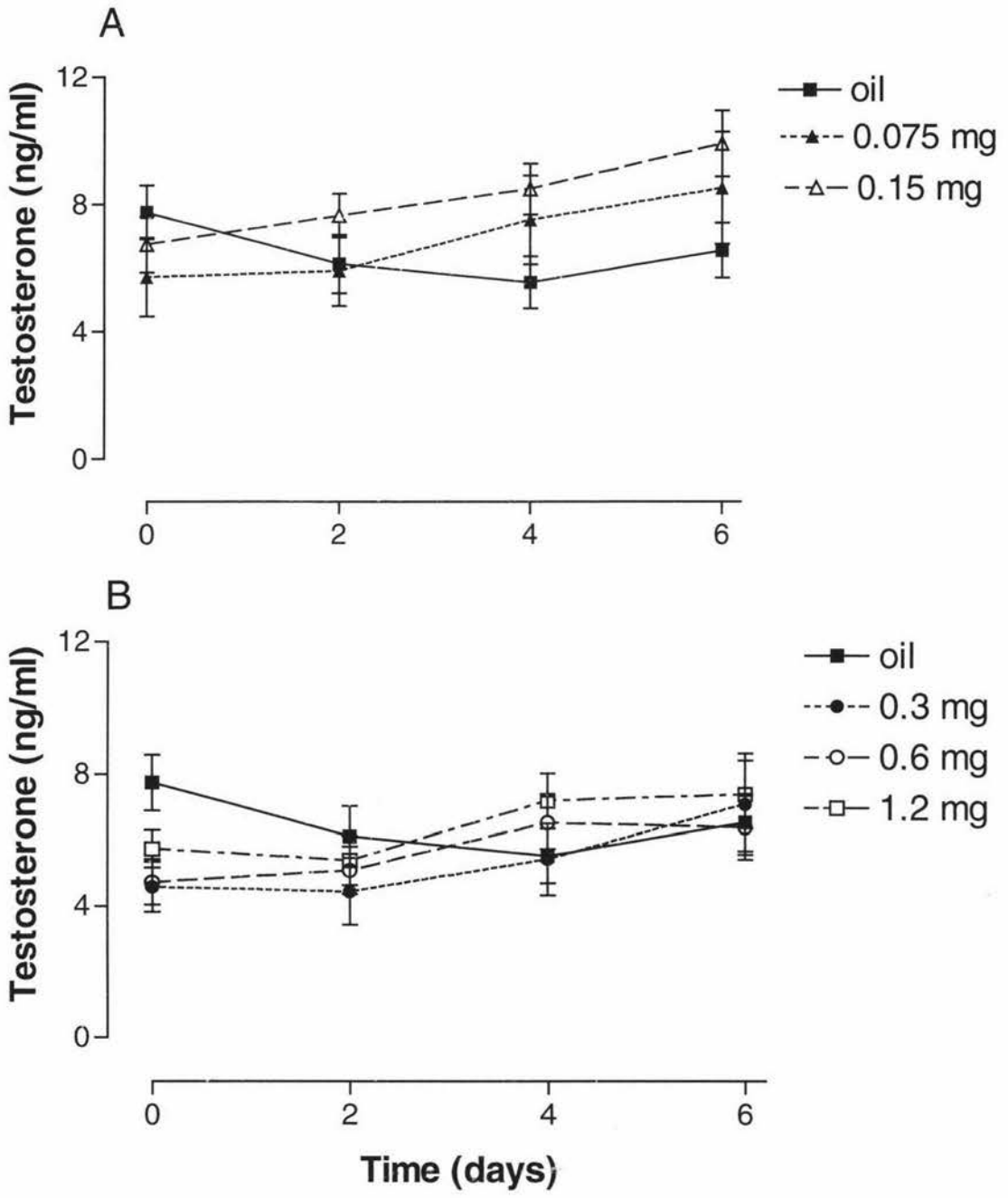


Fig. 2.20: Mean plasma testosterone levels on days 0, 2, 4 and 6. Each sample was collected approximately 24 hours after the last injection (mean ± SE; n=8 per group). A) Groups treated with oil or 0.075 or 0.15 mg corticosterone. B) Groups treated with oil or 0.3, 0.6 or 1.2 mg corticosterone.

Table 2.5: Summary of one-way repeat measures ANOVA for plasma testosterone levels on days 0, 2, 4 and 6.

	Plasma LH		
	F	DF	P
Effect of Day			
oil	4.592	3,18	0.015 *
0.075 mg	7.389	3,21	0.001 **
0.15 mg	10.112	3,21	<0.001 **
0.3 mg	1.348	3,18	0.290
0.6 mg	2.145	3,21	0.125
1.2 mg	3.705	3,18	0.031 *
Comparisons between days			
Day 0 vs 6			
oil	5.432	1,6	0.059
0.075 mg	15.575	1,7	0.006 **
0.15 mg	22.275	1,7	0.002 **
0.3 mg	1.503	1,6	0.266
0.6 mg	1.534	1,7	0.255
1.2 mg	7.127	1,6	0.037 *

Note: Variances were not homogeneous between treatments so a two-way repeat measures ANOVA could not be conducted. One way repeat measures ANOVAs were therefore performed for each treatment group. The first six rows show the overall effects of day (days 0, 2, 4 and 6) for each treatment. The following rows show post hoc comparisons between days 0 and 6 for each treatment group. Significant differences ($p<0.05$) are marked with asterisks, and highly significant differences ($p<0.01$) are marked with double asterisks.

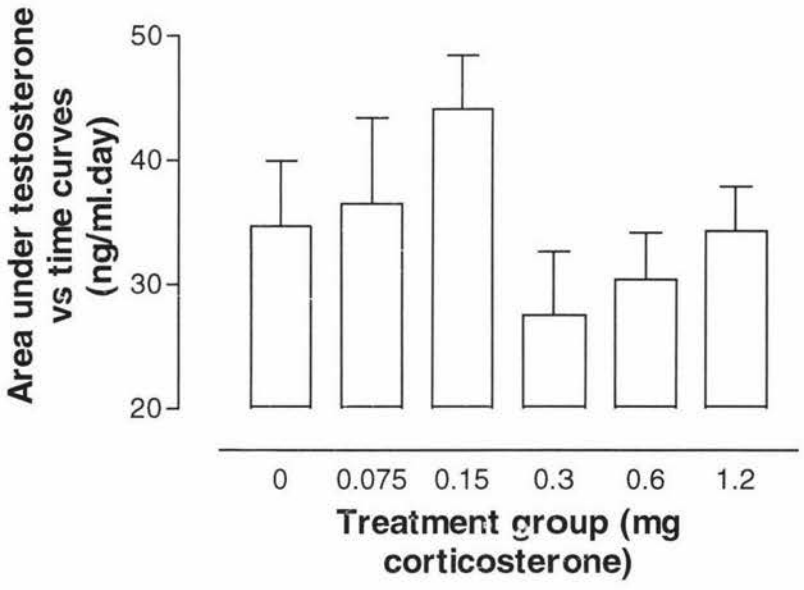


Fig. 2.21: Areas under the testosterone versus time curves over the treatment period (mean ± SE; n=8 per group).

Plasma testosterone levels in six month and seven week old birds were compared on day 0. Data from all birds in each experiment were combined as no birds had received any corticosterone treatment at this time. There was no significant difference in plasma testosterone between old and young birds on day 0 (2 sample t-test T_{58}=-1.184, p=0.241).

2.4.2.3 Body measurements

2.4.2.3.1 Body weight

There were no differences in relative body weight between groups on day 6 (Fig. 2.22, one way ANOVA $F_{5,41}$ = 1.351, p=0.263), or between oil injected birds and any of the corticosterone treatment groups (Bonferroni, p=0.242 for oil versus 0.3 mg corticosterone; p=1.000 for oil versus all other treatment groups).

2.4.2.3.2 Testes weight

There were no differences between groups in paired testes weight on day 6 (Fig. 2.23, one way ANOVA $F_{5,41}$=0.531, p=0.752).

2.4.2.3.3 Secondary sexual characteristics

Cloacal gland areas relative to areas on day 0 varied across days and between treatments (Fig. 2.24; see Table 2.6 for statistics). There was a dose-dependent effect of corticosterone treatment on cloacal gland area. The relative cloacal gland areas of birds treated with the two lowest doses of corticosterone (0.075 and 0.15 mg) remained similar to those of oil injected birds on all days (Table 2.6). Relative areas of birds treated with the three highest doses of corticosterone decreased over the 6 days of treatment. Birds treated with 0.3 mg corticosterone had significantly smaller relative cloacal gland areas than those of oil injected birds on day 4 only. However, relative cloacal gland areas of birds injected with the two highest doses of corticosterone were significantly smaller than oil injected birds on days 2, 4, and 6 (Table 2.6).

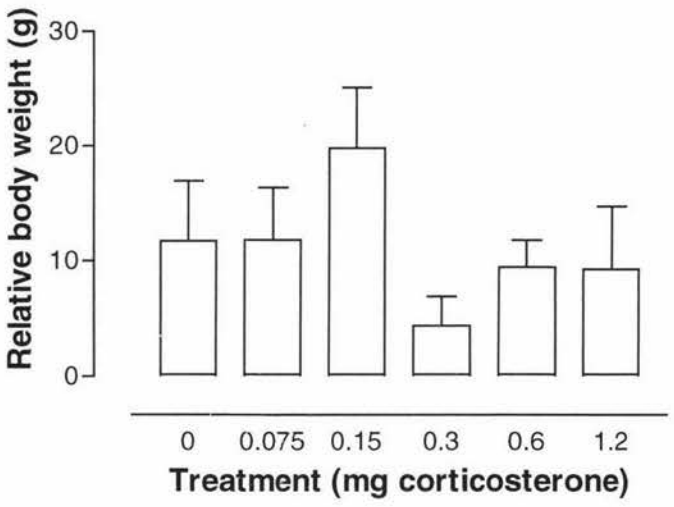


Fig. 2.22: Body weights on day 6 relative to weight on day 0 (mean ± SE; n=8 per group).

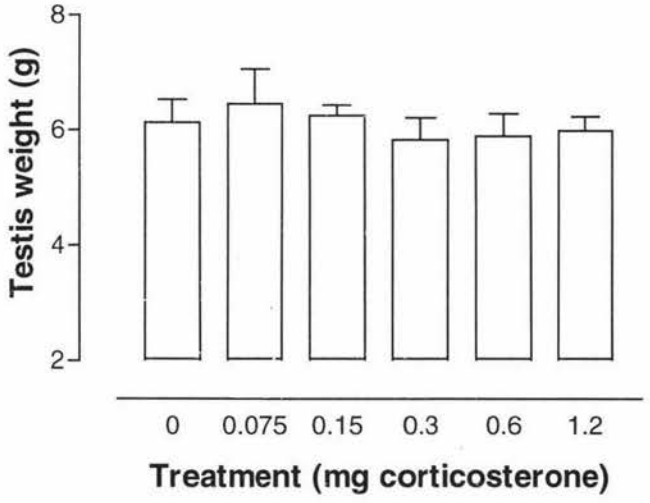


Fig. 2.23: Paired testes weights on day 6 (mean ± SE; n=8 per group).

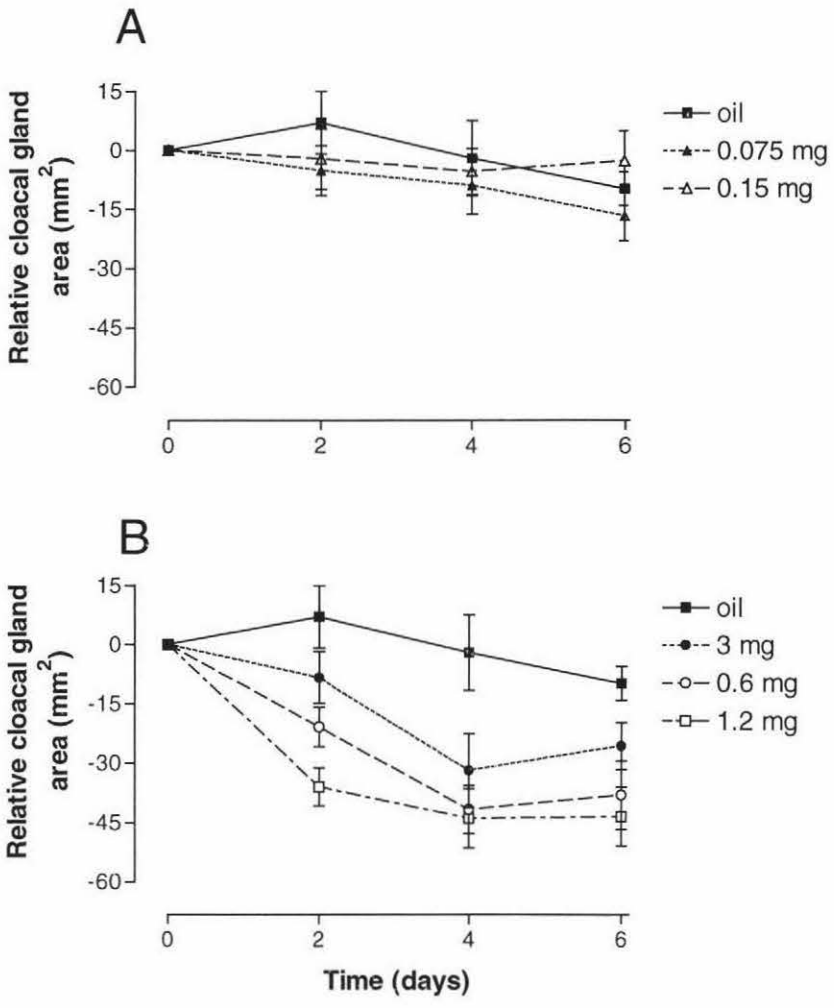


Fig. 2.24: Cloacal gland areas on days 0, 2, 4 and 6 relative to area on day 0 (mean ± SE; n=8 per group). A) groups treated with oil or 0.075 or 0.15 mg corticosterone. B) groups treated with oil or 0.3, 0.6 or 1.2 mg corticosterone.

Table 2.6: Summary of two-way repeat measures ANOVA for relative cloacal gland area on days 2,4 and 6.

	Plasma corticosterone			
Effect	F	DF	P	
Day	10.379	2,82	<0.00	**
Treatment	7.894	5,31	<0.00	**
Interaction	1.021	10,82	0.433	
Comparisons between				
Day 2				
oil vs 0.075 mg	1.754	1,41	0.193	
oil vs 0.15 mg	1.004	1,41	0.322	
oil vs 0.3 mg	2.794	1,41	0.102	
oil vs 0.6 mg	9.163	1,41	0.004	**
oil vs 1.2 mg	20.358	1,41	<0.00	**
Day 4				
oil vs 0.075 mg	0.41	1,41	0.525	
oil vs 0.15 mg	0.097	1,41	0.758	
oil vs 0.3 mg	7.543	1,41	0.009	**
oil vs 0.6 mg	13.318	1,41	0.001	**
oil vs 1.2 mg	13.877	1,41	0.001	**
Day 6				
oil vs 0.075 mg	0.522	1,41	0.474	
oil vs 0.15 mg	0.555	1,41	0.461	
oil vs 0.3 mg	2.73	1,41	0.106	
oil vs 0.6 mg	8.696	1,41	0.005	**
oil vs 1.2 mg	11.505	1,41	0.002	**

Note: The first three rows show the overall effects of day (days 0, 2, 4 and 6) and treatment and the interaction between the two. The following rows show post hoc comparisons between treatment groups on days 2, 4 and 6. Significant differences $p<0.05$) are marked with asterisks, and highly significant differences ($p<0.01$) are marked with double asterisks.

2.5 Discussion

There was a strong positive correlation between plasma and faecal concentrations of corticosterone in oil injected, six month old birds, indicating that faecal corticosterone levels can provide an indirect measure of basal plasma corticosterone levels. Daily injections of corticosterone were able to inhibit the reproductive systems of male Japanese quail, as shown by the decrease in cloacal gland area in both seven week old and six month old birds, and the decrease in plasma testosterone levels in the six month old birds. Administration of 1.5 mg of corticosterone daily was the lowest dose that resulted in a decrease in cloacal gland area in the younger birds.

2.5.1 Plasma corticosterone

Plasma corticosterone levels in seven week old quail were not elevated 24 hours after a corticosterone injection. This result is similar to that seen in previous studies in which injections were used to administer corticosterone. When three week old chicks were injected with 0.5 mg/kg corticosterone, all corticosterone had been cleared within two hours (Davison *et al.*, 1980). This dose is similar to the two lowest doses used in the current experiment. Studies on the clearance rate of corticosterone in male birds reported average corticosterone half-lives of 22 minutes in eight week old male broiler chickens (Birrenkott and Wiggins, 1984), 7.5 minutes in male ducks (*Anas platyrhynchos*, Holmes *et al.*, 1974) and 9.75 minutes (Kovacs and Peczely, 1983) or 10.2 minutes (Kovacs *et al.*, 1983) in male Japanese quail. Any of these clearance rates would result in complete clearance of corticosterone from the plasma by 24 hours if the doses used in the current experiment were administered. Therefore, in order to see changes in plasma corticosterone levels, blood samples would need to be collected during the hours immediately after injection.

Plasma corticosterone levels in some of the seven week old oil injected birds were considerably higher on day 0 than was expected (range = 3.84 - 24.87 ng/ml; mean = 13.28 ± 2.27 ng/ml). Levels were higher than those seen in

previous studies using male Japanese quail. Male Japanese quail in other studies had basal corticosterone levels of 1.51 ng/ml (Scatterlee *et al.,* 1983), 1-1.7 ng/ml (Kovacs *et al.,* 1983), 0.84 ng/ml (Jones *et al.,* 1994) and 1.5-2.4 ng/ml (Boissin and Assenmacher, 1970). In the current study, plasma corticosterone levels decreased over the six days of injections and on day 6 corticosterone levels were similar to those seen in previous studies (range = 1.01 - 5.59 ng/ml; mean = 2.79 ± 0.93). There could be several explanations for this. The birds had only been brought into our facility one week before treatment started, and the higher levels at the start of the experiment could indicate that they had not yet become accustomed to handling or human contact. If this was the case, removing birds for sampling could have initiated the corticosterone responses in neighbouring birds, resulting in corticosterone levels that were considerably higher than basal levels by the time these birds were bled. However, Webb and Marshaly (1984) reported no differences in basal plasma corticosterone levels in handled and non-handled chickens after seven days of regular handling, indicating that it is unlikely that corticosterone levels would decrease so dramatically with only four days of regular handling. Alternatively, the birds could have had increased basal corticosterone levels due to the novelty of their surroundings, as they had had only one week to acclimatise to the room and to their cages. The seven week old birds were also younger than those used previously, so it is possible that the younger males had higher basal corticosterone levels than older birds. There is some evidence that young (reproductively immature) birds can have basal corticosterone levels considerably higher than mature birds. For example, Schmeling and Nockels (1978) found that immature chickens of both sexes had basal corticosterone levels over 4.5 times higher than mature birds of the same sex. Although the seven week old birds used in the present study were sexually mature (i.e. had large cloacal glands, were secreting cloacal foam and were crowing), they had only recently reached maturity, and it is possible that basal corticosterone levels were still elevated due to their age. Another possibility is that the difference in basal corticosterone levels between the seven week old and six month old birds was related to differences between experiments in the number of birds kept in one room. There were 72 young

male quail in a single room compared with only 12 old quail. Aggressive interactions between male birds has been found to increase plasma corticosterone levels (Harding, 1981), therefore the presence of so many other mature male quail in close proximity could have resulted in elevation of plasma corticosterone levels in the younger birds. However, if this was the cause of elevated basal corticosterone levels, it is unclear why levels decreased over the six days of treatment.

2.5.2 Faecal corticosterone

Faecal corticosterone levels were measured in six month old birds. Levels remained low in oil injected birds throughout the experiment, whereas levels increased in all corticosterone treated birds. Faecal samples were collected after all the birds had been given their daily injections, so birds had been injected between five minutes and two and a half hours before faecal samples for that day were collected. Faecal corticosterone levels in some corticosterone injected birds were higher on day 0 (the first day of injections) than on the previous four days, indicating that some injected corticosterone had appeared in the faeces in the period between injection and collection of faeces. These results indicate that it takes less that two hours for injected corticosterone to begin to enter the faeces in the Japanese quail, which is similar to the reported lag times of one hour in the duck (Holmes and Slikker, 1976) and less than two hours in the northern spotted owl (*Strix occidentalis caurina*, Wasser *et al.*, 1997).

In all groups of corticosterone treated birds, there was a peak in faecal corticosterone excretion during the 24 hours after the first injection, and levels decreased thereafter to a lower level, where they remained for the duration of treatment. There was a dose-dependent relationship between corticosterone dose and the areas under faecal corticosterone excretion curves, indicating that the total amount of corticosterone excreted in the faeces was proportional to the amount present in the plasma. Although it was not possible to compare plasma and faecal corticosterone levels in treated birds (due to the contamination of plasma samples), this result provides support for the use of

faecal corticosterone measures as an indication of plasma corticosterone levels.

The percentage of corticosterone injected that was excreted into the faeces was low in all groups of treated birds. Within the first 24 hours, the amount of corticosterone excreted by individual birds varied between 2.08 and 6.20% with an average of 4.01% ± 1.09 (n=7) over all treated birds. Although this percentage seems very small, it has been suggested that normally less than 5% of endogenous hormone is excreted into the faeces unmodified (Bentley, 1998), and Holmes and Slikker (1976) found that less than 1% of injected corticosterone was excreted unmodified in the duck. The remaining 99% was thought to have been metabolised to corticosterone derivatives, conjugated to glucuronic or sulfuric acids (in the liver and kidneys) and excreted. By day 6, the percentage of injected corticosterone excreted over the previous 24 hours had decreased to an average of 1.31 ± 0.41% (range = 0.51- 2.60%, n=7). This decrease in percentage excretion indicates that the conversion rate of corticosterone to metabolites may have increased with treatment.

An increase in the metabolism rate of corticosterone with treatment has been reported in other bird species. Davison *et al.* (1985) found that after one week of corticosterone treatment by implants in four week old chickens, average corticosterone levels in the plasma had decreased. This decrease was not reversed with replacement of the implant, indicating that an increased rate of corticosterone clearance from the blood was occurring. A similar pattern was seen in six month old hens implanted with osmotic pumps delivering corticosterone (Etches *et al.,* 1984), and 18 week old pullets infused with corticosterone (Petitte and Etches, 1989). Although faecal corticosterone levels were not measured in any of these studies, the apparent increases in the rate of corticosterone metabolism agree with the results in the current experiment, in which less unmodified corticosterone was excreted with repeated corticosterone treatment.

2.5.3 Relationship between plasma and faecal corticosterone

Faecal corticosterone levels were measured in samples comprising the entire faecal production of each bird over 24 hours. The concentration of corticosterone in each sample was therefore a measure of the average level of faecal corticosterone over 24 hours. If plasma levels of corticosterone are relatively constant then there should be a clear relationship between corticosterone levels in a plasma sample and corticosterone levels in the faecal sample collected over the preceding 24 hours. A strong relationship was seen for oil injected birds when plasma corticosterone concentration was compared to faecal corticosterone concentration. However, no correlation was seen between plasma corticosterone and total faecal corticosterone excretion (ng) over the past 24 hours. As there were only seven time-points for comparison, this relationship needs to be examined further. However, this is an encouraging result as it indicates that plasma and faecal corticosterone concentrations are closely related in the Japanese quail. A similar result has been seen in the ring-tailed lemur (*Lemur catta*, Cavigelli, 1999), although the sample size was only four. The current result is the first step towards validating the use of faecal corticosterone measurements as a non-invasive measure of stress in birds.

As all plasma corticosterone samples for treated birds had to be excluded, a comparison between plasma and faecal corticosterone after corticosterone injections could not be made. However, when plasma corticosterone levels vary markedly over 24 hours (as occurs after a corticosterone injection), then a clear relationship between plasma corticosterone 24 hours after an injection and faecal corticosterone collected over the 24 hour period is less likely to be found. Therefore, it would be of more benefit to examine this relationship when plasma and faecal samples are collected several times over the 24 hours after an injection.

2.5.4 Plasma LH and testosterone

As all blood samples were collected 24 hours after the previous injection of corticosterone, it is not suprising that changes in plasma LH levels with treatment were not seen in either experiment. Although several studies have

shown that treatment using corticosterone implants results in decreased plasma LH in female chickens (Etches *et al.,* 1984; Petitte and Etches, 1988; Petitte and Etches, 1989), this is a very different situation to that seen when corticosterone is administered by daily injections. All corticosterone had been cleared from the plasma 24 hours after an injection, so even if there had been an acute effect of corticosterone on LH, it is likely that plasma LH had also returned to normal levels by this time. A similar study to the current experiment was conducted by Deviche *et al.* (1982). Four week old male Japanese quail were injected daily with 0.25 or 1 mg of corticosterone and there was no effect of this treatment on plasma LH levels 24 hours after an injection.

The testosterone response to a corticosterone injection was different in the six month old and seven week old birds. In the older birds, plasma testosterone levels remained constant in oil injected birds, whereas levels decreased markedly in all corticosterone treated birds. This is a clear indication that corticosterone was having an inhibitory effect on the reproductive system in these birds. This result prompted the second study in which a wider range of corticosterone doses was used. However, although basal testosterone levels in the younger birds used for the second experiment were similar to those seen in the six month old birds, plasma testosterone levels did not decrease with treatment in the younger birds. This difference could be due simply to experimental differences, as birds from the two age groups were not tested concurrently and had not been kept under identical conditions before treatment. Alternatively, this result could indicate an effect of age on the responsiveness of the reproductive system to corticosterone.

Little work has been carried out on the effect of age on the adrenal and gonadal axes of birds. In the current study, the older birds were only six months old, which is not old for a Japanese quail, who can live to over four years of age (Ottinger and Balthazart, 1986). The six month old birds also had similar basal testosterone levels to seven week old birds, had large testes and cloacal gland areas and were secreting cloacal foam. This is consistent with other studies of Japanese quail, in which six or nine month old birds still exhibit normal mating

behaviour, have large cloacal glands, and produce cloacal foam (Ottinger *et al.*, 1983; Ottinger and Balthazart, 1986). The results from the present experiment are therefore very interesting, as they suggest that younger quail may be more resilient to the effects of corticosterone on reproduction. More study is needed however, for definite conclusions to be drawn.

Plasma testosterone levels decreased in six month old birds 24 hours after a corticosterone injection, whereas LH levels were unchanged at this time. This indicates that a large proportion of the inhibition of testosterone was occurring at the level of the testes, rather than the hypothalamus or pituitary. Inhibition of testosterone at the level of the testis could be due to direct inhibition of testosterone release, a decrease in the amount of testosterone produced (i.e. decreased testosterone mRNA), or desensitisation of the testis to LH (i.e. down-regulation of LH receptors).

2.5.5 Faecal parameters

Corticosterone treatment resulted in increases in both faecal dry weight and water content in birds treated with 6 or 12 mg/kg corticosterone. Several previous studies have shown increases in both dry faeces weight and water content with corticosterone treatment. Siegel and Kampen (1984) found that faecal dry weight doubled, and the water content of the faeces increased by a factor of four when chickens were given daily 5 mg/kg corticosterone injections. This was accompanied by an increase in both food and water intake. Covasa and Forbes (1995) also found an increase in the amount of food and water consumed with corticosterone treatment. The increase in percentage water of the faeces is probably due to an increase in protein breakdown initiated by corticosterone, resulting in an increase in nitrogen excretion. The result of this process is an increase in the volume of urine produced and a consequent increase in the amount of water drunk (Siegel and Kampen, 1984; Covasa and Forbes, 1995).

2.5.6 Body measurements

2.5.6.1 Body weight and condition index

A condition index can be calculated to provide a measure of the relative amount of fat and muscle in a bird that is independent of its size. Condition indices are often calculated by dividing the body weight by a measure of the size of the bird such as the wing length or leg length. In the current study, the tarsus length was used as a measure of body size to avoid variation due to different degrees of wear of the outer primary feathers of the wing. Fat is the primary energy store, and changes in fat stores are responsible for most variation in body weight in adult birds. Therefore, birds with a high condition index are likely to have larger energy stores than those with a low condition index, and are likely to be better able to survive perturbations in their environment. As it takes into account the structural body size of the bird, a condition index also allows comparison between birds of different sizes.

Condition indices were calculated in the six month old birds as corticosterone has been seen to increase fat stored in birds (Bartov, 1982; Davison *et al.,* 1983; Simon, 1984; Buyse *et al.,* 1987; Bray, 1993; Covasa and Forbes, 1995). There was very little change in either body weight or condition index with corticosterone treatment in six month old birds. However, there was a trend towards decreased weight gain in the seven week old birds treated with the three highest corticosterone doses. Although the differences were not significant, more distinct differences may have been seen if treatment was carried out for a longer period. Most studies in which there was an effect of corticosterone treatment on body weight are studies in which young birds not yet at their adult weight were used (Davison *et al.,* 1983; Davison *et al.,* 1985; Buyse *et al.,* 1987; Bray, 1993; Hayashi *et al.,* 1994). The direct cause of this decreased growth rate is not known, but it may be due to inhibition of the expression of insulin-like growth factors (Bray, 1993). In studies in which adult birds were used, there was generally either no effect of treatment on body weight (Williams *et al.,* 1985; Wingfield and Silverin, 1986), or an increase in weight (Petitte and Etches, 1989).

2.5.6.2 Muscle to fat ratio

The muscle to fat ratio of a bird can give an indication of the effects of corticosterone on metabolism, as corticosterone has been reported to decrease muscle weight (Gross *et al.,* 1980; Davison *et al.,* 1983; Hayashi *et al.,* 1994) and increase the fat content (Davison *et al.,* 1983; Simon, 1984; Buyse *et al.,* 1987; Covasa and Forbes, 1995) of birds. Various methods have been used to estimate the effects of corticosterone on muscle and fat. Abdominal fat or total wet skin weight can be measured to give an estimate of total body fat (Whyte and Bolen, 1984). Japanese quail appear to store fat more in the neck region than the abdominal area, and it was thought that measurement of the neck skin would be easier and more accurate than measurement of abdominal fat. The wet weight of neck skin was measured after removal of feathers as an estimate of body fat in six month old birds. The effect of corticosterone on muscle is usually assessed by weighing either the pectoral or leg muscle. In the case of the quail, the pectoral muscle was used.

There were no differences in neck skin weight between groups, and there was a large amount of individual variation in weights. Some of this variation is likely to be because it is difficult to define the limits of the neck and the measure would also be affected by the exact place of decapitation. Our experience therefore indicates that neck skin weight is probably not a good measure of body fat to use in the quail. There were also no obvious differences in pectoral muscle weight between groups, although the average pectoral muscle weight of the 12 mg/kg group was slightly lower than that of the other groups. If treatment had been carried out for a longer period, this difference may have become more marked. When the muscle to fat ratio was calculated, there was a trend towards a decreased ratio with higher doses of corticosterone. This result is consistent with corticosterone injections decreasing muscle weight and increasing fat content of the birds. However, the significance of this difference could not be tested due to the small sample sizes.

The lack of any marked changes in pectoral muscle, neck fat or the ratio between them may be partially due to the relatively short treatment period.

However, Williams *et al.* (1985) found no effect of 14 days of corticosterone injections on abdominal fat in six month old hens, indicating that the age of the bird and the fact that they were no longer growing may have also been responsible for the lack of obvious changes seen.

2.5.6.3 Organ weights

2.5.6.3.1 Liver weight

Birds in all corticosterone treatment groups had heavier livers than oil injected birds after six days of corticosterone injections. The liver has previously been found to be greatly affected by treatment with corticosterone in birds. In studies on a wide range of birds of many different ages, corticosterone treatment at various doses resulted in increased liver weights (Gross *et al.,* 1980; Davison *et al.,* 1983; Simon, 1984; Davison *et al.,* 1985; Williams *et al.,* 1985; Buyse *et al.,* 1987). This effect on liver weight is thought to be due to an increase in lipogenesis and glycogenesis in the liver stimulated by increased plasma corticosterone levels (Siegel and Kampen, 1984; Buyse *et al.,* 1987).

2.5.6.3.2 Testes weight

When paired testes weights from all groups of six month old quail were compared, there was a small decrease in weight for birds treated with the highest corticosterone dose (12 mg/kg). No effect of corticosterone treatment on testis size was seen in the seven week old birds. In general, testis weight is correlated with cloacal gland area (Massa *et al.,* 1980). This was not the case in either of the current studies, probably because treatment was only carried out for six days, and injections were used to deliver the corticosterone (instead of a method of delivery that results in prolonged elevation of plasma corticosterone). The effects of corticosterone treatment on the testes may have been greater if treatment had continued for longer. Several other studies have examined the effects of corticosterone administration on the gonads of various bird species. In female birds, there was no effect of corticosterone treatment on ovary or oviduct weights in chickens fed corticosterone for 10 days (Gross *et al.,* 1980), or infused with 10 µg/h corticosterone for 14 days (Etches *et al.,* 1984). However, there was a significant decrease in gonadal weight in hens given 30

μg/h corticosterone for seven days (Petitte and Etches, 1991) or 14 days (Etches *et al.,* 1984). In male birds, there was no change in testis weight in Japanese quail injected with 0.25 or 1 mg corticosterone daily for 15 days (Deviche *et al.,* 1982). However, male ducklings given 3 mg injections of corticosterone daily for 19 days (Deviche *et al.,* 1979), maturing Japanese quail treated with ACTH for 12 days (Edens, 1987) and young Japanese quail subjected to social stress for seven days (Edens *et al.,* 1983; Edens, 1987) all showed significant decreases in testis weights. In male redheaded buntings (Chaturvedi and Suresh, 1990), treatment with corticosterone decreased testis weights only in the phases of the reproductive cycle when the testes were either growing or regressing. Therefore, effects of corticosterone on testis size in birds may be more apparent when the testes are growing or regressing than at other times.

2.5.6.4 Secondary sexual characteristics

2.5.6.4.1 Cloacal gland area

The cloacal gland area of both six month and seven week old birds treated with certain doses of corticosterone decreased over the course of the treatment, despite the lack of changes in plasma testosterone 24 hours after an injection in the younger quail. This effect of corticosterone administration has been seen before in male Japanese quail after four days of treatment with 1 mg corticosterone per day (Deviche *et al.,* 1982). However, testosterone levels were not measured in the earlier study. As the size of the cloacal gland is androgen dependent, it would be expected that a decrease in cloacal gland area would be accompanied by a decrease in plasma testosterone levels. This was the case in the current experiment with six month old birds, in which testosterone levels decreased within two days of corticosterone treatment, and remained depressed for the duration of treatment. However, when seven week old quail were treated with corticosterone, testosterone levels 24 hours after a corticosterone injection were not depressed, and yet cloacal gland area still decreased. This indicates that either testosterone levels did decrease with treatment, but had returned to normal by 24 hours, or that there is another mechanism to decrease cloacal gland area independently of plasma

testosterone levels. Deviche *et al.* (1982) found that corticosterone has an effect on the conversion of testosterone to various metabolites in both the pituitary and the cloacal gland. Overall, corticosterone appears to increase the proportion of testosterone that is transformed into metabolites such as 5β-dihydrotestosterone and 5β-androstane-3α,17β -diol. These β -metabolites were found by Massa *et al.* (1980) to have no stimulatory effect on the cloacal gland. The production of stimulatory metabolites such as androstenedione, 5α -dihydrotestosterone, and 5α -androstane-3α,17β -diol (Massa *et al.,* 1980) are decreased in the pituitary and cloacal gland with corticosterone treatment (Deviche *et al.,* 1982). These studies provide evidence of a mechanism for a decrease in cloacal gland area that is independent of changes in plasma testosterone. However, it is likely that fluctuations in plasma testosterone did occur in the young quail, and decreases in the cloacal gland area may have been due to a combination of these two factors.

2.5.6.4.2 Foam production

Cloacal foam is secreted by the cloacal gland of the male Japanese quail, and is thought to increase the likelihood of fertilisation (Adkins-Regan, 1999). The production of cloacal foam is androgen dependent and foam is produced only in reproductively active male quail. Foam production therefore provides an external measure of plasma testosterone and the reproductive state of the bird. The amount of cloacal foam produced by six month old birds in the present study decreased with corticosterone treatment, while remaining relatively constant in oil injected birds. There was a highly significant relationship between the amount of cloacal foam produced and plasma testosterone levels in the bird. This is the first demonstration of a clear relationship between plasma testosterone levels and cloacal foam production in the quail.

2.6 Conclusions

The preliminary experiment showed that daily injections of corticosterone have a negative effect on reproductive hormones and secondary sexual characteristics of the male Japanese quail. As the doses used all resulted in similar decreases in plasma testosterone, cloacal gland area and foam production, it was decided to repeat the experiment using a larger range of corticosterone doses. However, in the second experiment using younger birds, plasma corticosterone treatment did not result in changes in plasma testosterone levels 24 hours after an injection. This indicates that older birds may be more responsive to the negative effects of corticosterone on reproduction than younger birds. Although plasma testosterone levels where not depressed 24 hours after a corticosterone injection in the seven week old birds, changes in the cloacal gland area indicated that 0.3 mg/day was the lowest corticosterone dose able to inhibit the reproductive system. As cloacal gland area is a crude measure of reproductive status, this needs to be tested further. The a strong correlation between plasma and faecal corticosterone for basal levels of plasma corticosterone is the first demonstration of such a relationship in birds. This indicates that faecal corticosterone levels can be used as a measure of plasma corticosterone levels.

Chapter 3: Acute effects of a corticosterone injection

3.1 Abstract

In the experiment described in the previous chapter, daily corticosterone injections decreased cloacal gland area in young male Japanese quail (*Coturnix coturnix japonica*), however plasma testosterone levels 24 hours after injection were normal. The current study was designed to examine acute changes in plasma hormone levels after a corticosterone injection. Reproductively mature male Japanese quail were divided into three treatment groups. The first group received oil injections on days 0-6 (oil-treated). The second group received daily corticosterone (1.2 mg) injections (cort-treated), and the third group received a corticosterone injection (1.2 mg) on day 0, five days of oil injections, and a second corticosterone injection on day 6 (cort-oil-cort treated). Blood samples were collected during the 24 hours after injection on days 0 and 6, faecal samples were collected on day 0 only. On day 6, cort-treated birds had lower plasma corticosterone levels at 3 hours than did cort-oil-cort treated birds. Plasma corticosterone levels also returned to basal faster in birds receiving repeated corticosterone injections. This indicates that the rate of corticosterone clearance from the plasma increases after repeated corticosterone injections. Plasma testosterone levels were similar in both groups of corticosterone injected birds on both days 0 and 6. Levels were depressed at 3 and 6 hours, and had returned to basal by 12 or 24 hours. Plasma LH levels on day 6 did not vary in either corticosterone treatment group indicating that corticosterone was acting directly on the testes to decrease plasma testosterone levels. Faecal corticosterone levels in corticosterone injected birds were similar during the 0-3 and 3-6 hour collection periods, were lower but still elevated in the 6-12 hour period and were similar to levels in oil-treated birds for the 12-24 hour period. This result shows that injected corticosterone begins to be excreted in the faeces in considerably less than three hours. Overall, this experiment helped to define the acute hormonal changes that occur in response to a corticosterone injection.

3.2 Introduction

Many studies have examined the long-term effects of corticosterone administration on physiological parameters such as plasma hormone levels, body weight, ovary and oviduct development and body composition in birds (see chapter 2). However, little information is available on the acute effects of corticosterone administration on the reproductive hormones (LH and testosterone), or how these effects vary with repeated corticosterone administration. This study was carried out to determine the peak and duration of elevated corticosterone levels after a corticosterone injection, the effects of a single corticosterone injection on plasma LH and testosterone levels within the 24 hours after the injection, and whether these variables changed after repeated corticosterone injections. Faecal corticosterone levels after a single corticosterone injection were also measured on the first day of treatment for comparison with plasma corticosterone levels. This experiment was designed to extend the knowledge gained in the previous experiment of the effects of corticosterone injections on the reproductive system in male Japanese quail.

Corticosterone injections are commonly used to administer corticosterone to birds, as injections are cheap, they reliably deliver the desired dose, and they are less invasive than surgery. Corticosterone injections have been used to examine the effects of corticosterone on body weight, body composition and food intake (Simon, 1984; Bray, 1993; Covasa and Forbes, 1995), and various reproductive parameters (Deviche *et al.*, 1982; Chaturvedi and Suresh, 1990) in bird species. Few studies, however, have examined changes in plasma corticosterone levels after a corticosterone injection in birds. Davison *et al.* (1980) found that injection of chickens with 0.5 mg/kg corticosterone resulted in elevation of plasma corticosterone for approximately two hours, but no studies have examined plasma corticosterone levels after administration of larger doses of corticosterone. A decrease in the duration of elevated corticosterone levels was reported for birds given ACTH injections for seven days (Freeman and Manning, 1979), but the effect of repeated corticosterone injections on the duration of elevated corticosterone has not been examined. Therefore, there is

almost no information on peak corticosterone levels or the duration of elevated corticosterone after a single or repeated corticosterone injections in birds. Two studies in which corticosterone was administered to birds using implants found that corticosterone levels in the blood decreased after several days of corticosterone treatment (Davison *et al.,* 1985; Petitte and Etches, 1989). These results indicate an increase in the rate of corticosterone clearance from the blood over several days of constantly elevated corticosterone levels. It is not known whether the same effect occurs with repeated corticosterone injections, as these do not result in constant elevation of plasma corticosterone levels.

In the previous chapter, plasma and faecal corticosterone levels were measured during corticosterone treatment to determine the relationship between plasma and faecal levels of this hormone. This was the first step towards validating the use of faecal corticosterone as a measure of plasma corticosterone in the Japanese quail. A strong correlation between faecal and plasma corticosterone in basal corticosterone levels was seen in oil treated birds over the six days of treatment. These preliminary findings showed that basal levels of corticosterone in the faeces correspond to levels in the plasma, but further data is needed to confirm this and to determine whether the relationship is maintained when plasma corticosterone is elevated. Injections of corticosterone result in a peak of corticosterone which is rapidly cleared, so in this study I investigated the changes in faecal corticosterone levels using samples collected over the 24 hours following a corticosterone injection.

There is considerable evidence that elevations of plasma corticosterone levels can have a negative effect on the reproductive systems of birds. This has been seen in a number of studies where corticosterone was administered using injections (Deviche *et al.,* 1979; Deviche *et al.,* 1982; Chaturvedi and Suresh, 1990), osmotic pumps (Etches *et al.,* 1984; John *et al.,* 1987; Petitte and Etches, 1988; Petitte and Etches, 1989) or by the addition of corticosterone to the food (Gross *et al.,* 1980; Davison *et al.,* 1983). However, only Petitte and

Etches (1989) measured plasma levels of corticosterone, LH and the sex steroids together to give a comprehensive picture of how corticosterone (administered by osmotic pumps) was acting on the reproductive axis. Although it is clear that daily corticosterone injections can have a negative effect on reproductive parameters such as testis weight (Chaturvedi and Suresh, 1990), cloacal gland area (Deviche *et al.,* 1982 and chapter 2 of the current study) and testosterone metabolism (Deviche *et al.,* 1982) in various bird species, there have been no studies in birds of the effects of a corticosterone injection on plasma levels of LH or testosterone during the hours after the injection. Examination of plasma levels of the reproductive hormones over a short time-period (within the 24 hours after an injection) would allow close inspection of the effects of corticosterone on these hormones and may indicate at what level of the reproductive axis corticosterone is predominantly acting.

It is also not known whether corticosterone's effect on the reproductive hormones varies with repeated treatment, as this cannot be examined with administration of corticosterone using osmotic pumps, and has not been previously examined using corticosterone injections.

The experiment described in this chapter was designed to analyse the acute effects of corticosterone injections in Japanese quail. The experiment addressed the following questions:

1. What is the plasma profile of corticosterone over the 24 hours after a corticosterone injection?

2. Does the plasma corticosterone profile change after repeated corticosterone injections (i.e. does the rate of corticosterone clearance from the plasma change with repeated injections)?

3. Is there a relationship between plasma and faecal corticosterone when plasma corticosterone is elevated?

4. How do plasma levels of LH and testosterone vary over the 24 hours after a single injection of corticosterone?

5. Does the LH and testosterone response to a corticosterone injection change after repeated injections?

3.3 Materials and methods

3.3.1 Animals

Male Japanese quail were obtained from Rangatikei Game Birds Ltd (Bulls) at six weeks of age. They were transported to Massey University and held in individual cages in a light and temperature controlled room in the Veterinary Science Building. They were provided with food (quail pellets, Unifeeds, Massey University) and water *ad libitum*, and held under a long day photoperiod (16h light: 8h dark; lights on from 0800 - 0000) at an ambient temperature of 20°C.

3.3.2 Experimental design

Twenty-four male Japanese quail were divided into three treatment groups (n=8 each) when they were six weeks old so that each group had a similar mean body weight. Birds in the three groups were treated as follows:

Oil-treated: daily injections of corn oil for seven days (days 0-6)
Cort-treated: daily injections of corticosterone for seven days (days 0-6)
Cort-oil-cort: an injection of corticosterone (day 0), daily injections of oil (days 1-5) then a second injection of corticosterone (day 6).

Birds were injected each morning (from 9 am), in the same order each day. The birds were killed 24 hours after the final injection on day 6.

A blood sample was collected immediately before the first injection (on day 0), and further blood samples were collected at 3, 6, 12 and 24 hours after the injection. Faecal samples were collected for 24 hours following the injections on day 0. Birds were blood sampled in the same manner on day 6, although no faecal collections were carried on this day.

This study was approved by the Massey University Animal Ethics Committee.

3.3.3 Hormone administration

Injection solutions were made up on the day before treatment started and stored at 4°C. The same solutions were used throughout the treatment period.

A single corticosterone solution was made for all birds receiving corticosterone injections, with the corticosterone concentration of 1.2 mg/200 µl . The solution was made by dissolving 120 mg of corticosterone powder (Sigma) in 500 µl of benzyl alcohol (GPR, BDH), and adding 19.5 ml of corn oil (as described in Section 2.3.3.1). The 0 mg corticosterone (oil) solution consisted of 34.5 µl benzyl alcohol per ml of corn oil. The volume of each injection was 200 µl.

Injections were given every morning from 9 am. The corticosterone solution was stirred for at least 5 minutes before use to resuspend the corticosterone (magnetic stirrer, Chiltern Scientific). Injections were given subcutaneously in the abdominal area, with variation in the exact site to minimise skin irritation.

3.3.4 Data collection

3.3.4.1 Body weight and cloacal gland area

Body weight and cloacal gland area of quail were measured when the birds were six-weeks old. At this time all birds had large cloacal glands and were secreting cloacal foam.

3.3.4.2 Blood and faecal samples

Blood samples were collected immediately before the daily injection on days 0 and 6, and at 3, 6, 12 and 24 hours afterwards. All samples were collected by puncture of the brachial vein with a 27 gauge needle and collection of approximately 200 µl of blood into a heparinised 1 ml syringe. All samples were collected within three minutes of the initial handling, with most samples collected within two minutes. Blood was expelled into a heparinised 1 ml polystyrene tube, and kept on ice until centrifugation. Terminal samples were collected by decapitation following stunning, with blood samples collected into

heparinised 10 ml centrifuge tubes. Samples collected by venipuncture were centrifuged at 2 000 g for 15 minutes (Beckman GS-6R refrigerated centrifuge), whereas terminal samples were centrifuged at 1 900 g for 15 minutes (Beckman TJ-6). Plasma was removed with a glass Hamilton syringe, stored in 1 ml polypropylene titre tubes and frozen at -20°C until assay.

All faeces produced between 0 and 3 hours after the first injection were collected and pooled for each bird. This process was repeated for all faeces produced between 3-6 hours, 6-12 hours, and 12-24 hours after the injection. No samples were collected before treatment started. The quail were arranged so that there was an empty cage between adjacent birds to facilitate the collection of faeces. Faeces were collected on a plastic sheet, which was placed on top of a flat aluminium tray under each cage. The plastic sheet was changed immediately after the first injection, before the bird was returned to its cage, then at 3, 6, 12 and 24 hours. The bottom of each cage was checked before the sheet was changed, and any faeces that had not fallen through onto the plastic sheet were pushed through. The plastic sheet was then removed, and replaced with a new sheet. Pellets of quail food that had fallen onto the plastic were removed and all the faecal material was transferred to a 5 ml polypropylene screw-top vial (Labserv). Samples were then frozen at -20°C until assay. Average vial weights were used to determine the dry weights of the samples after freeze-drying.

3.3.5 Hormone assays

Plasma corticosterone and testosterone levels, and faecal corticosterone levels were measured by radioimmunoassay as described in Section 2.3.5. Plasma samples were divided evenly between two assays for each hormone. Corticosterone levels from all faecal samples were measured in a single assay.

3.3.6 Calculation of the area under the curve

Both the raw data and the area under the curves were used to compare plasma corticosterone, LH and testosterone values in different treatment groups. The

area under the curve was calculated for each bird with data points at each sampling time (0, 3, 6, 12 and 24 hours) by the trapezoid rule using GraphPad Prism version 3 (1999; GraphPad Software Inc.). All areas were calculated as the area between the curve and zero on the y-axis.

3.3.7 Statistics

Graphs were prepared and correlation analyses performed using GraphPad Prism version 3 (1999; GraphPad Software Inc.). Statistical analyses were performed using Systat version 5.0 and Systat version 8.0 (Systat Inc., Illinois). Normally distributed data with homogeneous variances (as determined by Bartlett's test) were analysed using one- or two-way ANOVA's (as defined in the results). Data was transformed by $\log_{10}$ if necessary to increase homogeneity for parametric analysis. Where parametric tests were not able to be performed, Kruskal-Wallis tests were used to compare independent data, and Friedman's or Sign tests were used to analyse repeat measures data. Non-parametric analysis was conducted using raw data.

3.4 Results:

3.4.1 Plasma corticosterone:

Plasma corticosterone levels measured in quail on the first day of treatment were generally very high (>50 ng/ml) in all three groups. It appears that many of the blood samples (including time 0 samples from before the injection) were contaminated with corticosterone from the corticosterone injection solution. The plasma corticosterone data from the first day of treatment was therefore discarded.

When sampling was repeated on day 6, a distinct pattern of plasma corticosterone levels was seen. Oil treated birds had low levels of corticosterone throughout the 24 hr period, with no values over 20 ng/ml (Fig. 3.1A). Plasma corticosterone levels in birds receiving corticosterone injections rose to a peak at 3 hours and had returned to basal levels by 6 (cort-treated birds) or 12 (cort-oil-cort) hours. Corticosterone levels at 3 hours varied between 71.0 and 216.6 ng/ml in the cort-oil-cort group (Fig. 3.1B), and between 25.0 and 147.8 ng/ml in birds treated with corticosterone for six days (Fig. 3.1C).

Mean plasma corticosterone levels in the oil-treated birds were uniformly low (Fig. 3.2). Levels did vary across the 24 hour period (Friedman's test statistic (t) = 10.267, p=0.036), and tended to be slightly higher at 0, 3 and 6 hours than at 12 and 24 hours. Mean plasma corticosterone levels in cort-oil-cort and cort-treated birds also varied over the 24 hours after the injection (Friedman's, t=22.857, p<0.001 and t=22.4, p<0.001 for cort-oil-cort and cort-treated groups respectively). Plasma corticosterone levels in cort-oil-cort birds were elevated at 3 and 6 hours (Sign test p=0.017 for both 0 versus 3 and 0 versus 6 hours), and had returned to basal levels by 12 hours (Sign test p=1.000 for 0 versus 12 hours). Plasma corticosterone levels in the cort-treated group were also significantly elevated at 3 hours (Sign test p=0.016), but were not significantly different from basal levels by 6 hours (Sign test p=0.125). Plasma

corticosterone levels differed between cort-oil-cort and cort-treated birds at 3 and 6 hours after an injection (Mann-Whitney U test, U= 47, p=0.028 and U= 54, p=0.003 respectively), and also at time 0 (Mann-Whitney U test; U= 6, p=0.018).

The areas under individual plasma corticosterone versus time curves on day 6 were calculated for 0-12 hours and for 12-24 hours. The areas under the curves for 0-12 hours differed between the three treatment groups (Fig. 3.3; one way ANOVA, $F_{2,17}$=39.62, p<0.001), but the curves for 12-24 hours did not vary between groups (one way ANOVA, $F_{2,17}$=2.98, p=0.078). The areas under the curves of both corticosterone injected groups were larger than those of oil-treated birds for 0-12 hours (Bonferroni p<0.001 for both cort-oil-cort and cort-treated), and the area under the curve for cort-oil-cort birds was larger than that of cort-treated birds (Bonferroni p=0.027).

The relationship between the time taken to collect blood samples and plasma corticosterone levels was examined in oil-treated birds to ensure that differences in plasma corticosterone were not due to the amount of time it took to collect the blood sample (data not shown). There was no effect of time taken to collect a blood sample on plasma corticosterone levels (Spearman correlation r^2=0.076, p=0.1321, n=31).

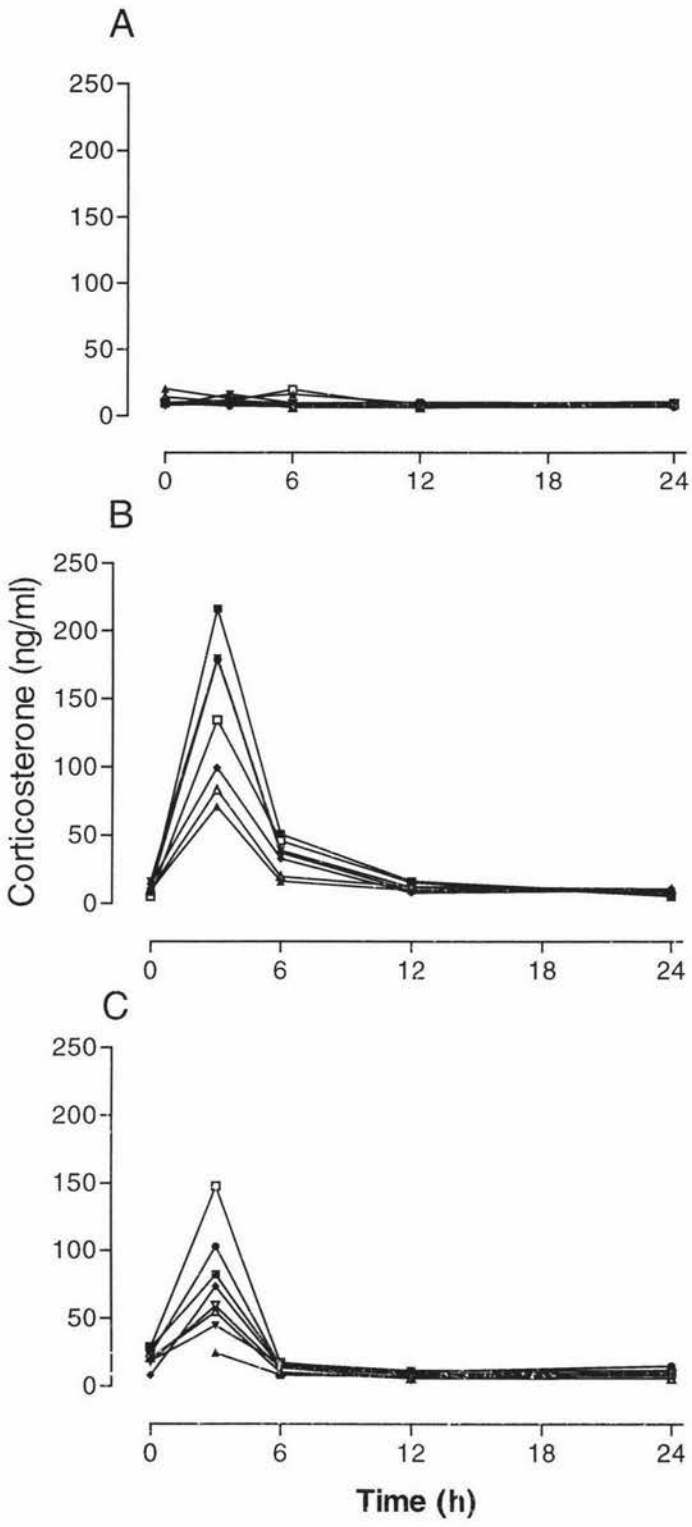


Fig. 3.1: Individual plasma corticosterone levels on day 6. A) oil treated B) cort-oil-cort treated C) cort treated.

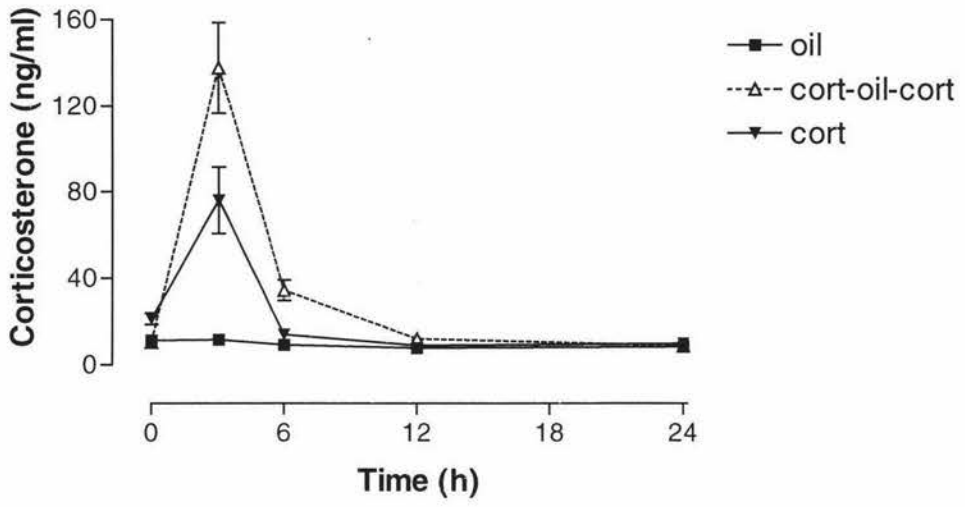


Fig. 3.2: Mean plasma corticosterone levels on day 6. Mean values were calculated from birds with complete data sets (mean ± SE; oil treated, n=6, cort-oil-cort and cort treated, n=7 each).

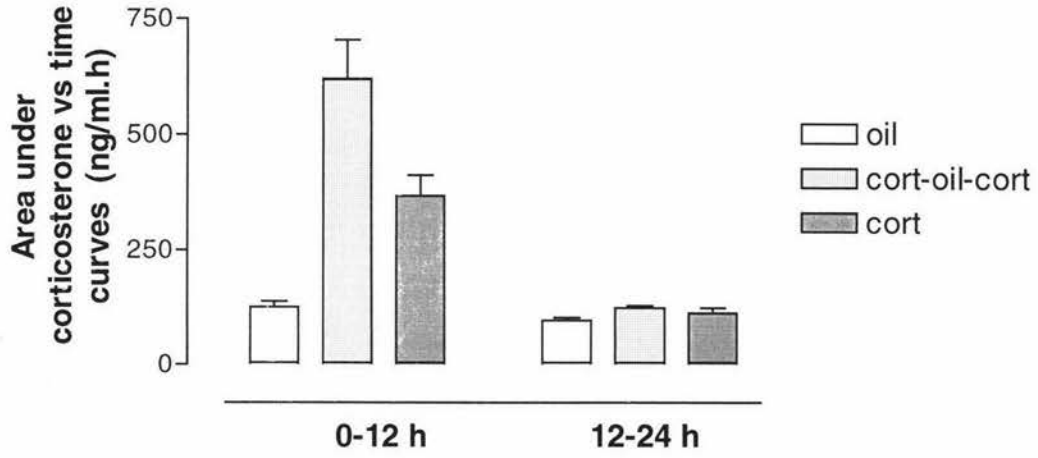


Fig. 3.3: Areas under the plasma corticosterone versus time curves for 0-12 hours and 12-24 hours on day 6. Areas under the curve were calculated from birds with complete data sets (mean ± SE; oil treated, n=6, cort-oil-cort and cort treated, n=7 each).

3.4.2 Faecal corticosterone

Faecal corticosterone levels were expressed as ng corticosterone per g of dry weight of the sample. This concentration was also multiplied by the dry weight of the sample and divided by the number of hours each sample was collected over to give the rate of corticosterone excretion in ng per hour. Finally, the percentage of injected corticosterone excreted by each bird over 24 hours was calculated. The faecal corticosterone concentration for each of the four collection periods was first multiplied by the dry weights of each faecal collection, to give the total corticosterone excreted within each period. These corticosterone excretion values for each of the four collection periods were added together for each bird to give the total corticosterone excreted over the 24 hours. This value was divided by 1.2 mg (the amount of corticosterone injected), and multiplied by 100 to give the percentage of injected corticosterone that was excreted.

Mean faecal corticosterone levels remained low in oil-treated birds throughout the 24 hour collection period on day 0 (Fig. 3.4A). There were no changes in faecal corticosterone concentration in these birds over the 24 hours after injection (Friedmans, $t=2.1$, $p=0.552$). However, the rate of corticosterone excretion (ng/h) decreased significantly over the 24 hours (Fig. 3.4B) Friedmans $t=8.1$, $p=0.044$).

On day 0, both of the groups of birds treated with corticosterone had been treated in an identical manner (both had received a single corticosterone injection of the same dose), and there were no differences between the two groups in faecal corticosterone concentration or excretion rate (see Table 3.1 for statistics). Therefore, these groups were combined for comparison with oil-treated birds. Faecal corticosterone concentrations in corticosterone injected birds decreased over the 24 hour sampling period (Fig. 3.4A; Friedmans, $t=32.4$, $p<0.001$), as did the rate of excretion of corticosterone into the faeces (Fig. 3.4B; Friedmans, $t=31.626$, $p<0.001$). Faecal corticosterone concentration

differed between oil-treated and corticosterone treated birds at 0-3, 3-6 and 6-12 hours, but were similar for the 12-24 hour collection period (Table 3.1). The rate of corticosterone excretion, however, differed between oil-treated and corticosterone-treated birds throughout the 24 hour collection period (Table 3.1).

The percentage of injected corticosterone that was excreted as unmodified corticosterone in the faeces over the 24 hour period following each bird's first injection was compared between six month old birds from chapter 2 and eight week old birds from the current experiment (Table 3.2). The percentage of injected corticosterone that was excreted was higher in six month old birds injected with either 3 mg/kg (0.82 ± 0.04 mg/ injection) or 6 mg/kg (1.65 ± 0.09 mg/injection) corticosterone than in eight week old birds injected with 1.2 mg/injection corticosterone. Basal corticosterone levels in oil-treated birds of both ages were similar during this period, however (data not shown).

It had been planned to compare plasma and faecal corticosterone levels on day 0, but the problem with plasma samples on day 0 meant that this comparison could not be performed.

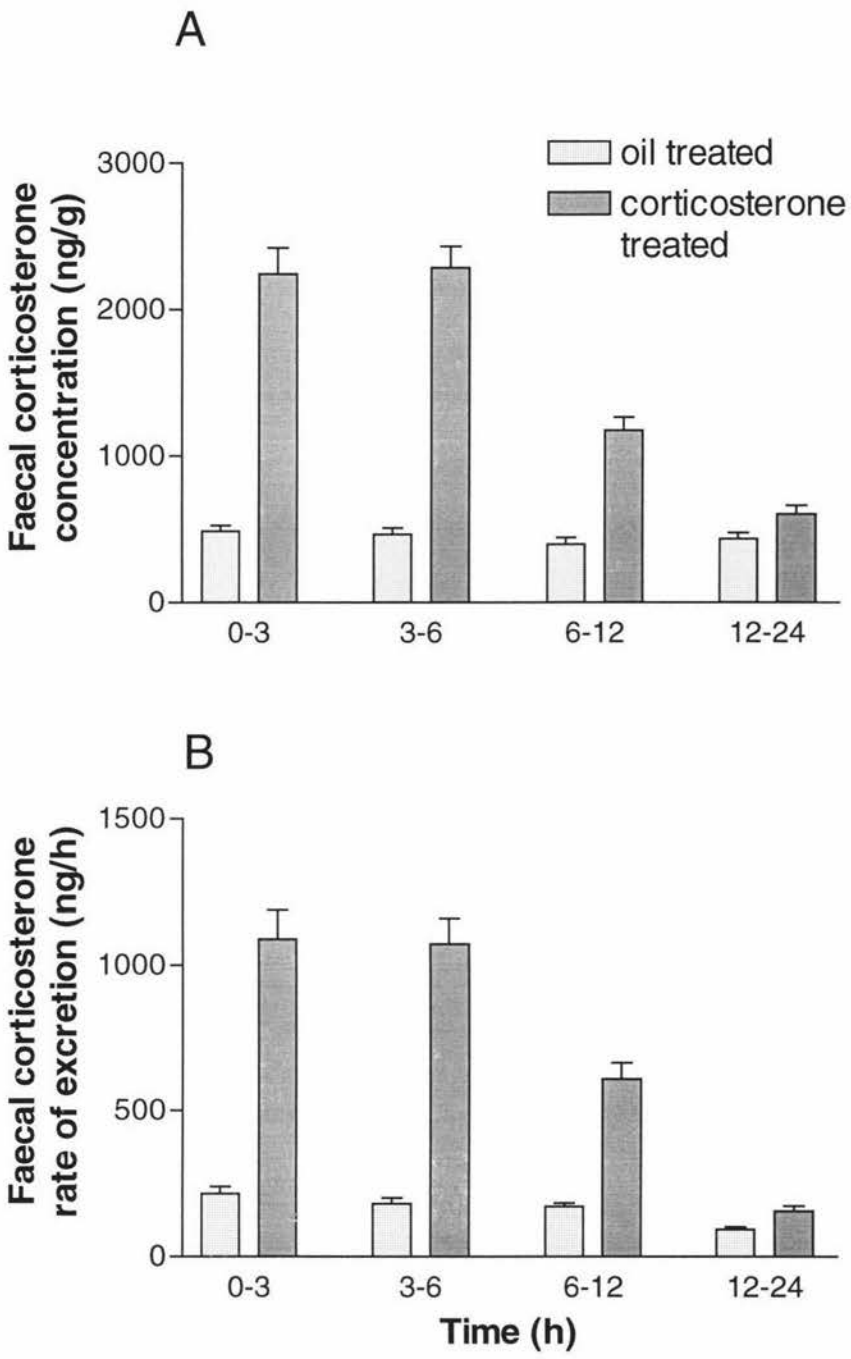


Fig. 3.4: Mean faecal corticosterone levels on day 0. (mean ± SE; oil treated, n=8; cort-oil-cort treated, n=8; cort treated, n=8). A) faecal corticosterone concentration (ng/g) B) faecal corticosterone rate of excretion (ng/h).

Table 3.1: Summary of Mann-Whitney U tests for faecal corticosterone concentration (ng/g) and rate of excretion (ng/h) on day 0

	Concentration (ng/g)		Excretion rate (ng/h)	
	U	P	U	P
Effect of treatment				
cort-oil-cort vs cort				
0-3 h	46	0.141	41	0.345
3-6 h	16	0.093	14	0.059
6-12 h	21	0.418	18	0.247
12-24 h	30	0.482	27	0.749
corticosterone vs oil				
0-3 h	3	<0.001 **	2	<0.001 **
3-6 h	0	<0.001 **	0	<0.001 **
6-12 h	1	<0.001 **	0	<0.001 **
12-24 h	24	0.062	8	0.002 **

Note: The first set of analyses compares the two groups of birds injected with corticosterone, and the second set of analyses compares all birds injected with corticosterone (cort-oil-cort and cort-treated) with oil-treated birds. Significant differences ($p<0.05$) are marked with an asterisk, and highly significant differences ($p<0.01$) are marked with a double asterisk.

Table 3.2: Percentage of corticosterone excreted in the faeces in six-month-old and seven-week-old male quail during the 24 hours after an injection of corticosterone on day 0.

Age of quail	Corticosterone injected (mg)	% excretion		
		mean	SE	n
6 months	0.83	4.73	0.88	3
	1.66	3.01	0.84	3
7 weeks	1.20	1.01	0.06	14

3.4.4 Plasma LH

Plasma LH did not vary greatly over the 24 hours after the injection on day 6 in birds from any of the three treatment groups (Fig. 3.5). Plasma LH levels in all of the oil-treated birds decreased from 0 to 3 hours, but a similar picture was not seen in either group of corticosterone treated birds.

There was a significant effect of time but not treatment group on plasma LH levels (Fig. 3.6, see Table 3.3 for statistics). Plasma LH levels in oil-treated birds had decreased significantly by 3 hours, and remained lower than levels at time 0 for the duration of the sampling period. However, plasma LH did not vary over the 24 hour period in either of the groups of birds treated with corticosterone. The areas under the LH versus time curves were similar for all treatment groups (Fig. 3.7; one way ANOVA $F_{2,12}=2.128$, $p=0.162$).

3.4.3 Plasma testosterone

Patterns of plasma testosterone levels did not vary consistently amongst oil-treated birds (Fig. 3.8A), whereas testosterone levels had decreased by 3 hours in all corticosterone injected birds (Figs. 3.8B and 3.8C). Plasma testosterone levels had returned to basal levels in most corticosterone injected birds by 12 or 24 hours after injection.

Mean plasma testosterone levels varied significantly over the 24 hour sampling period on day 0, with a significant interaction between time and treatment group (see Table 3.4 for statistics). Plasma testosterone levels in oil-treated birds had decreased by 12 hours, and remained depressed at 24 hours (Fig. 3.9). Levels in both corticosterone injected groups were depressed at 3 and 6 hours, with levels returning to basal by 12 hours in cort-treated birds and by 24 hours in cort-oil-cort birds (Table 3.4). Levels were lower than in oil-treated birds at 3 and 6 hours only. There were no differences in plasma testosterone levels between the two corticosterone treated groups at any time.

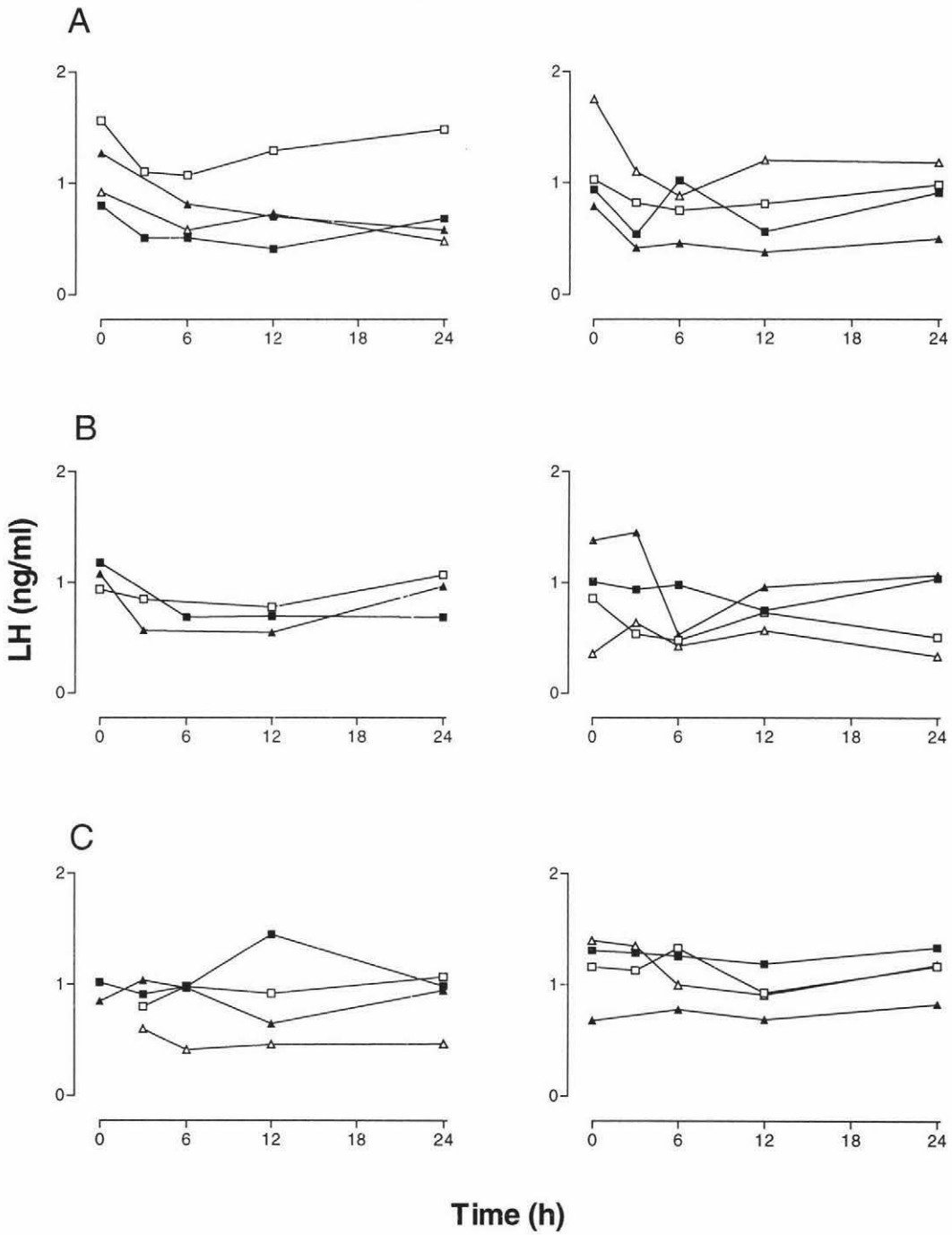


Fig. 3.5: Individual plasma LH levels on day 6. A) oil treated B) cort-oil-cort treated C) cort-treated.

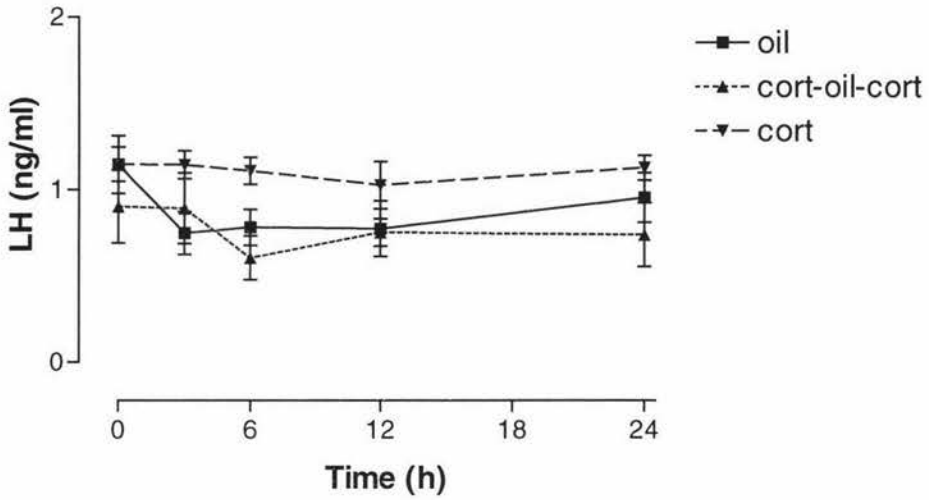


Fig. 3.6: Mean plasma LH levels on day 6. Mean values were calculated from birds with complete data sets (mean ± SE; oil treated, n=6; cort-oil-cort treated, n=4; cort treated, n=5).

Table 3.3: Summary of two-way repeat measures ANOVA for plasma LH levels on day 6.

	Plasma LH		
Effect	F	DF	P
Time	4.185	4,48	0.005 *
Treatment	1.908	2,12	0.191
Interaction	1.761	4,48	0.109
Comparisons between times			
Oil			
0 vs 3 h	31.711	1,12	<0.001 **
0 vs 6 h	7.915	1,12	0.016 *
0 vs 12 h	13.322	1,12	0.003 **
0 vs 24 h	6.686	1,12	0.024 *
Cort-oil-cort			
0 vs 3 h	0.013	1,12	0.910
0 vs 6 h	3.538	1,12	0.084
0 vs 12 h	1.460	1,12	0.250
0 vs 24 h	3.260	1,12	0.096
Cort			
0 vs 3 h	0.003	1,12	0.960
0 vs 6 h	0.080	1,12	0.782
0 vs 12 h	1.207	1,12	0.293
0 vs 24 h	0.075	1,12	0.789

Note: The first three rows show the overall effects of time and treatment and the interaction between the two. The following rows show post hoc contrasts carried out to compare between times within each treatment group. Significant differences ($p<0.05$) are marked with an asterisk, and highly significant differences ($p<0.01$) are marked with a double asterisk.

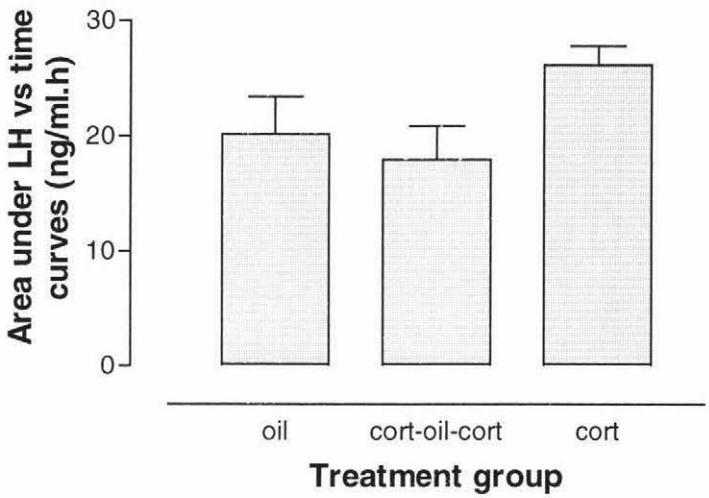


Fig. 3.7: Areas under the plasma LH versus time curves on day 6. Areas under the curves were calculated from birds with complete data sets (mean ± SE; oil treated, n=6; cort-oil-cort treated, n=4; cort treated, n=5).

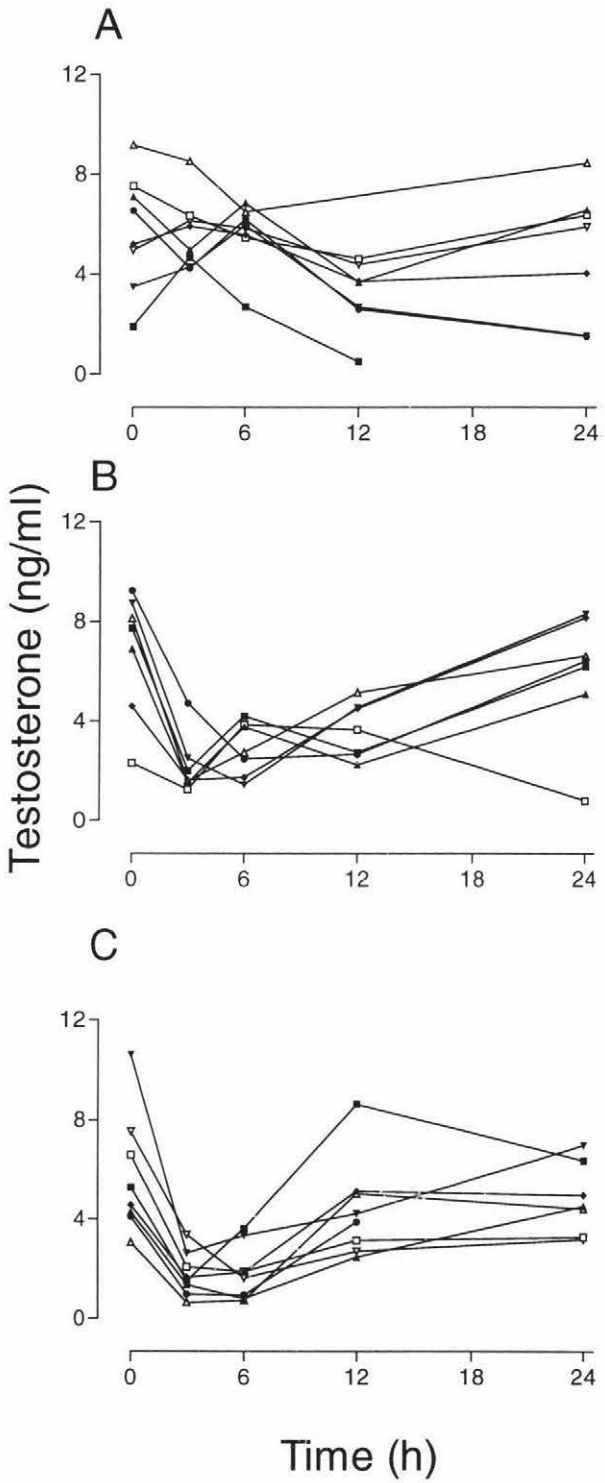


Fig. 3.8: Individual plasma testosterone levels on day 0. A) oil-treated B) cort-oil-cort treated C) cort-treated.

Table 3.4: Summary of two-way repeat measures ANOVA for plasma testosterone levels on day 0.

	Plasma testosterone		
Effect	F	DF	P
Time	11.660	4,68	<0.001 *
Treatment	3.224	2,17	0.065
Interaction	5.759	8,68	<0.001 *
Comparisons between times			
Oil			
0 vs 3 h	0.648	1,17	0.428
0 vs 6 h	0.047	1,17	0.832
0 vs 12 h	7.344	1,17	0.022 *
0 vs 24 h	5.336	1,17	0.044 *
Cort-oil-cort			
0 vs 3 h	92.092	1,17	<0.001 **
0 vs 6 h	17.705	1,17	0.001 **
0 vs 12 h	7.508	1,17	0.014 *
0 vs 24 h	1.449	1,17	0.245
Cort			
0 vs 3 h	83.804	1,17	<0.001 **
0 vs 6 h	23.681	1,17	<0.001 **
0 vs 12 h	2.523	1,17	0.138
0 vs 24 h	1.480	1,17	0.247
Comparisons between treatments			
0 hours			
oil vs cort-oil-	0.209	1,17	0.654
oil vs cort	0.001	1,17	0.971
cort-oil-cort vs	0.264	1,17	0.614
3 hours			
oil vs cort-oil-	17.487	1,17	0.001 **
oil vs cort	22.98	1,17	<0.001 **
cort-oil-cort vs	0.406	1,17	0.533
6 hours			
oil vs cort-oil-	10.122	1,17	0.005 **
oil vs cort	25.416	1,17	<0.001 **
cort-oil-cort vs	3.748	1,17	0.070
12 hours			
oil vs cort-oil-	0.004	1,17	0.952
oil vs cort	0.633	1,17	0.437
cort-oil-cort vs	0.795	1,17	0.385
24 hours			
oil vs cort-oil-	0.731	1,17	0.404
oil vs cort	0.475	1,17	0.500
cort-oil-cort vs	0.030	1,17	0.865

Note: The first three rows show the overall effects of time and treatment and the interaction between the two. The following rows snow post hoc comparisons between times and between treatments. Significant differences ($p<0.05$) are marked with an asterisk, and highly significant differences ($p<0.01$) are marked with a double asterisk.

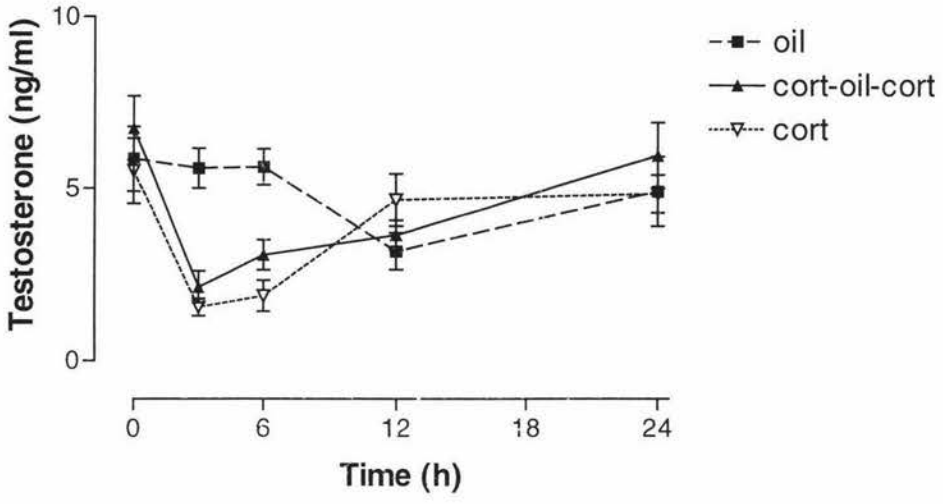


Fig. 3.9: Mean plasma testosterone levels on day 0. Mean levels were calculated from birds with complete data sets (mean ± SE; n=7 for each group).

On day 6, there was again no consistent pattern in plasma testosterone levels in oil-treated birds (Fig. 3.10). As seen on day 0, a corticosterone injection decreased plasma testosterone at 3 and 6 hours in all corticosterone injected birds, after which levels had again increased by 12 or 24 hours. Plasma testosterone levels were similar on days 0 and 6 within both the oil-treated and cort-treated groups (Fig. 3.11, see Table 3.5 for statistics), with no differences between the two days at any time. Although overall plasma testosterone levels were similar on days 0 and 6 in cort-oil-cort birds as well, levels at 6 hours were significantly lower on day 6 than day 0 (Table 3.5).

The areas under individual plasma testosterone versus time curves were calculated on both days 0 and 6 for 0-12 hours and 12-24 hours. The areas under the testosterone curves differed between treatment groups on day 0 for 0-12 hours (Fig. 3.12A; one way ANOVA; $F_{2,19}=6.693$, $p=0.006$), but not 12-24 hours (one way ANOVA, $F_{2,17}=0.755$, $p=0.485$). The areas under the curves on day 0 (0-12 hours) were significantly higher in oil treated birds than cort-treated birds (Bonferroni, $p=0.005$), but not cort-oil-cort birds (Bonferroni $p=0.101$). There was no difference between cort-oil-cort and cort-treated birds during this time period (Bonferroni $p=0.669$).

The areas under the testosterone versus time curves also varied between treatments on day 6 for 0-12 hours (Fig. 3.12B; one way ANOVA; $F_{2,17}=4.032$, $p=0.037$), but not 12-24 hours (one way ANOVA, $F_{2,17}=0.569$, $p=0.576$). On this day there was a significant difference between oil treated and cort-oil-cort groups for 0-12 hours (Bonferroni $p=0.038$), but not between oil-treated and cort-treated birds (Bonferroni $p=0.199$). Again, there was no difference between cort-oil-cort and cort-treated birds (Bonferroni, $p=1.000$).

There was a weak but significant inverse relationship between the areas under the plasma corticosterone and plasma testosterone curves on day 6 for all birds (Fig. 3.13; linear regression $r^2=0.2172$, $p=0.038$, $n=20$).

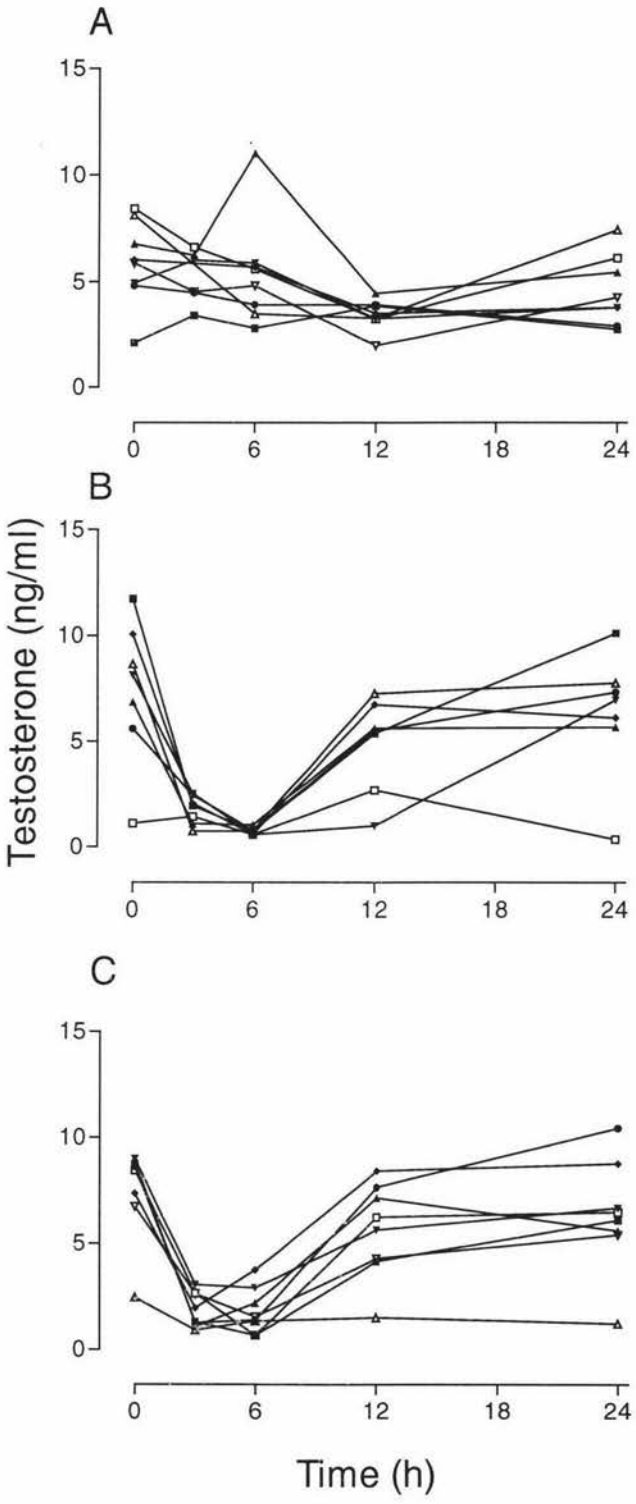


Fig. 3.10: Individual plasma testosterone levels on day 6. A) oil treated B) cort-oil-cort treated C) cort-treated.

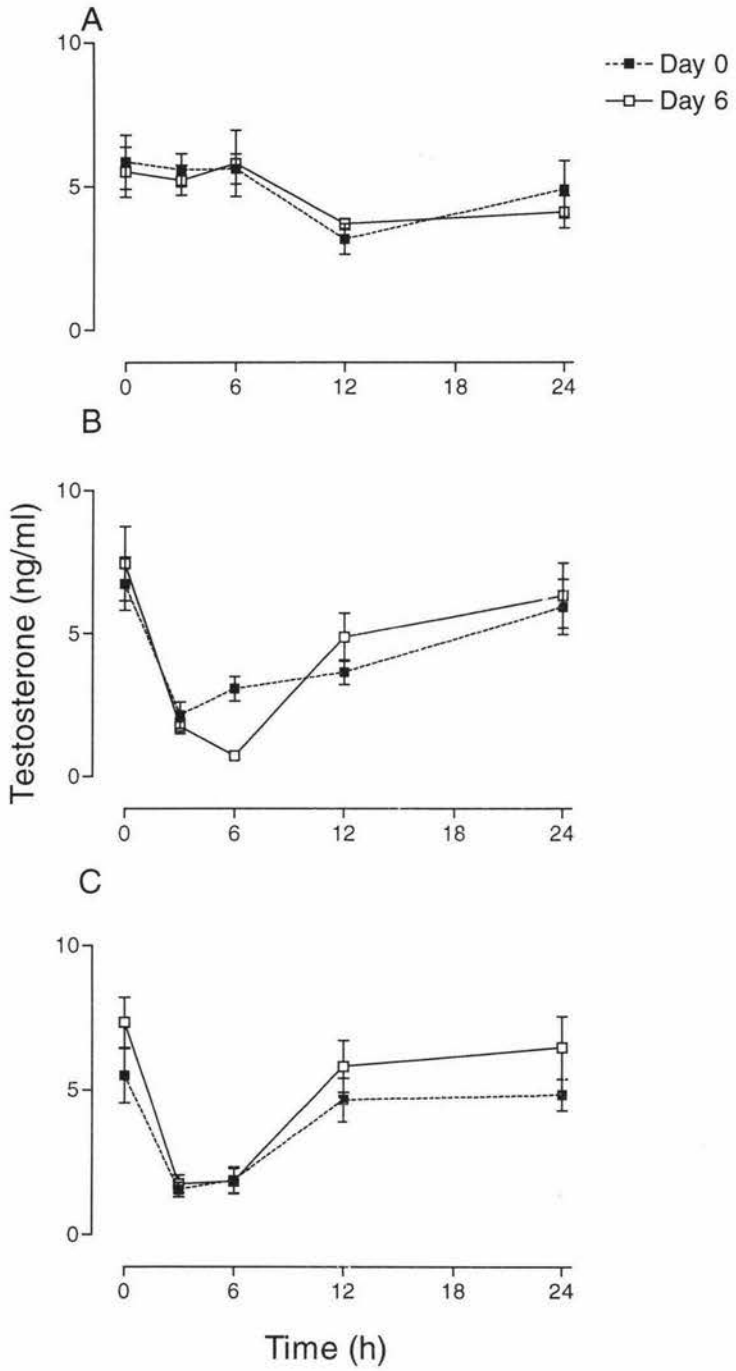


Fig. 3.11: Mean plasma testosterone levels on days 0 and 6 for each treatment group (mean ± SE; oil-treated day 0, n=7; oil-treated day 6, n=6; cort-oil-cort treated, n=7; cort-treated, n=7) A) oil-treated B) cort-oil-cort treated C) cort-treated.

Table 3.5: Summary of two-way repeat measures ANOVAs for plasma testosterone levels on days 0 and 6.

	Oil-treated			Cort-oil-cort			Cort-treated		
Effect	F	DF	P	F	DF	P	F	DF	P
Time	7.530	4,40	<0.001 **	24.089	4,48	<0.001 **	35.156	4,48	<0.001 **
Day	0.093	1,10	0.766	0.009	1,12	0.926	0.194	1,12	0.667
Interaction	0.022	4,40	0.999	1.812	4,48	0.141	0.443	4,48	0.777
Comparisons between days									
day 0 vs 6									
0 h	0.091	1,10	0.769	0.161	1,12	0.695	0.777	1,12	0.395
3 h	0.033	1,10	0.86	0.671	1,12	0.429	0.051	1,12	0.825
6 h	0.074	1,10	0.791	27.508	1,12	<0.001 **	0.177	1,12	0.682
12 h	0.130	1,10	0.726	1.685	1,12	0.219	0.335	1,12	0.573
24 h	0.011	1,10	0.92	0.072	1,12	0.794	0.381	1,12	0.549

Note: A separate ANOVA was carried out for each treatment group. The first three rows for each treatment show the overall effects of time and day and the interaction between the two. The following rows show post hoc comparisons between days for each time. Significant differences ($p<0.05$) are marked with an asterisk, and highly significant differences ($p<0.01$) are marked with a double asterisk.

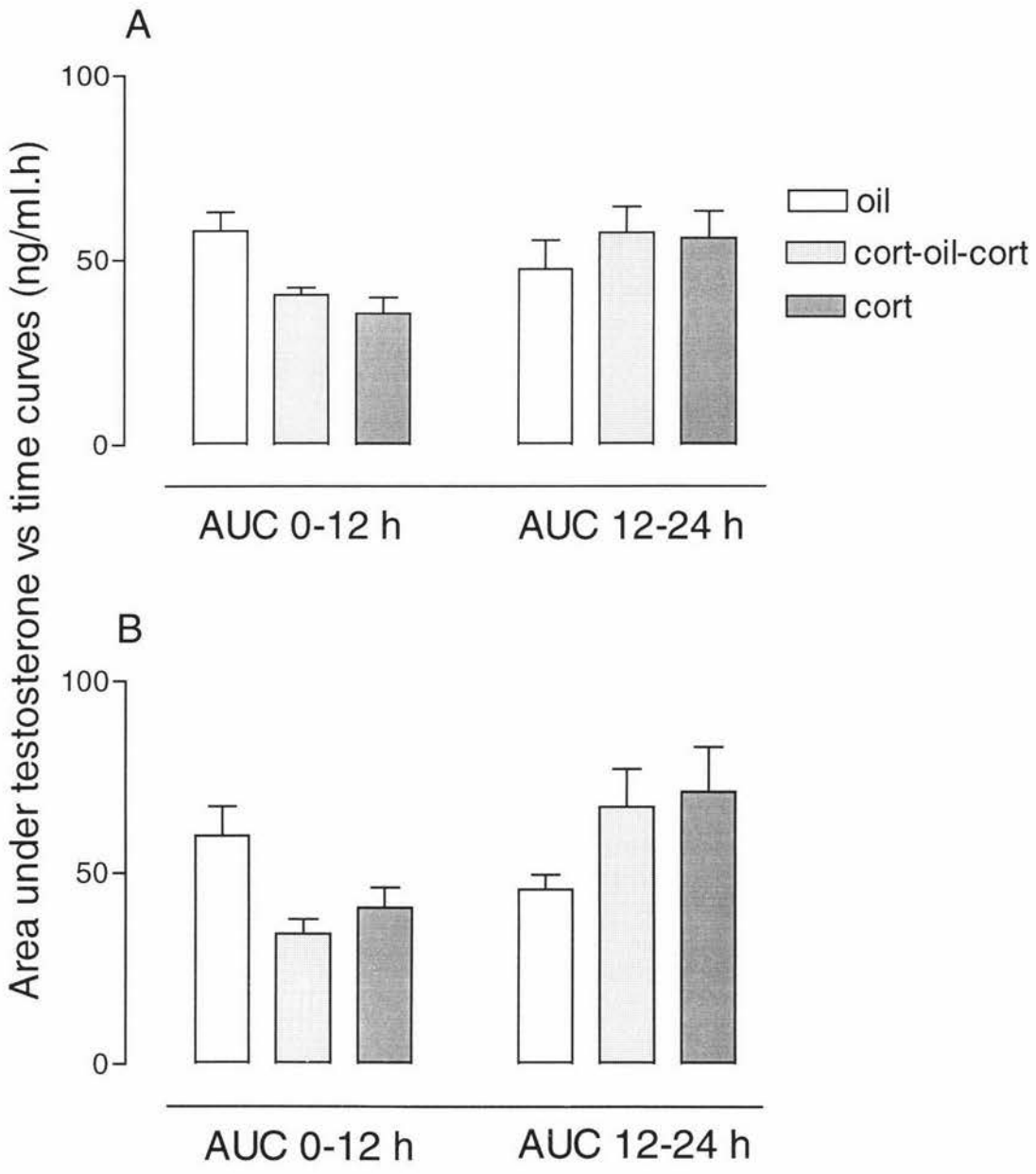


Fig. 3.12: Areas under the plasma testosterone versus time curves for 0-12 and 12-24 hours. Areas under the curve were calculated for birds with complete data sets (mean ± SE; oil-treated day 0, n=7; oil-treated day 6, n=6; cort-oil-cort and cort, n=7). A) Day 0 B) Day 6.

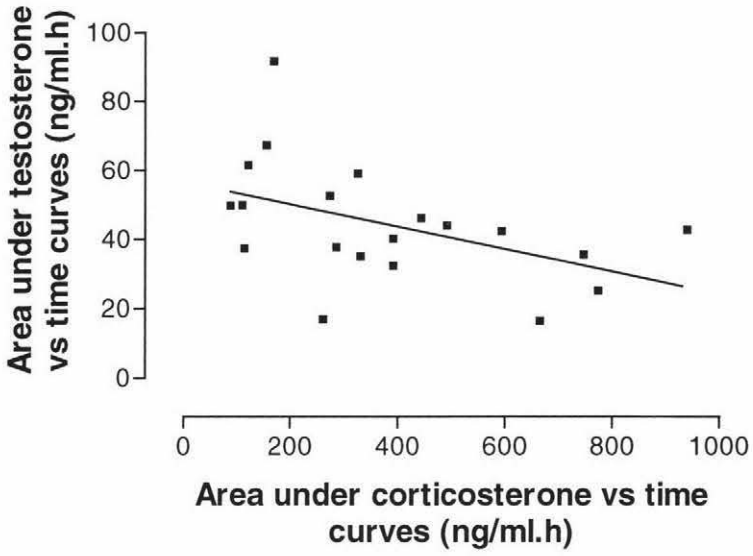


Fig. 3.13: Correlation between areas under the plasma corticosterone versus time curves 0-12 hours after a corticosterone injection and areas under the plasma testosterone versus time curves 0-12 hours on day 6 (data from all treatment groups, n=20).

3.5 Discussion

Administration of corticosterone by daily injections resulted in an increased rate of clearance of corticosterone from the plasma after six days of treatment. A single injection of corticosterone strongly decreased plasma testosterone levels for up to 12 hours, and the pattern of testosterone inhibition did not change after repeated injections of corticosterone. Changes in plasma testosterone levels after a corticosterone injection were not accompanied by variation in plasma LH levels.

3.5.1 Plasma corticosterone

An injection of corticosterone resulted in an increase in plasma corticosterone by 3 hours, and a return to basal levels by 12 hours. This result explains why birds of a similar age in a previous experiment (chapter 2, seven week old birds) did not have elevated corticosterone when sampled 24 hours after a corticosterone injection.

Birds who had been receiving daily corticosterone injections for the previous six days (cort-treated) had a lower levels of plasma corticosterone at 3 hours than those who received oil injections on all but the first and last days (cort-oil-cort). In chronically corticosterone treated birds, plasma corticosterone levels also returned to basal levels more rapidly, within 6 rather than 12 hours. These differences provide evidence for an increase in the clearance rate of corticosterone with repeated corticosterone injections over a six day period. An increased rate of clearance of corticosterone from the blood with chronic corticosterone treatment has been previously reported from studies in which corticosterone was administered to chickens using corticosterone implants (Davison *et al.,* 1985) or osmotic pumps (Petitte and Etches, 1989). Despite the fact that these treatments resulted in plasma corticosterone levels within the physiological range of <12 ng/ml (Davison *et al.,* 1985) and <10 ng/ml (Petitte and Etches, 1989), the rate of corticosterone clearance from the plasma still increased after several days of treatment. In another study, ACTH was injected for seven days instead of corticosterone (Freeman and Manning, 1979). In this

study, plasma corticosterone levels two hours after an injection were similar in chickens that had previously received daily injections of ACTH and those that had not, but levels then returned to basal faster in the pre-treated birds. In the current study, plasma corticosterone levels at 3 hours were significantly different in cort-oil-cort and cort-treated birds, unlike the result at 2 hours in the study by Freeman and Manning (1979). However, as corticosterone is elevated almost immediately after a corticosterone injection in chickens (Davison *et al.*, 1980), and levels have reached a peak by 30 minutes after a corticosterone injection in the Japanese quail (Chua *et al.*, unpublished data), it is likely that peak levels of corticosterone were similar in the two corticosterone injected groups in the current study. By 3 hours, however, the effects of increased clearance of corticosterone from the blood were evident in cort-treated birds, resulting in different plasma corticosterone levels in the cort-oil-cort and cort-treated groups.

3.5.2 Faecal corticosterone

The results described in the previous chapter (chapter 2) showed a definite relationship between basal plasma and faecal corticosterone in the male Japanese quail. A similar correlation between faecal and plasma levels when corticosterone is elevated was not able to found in the current experiment as plasma corticosterone data from day 0 had to be discarded. Although a correlation between faecal and plasma corticosterone levels when plasma corticosterone is elevated have not yet been reported for any species, faecal corticosterone levels are often presumed to reflect plasma corticosterone levels (Jurke *et al.*, 1997; Monfort *et al.*, 1998; Whitten *et al.*, 1998; Goymann *et al.*, 1999). The faecal corticosterone levels in the current study can therefore be used as an indication of the plasma corticosterone levels on day 0. Faecal corticosterone values for the cort-oil-cort and cort-treated groups were not significantly different at any time. This indicates that there were no differences between the two groups in the rates of corticosterone clearance from the blood on day 0. Although not conclusive, this is evidence that the differences in corticosterone clearance seen on day 6 were due to the corticosterone

treatment rather than intrinsic differences in clearance rate between the two groups.

Faecal corticosterone levels were high in the 0-3 hour samples in both corticosterone injected groups. The high corticosterone levels in the faeces during this period show that it takes considerably less than three hours for corticosterone from the plasma to be excreted in the faeces. This is far shorter than the six hour lag time seen in rodents (Harper and Austad, 2000), and is similar to the one hour lag time from plasma to faeces seen in ducks (*Anas platyrhynchos,* Holmes and Slikker, 1976), and two hour lag time in owls (*Strix occidentalis caurina*, Wasser *et al.,* 1997).

It is also of interest that faecal corticosterone concentrations (ng/g) were similar during 0-3 hours and 3-6 hours after an injection of corticosterone, as were rates of excretion (ng/h). Average plasma corticosterone levels over these intervals were likely to be higher for the 0-3 hour than 3-6 hour period, as corticosterone in the plasma peaks around 30 minutes after a corticosterone injection (Chua *et al.,* unpublished data), and clearance from the plasma is rapid. These results indicate that the rate of excretion of corticosterone into the faeces may not be directly proportional to the plasma concentration of corticosterone if plasma corticosterone levels are over a certain level. If a direct relationship was present, faecal corticosterone levels would be considerably higher in the 0-3 hour sample than the 3-6 hour sample.

A relatively small proportion of the injected corticosterone was excreted in the faeces over the 24 hour period (less than 1% of injected corticosterone). This means that around 99% of the corticosterone was cleared from the plasma in some manner other than excretion into the faeces as unmodified corticosterone. This is similar to the percentage of radioactive corticosterone injected that was excreted unmodified in ducks (Holmes and Slikker, 1976). Most of the other 99% of the corticosterone is likely to have been transformed into derivatives or excreted as conjugated corticosterone.

The total amount of corticosterone excreted during the 24 hours after the first corticosterone injection was compared between older birds given 6 mg/kg (chapter 2, six month old birds) and younger birds given a similar dose in the present study. Suprisingly, although basal faecal corticosterone levels in oil-treated birds were similar in both experiments, six month old birds excreted on average 3-4% of the injected corticosterone, whereas younger birds excreted around 1%. This could be because older birds tend to have slower rates of corticosterone clearance from the blood (Holmes and Kelly, 1976). This would result in plasma corticosterone being elevated for longer, and therefore more corticosterone would be excreted into the faeces. Alternatively, the modification and conjugation processes may be less efficient in the older birds, leading to a greater proportion of corticosterone being excreted in its native form.

3.5.3 Plasma LH

Corticosterone treatment did not significantly decrease plasma LH levels during the 24 hours after an injection. Another study on seven week old male Japanese quail (Chua *et al.,* unpublished data) also found no changes in plasma LH when blood samples were collected 30 minutes, 90 minutes, and 3 hours after an injection of 1.2 mg of corticosterone. As seen in the current study, plasma testosterone levels in the study by Chua *et al.* were depressed by corticosterone treatment despite the lack of changes in plasma LH levels. These results therefore indicate that elevated corticosterone does not reduce plasma LH levels in the male Japanese quail. A similar result was seen in male ducks (Deviche *et al.*, 1980b), where ACTH treatment decreased plasma testosterone levels without affecting plasma LH or FSH levels. However, in female chickens, corticosterone delivery by osmotic pumps decreased plasma LH and oestradiol levels (Etches *et al.,* 1984; Petitte and Etches, 1989) and inhibited the normal increase in plasma LH and oestradiol levels when birds were moved to a longer photoperiod (Petitte and Etches, 1988). Injections of GnRH returned plasma LH and oestradiol levels to control levels (Etches *et al.,* 1984) and injections of PMSG returned oestradiol to control levels (Petitte and

Etches, 1989), indicating that corticosterone was acting predominantly at the level of the hypothalamus or pituitary. However, in birds treated with corticosterone and PMSG, ovary and oviduct weights were significantly lower than in birds treated with PMSG alone, indicating an independent effect of corticosterone at the level of the gonads (Petitte and Etches, 1989). As these studies on female birds were carried out using implants or osmotic pumps to deliver corticosterone and the studies on male birds used injections, the differing results between male and female birds may be due to the method of corticosterone administration used.

In mammals, changes in the pulsatile secretion of LH (which results in changes in plasma levels of the sex steroids) can result from glucocortioid or ACTH administration. Studies in sheep have shown that injection of cortisol, the mammalian equivalent of corticosterone (Daley *et al.,* 1999) or ACTH (Van Lier *et al.,* 1999) both decrease LH pulse frequency without affecting mean plasma LH levels. If a similar situation occurred in birds, this could explain the apparent lack of changes in plasma LH seen in the current experiment. Few studies have been carried out on the pulsitility of LH in avian species. There is evidence, however, that LH is secreted in a pulsatile manner in cockerels (Wilson and Sharp, 1975), male Japanese quail (Glendhill and Follett, 1976), and male turkeys (*Meleagris gallopavo* , Bacon *et al.,* 1991; Bacon and Long, 2000), with pulses occurring 6-10 times per day (Glendhill and Follett, 1976) and lasting approximately 90-120 minutes (Wilson and Sharp, 1975). Although constantly elevated plasma corticosterone levels have been seen to decrease plasma LH levels in female birds (Etches *et al.,* 1984; Petitte and Etches, 1988; Petitte and Etches, 1989) and to decrease the pituitary's response to GnRH in male quail (Connolly and Callard, 1987), it is not known whether corticosterone can decrease the pulsitility of LH over a period of several hours, rather than days. As LH pulses in male birds occur less frequently than one per hour, the rapid changes in plasma testosterone levels after a corticosterone injection seen in the current experiment do not appear to be due to changes in LH pulse frequency.

The results from the current study seem to suggest that the acute effects of corticosterone on the reproductive system are predominantly due to direct effects on the testes (as no changes in plasma LH levels were seen). It is possible, however, that the effects of corticosterone on LH secretion from the pituitary were masked by the negative feedback effects of testosterone. Testosterone is known to suppress plasma LH by negative feedback in birds (Gibson *et al.,* 1975; Deviche *et al.,* 1979; Deviche *et al.,* 1980a; Knight *et al.,* 1983; Connolly and Callard, 1987) as well as mammals (Hileman and Jackson, 1999; Tilbrook *et al.,* 1999). In birds, this inhibition appears to occur both at the levels of the hypothalamus (Knight *et al.,* 1983) and the pituitary (Connolly and Callard, 1987). A decrease in plasma testosterone levels removes this negative feedback, and results in an increase in the plasma levels of LH, as seen after castration (Gibson *et al.,* 1975; Wilson and Sharp, 1975; Deviche *et al.,* 1980a). If corticosterone in the current study was acting at the level of the hypothalamus or pituitary gland as well as the testis, plasma LH levels might not change, as negative feedback inhibition of LH by testosterone could be replaced by inhibition of LH by corticosterone. If corticosterone had been acting entirely at the level of the testis, however, plasma LH levels may have risen (due to the loss of negative feedback from testosterone), whereas if corticosterone was acting entirely at the level of the hypothalamus or pituitary, plasma testosterone levels would have been dependent on plasma LH levels, and plasma testosterone levels would not have decreased without plasma LH levels first decreasing. In a previous study, Deviche *et al.* (1979) found that an injection of 3 mg corticosterone significantly decreased plasma LH levels in one day old ducklings. This is consistent with the above hypothesis, as plasma testosterone levels were already low in these birds (as they were reproductively immature). There would be little negative feedback of testosterone on LH, and this would not change with corticosterone treatment. Therefore, an injection of corticosterone would decrease plasma LH levels.

The results of this study are therefore consistent with corticosterone acting independently at the testis and possibly higher in the reproductive axis as well. However, the effects of testosterone and corticosterone on plasma LH in birds have not been quantified, so it is not known whether a similar situation would be seen with endogenous elevated corticosterone during the stress response.

3.5.4 Plasma testosterone

Plasma testosterone levels in oil-treated birds remained constant for 0, 3 and 6 hours. By 12 hours, however, there was a small but significant decrease in plasma testosterone levels and a slight rise again by 24 hours. This decrease was probably not due to random factors, as the same pattern was seen on both day 0 and day 6. Two explanations for this decrease are that it was either due to the repeated blood sampling, or to a circadian rhythm in testosterone. A study on mature male Japanese quail held on a photoperiod of 16 light : 8 dark (lights on at 0600), and sampled once only each showed that the nadir of basal testosterone levels was in the late afternoon, around 5 pm, and levels had reached a peak by 8 pm (Ottinger and Follett, 1987). Other, similar studies in male quail also found troughs of plasma testosterone levels during the afternoon and peaks at night (Ottinger and Brinkley, 1979; Kosutzky *et al.,* 1983). Studies carried out on ducks showed either lower levels of testosterone in the afternoon than the morning (Balthazart *et al.,* 1980), or no distinct pattern throughout the day (Balthazart and Hendrick, 1979), and a study in male chickens found higher average plasma testosterone levels at night than during the day (Schanbacher *et al.,* 1974). The results of the current study are not consistent with these previous studies, as the decrease in plasma testosterone levels was seen at 9 pm, at the time when plasma testosterone levels were rising in the majority of the previous studies. There is evidence from both our lab (Chua *et al.,* unpublished data) and previous studies (Wilson *et al.,* 1979) that handling and the collection of blood samples can decrease plasma testosterone levels, making this the most likely explanation for the results of the current experiment.

Plasma testosterone levels decreased by 3 hours after a corticosterone injection and had returned to basal levels by 12 hours. This pattern was similar on both days 0 and 6, both in birds that had received two corticosterone injections only and those that had received daily injections of corticosterone for the previous six days. This result shows that birds receiving daily corticosterone injections would have experienced a daily decrease in plasma testosterone. This result helps to explain the results of the previous experiment (chapter 2, seven week old birds). In this experiment, the cloacal gland area of treated quail decreased over six days of corticosterone treatment without any changes in plasma testosterone levels 24 hours after an injection. However, if plasma testosterone levels were depressed for up to 12 hour each day, this could have caused the decrease in cloacal gland area. The cloacal gland area may provide an integrated measure of plasma androgen levels over a period of days, with a number of daily decreases in plasma testosterone levels resulting in an overall decrease in area of the gland's protuberance.

Although plasma corticosterone returned to basal levels within a shorter time after repeated corticosterone injections, there were no changes in the duration of the plasma testosterone response to a corticosterone injection. Although plasma corticosterone levels were different between the two corticosterone treatment groups by 3 hours, the peak corticosterone levels after the injection were probably similar in both groups. An injection of corticosterone results in a peak of plasma corticosterone within 30 minutes (Chua *et al.,* unpublished data) and within this time there is not likely to have been much effect of different rates of corticosterone clearance from the blood. An alternative explanation is that the dose of corticosterone administered was too large for a difference to be seen. Although plasma corticosterone at 3 hours after injection was lower in chronically treated birds, levels were higher than physiological levels. If smaller doses of corticosterone were administered, if treatment had been carried out for a longer period, or if blood samples had been collected more frequently, differences in the magnitude and duration of testosterone inhibition may have emerged.

3.6 Conclusions

The rate of corticosterone clearance from the plasma increased with repeated corticosterone injections, but the effect of a corticosterone injection on plasma testosterone levels did not change over this period, possibly due to the large dose of corticosterone administered in each injection. Plasma corticosterone levels were elevated and plasma testosterone levels were depressed for approximately six hours after a corticosterone injection, these changes in plasma testosterone levels were independent of changes in plasma LH levels. These findings, together with those from the previous chapter, have helped to define the acute and chronic effects of corticosterone on the reproductive system of the male quail. Faecal corticosterone levels were elevated in the 0-3 hour collection period, which shows that corticosterone enters the faeces within three hours of an injection in the Japanese quail. Finally, older birds excreted larger amounts of corticosterone into the faeces over the 24 hours after an injection than did young birds, which may be due to differences in the clearance rate of corticosterone from the plasma.

4 Corticosterone responses to handling

4.1 Abstract

Corticosterone responses to the stressor of handling can differ in birds tested on several occasions, and in birds held in different conditions. This study was designed to examine the corticosterone responses to handling in the female Japanese quail (*Coturnix coturnix japonica*). In the first experiment, female Japanese quail were handled, and blood samples were collected immediately and then either at 15, 30, 60, 120 or 240 minutes afterwards to define the duration of the corticosterone response in these birds. In a second experiment, individual variation in the corticosterone response was quantified by handling reproductively immature quail, and collecting serial blood samples at 0, 15, 30 and 60 minutes. This was repeated twice, at two week intervals, to allow examination of within and between bird variation. In the third experiment, serial samples were collected at 0, 15, 30 and 60 minutes after handling during the afternoon and at night in order to compare corticosterone responses during the active and inactive periods of the day. A final experiment tested whether corticosterone responses were similar in birds of the same age with large and small gonads.

After the stressor of handling, plasma corticosterone levels rose to a peak at 15 minutes, and returned to basal levels by 30 minutes. The variation in plasma corticosterone responses between birds was, on average, less than variation within birds, and overall levels of variation were similar to those seen in chickens. Corticosterone responses were similar during the day and night, although basal corticosterone levels were lower and the areas under the corticosterone response curves were smaller during the night sampling. Birds with large gonads had higher basal corticosterone levels and higher levels at 15 minutes than birds with small gonads. These results have defined the corticosterone response to handling in the female Japanese quail.

4.2 Introduction

The corticosterone response to a stressor differs between birds in various situations. Corticosterone responses to handling have been examined in a number of species of wild birds in order to responses between birds of different ages (Schmeling and Nockells, 1978; Schwabl, 1995), genders (Jones *et al.,* 1994; Astheimer *et al.,* 1995; Schwabl, 1995), reproductive status (Wingfield *et al.,* 1982; Astheimer *et al.,* 1994; Astheimer *et al.,* 1995; Romero *et al.,* 1997; Romero *et al.,* 1998a,b), or habitat (Wingfield, 1994). Similar, comprehensive studies have not been carried out in a domesticated species, kept in captivity. The current study therefore examined corticosterone responses in female Japanese quail, including examination of the duration of the corticosterone response, the degree of individual variation, differences in corticosterone responses during the day and night, and the differences between birds of different reproductive states.

The duration of the corticosterone response to handling varies between different bird species. Measurements of plasma corticosterone levels are usually carried out over the 60 minutes after the initiation of handling, but in many species, corticosterone has not returned to basal levels within this time (Wingfield *et al.,* 1982; Astheimer *et al.,* 1994; Smith *et al.,* 1994; Schwabl, 1995; Dufty and Beltoff, 1997). As corticosterone responses to handling have not previously been examined in Japanese quail, the duration of the corticosterone response in this species is not known. However, similar studies in chickens (Freeman and Flack, 1980) and ducks (*Anas platyrhynchos,* Harvey *et al.,* 1980) have found that plasma corticosterone was elevated 15 minutes after the initiation of handling, and had returned to basal levels by 60 minutes.

The magnitude and duration of a corticosterone response can differ between individual animals, even when exposed to the same stressor (Beuving and Vonder, 1986; Sapolsky, 1992). For example, some birds will show almost no corticosterone response to handling, whereas others will have greatly increased plasma corticosterone levels (Beuving and Vonder, 1978). If the corticosterone

response to a stressor is to be used to compare birds held in different conditions, the degree of variability both within a bird and between different birds must first be established. The only studies on the repeatability of the corticosterone response within individual birds are those conducted in our laboratory on chickens (Litten, 1998) , grey ducks (*Anas superciliosa,* Cockrem *et al.,* unpublished data) and great tits (*Parsus major,* Cockrem and Silverin, submitted). Maximum plasma corticosterone levels were almost identical within and between birds in the chicken (Litten, 1998) and were slightly higher within than between birds in the great tit (Cockrem and Silverin, submitted). The variation over the entire corticosterone response (average of variations at all times) were similar within and between birds both for chickens (Litten, 1998) and great tits (Cockrem and Silverin, submitted).

A diurnal rhythm in basal corticosterone levels has been seen in many species of bird, including chickens (Beuving and Vonder, 1977; Etches, 1979), pigeons (*Columba Livia domestica*, Joseph and Meier, 1973; Westerhof *et al.*, 1994), white-crowned sparrows (*Zonotricha leucophrys gambelii*, Astheimer *et al.*, 1994; Breuner *et al.,* 1999), white-throated sparrows (*Zonotrichia albicollis*, Dusseau and Meier, 1971), starlings (*Sturnus vulgaris*, Romero and Remage-Healey, 2000) and Japanese quail (Boissin and Assenmacher, 1970; Kovacs and Peczely, 1983). However, few studies have examined the corticosterone response to handling at different times of the day. Beuving and Vonder (1978) found slightly higher corticosterone responses to immobilisation in the afternoon than the morning in chickens, and Freeman and Flack (1980) found that differences between morning and afternoon depended on the strain of chicken used. However, Beuving and Vonder (1986) found no differences in corticosterone responses in the morning compared to the evening. As the magnitude of the corticosterone response is thought to be partially dependent on the animal's perception of the stressor (Sapolsky, 1992), corticosterone responses would be expected to be greater during the inactive period of the day when the bird was woken from sleep than during the active period. Only three studies have examined the differences in corticosterone responses during the

active and inactive periods in birds. Romero and Remage-Healey (2000) reported larger corticosterone responses in captive starlings at 2 am than at any time during the active period of the day. In the western screech owl (*Otus kennicottii*, Dufty and Beltoff, 1997), however, there were no significant differences in corticosterone responses between the day and night and in the white-crowned sparrow (Breuner *et al.,* 1999) corticosterone responses were larger in the morning than in the afternoon or at night. However, all of these studies were carried out on non-domesticated birds kept in captivity and it is not clear to what extent captivity affects corticosterone responses in free-living birds. Comparisons between corticosterone responses during the day and night have not previously been examined in a domesticated species.

There is evidence in a number of species of wild birds that corticosterone responses to handling differ depending on the season. Different species of birds have been reported to have decreased (Wingfield *et al.,* 1982; Wingfield *et al.,* 1992) or increased (Romero *et al.,* 1998a; Astheimer *et al.,* 1994; Wingfield, 1994; Astheimer *et al.,* 1995; Breuner *et al.,* 1999) corticosterone responses to handling during the breeding season compared to when their gonads are regressed. The effect of season on corticosterone responses often differs between males and females of the same species, in some species it is only present in female birds (Wingfield *et al.,* 1992). It is not clear what the cause of these differences are, especially as they are not consistent within each species. For example, white-crowned sparrows have been seen to have both increased (Romero *et al.*, 1997) and decreased (Wingfield *et al.*, 1982) corticosterone responses during the breeding season. In several of the studies in which corticosterone responses were lower during the breeding season, it was suggested that the differences were due to the restricted length of the breeding season in these birds, who generally breed in desert or arctic environments (Wingfield *et al.*, 1992). However, other species who also breed in harsh conditions such as snow buntings (*Plectrophenax nivalis*, Romero *et al.*, 1998b) and lapland longspurs (*Calcarius lapponicus*, Romero *et al.,* 1998a) still have increased corticosterone responses during the breeding season.

Similar studies have not previously been carried out in any species of domesticated bird.

The primary aim of this study was to define the corticosterone responses to handling in Japanese quail in several different situations. The study addressed the following questions:

1) What is the duration of the corticosterone response in the Japanese quail?

2) What degree of variation is there within and between birds tested on three separate occasions?

3) Does the corticosterone response to handling differ during the day (when the bird is awake) and at night (when the bird is asleep)?

4) Do Japanese quail with large gonads have a larger or smaller corticosterone response to handling than birds with small gonads?

4.3 Materials and methods

4.3.1 Animals

Female Japanese quail were obtained from Rangitikei Game Birds Ltd (Bulls) at three weeks of age. They were transported to Massey University and held in individual cages in a light and temperature controlled room in the Veterinary Science Building. They were provided with food (quail pellets, Unifeeds, Massey University) and water *ad libitum*.

4.3.2 Experimental Design

4.3.2.1 Duration of the corticosterone response

An experiment was conducted to describe plasma corticosterone levels in quail up to four hours after a corticosterone response was initiated.

Forty six female quail were kept under long days (16 h light: 8 h dark; lights on 0800-0000) at an ambient temperature of approximately 20-25°C from three weeks of age. The birds were divided into five groups when they were six weeks old so that the average body weight in each group was similar. Each bird was taken from its cage into a separate room and a blood sample was collected. The bird was then placed into a small cardboard box. Every minute for 15 minutes the bird was removed from the box, held briefly, then replaced into the box. This handling procedure was used to ensure that a corticosterone response was initiated, as we did not know whether the collection of the initial blood sample would be a sufficient stressor to initiate a corticosterone response in the Japanese quail. Each bird was bled a second time, either at 15, 30, 60, 120 or 240 minutes after the initial blood sample. Birds were left undisturbed in their individual boxes between the initial 15 minutes of handling and the second sample, except for birds in the groups that were bled at 120 or 240 minutes. These birds were returned to their cages after 60 minutes. Sampling was conducted from 1 pm each day, with birds sampled in a random order.

4.3.2.2 Individual variation

A second study was conducted to quantify the variation within and between reproductively immature quail in corticosterone responses to handling.

Fourteen female quail were kept on short days (8 h light: 16 h dark; lights on from 0900-1700) at an ambient temperature of 13°C from three weeks of age. The corticosterone response to handling was measured in each bird when it was six weeks old. Each bird was taken from its cage to a separate room and a blood sample was collected. The bird was then placed in a small cardboard box. Each bird was removed from the box, held briefly and then replaced in its box at one minute intervals for 15 minutes. A second blood sample was collected 15 minutes after the original sample. The bird was then replaced in its box, and not disturbed again until 30 minutes after the first blood sample, when a third sample was collected. A final sample was collected at 60 minutes. After the final sample, the bird was returned to its cage. The corticosterone response to handling was measured on two subsequent occasions, when the birds were eight and ten weeks old. Blood sampling was conducted from 1 pm each day, with birds sampled in the same order on each day.

4.3.2.3 Day versus night

A third study was carried out to determine whether basal corticosterone levels and the corticosterone response to handling differed between the afternoon and night in reproductively imrnature quail.

Ten female quail were obtained at the same time as those in which individual variation was measured, and were housed on short days under the same conditions. Their corticosterone responses to handling were measured at six weeks of age using the procedure described for birds tested for the individual variation of the corticosterone response. Blood sampling was carried out in the afternoon from 1 pm (four hours after lights on). The sampling process was repeated two weeks later (when the birds were eight weeks old), starting at 9 pm (four hours after lights off). The corticosterone responses of these quail

were compared with those of quail from the same batch that were sampled during the day at both six and eight weeks old (Table 4.1).

4.3.2.4 Effect of reproductive status

A final study was conducted out to compare the corticosterone responses of female quail of the same age with inactive or active reproductive systems.

Eight female Japanese quail were kept under long days (16 h light: 8 h dark; lights on 0800-0000) at an ambient temperature of 20^0C from three weeks of age.

The corticosterone response to handling was measured in these birds following the procedure described for the individual variation study when they were six weeks old. Each bird was bled at 0, 15, 30 and 60 minutes. The corticosterone responses of these birds with large gonads were compared with the corticosterone responses of birds of six weeks of age that had small gonads (from the studies of individual variation and the comparison of responses during the day and at night).

Table 4.1: Sampling times for birds from the studies examining individual variation in corticosterone responses and responses during the day and at night.

Study	Sampling time	
	six weeks old	eight weeks old
Individual variation	afternoon	afternoon
Day versus night	afternoon	night

4.3.3 Data Collection

4.3.3.1 Cloacal diameter, gonad weight and egg laying

The diameter of the cloacal opening in female quail can be measured to give an indication of reproductive development (Wakabayashi *et al.,* 1992) and is related to oviduct size. Body weight (Mettler P1200; ± 0.05 g) and the width of the cloacal opening (vernier callipers ± 0.05 mm) were measured weekly from three weeks of age until the end of each experiment.

At the end of each experiment, the birds were killed and the ovaries and oviduct were removed and weighed.

Records of egg laying were also made for birds kept on long photoperiods.

4.3.3.2 Blood samples

Serial blood samples (up to 200 µl) were collected by puncture of the brachial vein with a heparinised 27 g needle and collection of blood into a heparinised 1 ml syringe. Heparinised capillary tubes were also used to collect blood when necessary. Most samples were collected within two minutes of the time the bird was removed from its cage. However, due to the effects of serial sampling on the veins, some samples took up to five minutes to collect. Blood was expelled from syringe and capillary tubes into a heparinised 1 ml polystyrene test tube, and kept on ice until centrifugation. Terminal blood samples were collected by decapitation after stunning. These blood samples were collected into heparinised 10 ml polypropylene centrifuge tubes. Samples collected by venipuncture were centrifuged at 2 000 g for 15 minutes (Beckman GS-6R refrigerated centrifuge), whereas terminal samples were centrifuged at 1 900 g for 15 minutes (Beckman TJ-6). Plasma was removed with a 500 µl glass Hamilton syringe, stored in 1 ml polypropylene titre tubes and frozen at -20°C until assay.

4.3.4 Hormone assays

Corticosterone was measured in all plasma samples by radioimmunoassay as described in Section 2.3.5. Corticosterone was the only hormone to be measured in these samples, so only 20 µl of plasma was extracted from each sample (instead of 40 µl), and half the volumes of dichloromethane and PBSG were used.

Samples from the study of duration of the corticosterone response were randomly divided between two assays. Samples from the studies of individual variation and the comparison between day and night were randomly divided between three assays, and samples from long day birds from the study on the effects of reproductive status were assayed in a single assay.

4.3.5 Calculation of the area under the curve

Both the raw data and the area under the curves were used to compare the corticosterone response curves of birds. The area under the curve was calculated for birds with data points at each sampling time (0, 15, 30 and 60 minutes) by the trapezoid rule using GraphPad Prism version 3 (1999; GraphPad Software Inc.). All areas were calculated as the area between the curve and zero on the y-axis.

4.3.6 Statistics

Graphs were prepared and correlation analyses performed using GraphPad Prism version 3 (1999; GraphPad Software Inc.). Statistical analyses were performed using Systat version 5.0 and Systat version 8.0 (Systat Inc., Illinois). Normally distributed data with homogeneous variances (as determined by Bartlett's test) was analysed using one- or two-way ANOVA's, or t-tests (as defined in the results). Data was transformed by $\log_{10}$ if necessary to increase homogeneity of variance for parametric analysis. Where parametric tests were not able to be performed, Mann-Whitney-U tests were performed on raw data.

4.4 Results

4.4.1 Duration of the corticosterone response

Plasma corticosterone levels were measured in birds sampled at time 0 (within two minutes of being picked up) and once thereafter. Plasma corticosterone levels had increased four fold by 15 minutes after the birds were first picked up, after which plasma corticosterone levels fell and remained similar to basal levels for all other sampling times (Fig. 4.1, paired t-tests, t=-5.131, p=0.001; t=0.011, p=0.991; t=-1.594, p=0.150; t=-0.732, p=0.485; t=-2.184, p=0.061 for 0 minutes versus 15, 30, 60, 120 and 240 minutes respectively).

4.1.2 Effect of sampling speed

The corticosterone response to handling is initiated when a bird is picked up, but it takes between one and three minutes for a measurable increase in plasma corticosterone levels to be seen. The relationship between the time elapsed from when a bird is picked up and the first rise in plasma corticosterone must therefore be defined for each species. An elapsed time can then be chosen within which all plasma corticosterone levels are considered to be basal.

In the current experiments the time it took to collect each blood sample was influenced by how much each bird struggled, how many previous samples had already been collected from that bird and my experience with the collection of blood samples. The relationship between plasma corticosterone levels and the time taken to collect blood samples was examined for samples from the studies of individual variation and of corticosterone responses during the day and at night. There were significant correlations between plasma corticosterone and the time taken to collect the samples at all sampling times (0, 15, 30 and 60 minutes) when all samples were included (Table 4.2). However, for samples collected in $\leq$ 2 minutes, there was a weak correlation only for samples collected at time 0.

Corticosterone levels in samples collected in $\leq$ 2 minutes or $>$ 2 minutes and the average time taken to collect samples in each category were compared at

each sampling time (0, 15, 30 and 60 minutes) (Fig. 4.2). There was a trend towards higher corticosterone levels in samples collected in > 2 minutes at each time, with significant differences at 15 and 30 minutes (two group t-tests, t_{60}=-1.914, p=0.060; t_{60}=-2.717, p=0.009; t_{60}=-2.712, p=0.009; t_{55}=-1.879, p=0.065 for 0, 15, 30 and 60 minutes respectively). The average time taken to collect samples in the > 2 minutes group were similar for each time (0, 15, 30 and 60 minutes), although there was considerable variation in the number of samples that were collected in > 2 minutes at each time.

It was concluded that the plasma corticosterone levels measured in blood samples collected in ≤ 2 minutes were suitable for inclusion in further analyses, whereas samples that took longer than two minutes to collect were excluded.

4.4.3 Individual variation

Corticosterone response curves for the 14 birds bled on three occasions are shown in Fig. 4.3. The exclusion of samples which took more than two minutes to collect meant that limited numbers of complete corticosterone response curves were obtained. However, the data are still sufficient to show that basal corticosterone levels were uniformly low (<2 ng/ml). Most birds showed a peak in plasma corticosterone at 15 minutes, and a decrease again by 30 minutes. In some birds, plasma corticosterone levels remained high at 30 and 60 minutes (e.g. G4, G20, G1), in others levels decreased at 30 minutes but rose again by 60 minutes (G25, G5) on one sampling occasion. Some other birds showed no increase in plasma corticosterone levels after handling on some (G17, G7, G25) or all (G9) sampling occasions. A number of birds had similar corticosterone responses at each sampling session (e.g. G1, G10, G18, G4, G5, G9), while the corticosterone responses in other birds differed markedly between sampling sessions (e.g. G17, G25, G7).

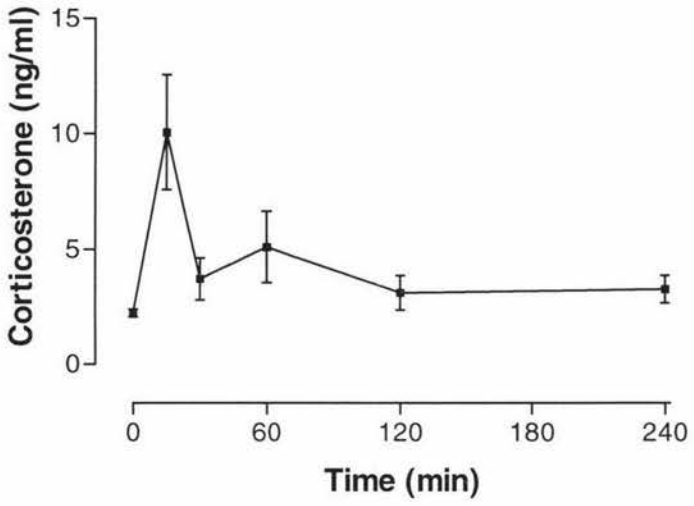


Fig. 4.1: Corticosterone response to handling curve for birds held on long days (LD 16:8) and sampled at time 0 and one subsequent time (mean ± SE; 0 minutes, n=36; 30 minutes, n=10; all other times, n=9).

Table 4.2: Correlations between plasma corticosterone levels and the length of time taken to collect each blood sample at 0, 15, 30 and 60 minutes.

Sample time (min)	r^2	p	n
All blood samples			
0	0.0652	0.0452 *	62
15	0.0989	0.0128 *	62
30	0.3712	<0.0001 **	62
60	0.1739	0.0013 **	57
Samples collected in ≤ 2 minutes			
0	0.0965	0.0187 **	57
15	0.0263	0.2705	48
30	0.0001	0.9503	41
60	0.0522	0.1565	40

Note: All samples from the studies of individual variation (samples collected from birds at 6, 8 and 10 weeks old) and of differences between day and night (samples collected from birds at 6 and 8 weeks old) are included in these results. Significant correlations ($p<0.05$) are marked with asterisks, and highly significant correlations ($p<0.01$) are marked with double asterisks.

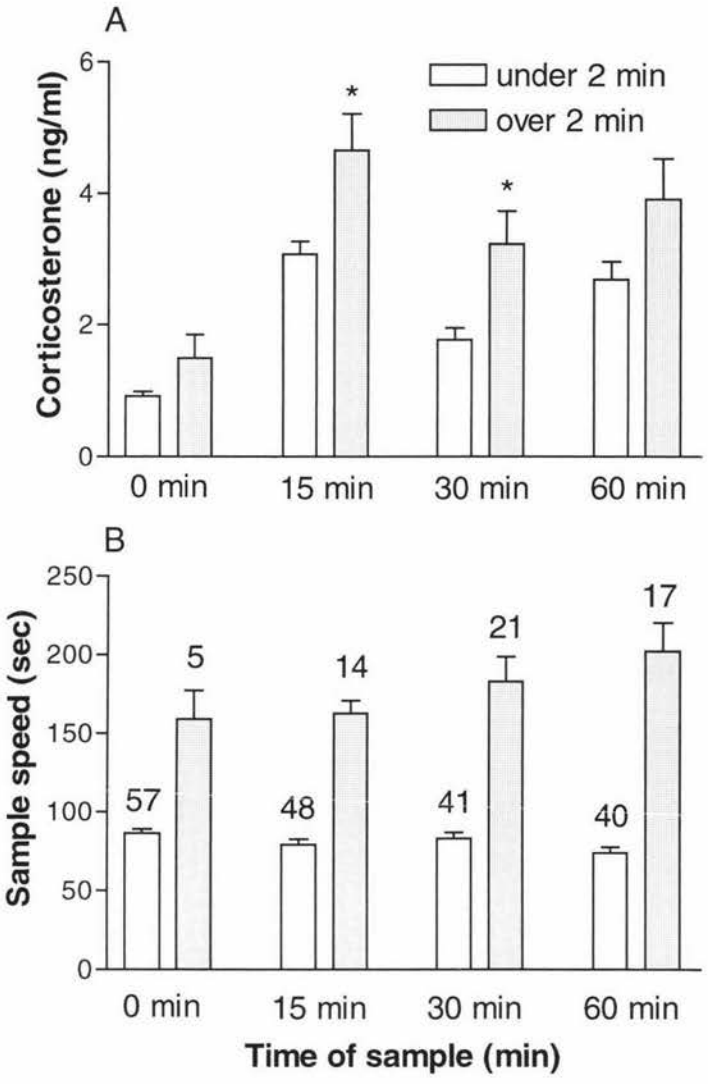


Fig. 4.2: Plasma corticosterone levels in samples collected in ≤ 2 minutes or > 2 minutes at 0, 15, 30 and 60 minutes (mean ± SE; sample sizes are indicated on the bottom graph).. A) Plasma corticosterone levels of samples collected in ≤ 2 minutes or > 2 minutes. B) Average times taken to collect blood samples. All samples from the studies of individual variation (samples collected at 6, 8 and 10 weeks) and differences between day and night (samples collected at 6 and 8 weeks) are included in these results. Significant differences ($p<0.05$) in plasma corticosterone levels between samples collected in ≤ or > 2 minutes are marked with an asterisk.

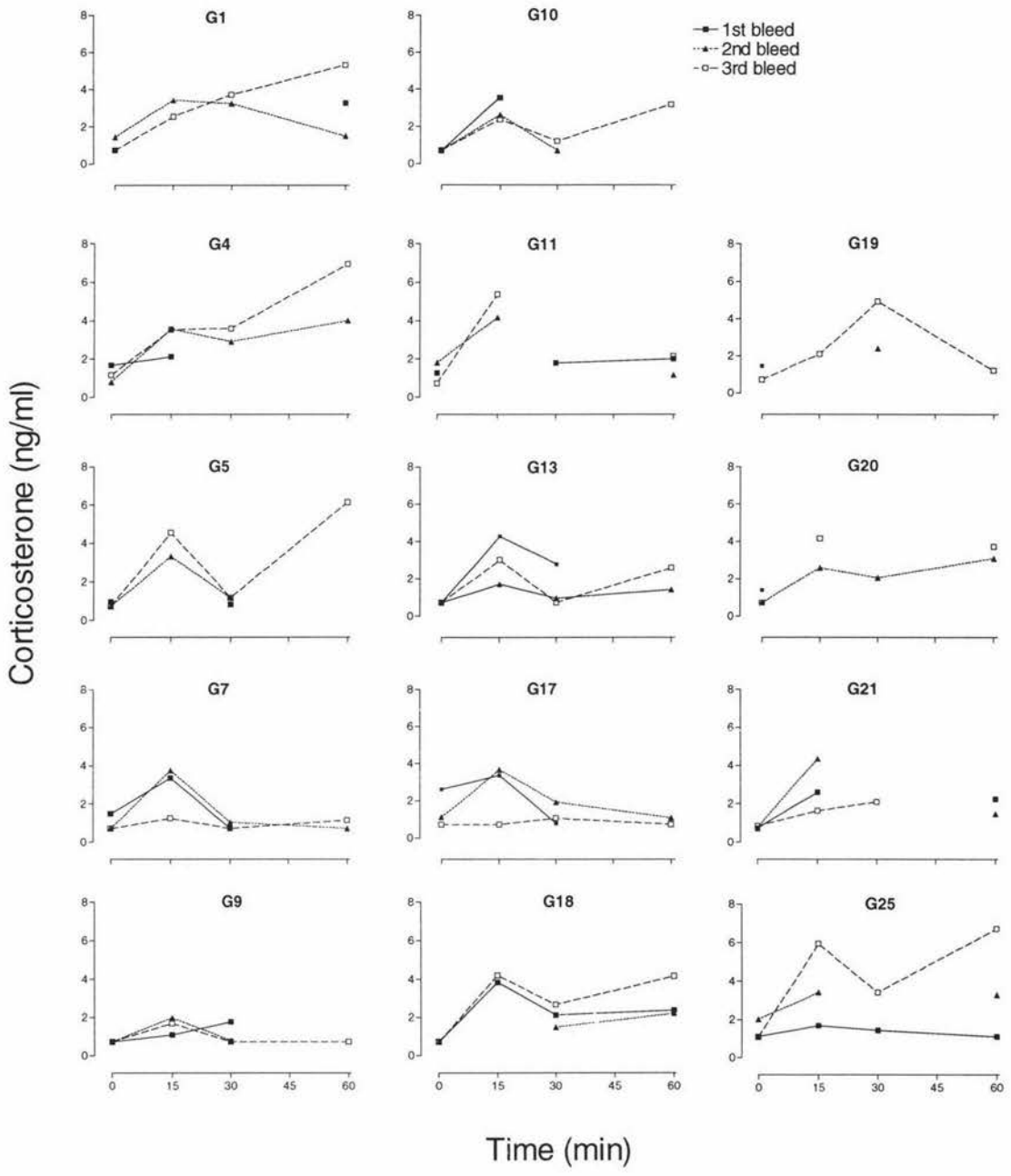


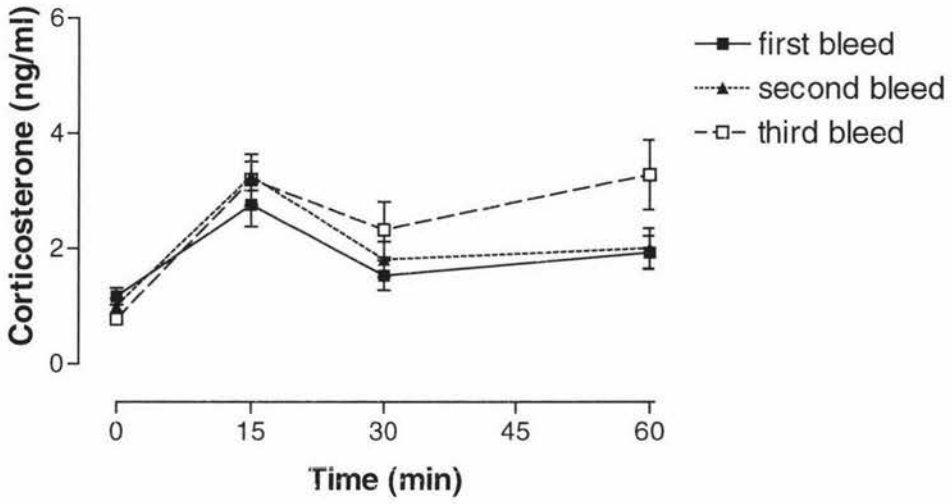
Fig. 4.3: Individual corticosterone response to handling curves for birds bled on three occasions. Individual points are shown where the preceding or following sample was excluded because they took >2 minutes to collect.

There was a significant effect of time on plasma corticosterone levels, but no differences between bleeding sessions (Fig. 4.4, Table 4.3 for statistics). Over all three bleeds, corticosterone levels were low at 0 minutes, increased increased three-fold by 15 minutes, and did not decline significantly between 15 and 30 minutes or between 30 and 60 minutes. Therefore, plasma corticosterone levels were still elevated above basal levels at 60 minutes (Table 4.3).

The areas under the corticosterone response curves for birds with complete data sets were similar for each sampling session (Fig. 4.5; one way ANOVA $F_{2,15}=0.256$, $p=0.777$). Three birds had complete curves at the first bleed and three different birds had complete curves for the second bleed. Ten birds had complete curves for the third bleed.

Complete corticosterone response curves were not obtained from any of the birds on all three sampling sessions, but three samples at either 0, 15, 30 or 60 minutes were obtained from some of the birds. Plasma corticosterone levels for each bird at each time for which samples were collected on three occasions were used to calculate coefficients of variation for each bird (variation within birds). The mean plasma corticosterone levels for each bird were averaged to determine the coefficient of variation between birds (Table 4.4).

Variation between birds was greatest at 30 minutes (33.9%) and 60 minutes (34.6%), smaller for basal levels at 0 minutes (27.8%) and least at 15 minutes (21.0%). Variation within birds was greatest at 60 minutes (47.9%), similar at 15 minutes (39.6%) and 30 minutes (40.8%), and least at 0 minutes (25.9%). Variation within and between birds was similar at 0 minutes, whereas variation within birds was higher than variation between birds at 15, 30 and 60 minutes. The variation within birds was double the variation between birds at 15 minutes.



Sample sizes at each time

	Bleeding session		
Time (min)	First	Second	Third
0	14	13	14
15	8	12	13
30	8	9	10
60	4	10	14

Fig. 4.4: Mean plasma corticosterone response to handling curves for birds bled on three occasions (mean ± SE, sample sizes shown in table).

Table 4.3: Summary of two-way repeat measures ANOVAs for plasma corticosterone response curves on three bleeding sessions.

	Plasma corticosterone		
Effect	F	df	P
Time	6.434	3,39	0.001 **
Bleed	0.262	2,13	0.774
Interaction	1.316	6,39	0.273
Comparisons between bleeding sessions			
First vs second bleed			
0 min	0.504	1,13	0.490
15 min	2.814	1,13	0.117
30 min	0.220	1,13	0.645
60 min	0.285	1,13	0.603
First vs third bleed			
0 min	1.194	1,13	0.294
15 min	1.389	1,13	0.260
30 min	0.239	1,13	0.633
60 min	1.172	1,13	0.299
Comparisons between times for all bleeds			
0 vs 15 min	48.323	3,13	<0.001 **
0 vs 30 min	11.637	3,13	0.001 **
0 vs 60 min	8.826	3,13	0.002 **
15 vs 30 min	2.855	3,13	0.078
30 vs 60 min	0.840	3,13	0.495

Note: The first three rows for each treatment show the overall effects of time and bleeding session and the interaction between the two. The following rows show post hoc comparisons between bleeding session and between times (over all bleeding sessions). Significant differences ($p<0.05$) are marked with asterisks, and highly significant differences ($p<0.01$) are marked with double asterisks.

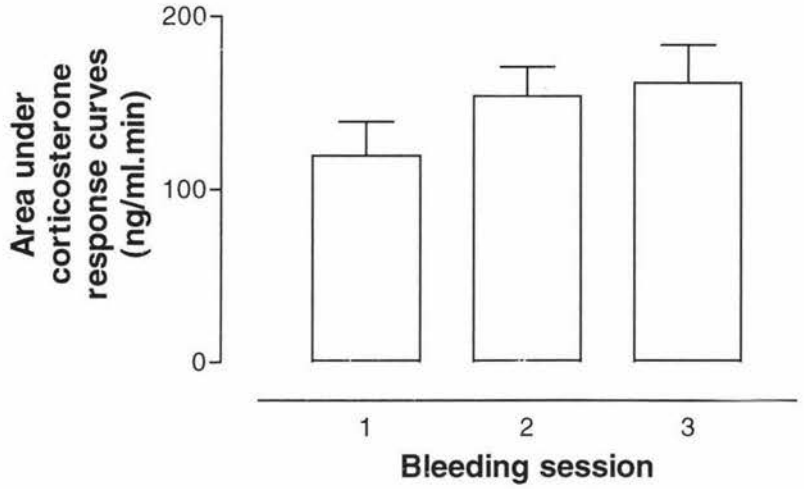


Fig. 4.5: Areas under corticosterone response to handling curves on three bleeding sessions. The area under the curve was calculated for birds that had complete corticosterone response curves at any of the bleeding sessions (mean ± SE; first bleed, n=3; second bleed, n=3; third bleed, n=10).

Table 4.4: Variation in plasma corticosterone levels within and between birds at 0, 15, 30 and 60 minutes after handling.

	0 min corticosterone				15 min corticosterone				30 min corticosterone				60 min corticosterone			
Bird	mean	SE	CV	n	mean	SE	CV	n	mean	SE	CV	n	mean	SE	CV	n
G1	0.97	0.23	41.53	3												
G10	0.73	0.00	0.00	3	2.85	0.36	21.86	3								
G11	1.27	0.31	42.63	3									1.78	0.31	29.98	3
G13	0.73	0.00	0.00	3	3.00	0.74	42.62	3	1.49	0.65	75.59	3				
G17	1.49	0.57	66.47	3	2.59	0.93	62.46	3	1.26	0.34	46.96	3				
G18	0.73	0.00	0.00	3					2.09	0.34	28.21	3	2.92	0.62	37.03	3
G20	0.95	0.22	40.58	3												
G21	0.77	0.04	8.28	3	2.87	0.80	48.03	3								
G25	1.39	0.31	38.64	3	3.69	1.24	58.31	3					3.71	1.64	76.57	3
G4	1.22	0.26	36.32	3	3.08	0.13	7.14	3								
G5	0.81	0.08	17.11	3					1.06	0.12	19.00	3				
G7	0.98	0.25	44.62	3	2.79	0.78	48.34	3	0.83	0.10	21.48	3				
G9	0.73	0.00	0.00	3	1.59	0.26	28.20	3	1.10	0.34	53.61	3				
All birds (mean of all individual corticosterone values)																
mean	0.98				2.81				1.31				2.80			
SE	0.07				0.44				0.16				0.59			
CV	44.49				44.00				33.90				62.64			
n	39				24				18				9			
Average variation between birds (mean of individual bird means)																
mean	0.98				2.81				1.31				2.80			
SE	0.08				0.21				0.18				0.56			
CV	27.75				20.96				33.92				34.58			
n	13				8				6				3			
Average variation within birds (mean of individual bird CV's)																
mean	25.86				39.62				40.81				47.86			
n	13				8				6				3			

Samples collected ≤ 2 min only. At each sampling time, only birds with samples collected on all three occasions included.

4.4.4 Day versus night

Complete corticosterone response curves were obtained from some of the quail sampled during the day and then at night two weeks later (Fig. 4.6). Corticosterone responses differed between birds, with the response being higher during the day than at night in some birds (e.g. G2, G16) but similar at both times in others (e.g. G6, G8).

The corticosterone response curves for the birds bled during the day and the night were compared with curves from birds from the same batch of quail that were bled twice during the day (from the experiment of individual variation in corticosterone responses). There were no differences in plasma corticosterone levels when corticosterone responses to handling were measured twice during the day (Fig. 4.7B, see Table 4.3 for statistics). In birds from the current study, mean plasma corticosterone levels at 0 minutes were higher during the day than at night (Fig. 4.7A; two group t-test $t_{14}=2.346$, $p=0.034$[1]). There were however no significant differences in plasma corticosterone levels between day and night at 15, 30 or 60 minutes (two group t-test, $t_{13}=2.045$, $p=0.062$; $t_{12}=1.548$, $p=0.148$ and $t_{10}=0.174$, $p=0.865$ for 15, 30 and 60 minutes respectively).

The areas under the corticosterone response curves were also similar for both bleeding sessions in birds from the study of individual variation in corticosterone responses (Fig. 4.5; one way ANOVA, $F_{1,4}=1.873$, $p=0.243$). However, the mean area under the corticosterone response curves was significantly lower at night than during the day in birds from the study of day versus night (Fig. 4.8; two group t-test, $t_{55}=2.135$, $p=0.037$). The areas under the curves could be calculated for three birds with complete corticosterone curves during the day and for four birds with complete curves at night. Only two of these birds had complete data sets at both times.

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[1] A 2 way repeat measures ANOVA was not able to be carried out on this data as variances were homogeneous across bleeding sessions but not across times within each session.

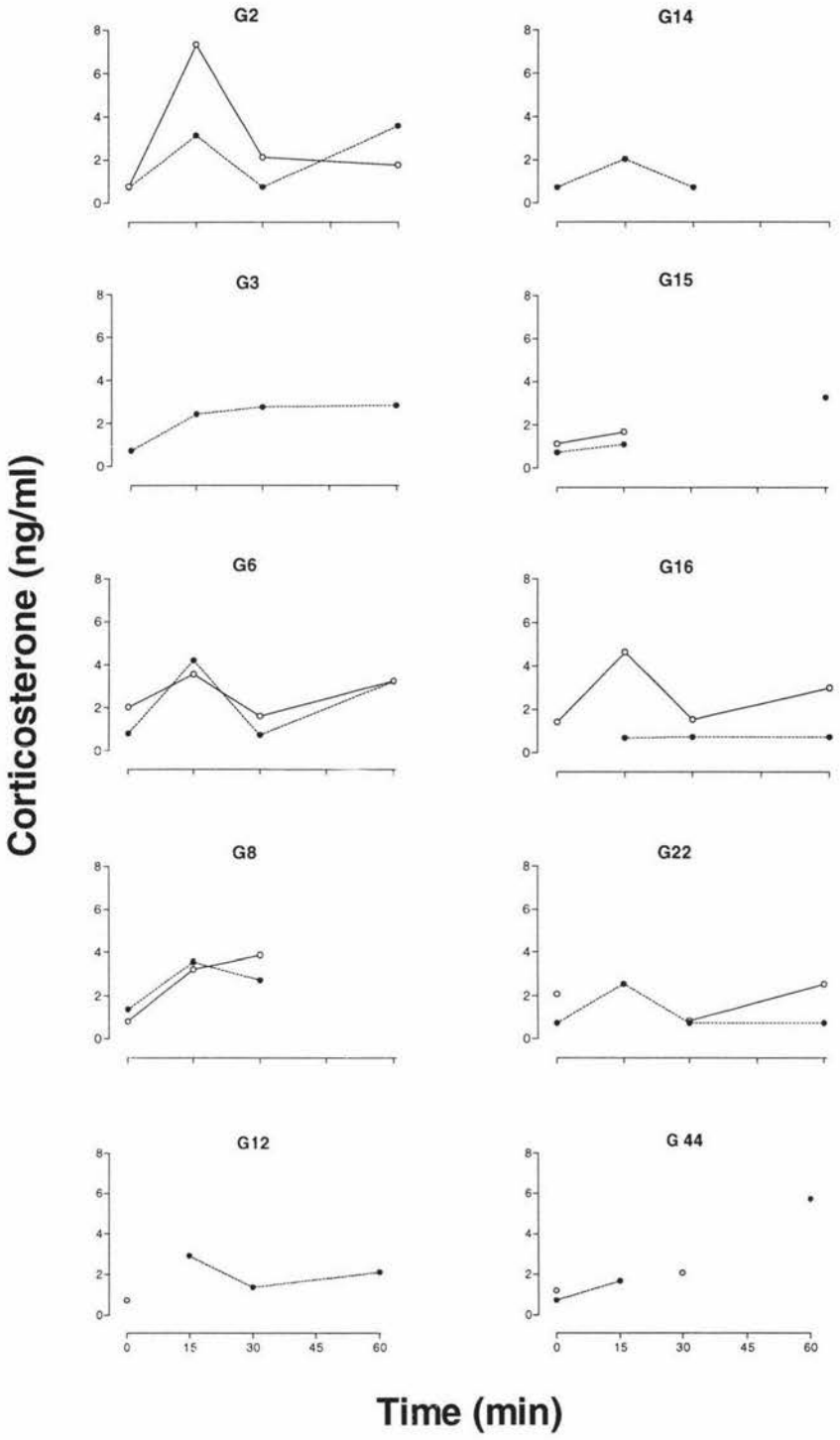
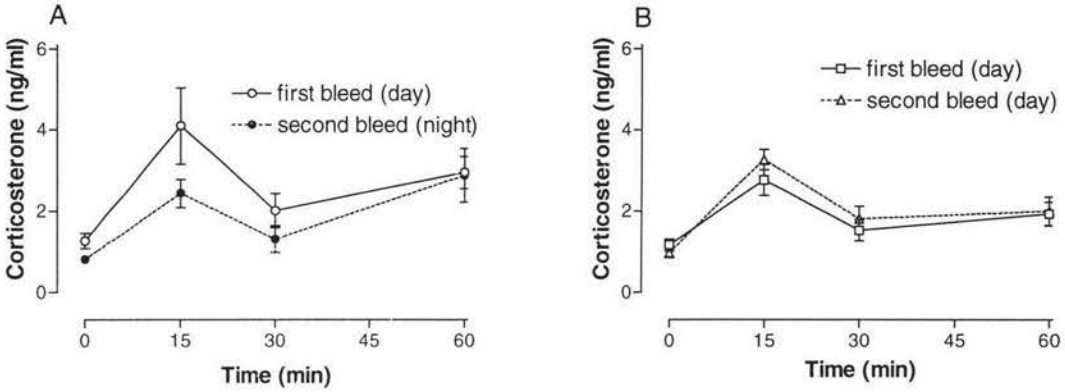


Fig. 4.6: Individual corticosterone response to handling curves for birds sampled during the day and at night. Individual points are shown where the preceding or following sample was excluded because it took > 2 minutes to collect.



Sample sizes at each time

	Bleeding session	
Time (min)	First	Second
0	8	8
15	5	10
30	6	8
60	5	7

Sample sizes at each time

	Bleeding session	
Time (min)	First	Second
0	14	13
15	8	12
30	8	9
60	4	10

Fig. 4.7: Mean corticosterone response to handling curves for quail bled on two occasions (mean ± SE; sample sizes shown in tables). A) Samples were collected during the afternoon for the first bleed, and during the night for the second bleed. B) Samples collected during the afternoon for both bleeds.

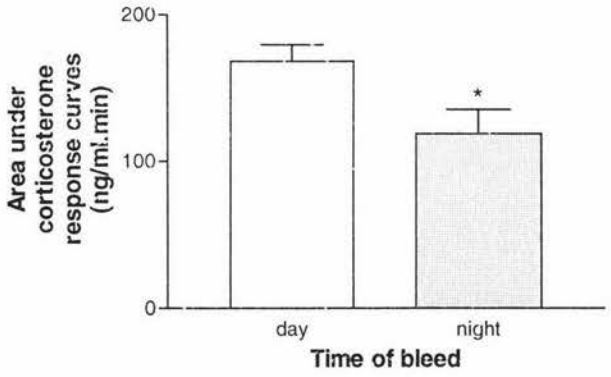


Fig. 4.8: Areas under corticosterone response to handling curves during the day and at night. The area under the curve was calculated for birds with complete corticosterone response curves at either time (mean ± SE; day, n=3; night, n=4). The asterisk indicates a significant difference ($p<0.05$).

4.4.5 Effect of reproductive status

4.4.5.1 Cloacal diameter and gonad weights

Cloacal diameters of birds held on short days (from the studies of individual variation and day versus night) and birds held on long days were similar at three weeks of age (Fig. 4.9; two group t-test; t_{30}=-0.390, p=0.699), but by six weeks of age cloacal diameters were much larger in the birds held on the long photoperiod (two group t-test; t_{30}=-13.109, p<0.001).

Weights of the ovary and oviduct were measured after the birds were killed at the end of each experiment (long day birds at six weeks old, short day birds from the study of day versus night at eight weeks old, and short day birds from the study of individual variation at ten weeks old). Ovaries and oviducts were large in all of the long day birds (Fig. 4.10) and all but two of these birds were laying eggs at the time of sampling. Ovaries and oviducts were small in all except one of the short day birds. As birds held on short days had small cloacal diameters throughout the experiment, and small gonads when they were measured at either eight or ten weeks of age, it was assumed that they also had small gonads at six weeks when comparisons between their corticosterone response curves were conducted. Therefore, birds held on short days were defined as having small gonads, and birds on long days as having large gonads.

4.4.5.2 Plasma corticosterone

Complete corticosterone response curves were obtained from all eight birds with large gonads (Fig. 4.11). As seen in the birds with small gonads, there was considerable variation between birds in the corticosterone responses to handling. Most birds (e.g. W28, W33, W35, W38) had a peak in plasma corticosterone levels at 15 minutes, after which corticosterone returned to basal levels. Three birds (W25, W32 and W37) showed no obvious rise in corticosterone with handling, and in one bird (W29), plasma corticosterone had not returned to basal levels by 60 minutes. Overall, there was a significant effect of time on plasma corticosterone (one way repeat measures ANOVA

$F_{3,21}$=5.836, p=0.005). Mean corticosterone levels rose significantly from 0 to 15 minutes (post hoc test; $F_{1,7}$=14.115, p=0.007), and had returned to basal levels at 30 and 60 minutes (post hoc test; $F_{1,7}$=0.037, p=0.854 and $F_{1,7}$=0.082, p=0.782 for 30 and 60 minutes respectively).

Corticosterone responses in birds with large gonads were compared with responses in the two groups of birds of the same age that were held on short days and therefore had small gonads (from the studies of individual variation and the day versus night comparison). The two groups of birds with small gonads were treated identically at six weeks and there were no differences in plasma corticosterone levels between them (Mann-Whitney U test, U=45.0, p=0.453; U=12.0, p=0.242; U=16.0, p=0.302; U=3.0, p=0.086 for 0, 15, 30 and 60 minutes respectively). Therefore, corticosterone responses in these groups were combined for comparison with birds with large gonads.

Quail with large gonads had higher corticosterone levels than birds with small gonads at 0 and 15 minutes (Figure 4.12; Mann-Whitney U test; U=45.5, p=0.041 and U=15.0, p=0.007 respectively), but not at 30 or 60 minutes (U=45.0, p=0.452 and U=43.0, p=0.500 respectively). However, there was no difference between birds with large and small gonads in the areas under the corticosterone response curves (Fig. 4.13; two group t-test; t_{11}=-1.278, p=0.228).

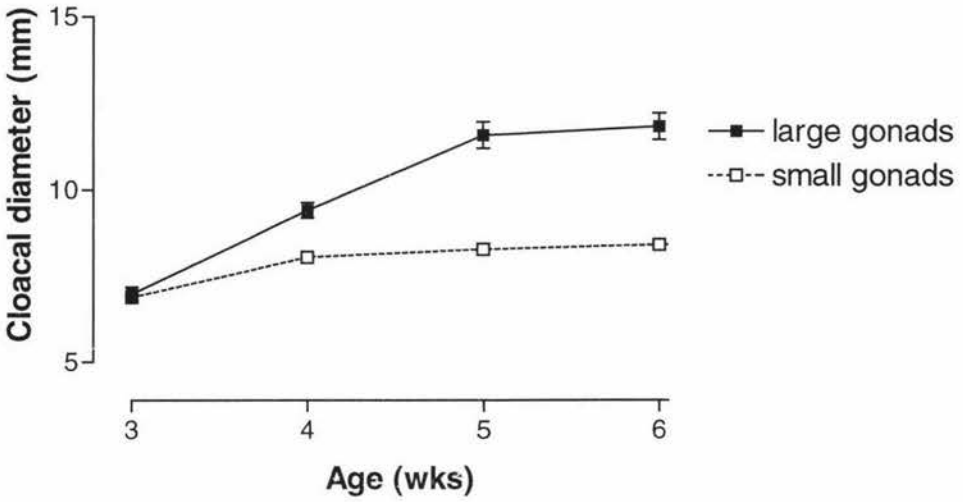


Fig. 4.9: Cloacal diameters from three to six weeks of age for birds with small gonads (from the studies of individual variation and day versus night) and large gonads (mean ± SE; small gonads, n=24, large gonads, n=8).

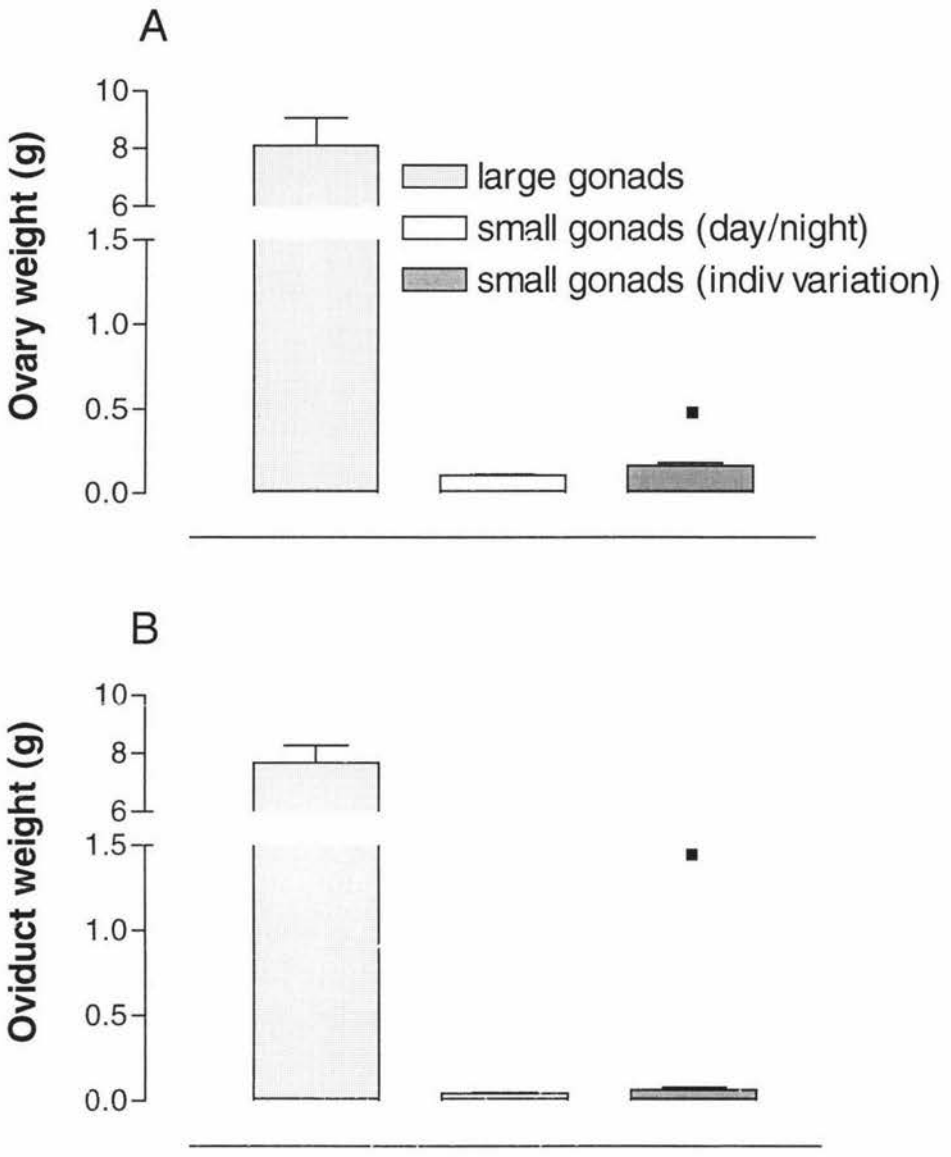


Fig. 4.10: Ovary and oviduct weights for birds with small gonads (from the studies of individual variation and day versus night) and large gonads (mean ± SE; individual variation, n=14; day/night, n=10; large gonads, n=8). A) ovary weights. B) oviduct weights.

One bird held on short days in the study of individual variation had undergone some gonadal growth. The data for this bird are presented as dots and were not included on the calculations of the means.

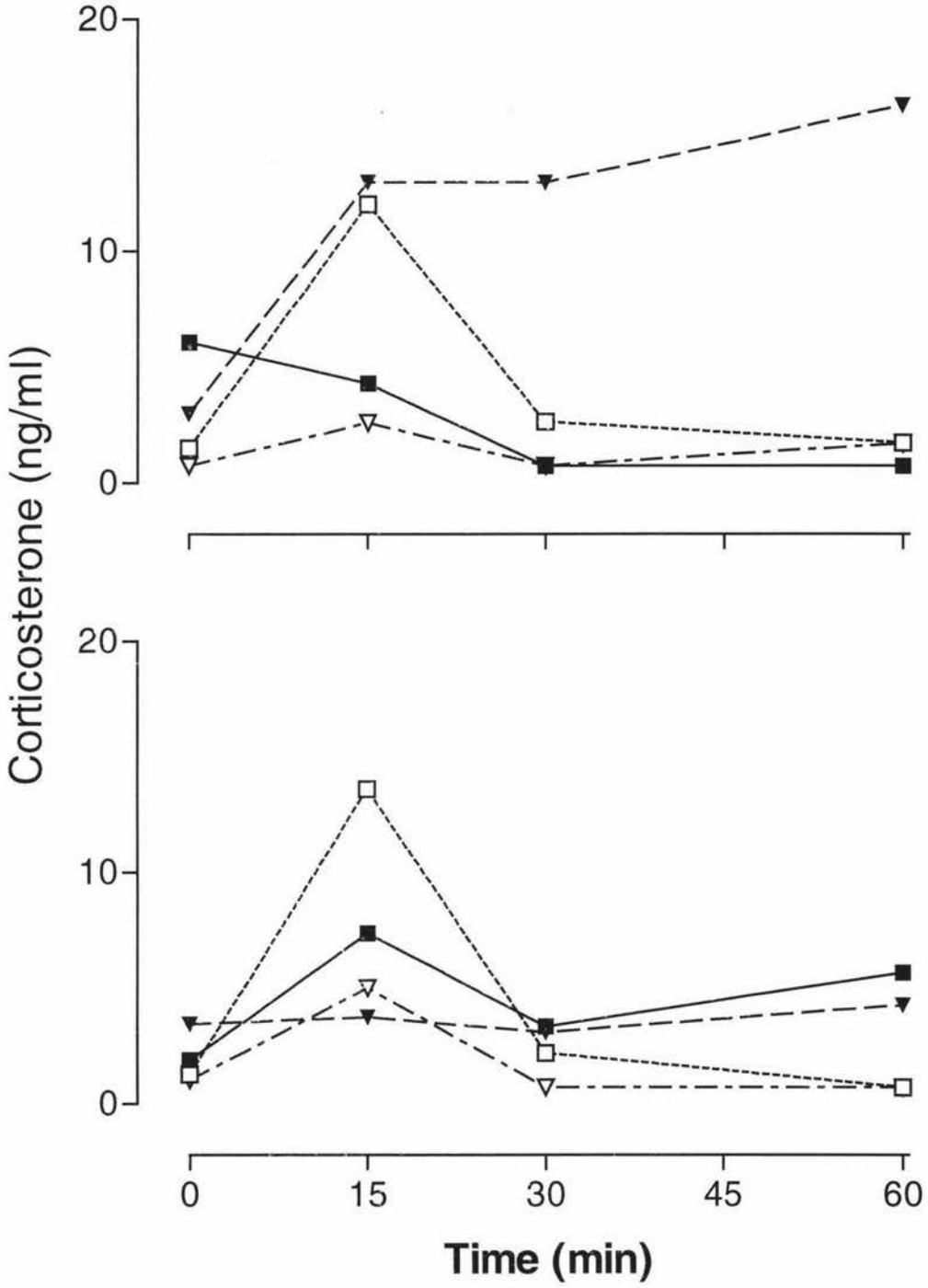
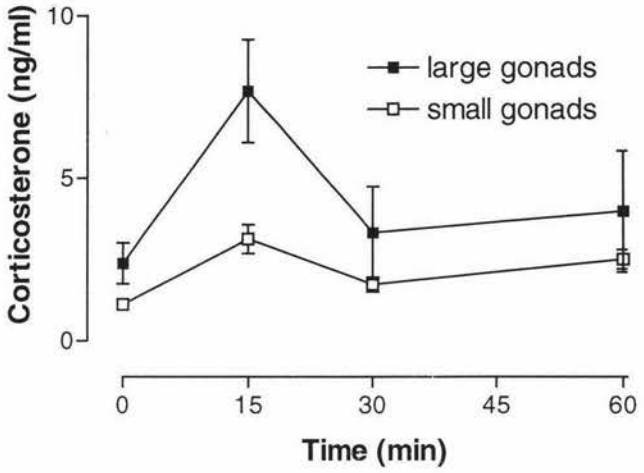


Fig. 4.11: Individual corticosterone response to handling curves for birds with large gonads. All samples were collected during the afternoon.



	Sample sizes	
Time (min)	Large gonads	Small gonads
0	8	25
15	8	14
30	8	15
60	8	9

Fig. 4.12: Corticosterone response to handling curves for birds with small gonads (from the studies of individual variation and day versus night) and large gonads (mean ± SE; sample sizes shown in table).

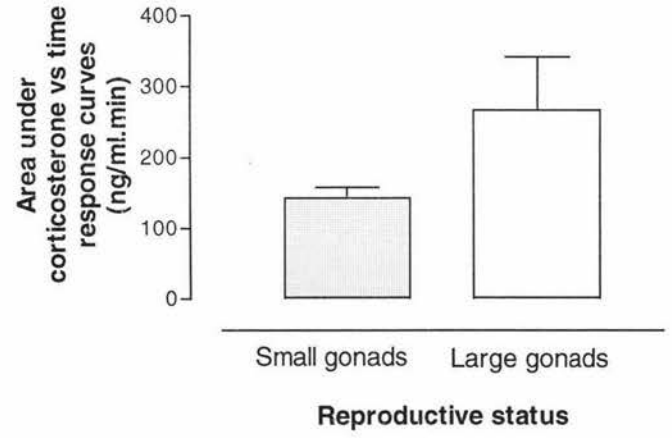


Fig. 4.13: Areas under corticosterone response to handling curves for birds with small gonads (from the studies of individual variation and day versus night) and large gonads (mean ± SE; small gonads, n=6; large gonads, n=8).

4.5 Discussion

The corticosterone response to handling in the female Japanese quail lasts less than 30 minutes. The coefficients of variation in the corticosterone response to handling within and between birds were similar to those found in our laboratory for chickens, and mean plasma corticosterone response curves were identical on the three different sampling occasions. Corticosterone response curves were similar during the afternoon and evening, although areas under the curves were smaller at night. Birds with large gonads had higher plasma corticosterone levels 15 minutes after handling than those with small gonads, but the two groups of birds had similar areas under the corticosterone response curves.

4.5.1 Duration of the corticosterone response

Corticosterone levels in Japanese quail rose in response to handling, to reach a peak 15 minutes after handling began. Plasma corticosterone then decreased to levels similar to basal levels by 30 minutes and remained low thereafter.

This study shows that the duration of the corticosterone response in the female Japanese quail is less than 30 minutes. This duration is similar to that seen in chickens (Freeman and Flack, 1980). However, corticosterone responses in free-living birds generally have a much greater duration, with corticosterone levels often being elevated for more than 60 minutes (Wingfield *et al.,* 1982; Astheimer *et al.,* 1994; Smith *et al.,* 1994; Schwabl, 1995). Peak levels of corticosterone also tend to be much higher in wild birds than in domesticated birds. For example, peak corticosterone levels were 40 ng/ml in ducks (Harvey *et al.,* 1980), 32 ng/ml in the lapland longspur (Astheimer *et al.,* 1995) and 25 ng/ml in the white throated sparrow (*Zonotrichia albicollis*, Schwabl, 1995), compared to 4 ng/ml in the chicken (Beuving and Vonder, 1986), and 3 ng/ml (Scatterlee and Johnson, 1988) or 4 or 10 ng/ml (birds with small and large gonads in the current study) in Japanese quail. The differences in corticosterone responses between free-living and domesticated birds are presumably a consequence of the domestication process, as birds who are less

responsive to the stress of captivity will be more likely to breed successfully than those showing large corticosterone responses. Therefore, birds with low corticosterone responses would be unintentionally selected for.

4.5.2 Effect of sampling speed

In the studies of individual variation in the corticosterone response and differences in the day versus night, plasma corticosterone levels were higher in samples that took more than two minutes to collect than in samples collected within a shorter time-period. This result shows the importance of rapid sampling in the Japanese quail if plasma corticosterone levels are to be measured. A similar relationship between sampling speed and plasma corticosterone levels has been seen previously when basal corticosterone levels were measured in a number of different bird species. Corticosterone began to rise within one minute in ducks (Harvey *et al.,* 1980) and Japanese quail (Scatterlee and Johnson, 1988), within two minutes in chickens (Beuving and Vonder, 1978), and between 50-80 seconds in free-living starlings (*Sturnus vulgaris,* Dawson and Howe, 1983). However, other studies showed that corticosterone did not change within 150 seconds of handling in chickens (Lagadic *et al.,* 1990), or within 350 seconds in captive white-crowned sparrows (Wingfield *et al.,* 1982). Obviously, there can be considerable variation in the time it takes for corticosterone to begin to rise depending on the species and environment.

Corticosterone levels were higher in samples that took longer than two minutes to collect both for the first blood sample (0 minutes) and for subsequent samples (15, 30 and 60 minutes). This may indicate that plasma corticosterone had not reached peak levels by 15 minutes, and therefore was still rising, or that the collection of a second serial blood sample at 15 minutes initiated a second corticosterone response. The former is unlikely, as several other studies have found that the corticosterone peak occurs within 10 minutes of handling in chickens (Freeman and Flack, 1980) and Japanese quail (Scatterlee and Johnson, 1988). It is possible that in domesticated birds such

as the Japanese quail, a short period of handling (under two minutes) may not result in a re-initiation of the corticosterone response, whereas a longer period of handling may. This appears to be the first report of an effect of the time taken to collect a blood sample on plasma corticosterone levels in samples collected after initiation of the corticosterone response (i.e. non-basal samples).

It is possible that the effect of time taken to collect a blood sample on basal plasma corticosterone levels was due to birds who had larger basal corticosterone levels struggling more, resulting in longer times for sample collection from these birds. However, a study in Japanese quail showed that there was no relationship between the duration of tonic immobility (which would result in faster sampling) and plasma corticosterone levels (Jones *et al.,* 1994). Also, in other studies (e.g. Dawson and Howe, 1983), blood samples were collected by decapitation, and were deliberately delayed after initiation of handling to determine when corticosterone started to rise in the plasma. In studies like this, the time of blood sample collection would be independent of whether or not the bird struggled. Corticosterone levels in birds in these studies still rose several minutes after the birds were first handled, indicating that higher corticosterone levels after several minutes were due to the initiation of a corticosterone response when the birds were first handled.

4.5.3 Individual variation

The main aim of this study was to determine whether variations in plasma corticosterone were more pronounced between different birds or between different sampling sessions for the same birds. It was thought that individual birds would have similar responses to handling at each time, resulting in a smaller coefficient of variation within birds than between birds. However, the means of the coefficients of variation for the first three blood samples each day (the 60 minute sample was omitted because of small sample sizes) were 27.5 ± 3.7% for variation between birds and 35.4 ± 4.8% for variation within birds. The variation between maximum plasma corticosterone levels (at 15 minutes) within birds was double the variation at this time between birds. These results indicate that the peak corticosterone levels at 15 minutes vary more between

sampling sessions for individual birds than between birds, as do levels at 30 and 60 minutes after the initiation of the corticosterone response. The larger degree of variation within birds than between birds in the current study may have been due to differences in the amount of time needed to collect samples and the large number of samples that were excluded.

The results of the current experiment were different to those seen in chickens (Litten, 1998), in which variations similar within and between birds. In the study by Litten (1998), the average variation between birds for samples collected at 0, 15 and 40 minutes (42.8 ± 6.4%) was almost identical to the variation within birds (42.3 ± 4.8%), and the variation in maximum plasma corticosterone levels at 15 minutes were also similar. However, both within bird and between bird variations were generally lower for corticosterone responses of the Japanese quail (from the current study) than were seen in the chicken (Litten, 1998). More data is needed from both species to determine whether the corticosterone responses of quail are generally more consistent than those of chickens.

There were no significant differences in overall corticosterone levels between the three bleeding sessions. This indicates that there was no effect of age on corticosterone response and that there was no adaptation to the handling and sampling procedure. The quail are reared in an intensive production system and are reasonably accustomed to the presence of people. It is therefore not suprising, therefore, that these birds did not show any adaptation to the fortnightly blood sampling procedure. A similar result has been reported for chickens, with no noticeable adaptation to handling in chickens handled once daily (Webb and Marshaly, 1984) or twice daily (Freeman and Manning, 1979) for several weeks.

4.5.4 Day versus night

Corticosterone levels were lower during the night than the day for the initial (basal) sample only. The area under the corticosterone response curve was, however, smaller at night. Different birds had complete curves at the two sampling sessions and the sample sizes were small. Therefore, this result

needs to be confirmed in a larger study before conclusions on corticosterone responses during the day and night in our quail can be drawn. Although there may be small differences between basal corticosterone levels and the corticosterone response to handling during the day and at night, these results do not support the hypothesis that being woken and sampled during the inactive period of the day is more stressful for the bird than being sampled during the active period.

Corticosterone responses to handling have been found to vary over 24 hours in several other studies, but no consistent pattern has been found. Freeman and Flack (1980) found that different strains of chickens had greater corticosterone responses during either the morning or afternoon, depending on the strain. There were no significant differences in corticosterone responses between morning and evening in another study of chickens (Beuving and Vonder, 1986), or between responses during the day and at night in western screech owls (Dufty and Belthoff, 1997). However, Breuner *et al.* (1999) reported a greater response at 10 am (just after lights-on) compared to 4 am, 4 pm, and 10 pm in white-crowned sparrows and Romero and Remage-Healey (2000) found the greatest response in starlings at 2 am (just before lights-on), compared to 8 am, 2 pm and 8 pm. The results of these two experiments may indicate a circadian rhythm in the sensitivity of the hypothalamo-pituitary-adrenal axis, resulting in larger responses at the beginning of the active period than throughout the rest of the day. However, more study on this is needed.

There is considerable evidence for a diurnal rhythm in basal corticosterone levels in birds, although the exact rhythms vary with the species. Different basal corticosterone levels during the light and dark periods have been seen in Japanese quail (*Boissin and Assenmacher*, 1970; Kovacs and Peczely, 1983), hens (Beuving and Vonder, 1977; Etches, 1979; Johnson, 1981; Wilson and Cunningham, 1981), pigeons (Joseph and Meier, 1973; Westerhof *et al.,* 1994), white-throated sparrows (Dusseau and Meier, 1971), white-crowned sparrows (Breuner *et al.,* 1999), starlings (Romero and Remage-Healey, 2000), western screech owls (Dufty and Beltoff, 1997) and turkeys (*Meleagris gallopavo*,

Proudman, 1991). Plasma corticosterone levels were most commonly at their lowest point at the beginning of the dark period, thereafter increasing to peak levels either several hours before the light period began (Boissin and Assenmacher, 1970; Joseph and Meier, 1973; Etches, 1979; Westerhof *et al.*, 1994; Romero and Remage-Healey, 2000), just after lights-on (Breuner *et al.*, 1999), or several hours after lights-on (Johnson, 1981, Proudman, 1991). This usually resulted in plasma corticosterone levels that were higher several hours after lights-on than at several hours after lights-off (Boissin and Assenmacher, 1970; Beuving and Vonder, 1977; Proudman, 1991; Breuner *et al.*, 1999). My results agree with the above studies, with basal corticosterone levels being lower early in the night than early in the afternoon. However, Kovacs and Peczely (1983) showed increasing basal corticosterone during the light period, a peak in corticosterone at the beginning of the dark period, and then decreasing corticosterone throughout the dark in the Japanese quail, and Dusseau and Meier (1971) found a similar pattern in white-throated sparrows.

4.5.5 Effect of reproductive status

Both basal plasma corticosterone levels and the corticosterone response to handling were greater in quail with large gonads than in birds with small gonads. This is apparently the first study of corticosterone responses in domesticated birds (quail or chickens) in relation to reproductive status. This difference could be due to the effects of photoperiod, temperature or reproductive hormones. As the corticosterone responses of birds with large and small gonads were not measured in birds from the same batch, it is also possible that the variation was due to a difference between groups of birds rather than an effect of treatment. However, the birds with large gonads had similar corticosterone responses to birds tested for the duration of the corticosterone response (who were also held on long days and therefore had large gonads). This similarity suggests that the difference in the corticosterone responses was an effect of the conditions rather than a difference between batches of quail.

A number of previous studies have examined the effects of reproductive status on basal corticosterone levels in species including the Japanese quail (Boissin and Assenmacher, 1970), starlings (Dawson and Howe, 1983; Romero and Remage-Healey, 2000), American kestrels (*Falco sparverius*, Rehder *et al.,* 1984; Rehder *et al.,* 1986), lapland longspurs (Romero *et al.,* 1998a), white-crowned sparrows (Wingfield *et al.,* 1982; Astheimer *et al.,* 1994; Romero *et al.,* 1997), rooks (*Corvus frugilegus*, Peczely and Pethes, 1982) and the snow bunting (Romero *et al.,* 1998b). As seen in the current study, a number of these authors reported higher basal corticosterone levels during the breeding season when birds had large gonads than in winter when their gonads were regressed (Peczely and Pethes, 1982; Dawson and Howe, 1983; Astheimer *et al.*, 1994; Romero *et al.,* 1997). However, little difference was seen in basal corticosterone levels between starlings held on long days (to imitate the breeding season) and short days (to imitate winter) (Romero and Remage-Healey, 2000). Within the breeding season, laying American kestrels also had higher basal corticosterone levels than non-layers (Rehder *et al.,* 1984; Rehder *et al.,* 1986), indicating that at least in this species, basal corticosterone levels are dependent on the reproductive state of the bird (i.e. the size of their gonads) and whether or not they are laying, rather than a direct effect of temperature or photoperiod.

Renden *et al.* (1994) examined the effect of photoperiod independently of reproductive status by holding young, immature chickens on different photoperiods. In this study, no differences between basal corticosterone levels of birds held on different photoperiods was seen. However, a study on gonadectomised and thyroidectomized quail (Peczely, 1985) found that increasing the photoperiod (from 6h light-18h dark to 18h light-6h dark) increased basal corticosterone in both males and females, similar to results seen in intact birds. Therefore, it is not clear whether or not photoperiod alone has an effect on basal corticosterone levels.

The increase in basal corticosterone levels in female birds during the breeding season may partially be caused by the process of egg-laying. Several studies have found increases in plasma corticosterone levels around the time of ovulation (Etches, 1979; Wilson and Cunningham, 1981) and during egg-laying itself (Beuving, 1980; Rehder *et al.,* 1986). In chickens, Beuving (1980) stated that corticosterone is doubled (from 2 ng/ml to 4 ng/ml) by egg laying. Similarly, Beuving and Vonder (1977) found that plasma corticosterone was elevated from 45 minutes before to one hour after laying, and Assenmacher and Jallageas (1980) stated that plasma corticosterone levels were generally elevated from 1.5 hours before to one hour after laying. However, the increase in basal corticosterone seen in the present study is probably not due to egg laying, as our strain of Japanese quail generally lay eggs between 5-8 pm (Bennett *et al.,* unpublished data), and blood sampling in the current study was carried out from 1 pm.

Environmental temperature could also have an effect on basal corticosterone levels independent of the reproductive state. In the current study, the birds held on short days were kept at 13^{0}C, whereas those on long days were held at 20^{0}C. The birds were kept at different temperatures as low temperatures in addition to short days are often necessary to keep quail's gonads small (Chaturvedi *et al.,* 1992, Chua *et al.,* unpublished data). It has long been known that low temperatures result in increased circulating levels of the thyroid hormone, T_3, in the Japanese quail (Oishi and Konishi, 1978). More recently, it has also been found that T_3 has a negative effect on circulating ACTH and corticosterone in chickens (Carsia *et al.,* 1997). Therefore, in the present study, the low temperatures used to keep the short day birds reproductively immature could have resulted in elevated T_3 levels and consequently lower basal corticosterone levels. In most of the free-living species mentioned previously, temperatures during the non-breeding season are lower than in the breeding season, so a similar interaction between T_3 and corticosterone may be occurring.

4.6 Conclusions

The duration of the corticosterone response was less than 30 minutes in the Japanese quail and corticosterone begins to rise two minutes after the handling stimulus is applied. The individual variation within and between birds was quantified, and was found to be similar to that seen in chickens. Plasma corticosterone curves were identical when the corticosterone response to handling was measured in three occasions. There was little difference in the corticosterone responses during the day (active) and night (inactive) periods of the day, although basal corticosterone levels were lower at night, and the area under the corticosterone response curves was smaller. Finally, both basal corticosterone and peak levels at 15 minutes after handling were higher in birds with large gonads than those with small gonads.

5 General Discussion

5.1 Conclusions

This study was divided into two parts. The first set of experiments (chapters 2 and 3) studied the acute and chronic effects of corticosterone injections on the reproductive systems of Japanese quail, and examined the relationship between faecal and plasma corticosterone levels. The second set of experiments (chapter 4) examined corticosterone responses to handing in the Japanese quail. The major results and conclusions can be summarised as follows:

1. Corticosterone injections can reduce plasma testosterone levels (and consequently cloacal gland area and cloacal foam production) in male Japanese quail, the extent of which may depend on the age of the bird. Increased plasma corticosterone levels result in a rapid decrease in plasma testosterone levels. This decrease in plasma testosterone levels appears to be independent of plasma LH levels, which did not change during the hours after a corticosterone injection. This indicates a direct effect of corticosterone on the testes of the male quail, but does not rule out further effects at the levels of the hypothalamus or the pituitary.

2. Corticosterone can be measured in the faeces after an injection of corticosterone and faecal corticosterone levels are highest from 0-3 and 3-6 hours after an injection. Therefore, it takes less than three hours for corticosterone from the plasma to be excreted into the faeces in quail. There is a strong relationship between plasma and faecal corticosterone concentrations when plasma corticosterone is at basal levels and a significant relationship between the area under the faecal corticosterone excretion curve and corticosterone dose. These results indicate that faecal corticosterone measurements can provide a non-invasive measure of plasma corticosterone levels.

3. Corticosterone responses to handling were compared in several different situations in female Japanese quail. The degree of individual variation of the corticosterone response to handling within and between birds was quantified, and was found to be similar to that seen in several other species of bird. The corticosterone response was similar during active (afternoon) and inactive (night) periods, possibly indicating that quail do not perceive being woken and blood sampled at night as being more stressful than being sampled during the day. Female Japanese quail with large gonads (kept on a long photoperiod at 20^0C) had higher plasma corticosterone levels at 15 minutes after handling than did birds with small gonads (kept on short days at 13^0C). This difference most likely being due to the reproductive state of the bird rather than the photoperiod or temperature alone.

5.2 Future work

5.2.1 Corticosterone and reproduction

There is little information available on how corticosterone can inhibit the reproductive system in birds. Results from the current study are the first data to show the changes in plasma LH and testosterone during the hours after a corticosterone injection in an avian species. The current experiments therefore lead on to further studies to clarify the level of the reproductive system most affected by corticosterone and the relationship between plasma corticosterone and testosterone levels with lower doses of corticosterone.

Much further work is needed to clarify the level of the reproductive axis that corticosterone predominantly inhibits. From the results of the current experiment (chapter 3) it was suggested that corticosterone was acting both at the level of the gonads and higher in the reproductive axis. This hypothesis could be tested by treating castrated quail with either oil or corticosterone. In castrated quail, there is no negative feedback by endogenous testosterone on LH, so if corticosterone is acting at the hypothalamus or pituitary, corticosterone treatment should decrease plasma LH levels. A similar experiment in which intact birds are treated with corticosterone or LH, or both hormones together

would confirm a direct action of corticosterone on the testes. If birds that received both corticosterone and LH had lower levels of plasma testosterone than those receiving LH only, this would indicate that corticosterone was acting directly on the testes to decrease plasma testosterone levels. As in the current experiment, blood samples would need to be collected repeatedly within the 12 hours after an injection to ensure changes in plasma hormone levels were not missed.

Corticosterone was shown to decrease plasma testosterone levels in the male quail in the current study, but it is not known whether the same effect occurs with physiological levels of corticosterone. The results presented in chapter 2 showed that 0.3 mg corticosterone per day was the lowest dose of corticosterone that resulted in a decrease in the cloacal gland areas of young male quail. However, the cloacal gland area is only a crude measure of reproductive status and changes in plasma hormone levels during the hours after injection were not measured in this study. In order to further understand the relationship between corticosterone and plasma testosterone levels, changes in plasma levels of these hormones should be measured after injections of low doses of corticosterone (e.g. 0.075, 0.15 and 0.3 mg per day). Such an experiment could find the lowest corticosterone dose and plasma corticosterone level that results in a short-term decrease in plasma testosterone levels, and whether the size of the decrease in plasma testosterone is proportional to plasma corticosterone concentrations.

5.2.2 Non-invasive measures of corticosterone

A strong, positive relationship was seen between basal plasma and faecal corticosterone levels in 6 month old birds (see chapter 2, Fig. 2.7). This is the first such correlation demonstrated for any avian species. A similar correlation has been seen before in the ring-tailed lemurs (*Lemur catta*, Cavigelli, 1999), but only four pairs of plasma and faecal samples were compared. The current study therefore produced the best correlation seen so far between plasma corticosterone in the plasma and the faeces, and is the first step towards the

validation of faecal corticosterone as a non-invasive measure of plasma corticosterone in the Japanese quail.

Further study is needed both to confirm the relationship between plasma and faecal measures of corticosterone when plasma corticosterone is at basal levels, and to examine the relationship when corticosterone is elevated by injections of corticosterone or ACTH, or by a stressor. Before further validation of the plasma and faecal relationship in quail is carried out, however, it should be confirmed that the corticosterone response to a stressor results in a measurable change in faecal corticosterone levels. The results of the current study on corticosterone responses in the Japanese quail (chapter 4) showed relatively small changes in plasma corticosterone after handling for 15 minutes. It is not known whether these changes are large enough to be measurable in the faeces. An experiment in which plasma and faecal samples are collected and corticosterone levels measured every 15 or 30 minutes for several hours after a stress response is initiated would confirm that changes in plasma corticosterone levels during the stress response were also present in the faeces. If faecal corticosterone levels did increase in response to the stressor, this experiment would also accurately define the lag time between peak corticosterone levels in the plasma and in the faeces. The length of this lag is not presently known in the Japanese quail, but has been reported to be one to two hours in two other avian species (Holmes and Slikker, 1976; Wasser *et al.*, 1997). As the corticosterone response to handling in the quail lasts less than 30 minutes, plasma corticosterone is likely to have returned to basal levels before faecal corticosterone begins to rise. This problem may be overcome, however, by correlating the area under the plasma corticosterone curve and the area under the faecal corticosterone curve after the stressor.

If further evidence for the correlation between plasma and faecal corticosterone is needed, similar experiments to the ones described in chapter 3 of the current study could be carried out, with the administration of either corticosterone or ACTH. Because of the lag time for corticosterone to be excreted in the faeces,

it may be necessary to administer corticosterone by implants, or to give injections of long-acting ACTH to provide constant levels of plasma corticosterone over several hours. A direct positive relationship between plasma and faecal corticosterone is most likely to be seen if plasma corticosterone is not fluctuating greatly. Alternatively, the areas under the plasma and faecal corticosterone curves could be compared. Once faecal corticosterone measurements have been validated in the Japanese quail, the validation techniques used could then be applied to other species of bird.

5.2.3 Corticosterone responses in the Japanese quail

Chapter 4 of the current study examined differences in the corticosterone responses of female quail within and between birds tested on several occasions, between birds tested during the day and the night, and between birds with large and small gonads. Many of the blood samples took longer than two minutes to collect and were discarded, so was difficult to draw definite conclusions from the results. However, several areas for further work were identified from the results of these studies.

It would be interesting to confirm the result that female birds with large gonads had greater corticosterone responses than females with small gonads. Since these experiments were carried out, it has been found that the strain of Japanese quail we use may not need cold temperatures in addition to a short photoperiod in order to maintain small gonads in most birds (Henare *et al.*, unpublished data). Therefore, it may be possible to keep birds under a variety of different photoperiods and temperatures (some which allow gonadal growth and others which do not), to determine whether the difference in the magnitude of the corticosterone responses was due to the long photoperiod, high temperatures, the size of the gonads and related changes in levels of reproductive hormones, or a mixture of the above.

The level of the HPA axis that the difference in plasma corticosterone responses was occurring at could also be examined by measuring plasma

corticosterone levels after an ACTH injection in birds with large and small gonads. If the differences in plasma corticosterone levels were due to differences in adrenal sensitivity to ACTH, an ACTH injection would result in higher plasma corticosterone levels in birds with large gonads. If it was due to different levels of ACTH release in response to a stressor, an ACTH injection would result in similar levels of corticosterone in both groups of birds.

To complete a comprehensive picture of corticosterone responses in the Japanese quail, studies would need to be carried out on male birds as well as females. For example, corticosterone responses in male birds with large or small gonads could be compared to those of females with large or small gonads. Corticosterone responses in relation to age could also be examined in both females and males. One experiment from the current study of the effects of corticosterone on reproduction (chapter 2) found that corticosterone administration had a more pronounced effect on plasma testosterone in older males than younger males. It would be interesting to examine changes in both plasma corticosterone and testosterone levels during a stress response in old and young male quail. This would determine first whether plasma testosterone levels decrease in response to endogenous corticosterone during the stress response, and second whether changes in plasma levels of corticosterone and testosterone during the stress response vary with age.

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