Faecal steroid measurements for the assessment of reproductive function in Japanese quail (*Coturnix coturnix japonica*) and kakapo (*Strigops habroptilus*).

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Abstract

The kakapo (*Strigops habroptilus*) is an endangered parrot endemic to New Zealand and little is known of its reproductive physiology. Reproductive function is conventionally determined by the measurement of reproductive steroids in plasma samples. This is impractical and invasive in endangered, free-living species. However, the measurement of reproductive steroids in avian faecal samples is practiced. Few studies have documented strong relationships between faecal and plasma steroid concentrations. The objectives of this study were to develop and validate a faecal extraction method for the measurement of oestradiol, progesterone and testosterone in Japanese quail (*Coturnix coturnix japonica*); determine the relationships between steroid concentrations and gonadal development in quail; and define annual faecal hormone cycles of kakapo in relation to their breeding status.

Groups of male and female quail were held on different photoperiodic and temperature regimes to produce birds with a range of gonad sizes and steroid concentrations. Steroid concentrations were measured in faeces and plasma by radioimmunoassay. Positive relationships were demonstrated between plasma and faecal steroid concentrations. Faecal steroid concentrations had strong positive relationships with ovary and testis size in female and male quail respectively.

The extraction method developed was then applied to faecal samples, which were collected from kakapo in their free-living environment on Whenua Hou (Codfish Island). The samples were collected from identified birds over three potential breeding seasons. There were annual cycles of hormone concentrations that corresponded with cycles of breeding activity in females and males. No significant differences were found between breeding and non-breeding years for faecal concentrations of all three hormones, suggesting that kakapo undergo a degree of gonadal development each year. Annual hormone profiles for individual birds supported this finding.

This study quantifies the value of collecting multiple faecal samples in both captive and wild situations and demonstrates the power and value of faecal steroid analysis.

Author with Sirocco, photo: R.Cole.

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Kakapo chicks, photo: Department of Conservation, New Zealand.

1 General Introduction

The use of faecal steroid analysis for the non-invasive measurement of reproductive steroids has increased considerably over the last two decades. Faecal steroid measurements are now used as a tool for monitoring breeding in a diverse range of species, both in captivity and in free-living situations. The survival of endangered species relies on knowledge of reproductive status and physiology. The non-invasive nature of faecal steroid analysis allows this, while reducing disturbance to individuals during the breeding season.

1.1 Kakapo

The kakapo is an endangered parrot species endemic to New Zealand. Like several other threatened bird species that have evolved on these isolated mammal free islands, it is nocturnal and flightless. Furthermore, it is the largest parrot in the world, weighing up to four kg. Breeding attempts are infrequent and occur every two to five years, with two to four eggs being laid, sometimes in multiple clutches.

Subfossil and midden records show that until around 1 000 years ago, kakapo were distributed throughout the North and South Islands of New Zealand, inhabiting areas from sea level to subalpine tussock (Merton and Clout, 1998). Populations had disappeared from the North Island by the early 1900's, and by the 1950's only fragmented populations remained in Fiordland and the south west of the South Island. This rapid decline in numbers is attributed to habitat loss, hunting and introduced predators, which resulted from European settlement (Merton and Empsom, 1989).

Conservation efforts began in 1894, and by 1910 several hundred birds were translocated to Resolution Island, Fiordland by a pioneer conservationist, Richard Henry (Merton and Clout, 1998). However, predation from stoats continued and it was not until the 1950-60s that a census was conducted to determine the status of the remaining mainland population. Only a small number of birds was found. In the 1970s efforts

continued, and a new population was discovered on Stewart Island. During the 1980s, predation from cats became apparent, and all birds were removed from Stewart Island and translocated to Little Barrier, Maud Island and Whenua Hou (Codfish Island). Since then, an intensive management regime has been undertaken by the Department of Conservation (Powlesland, 1991). This regime is primarily focused on increasing the breeding population. Research is aimed at increasing the breeding frequency and productivity of breeding attempts and determining the cause of infrequent breeding (Cresswell, 1996).

Kakapo are a solitary species with home ranges that vary from 15 – 30 ha in the non-breeding season in males and from 35 – 50 ha in females (Cresswell, 1996). Kakapo are lek breeders and in late spring and early summer males establish miniature display territories often within 15 to several hundred meters of each other on ridges or hill tops where calls can be projected a long distance (Merton *et al.*, 1984). The display area is a 'track and bowl' system consisting of one or more shallow depressions or bowls about 30 – 60 cm in diameter, connected by tracks of a similar width. From these bowls males emit several calls in order to attract females for mating. The two most common are a low frequency, resonant 'boom' interspersed with a higher-pitched 'ching'. Males call for six to eight hours per night and can continue every night for up to four months. Elaborate wing displays and whetting of the bill are also common. Booming seldom occurs annually, although low intensity booming was recorded on Whenua Hou during late 1995 to early 1996 and again from late 1996 to early 1997. Once a female has approached the track and bowl system and mating has occurred, the males' contribution to the breeding cycle is complete. Males continue to boom after mating and one male may mate with more than one female (Trewick, 1998). The female returns to her home range and selects a suitable area for nesting. Nest preparation is limited, with decaying centres in fallen trees, holes in stumps or areas under tussock clumps used as nest sites. Up to four eggs are laid at intervals of three to five days from late January to mid March. The female provides sole incubation lasting about 30 days and care for the young chicks. Chicks fledge at about ten weeks but remain associated with the female for at least a month (Merton and Empsom, 1989).

Little is known of kakapo reproductive physiology, and the assessment of breeding status and gonadal activity in individual birds would be of great value to conservation management practices. Details of kakapo reproductive physiology may also help to understand the factors that control the onset of breeding in this endangered bird.

1.2 The timing of seasonal breeding in birds

Reproduction in birds is seasonal and occurs when specific environmental conditions are appropriate. Seasonal breeding is primarily controlled by two types of environmental information, defined as ultimate and proximate factors (Baker, 1938). Ultimate factors are those that ensure young are produced at the optimum time for survival, for example food availability. Proximate factors are those that regulate the timing of gonadal development and the breeding cycle. These factors have been divided into four different types of information: initial predictive, essential supplementary, synchronising and integrating, and modifying (Wingfield, 1980; reviewed in Wingfield and Farner, 1993; and Cockrem, 1995). Initial predictive information includes factors such as the annual cycle of day length, endogenous reproductive rhythms and rainfall. These factors prime the birds physiologically for breeding. Virtually all birds have been shown to be photoperiodic and respond to increasing day lengths by increasing LH and FSH secretion (Follett and Maung, 1978). Essential supplementary information includes factors such as social cues, possession of a territory and availability of specific foods. These factors stimulate the final stages of gonadal development, nest building and egg laying and prevent the expenditure of valuable energy if conditions are not ideal. Once nesting behaviour has been initiated, internal and external factors synchronise and integrate the remaining events of the breeding season. Modifying information such as predation, loss of a nest site or food source can also enhance or disrupt the cycle at any stage.

1.3 Reproduction and the gonadal axis in birds

The hypothalamo-pituitary-gonadal (HPG) axis is the central control system for reproduction. The release of gonadotrophic hormones (LH and FSH) from the anterior pituitary gland is controlled by

gonadotropin-releasing-hormone (GnRH) secreted by the hypothalamus in response to external and internal stimuli. The roles of LH and FSH in avian reproduction are becoming increasingly well understood. LH stimulates steroidogenesis in both male and female birds (Phillips *et al.*, 1985), as well as playing a major role in the onset of ovulation in females (Wingfield and Farner, 1993; Scanes, 2000). FSH controls the functioning and development of the less mature ovarian follicles in females and spermatogenesis in males (Wingfield and Farner, 1993; Scanes, 2000). Steroidogenesis of oestradiol, progesterone and testosterone occurs in the ovaries and testes of reproductively active birds. Therefore, the measurement of these hormones can give valuable information on the reproductive status of individual birds.

The avian ovary differs significantly from the mammalian ovary. In the majority of birds only the left ovary is functional, although certain birds of prey and the kiwi are exceptions (Phillips *et al.*, 1985). A functional mature ovary contains large numbers of follicles arranged in a hierarchy according to size. Commonly there are four to six large yolk-filled follicles, a greater number of smaller follicles in which yolk deposition has been initiated, and many even smaller white follicles. Once yolk deposition begins, the follicle grows rapidly. For example, in domestic fowl (*Gallus domesticus*), a follicle increases in size slowly over many months, from 0.06 mm to 1.0 mm in diameter. Increasingly rapid growth occurs over several months until it reaches 8.0 mm. In the final stage, rapid yolk deposition occurs and the follicle reaches the mature size of 37 mm diameter in 6 - 11 days (Scanes, 2000). Once the deposition of yolk is complete, the largest follicle ruptures or ovulates and reveals a fully developed oocyte. Fertilisation and shell production occur in the oviduct before laying. The post-ovulatory follicle is reabsorbed. So, in contrast to mammals, there is no formation of a corpus luteum. In the male bird, internal paired testes vary in size according to the season. Maximal size occurs during the breeding season when they may be 200 to 300 times larger than other times of the year (Welty, 1962).

1.4 Role of gonadal steroids in birds

The roles of oestradiol, progesterone and testosterone in birds have been well documented (Wingfield and Farner, 1980; Scanes, 2000). The role of progesterone is to

initiate a positive feedback mechanism on LH secretion, which causes ovulation. Progesterone is secreted from the largest pre-ovulatory follicle and peak secretion occurs approximately six to four hours before ovulation. Progesterone has a minimal influence on the male avian reproductive system. Together with progesterone, oestradiol primes the hypothalamus and pituitary so that progesterone can cause LH secretion for ovulation. By itself, oestradiol has a negative feedback effect on LH secretion, as does progesterone if the hypothalamus has not been primed by itself or oestradiol. In addition, oestradiol regulates calcium metabolism for shell formation, enhances growth of the oviduct, controls secondary sex characteristics and sexual behaviour and influences sexual differentiation of the female brain Although oestradiol is mainly produced by pre-hierarchal follicles, the pre-ovulatory follicle produces two ovulatory-associated peaks six to four hours before and one to three hours after ovulation (Wingfield and Farner, 1980; Scanes, 2000).

Testosterone is essential for spermatogenesis and controlling GnRH secretion from the hypothalamus. Testosterone exerts an inhibitory feedback mechanism on the hypothalamus after being aromatised to oestrogen. Testosterone and its metabolites are also important in the control of secondary sex characteristics and the expression of specific sexual behaviours associated with courtship and mating (Adkins *et al.*, 1980; Scanes, 2000).

1.5 Steroid metabolism

1.5.1 Biosynthesis

Cholesterol is the biosynthetic source of all steroid hormones (McKerns, 1969; Goad, 1975). The pathways of steroid hormone synthesis from cholesterol are generally similar in both birds and mammals and are controlled by enzymes. Cholesterol circulates in the blood attached to low-density lipoproteins and is taken into the cell cytoplasm and stored as cholesterol esters in lipid droplets. Cholesterol can also be synthesised in the smooth endoplasmic reticulum or cytoplasm. Cholesterol is then taken up by the mitochondria of the target cells and is cleaved to yield pregnenolone, which is converted to other steroid hormones depending on the type of organ cell it is

in, for example testes, ovaries, placenta or adrenal glands. Progesterone is synthesised directly from pregnenolone and is then converted to testosterone, before conversion to oestradiol. Some steroids can be carried in the blood and further modified in other tissues. For example testosterone can be synthesised in the adrenals and then converted to oestradiol by ovarian follicles (McKerns, 1969; Goad, 1975). Only small amounts of the actual steroid hormones are stored in cells at any one time. Once synthesis has been stimulated, cholesterol conversion takes place and the steroid is secreted (Guyton, 1991).

1.5.2 Steroid action and transport

Steroids are secreted directly into the blood and are transported bound to specific carrier proteins, due to their insolubility in water. Once at the target tissue, steroid hormones dissociate from the carrier protein and bind to receptor proteins in the cytoplasm or cell membrane. This steroid-receptor complex is then transported to the cell nucleus where it binds to specific DNA sites and either activates or inhibits the transcription of target genes. This leads to the synthesis of new proteins or enzymes that produce a physiological response (Hadley, 1996).

1.5.3 Catabolism and excretion

Any steroid hormones left unbound in the blood are deactivated and excreted by the liver or kidneys (McKerns, 1969). Deactivation in birds occurs mainly in the liver and although steroids can be catabolised by oxidation, reduction, degradation or methylation, conjugation is the most common form of deactivation in birds (Holmes and Slikker, 1976; Gorsline et al., 1989). Deactivated steroids and their conjugates are secreted from the liver in bile. Bile salts increase the solubility of the deactivated steroids and conjugates and allow passage through the gut and excretion in faeces. Both conjugated and unconjugated steroid can be excreted via the kidney in urine.

The rate of hormone excretion can vary among species and also between steroids within the same species (Schwarzenberger et al., 1996). In mammals it has been shown that the time delay between circulating steroids in the plasma and their detection in the urine

samples is significantly shorter than between plasma and detection in faecal samples. It is thought that this is influenced by the intestinal passage of bile to the rectum (Palme et al., 1996). Although the pattern of hormone excretion generally follows the pattern of secretion (Carlstead et al., 1992; Lasley and Kirkpatrick, 1991; Wasser et al., 1997), intestinal reabsorption of bile and microbial action, timing of peristaltic contractions and amount and timing of food and water intake may affect the rate of hormone secretion (King and McLelland, 1975).

1.6 Faecal steroids

Conventionally endocrine data has been obtained through analysis of plasma samples. By definition, "a hormone is a chemical messenger that exerts its effect as a result of the circulatory system" (Hadley, 1996). For that reason, the measurement of circulating concentrations of hormones has been the preferred method for the evaluation of endocrine function. In reproductive endocrinology, the application of plasma hormone analysis has been favoured in a wide variety of situations over previously developed urinary metabolite analysis. Plasma analysis has been commonly used to monitor gonadal function, the onset of sexual maturity, reproductive dysfunction, fertility, characterise physiological events such as ovulation, produce seasonal hormone profiles, and to correlate hormone concentrations with behavioural patterns.

In recent years knowledge of the reproductive patterns of non-domesticated animals, especially those that are endangered, has become of increasing importance. Plasma analysis used for domestic animals is not ideal for many non-domesticated species. The risks involved with invasive procedures are considered high, even more so when serial samples are needed from a non-tractable species with limited numbers. Furthermore, capture and handling can cause stress, which can alter the production of hormones being monitored (Kirkpatrick *et al.*, 1979). Eliminating the risks of induced stress from invasive procedures led to the resurgence of monitoring hormone metabolites in urine and faeces. Urinary or faecal analyses offer several advantages when compared to plasma monitoring. Protocols involving infrequent sampling or sequential sampling over prolonged periods pose fewer risks to the subject and researcher when non-invasive sampling is adopted (Lasley and Kirkpatrick, 1991). Daily urinary and in

particularly faecal collections are easier than daily plasma collections, especially in non-tractable species. Concentrations of metabolites are often two to four times higher in faeces or urine than the circulating parent steroid (Peter et al., 1996). Finally, faecal and urinary hormone concentrations reflect the change of hormone production over time, rather than an instantaneous measurement in time, which is preferable in many investigations.

1.6.1 History and development of faecal steroid analysis

Faecal steroid analysis was originally developed for use in avian species by Czekala and Lasley in 1977, for determining gender in monomorphic bird species. Many captive breeding programmes were encountering problems identifying the sex of individuals, in order to pair birds for mating. Czekala and Lasley (1977) adapted human urinary analysis procedures used for pregnancy testing, for the measurement of total immunoreactive steroids in faecal samples from Indian jungle fowl (*Gallus gallus murghi*). The ratio of oestrogen to testosterone gave a reliable, non-invasive method for distinguishing gender in adult birds. This technique was then applied to birds from many orders, in particular Psittaciformes (Bercovitz *et al.*, 1978; Stavy *et al.*, 1979; Erb and Bercovitz, 1980). In addition to gender identification, oestrogen: testosterone ratios were used for assessing gonadal status in individual birds. Gonadal tissue and cloacal faecal samples were collected from a wide variety of species found dead within the San Diego zoo grounds. Indications of differences between birds with varying degrees of gonadal activity were found (Bercovitz *et al.*, 1978).

In the 1980's, immunoassays were developed that could accurately measure faecal and urinary steroid metabolites so excretory steroid analysis was applied to numerous mammalian species in zoos and in farm animals (reviewed in Lasley and Kirkpatrick, 1991, Schwarzenberger *et al.*, 1996). In the last 20 years the use of faecal and urinary steroid analysis has increased from the early use with mammalian species in zoos, to applications in a wide variety of mammalian and avian species, both captive and wild.

1.6.2 Applications of faecal steroid analysis

The use of faecal steroid analysis has been reported in well over 100 studies in the last 20 years and is accepted as a diagnostic tool in reproductive endocrinology. Research has been directed in four major areas; sex determination and gender differences, evaluation of breeding potential and reproductive activity, correlation of endocrine changes with behavioural changes, and glucocorticoid responses to stress.

1.6.2.1 Sex determination and gender differences

The use of faecal steroid analysis for sex determination has primarily been with avian species (Czekala and Lasley, 1977; Bercovitz *et al.*, 1978; Stavy *et al.*, 1979; Erb and Bercovitz, 1980; Tell and Lasley, 1991). This is probably because gender in many mammalian species can be identified from afar, by morphological or behavioural differences. However, its application with giant pandas (*Ailurpoda melenoleuca*) has emphasised its role when dealing with individuals from species where morphological differences are not apparent. Testosterone and oestradiol seasonal profiles were measured in faecal samples from an adult female and an adult male panda, as well as from two juvenile pandas. The range of concentrations (8-880 pg/g female, 105-8 910 pg/g male) and the difference in seasonal profiles of testosterone from the two adult pandas, suggested a possible sexing method. Concentrations of both hormones in the young pandas were relatively low and too variable to be used as a reliable sexing method (Kubokawa *et al.*, 1992).

1.6.2.2 Evaluation of breeding potential and reproductive activity

Urinary and faecal steroid analysis has most widely been used to monitor reproductive activity and evaluate breeding potential of many exotic and endangered mammalian species, both captive and wild. Examples of species in which this technique has allowed the detection of pregnancy, timing of ovulation, or seasonality includes African wild dog (*Lycaon pictus*, Monfort *et al.*, 1997), baboon (*Papio cynocephalus*, Stavisky *et al.*, 1995), domestic cattle (*Bos taurus*, Glatzel, 1999), cynomolgus monkey (*Macaca fascicularis*, Matsumuro *et al.*, 1999), giant panda (Kubokawa et al., 1992), Kaimanawa feral mare (*Equus caballus*, Linklater et al., 2000), maned wolf (*Chrysocyon brachyurus*, Wasser *et al.*, 1995), marmoset (*Callithrix jacchus*, Sousa and Ziegler,

1998), moose (*Alces alces*, Monfort *et al.*, 1993; Berger *et al.*, 1999), tufted capuchin monkey (*Cebus apella nigritus*, Lynch *et al.*, 2002), and various felids (Brown *et al.*, 1994).

Seasonal and developmental changes of reproductive function have also been studied in a variety of avian species. Valuable information on the timing of breeding cycles that has been used to aid conservation management programmes has been gained in several species. Seasonal changes in faecal oestradiol and testosterone in captive bald eagles (*Haliaeetus leucocephalus*) were used to synchronise individual male and female birds in an artificial insemination project (Bercovitz *et al.*, 1982). Breeding cycles of reproductive steroids have also been identified in free-living brown dippers (*Cinclus pallasii*, Kofuji *et al.*, 1993), greylag geese (*Anser anser*, Hirschenhauser *et al.*, 1999a), kakapo (*Strigops habroptilus*, Cockrem and Rounce, 1995), and rock ptarmigan (*Lagopus mutus*, Kikuchi *et al.*, 1996). In all cases, annual cycles of steroids that peak during the breeding season were identified. Faecal steroids have also been used to provide detailed information on the timing and onset of ovulation in female Japanese quail (*Coturnix coturnix japonica*, Yamaguchi and Ishii, 1991, in Ishii *et al.*, 1994) and canaries (*Serinus canaria*, Sockman and Schwabl, 1999).

1.6.2.3 Correlation of endocrine changes with behavioural changes

Changes in hormone concentrations are often associated with changes of behaviour. These are often linked to sexual behaviour and are associated with social dominance, mate selection or aggression. Relationships between steroid concentrations and behavioural indices have been well documented in relation to reproduction in mammals (Strier *et al.*, 1999; Lynch et al., 2002). In avian studies, faecal hormone concentrations are commonly related to behavioural changes associated with the breeding cycle (Bercovitz *et al.*, 1982; Kofuji *et al.*, 1993; Cockrem and Rounce, 1995; Kikuchi *et al.*, 1996; Hirschenhauser *et al.*, 1999a, 1999b; Sockman and Schwabl, 1999).

1.6.2.4 Glucocorticoid responses to stress

In addition to oestradiol, progesterone and testosterone, faecal steroid analysis can be applied to glucocorticoid (cortisol or corticosterone) measurement. Prolonged elevated

concentrations of cortisol and corticosterone can have inhibitory effects on reproduction, which is of concern in many endangered species. The effects of stress on cortisol concentrations caused by environmental conditions or husbandry practices has been investigated in various mammalian species including cheetah (*Acinonyx jubatus*, Jurke *et al.*, 1997) and other felids (Carlstead *et al.*, 1992; Graham and Brown, 1996); hares (*Lepus europaeus*, Teskey-Gerstl *et al.*, 2000), roe deer (*Capreolus capreolus*, Dehnhard *et al.*, 2001), Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*, Miller *et al.*, 1991) and various rodents (Harper and Austad, 2000).

Faecal corticosterone has been used to investigate relationships between the behavioural stages of the reproductive cycle in rock ptarmigan (Kikuchi *et al.*, 1996), as well as seasonal relationships between social stress and dominance in greylag geese (Kotrschal *et al.*, 1998). In both species, corticosterone concentrations were elevated in males during periods of male aggression in the mating season. Faecal corticosterone has also been used as a measure of environmental disturbance in the northern spotted owl (*Strix occidentalis caurina*, Wasser *et al.*, 1997).

1.6.3 Limitations of urinary and faecal steroid analysis

As with any procedure, there are disadvantages and problems with the application of urinary and faecal steroid analysis. Excretory hormone profiles differ between taxa and sometimes between related species. Bercovitz *et al.* (1978) illustrated the variation in faecal oestradiol (O)/testosterone (T) ratios between different species of Psittaciformes and between a variety of other unrelated species. An average O/T value of 1.30 for female Timneh Grey parrots (*Psittacus erithacus timneh*) compared to 1.25 for male Barraband's parrots (*Pionopsitta barrabandi*) demonstrates the importance of establishing profiles for each species, to avoid diagnostic mistakes through extrapolation based on other species' profiles.

Hormone metabolites are excreted in different forms and by different routes (O'Malley and Schrader, 1976). For example, geese excrete steroids predominantly in conjugated forms in contrast to chickens, which excrete free-steroids (Krawany, 1996 and Wallpach, 1998 in Hirschenhauser *et al.*, 1999a). Therefore, it is important to identify the steroid or steroid equivalent of interest to ensure appropriate extraction and assay

techniques are used. In mammals, it is also important to identify the route of excretion. Some hormones are excreted in high concentrations in faeces but low concentrations in urine (Peter et al., 1996). For example, in domestic cats (*Felis catus*), greater than 95% of metabolised oestradiol is excreted in faeces as opposed to urine (Shille *et al*, 1984 in Brown *et al*, 1994). In contrast, horses excrete only 2% of oestradiol in faeces (Schwarzenberger et al., 1996, 1997). Identification of the route of excretion is not required for avian studies, as urine and faeces are combined prior to excretion in the urodaeum.

Investigations into reproductive endocrinology that utilise faecal or urinary steroid analysis assume a positive relationship between plasma and faecal or urinary steroid concentrations. Validation of faecal and urinary steroid measurements is important to ensure that concentrations measured in faeces or urine reflect circulating plasma concentrations. This relationship is generally accepted for most mammalian species (Lasley and Kirkpatrick, 1991), including felids (Brown et al., 1994), primates (Shideler *et al.*, 1993; Brown et al., 1997) and ungulate species (Brown *et al.*, 1997). Urinary and faecal steroids have also been shown to reflect reproductive status in a variety of species as discussed previously. Similarly in birds, investigators have assumed a positive correlation between plasma and faecal steroid concentrations. Only three investigations have successfully validated faecal gonadal steroids by comparing plasma and faecal hormone concentrations (Bishop and Hall, 1991; Ishii *et al.*, 1994; and Cockrem and Rounce, 1994). Bishop and Hall compared plasma testosterone and progesterone in male and female Japanese quail to faecal testosterone and pregnanediol-3α-glucuronide, respectively. Faecal samples were pooled for individual birds over six hours and compared to individual plasma samples. Significant correlations of $r = 0.59$ and 0.79 were reported for testosterone and progesterone respectively. Ishii *et al.* reported a summary of results for oestradiol and testosterone validations in female and male Japanese quail. Increases in plasma steroid concentration were accompanied by increases in faecal steroid concentrations. Cockrem and Rounce compared faecal and plasma oestradiol and testosterone in domestic fowl. Both individual droppings and pooled three-hour samples were compared to individual plasma samples. Significant correlations of $r = 0.49$ and 0.59 for individual droppings were reported for testosterone and oestradiol respectively. A recent validation was attempted in domestic ganders (*Anser domesticus,* Hirschenhauser *et al.*, 2000). Plasma and faecal concentrations of

testosterone and its metabolites were compared across seasons and in response to a GnRH challenge. Plasma testosterone increased in response to GnRH and this was followed by an increase in faecal testosterone metabolites. However, high variation in faecal testosterone metabolite concentrations resulted in an unsuccessful validation.

Because there is a lag-time between observing a change in plasma concentration to observing the same faecal change, isolating certain physiological events can be difficult. Using radiolabelling techniques, this lag-time can be determined. In birds, there is evidence to support a more rapid clearance rate than in mammals. In the cockatiel (*Nymphicus hollandicus*), radio labelled oestradiol and testosterone were first detected in faecal samples within a few hours (≤4 hours), which was also the time of peak excretion (Tell, 1997). In free-ranging African elephants, the lag time from injection to peak excretion for ^{14}Cprogesterone was 48 hours (Wasser *et al.*, 1996). Similar lag times have been reported in several primate species (Wasser *et al.*, 1993). Correlations between urinary or faecal concentrations of hormone metabolites and the circulating parent steroid are often improved when daily hormone values are adjusted to account for this delay (Monfort *et al.*, 1987).

Dietary fibre has been shown to influence faecal steroid measurements. Wasser *et al.* (1993) report a negative effect of increased dietary fibre on progesterone excretion in female baboons. Expressing faecal steroids as g^{-1} dry faeces rather than g^{-1} of equivalent wet faeces appears to be sufficient to control for most dietary effects when monitoring longitudinal endocrine status. The effects of faecal lipid content has not been widely studied, although there is evidence that high lipid content can interfere with the extraction process and cause low extraction efficiencies in faecal samples from rock ptarmigan (*Lagopus mutus*; M. Kikuchi, pers. comm.). Therefore, extraction processes that eliminate lipid from the sample may be beneficial. The effects of foreign substances such as phytoestrogens present in the environment on faecal or urinary steroid concentrations has also had limited investigation. A recent study of phytoestrogens present in supplementary food and natural food sources of kakapo identified only very low levels of oestrogenic activity (Fidler *et al.*, 2000). The interference that these phytoestrogens may cause with measuring faecal oestradiol in kakapo is probably low and the use of specific antibodies can minimise any cross-reactivity.

Urine samples can be difficult to collect from free-ranging animals, so faecal sampling is often preferred. When monitoring hormone profiles in known individuals, identification of faecal deposits can be challenging. Direct observation is the most common method employed, although techniques involving marking the faeces by way of dyes via food (H. Hesterman, pers. comm.) or the systemic injection of short-lasting radio labelled tracers (Pelton and Marcum, 1977, in Bishop and Hall, 1991) have been used. Any sample used must be representative of the entire waste deposit. This is achieved by thorough mixing of faecal samples, but urine samples are more complicated as they must be collected mid-flow.

Synchronisation of collection time with seasonal patterns, photoperiodic patterns and reproductive status is important when monitoring certain reproductive events. For example, sexing of birds needs to be done during the breeding season, when gonadal activity and hormone concentrations are at their peak.

The use of urinary and faecal steroid analysis is becoming an increasingly popular tool for monitoring reproductive status in endangered species. The number of individuals from which to sample is often limited to only a few, or in some cases single animals. An investigation into using faecal steroids for sex-determination and monitoring reproductive activity in giant pandas only had one adult male, one adult female and two infants to sample (Kubokawa *et al.*, 1992). Similarly, an investigation into breeding cycles of brown dippers (*Cinclus pallasii*) had only three female and two male birds to sample (Kofuji *et al.*, 1993). In such situations, it is important to interpret results carefully, especially when new techniques are being developed.

1.7 Thesis outline

In this study, a non-invasive method for measuring reproductive steroids was applied to a free-living species, the kakapo. Japanese quail were used to develop and validate a faecal extraction method for measuring oestradiol, progesterone and testosterone. Japanese quail were used as they are an easily maintained domestic species, which have been extensively studied. The relationships between faecal and plasma steroid concentrations and gonadal development in quail were examined, as well as the value of

individual and pooled sample collections. Finally, the extraction method was applied to kakapo faecal samples and the annual hormone cycles in relation to breeding status were described.

Female Japanese quail, photo: W.H. Chua.

2 Development of methods to measure faecal steroids in Japanese quail

2.1 Introduction

Studies of reproductive endocrinology in free-living birds can be difficult because the birds must be captured for blood samples to be collected. The breeding season is the most important period for sample collection, but this is also the period when capture is most likely to disrupt events of the annual reproductive cycle. The problems of sample collection from free living birds can be even greater for endangered birds as conservation managers wish to minimise disturbances during the breeding season.

An alternative approach to the measurement of steroid hormone concentrations in free-living birds is to use faecal rather than plasma samples (Cockrem and Rounce, 1995). Studies in which faecal steroid measurements are made presume that the faecal concentrations are reliable indicators of plasma steroid concentrations. The validation of faecal steroid hormone measurements against the parent steroid in plasma has been shown in a number of mammalian species (Schwarzenberger *et al.*, 1996, reviewed in Brown *et al.*, 1997). Although faecal gonadal steroids have been investigated in a variety of avian species, only three investigations have successfully validated faecal gonadal steroids (Bishop and Hall, 1991, Japanese quail, *Coturnix coturnix japonica*; Ishii *et al.*, 1994, Japanese quail; Cockrem and Rounce, 1994, domestic fowl, *Gallus domesticus*). In all three studies, an increase in plasma steroid concentration was followed by an increase in faecal steroid concentration. In most cases where avian faecal gonadal steroids have been investigated, individual faecal samples have been collected as opposed to multiple faecal samples which are pooled (for example, individual samples: Bercovitz *et al.*, 1982; Kofuji *et al.*, 1993; Cockrem and Rounce, 1994; Ishii *et al.*, 1994; Cockrem and Rounce, 1995; Kikuchi *et al.*, 1996; Hirschenhauser *et al.*, 1999a, 1999b; Sockman and Schwabl, 1999; Hirschenhauser *et al.*, 2000; Kotrschal *et al.*, 2000; pooled samples: Bishop and Hall, 1991; Tell, 1997). Two of the three successful validations were conducted on individual faecal samples

(Ishii *et al.*, 1994, and Cockrem and Rounce, 1994). When a mean faecal hormone concentration was used instead of an individual value, the relationship between plasma and faecal steroid did not improve (Cockrem and Rounce, 1994). An attempt at validation with individual samples from domestic ganders (*Anser domesticus*) was unsuccessful (Hirschenhauser *et al.*, 2000).

An alternative method of validation has been demonstrated in the cockatiel (*Nymphicus hollandicus*, Tell 1997). Radiolabeled oestradiol and testosterone were injected intramuscularly and within 24 hours, 79% of the original radiolabel was measured in faecal samples. This indicates that daily changes in the production of oestradiol and testosterone can be detected in faecal samples. Cockrem and Rounce (1995) compared faecal oestradiol and testosterone measurements from kakapo (*Strigops habroptilus*) to data from other species as a form of validation. Sockman and Schwabl (1999) investigated relationships between the onset of incubation and the regulation of clutch size by measuring faecal oestradiol and progesterone in laying canaries (*Serinus canaria*). Validation was offered in the form of a comparison of the trends in faecal concentrations to those from a study in which validation against the parent steroid in plasma was carried out. Most of the studies have made comparisons between faecal hormone concentrations and behavioural observations in relation to the breeding cycle. Changes in faecal hormone concentrations that correspond to stages of the breeding cycle validate, to a limited extent, faecal steroid measurement techniques. These methods of validation are not as robust as a direct comparison between plasma and faecal steroid measurements. The measurement of faecal steroid concentrations in free-living kakapo was planned, but the collection of plasma samples from kakapo for validation of the faecal steroid method was not possible. An alternative species, the Japanese quail was used to determine the reliability of faecal measurements as indicators of plasma steroid concentrations in birds.

A wide variety of extraction methods have been used in other studies and improved extraction methods are being developed to maximise extraction efficiency and minimise effort. Three types of extraction method have been used for faecal steroid analysis. Early studies of androgens and oestrogens in avian species used variations of a phosphate-buffer extraction followed by enzymatic hydrolysis to de-conjugate the

steroids (Czekala and Lasley, 1977; Bercovitz *et al.*, 1978; Stavy *et al.*, 1979; Erb and Bercovitz, 1980; Bercovitz *et al.*, 1982). More recently, variations on the phosphate-buffer extraction method without enzyme hydrolysis have been successfully used in a variety of avian species (Japanese quail, Bishop and Hall, 1991; cockatiel, Tell and Lasley, 1991; domestic fowl, Cockrem and Rounce, 1994; kakapo, Cockrem and Rounce, 1995; white-crowned sparrow, *Zonotrichia leucophrys oriantha*, Lee *et al.*, 1995). The third method involves the extraction of faecal samples by ethanol or methanol with or without phosphate buffer, and sometimes includes an additional fat extraction by dichloromethane. The addition of ethanol prevents storage-dependent changes in steroid content in the faeces (Peter *et al.*, 1996). Variations of this method are most commonly used with mammalian species (for example, Monfort *et al.*, 1993, 1997; Brown *et al.*, 1994; Wasser *et al.*, 1994, 1995; Stavisky *et al.*, 1995). The phosphate-buffer extraction method without enzyme hydrolysis has also been developed for use in cynomolgus monkeys (*Macaca fascicularis*) in an attempt to eliminate the use of organic solvents and develop simple field assays (Shideler *et al.*, 1993). Both radioimmunoassay (RIA) and enzymeimmunoassay (EIA) techniques are used. Although RIA has higher specificity and repeatability, EIA offers a faster, easier, more economical method for measuring hormone concentrations (Meyer, 1992; Tell, 1996).

In this study testosterone, oestradiol and progesterone were measured in quail faecal samples extracted by an ethanol/buffer method and the results compared with plasma measurements of the hormones. The relationship between faecal and plasma steroid concentration is best examined over a wide range of concentrations. A clear relationship between plasma steroid hormone concentrations and gonadal development has been established in many species of bird, including Japanese quail (Follett and Maung, 1978; Follett and Robinson, 1980; Wingfield *et al.*, 1987). A range of steroid concentrations is therefore likely to be obtained by sampling birds at various stages of gonadal development. It is also well established that exposure to long day lengths stimulates the hypothalamo-pituitary-gonadal axis, increasing gonadotropin secretion (follicle-stimulating hormone, luteinising hormone) and gonadal growth in Japanese quail (Follett and Sharp, 1969; Follett and Robinson, 1980).

The Japanese quail is a relative photorefractory species in which decreases in day length are needed to suppress gonadotropin secretion and terminate reproduction (Robinson and Follett, 1982). In contrast, absolute photorefractory species experience a decline in gonadotropin secretion and undergo gonadal regression while day lengths are still long (e.g. European starling, *Sturnus vulgaris,* Nicholls *et al.* 1988). For this reason, the Japanese quail is an ideal species to use for photoperiodic manipulation of reproductive steroid concentrations and gonadal development.

The aims of this study were to:

- Adapt the faecal extraction method currently used in the laboratory, for use with avian faecal samples.

- Validate this faecal extraction method in Japanese quail for three reproductive steroids (testosterone, oestradiol and progesterone).

- Determine if the relationships between steroid concentration and gonad size are similar for plasma and faecal samples.

- Determine the reliability of individual faecal samples for the assessment of plasma hormone concentrations.

2.2 Methods

2.2.1 Birds

Male and female Japanese quail were purchased from Rangitikei Game Birds Ltd (Bulls, Manawatu, New Zealand). Birds were housed individually in custom-made cages, in photoperiod-controlled rooms in the Veterinary Science building at Massey University, Palmerston North, New Zealand. Food (quail layer mash, Feed Processing Unit, Massey University) and water were supplied *ad libitum*.

2.2.2 Experimental design

Birds were purchased at three or seven weeks of age and divided into groups of ten birds. One group of males and one group of females purchased at three weeks of age were held under a short day photoperiod (8h light: 16h dark; lights on from 0800 - 1600). The remaining two groups, one of females and one of males, were purchased at seven weeks of age and held under a long day photoperiod (16h light: 8h dark; lights on from 0800 – 0000). All birds were held at an ambient temperature of 20 - 26°C until they reached 11 weeks of age to allow time for gonadal regression or development to occur in response to photoperiod. Individual faecal samples were collected over a three-hour period, and a single plasma sample was collected at the end of the three hours. The birds were killed and the reproductive system dissected on the day of faecal sample collection (short day birds) or the following day (long day birds). This study was approved by the Massey University Animal Ethics Committee.

2.2.3 Sample collection

2.2.3.1 Faeces

Droppings produced by each bird between 0900 and 1200 were individually collected and the time of production recorded. Droppings were collected from a plastic sheet placed on top of an aluminium tray under each cage. Any droppings that did not fall through the bottom of the cage were pushed through. A dropping consistency grading

scale[#] (Table 2.1) was used to grade each dropping. Droppings of grade 5 were not collected. Droppings were collected into pre-weighed 5ml polypropylene screw top vials, re-weighed and kept on ice until the end of the three-hour collection period. Samples were then frozen at -20°C until extraction. Wet weight was calculated and recorded for each sample.

Table 2.1. Dropping consistency grading scale (taken from Littin, 1998).

Grade	Consistency
1	Solid
2	Soft
3	Loose
4	Liquid incorporating some solid particles (liquid and solids)
5	Liquid

2.2.3.2 Plasma

Blood samples were collected immediately after the three-hour faecal collection period. Birds held on short days were sampled by puncture of the brachial vein and blood was collected into heparinised capillary tubes. All samples were collected within three minutes from the time the bird was removed from its cage. Birds held on long days were decapitated (after stunning) and blood collected into heparinised centrifuge tubes. Blood was expelled from the capillary tubes into heparinised 1 ml polystyrene test tubes (BDH) and kept on ice for up to 1.5 hours until centrifugation. Samples were centrifuged at 1 900g for 15 minutes (Beckman GS-6R refrigerated centrifuge). Plasma was removed with a glass Hamilton syringe and stored at –70°C in 1 ml polypropylene titre tubes until assay.

[#] This scale was developed by Littin (1998) and used to identify droppings of a certain consistency that were not suitable for steroid measurement.

2.2.3.3 Reproductive system

All birds were killed by stunning and decapitation within a day of completion of the experiment. Gonadal tissue was removed and various measurements were recorded. In males individual testis weight was recorded and in females the oviduct and ovary weight, and diameter of the five largest ovarian follicles was recorded. Tissue was then stored in formol saline for future reference.

Egg production in females and the presence of cloacal foam under the cages of males was recorded daily from arrival.

2.2.4 Steroid extraction from plasma

Plasma samples were thawed and centrifuged at 14 000 g for five minutes (IEC Micromax ventilated microcentrifuge OM3590) to separate lipid from the plasma. Testosterone, oestradiol and progesterone were extracted separately from each sample. Table 2.2 below summarises the volumes and solvents used for each hormone. Clear plasma was removed from below the lipid layer with a glass Hamilton syringe and transferred to a glass screw-top test tube (13 mm x 100 mm) and the appropriate volume of solvent (Analar, BDH, Poole, England) added using a Merck bottle-top dispenser. Plasma and solvent were vortexed for 10 seconds, shaken for one hour on an orbital shaker, then centrifuged at 2 300 g (Haraeus Christ 50002 refrigerated centrifuge) for 15 minutes to separate the organic and aqueous phases. An aliquot of the organic solvent phase was removed into an open-top glass test tube (Kimax, 13 mm x 100 mm). The solvent was completely evaporated by placing test tubes in a heating block at 37°C under a stream of air. Extracts were left to cool and reconstituted in an aliquot of (PBSG), vortexed for 30 seconds five times at five minute intervals, shaken for one hour on an orbital shaker and refrigerated overnight at 4°C. The following day, extracts were vortexed for 30 seconds then transferred into a 1.0 ml polypropylene titre tube and stored at –20°C until assay.

Extraction efficiencies were obtained for testosterone, oestradiol and progesterone by adding 100 µl (~ 5 000 cpm; 20 µl, ~ 500 cpm for progesterone) of the respective

tritiated hormone to samples at the start of extraction. Tritiated hormone was also added to four scintillation vials (polypropylene, 5 ml) for total counts. After extraction, a 100 µl (80 µl for progesterone) aliquot of the reconstituted extract containing tritiated hormone was transferred into a 5 ml polypropylene scintillation vial. 3 ml of scintillant (5 g/l PPO (2,5-diphenyl-oxazole, Sigma), 0.3 g/l dimethyl POPOP (1,4-bis-[4-methyl-5-phenyl-2-oxazoly]-benzene, Sigma) in toluene (Mobil)) was added, vials capped and shaken for one hour on an orbital shaker. Samples were left at room temperature for one hour, before being counted for five minutes in a Wallac 1409-411 liquid scintillation counter.

Extraction efficiencies were determined for testosterone and oestradiol from 15 samples from quail plasma pools. The mean efficiencies were 90.8 ± 3.6% for oestradiol and 96.6 ± 4.0% for testosterone. Mean extraction efficiencies were used for the calculation of oestradiol and testosterone concentrations in individual samples. Progesterone extraction efficiencies were determined for each sample due to high variation between samples (mean efficiency 73.9 ± 32.8%).

Table 2.2. Volumes and solvents used for testosterone, oestradiol and progesterone extraction from quail plasma samples.

Hormone	Plasma volume (µl)	Solvent	Solvent added (µl)	Solvent removed (µl)	PBSG added (µl)
Testosterone	20	Dichloromethane	1 000	800	450
Oestradiol	200	Dichloromethane	2 000	1 600	240
Progesterone	60	N-Hexane	1 000	800	300

2.2.5 Steroid extraction from faecal samples

The original faecal extraction method that was being used in the Institute of Veterinary and Biomedical Science (IVABS) radioimmunoassay laboratory for mammalian faecal samples was a modified method of Wasser *et al.* (1994). A trial was conducted to refine this extraction method for avian faecal samples. A method was selected from this trial and used for extracting samples in this study. Further development of the faecal extraction method was required to optimise the extraction efficiencies and to make the method more practical for processing large numbers of samples. Trials were conducted and an improved method was selected for use in subsequent studies.

2.2.5.1 Original extraction method

The modified method of Wasser *et al.* (1994) that was being used in the IVABS radioimmunoassay laboratory for mammalian faecal samples was as follows:

Frozen faecal samples were lyophilised (Cuddon 0610 Freeze Drier) for 70 hours. Samples were crushed to an homogenous texture with a spatula. 0.1 g of dried sample was added to 5.0 ml of 90% absolute ethanol (AnalaR BDH, Poole, England) in a glass screw-top tube (13 x 100 mm). The level to which the liquid came was then marked. Tubes were vortexed for 30 seconds and the suspension boiled for 20 minutes in a boiling bath heated to 80°C. The level of ethanol was checked and extra ethanol was added during the boiling process to replace losses due to evaporation. Tubes were then removed and refilled to the 5.0 ml level before being capped and centrifuged at 1 000 g (Haraeus Christ 5000S refrigerated centrifuge) for 20 minutes at room temperature. The supernatant was pipetted into a second glass screw-top test tube. The pellet was rinsed with a further 1.25 ml of 90% ethanol, vortexed for 30 seconds and re-centrifuged at 1 000 g for 20 minutes at room temperature. The supernatant was removed and added to the previous aliquot. The faecal pellet was discarded. The ethanol was completely evaporated from the supernatant by placing test tubes in a heating block at 37°C under a stream of air. The sides of the tubes were rinsed with 90% ethanol twice during this process to ensure that the final level of extract residue was below the level of 1 ml. Extracts were left to cool and reconstituted in 1 ml of

PBSG (0.1M, pH 7.0, 0.1% gelatin), gently vortexed for 30 seconds and refrigerated overnight at 4°C. The following day, tubes were vortexed for 30 seconds and extracts were removed into 1.5 ml polypropylene eppendorf tubes before centrifugation for 10 minutes at 14 000 g (IEC Micromax ventilated microcentrifuge). The supernatant was transferred into a 1.0 ml polypropylene titre tube and stored at –20°C until assay.

2.2.5.2 Refinement of original extraction method

A trial with five treatments, each with ten faecal samples (four from female quail, six from male quail), was conducted to determine how best to adapt the extraction method for avian faecal samples.

The quail faecal samples were often smaller than 0.1 g, so the first treatment was 0.05 g of dried dropping added to 2.5 ml of 90% ethanol. The second treatment was the original method with the addition of a 3 mm glass boiling ball to the faecal sample – ethanol mixture before boiling. The third treatment was the same as the second treatment, except the dried extract was reconstituted overnight in only 0.8 ml of PBSG. The following day, tubes were vortexed for 30 seconds and the extracts were removed into 1.5 ml polypropylene eppendorf tubes. The extraction tube was rinsed with a further 0.2 ml of PBSG, vortexed and the extract added to the previous aliquot. The fourth treatment was the same as the second treatment, with the addition of a 30 second vortex after 10 minutes of boiling. The fifth treatment was the same as treatment two, with an additional 1-hour of shaking on an orbital shaker (Chiltern Scientific SS70), on the second day of the process, before reconstituted extracts were removed into eppendorf tubes.

Extraction efficiencies were obtained for oestradiol by adding 100 µl (~ 5 000 cpm) of tritiated hormone (^{3}H-oestradiol, TRK.332 Amersham, UK) to samples before boiling. Tritiated hormone was also added to four scintillation vials (polypropylene, 5 mL) for total counts. After extraction a 100 µl aliquot of the reconstituted extract was used to determine the extraction efficiency (as described in section 2.2.4 for plasma).

2.2.5.2.1 Results

Mean quail faecal oestradiol extraction efficiencies were high (≥ 69.90 %) for all five extraction methods (Table 2.3). Treatment three (original extraction method with the addition of a boiling stone and a 800 ul reconstitution/200 ul rinse) had the highest mean oestradiol extraction efficiency (One-way ANOVA, with contrasts, $F = 16.84$, $p = 0.001$). There were no significant differences between the remaining treatments. Treatment four involved the original extraction method with the addition of a boiling ball and an additional vortex after ten minutes of boiling. This method was discounted because of safety issues. Upon replacement in the water bath following the mid-boil vortex, ethanol from several samples exploded out the top of the test tubes. The increased time required to achieve the mean difference of 8.52% between treatment two and treatment three (the treatment with the next highest mean extraction efficiency) was considered unnecessary (treatment three required an extra 12 hours). From the remaining treatments, the faecal extraction method with the smallest coefficient of variation (CV %) was chosen as the preferred method. Treatment two (original method with the addition of a boiling ball) was selected, and details of the method are described in section 2.2.5.3 below.

No difference in mean oestradiol extraction efficiency was found between male and female samples, except in treatment one, where male samples had significantly higher extraction efficiencies than female samples (Independent samples T Test, $t = -4.917$, $df = 8$, $p = 0.001$; Table 2.4). Therefore, overall mean extraction efficiencies were calculated and applied to both male and female samples for testosterone and oestradiol in section 2.2.5.3.

Table 2.3. Mean quail faecal oestradiol extraction efficiencies (%) for five different extraction methods (n=10).

Treatment	**1**	**2**	**3**	**4**	**5**
Mean	69.90	71.99	80.51	73.68	71.07
SE	2.73	1.88	1.88	1.14	2.36
% CV	12.35	8.28	7.39	4.91	10.48

Table 2.4. Mean faecal oestradiol extraction efficiencies (%) for male and female quail for five different extraction methods.

Treatment	1	2	3	4	5
Male: mean	75.70	71.57	79.28	72.43	72.31
SE	1.92	2.60	2.25	0.66	2.48
%CV	6.20	8.90	6.96	2.23	8.42
Female: mean	61.21	72.60	82.36	75.56	69.19
SE	2.17	3.09	3.45	2.60	4.92
%CV	7.08	8.51	8.38	6.88	14.23

2.2.5.3 Faecal extraction method

The extraction method developed in section 2.2.5.2 and used for the extraction of avian faecal samples in this chapter was as follows. Frozen faecal samples were lyophilised (Cuddon 0610 Freeze Drier) for 70 hours. Samples were crushed to an homogenous texture with a spatula. 0.05 g of dried sample was added to 2.5 ml of 90% absolute ethanol (AnalaR BDH, Poole, England) with a glass boiling ball (3 mm diameter) in a glass screw-top tube (13 x 100 mm). Samples weighing under 0.05 g were not used for individual hormone measurement, but were used for determining extraction efficiencies (see below). The level to which the liquid came was then marked on each tube. Tubes were vortexed for 30 seconds and the suspension boiled for 20 minutes in a boiling bath heated to 80°C. The level of ethanol was checked and extra ethanol was added during the boiling process to replace losses due to evaporation. Tubes were then removed and refilled to the 2.5 ml level before being capped and centrifuged at 1 000 g (Haraeus Christ 5000S refrigerated centrifuge) for 20 minutes at room temperature. The supernatant was pipetted into a second glass screw-top test tube. The pellet was rinsed with a further 1.25 ml of 90% ethanol, vortexed for 30 seconds and re-centrifuged at 1 000 g for 20 minutes at room temperature. The supernatant was removed and added to the previous aliquot. The faecal pellet was discarded. The ethanol was completely evaporated from the supernatant by placing test tubes in a heating block at 37°C, under a stream of air. The sides of the tubes were rinsed with 90% ethanol twice during this process to ensure that the final level of extract residue was below the level of 1 ml.

Extracts were left to cool and reconstituted in 1 ml of PBSG (0.1M, pH 7.0, 0.1% gelatin), gently vortexed for 30 seconds and refrigerated overnight at 4°C. The following day, tubes were vortexed for 30 seconds, extracts removed into 1.5 ml polypropylene eppendorf tubes and centrifuged for 10 minutes at 14 000 g (IEC Micromax ventilated microcentrifuge). The supernatant was transferred into a 1.0 ml polypropylene titre tube and stored at –20°C until assay.

Extraction efficiencies were measured for testosterone, oestradiol and progesterone by adding 100 µl (~ 5 000 cpm) of the respective tritiated hormone (^{3}H-testosterone, TRK.406 Amersham, UK; ^{3}H-oestradiol, TRK.332 Amersham, UK; ^{3}H-progesterone, TRK.413, Amersham, UK) to samples before boiling. Tritiated hormone was also added to four scintillation vials (polypropylene, 5 ml) for total counts. After extraction, a 100 µl aliquot of the reconstituted extract containing tritiated hormone was transferred into a 5 ml polypropylene scintillation vial. 3 ml of scintillant (5 g/l PPO (2,5-diphenyl-oxazole, Sigma), 0.3 g/l dimethyl POPOP (1,4-bis-[4-methyl-5-phenyl-2-oxazoly]-benzene, Sigma) in toluene (Mobil)) was added, vials capped and shaken for one hour on an orbital shaker. Samples were left at room temperature for one hour, before being counted for five minutes in a Wallac 1409-411 liquid scintillation counter.

Extraction efficiencies were determined for testosterone and oestradiol from 20 samples each of which was created by pooling droppings weighing under 0.05 g. The mean efficiencies were 72.8 ± 1.5% for oestradiol and 77.3 ± 0.9% for testosterone. Progesterone extraction efficiencies were determined for each sample due to high variation between samples (mean efficiency 62.4 ± 14.1%).

Each batch of samples extracted also included quality control samples. Two screw-top test tubes containing only ethanol and tritiated hormone (no dried dropping) were 'extracted' in exactly the same manner as above. A 100 µl aliquot of the final supernatant was used to determine a total count for the extraction.

2.2.5.4 Further development of faecal preparation methods

Further development of the faecal extraction method was required to optimise the extraction efficiency and to make the method practical for processing the large numbers of samples in future studies.

2.2.5.4.1 Grinding methods

Crushing large faecal samples with a spatula did not always produce an homogenous texture and was time consuming. Five quail faecal samples (consisting of 5-10 individual droppings combined) were randomly collected and processed by one of four methods; spatula, coffee grinder (Russell Hobbs 9702), hand-held blender (Cascade, FP332) or a centrifugal grinder (RETCH 2M100; 0.25 sieve). Each method was scored on a scale from 1 – 5 (bad – excellent) based on the homogeneity of the sample, minimal wastage, time efficiency and ease of cleaning.

Overall, the centrifugal grinder scored the highest (Table 2.5). Samples were ground to an homogenous texture, with reasonable recovery of material, time efficiency and ease of cleaning. The centrifugal grinder was selected as the most efficient instrument for following experiments.

Table 2.5. Scores for grinding capabilities for various instruments (scored 1 – 5, bad – excellent)

Instrument	Consistency	Low wastage	Minimal time	Easy cleaning	Total
Spatula	1	5	1	5	12
Coffee grinder (Russell Hobbs 9702)	5	3	3	2	13
Hand held mixer (Cascade, FP332)	2	2	2	2	8
Centrifugal grinder (RETCH 2M100)	5	3	4	3	15

2.2.5.4.2 Extraction methods

Two series of extraction methods were trialed, with four methods in each series. The results from the first series were used to design the second series of trials. Each method used the same six quail faecal samples (three with expected high hormone concentrations, three with expected low hormone concentrations) except treatment one and two, which had 14 samples (seven with expected high hormone concentrations, seven with expected low hormone concentrations). Each sample consisted of the combination of all droppings produced by an individual bird over 24 hours. Extraction efficiencies were determined for testosterone, oestradiol and progesterone for each sample separately, as described in section 2.2.5.3.

All treatments followed the method as described in section 2.2.5.3, with the following modifications:

Series 1:

Method 1: 0.1 g dried, ground sample added to 5 ml ethanol in 13 x 100 mm glass screw-top test tubes.

Method 2: Same as method 1, except tritiated hormone for extraction efficiency was added to dried sample before ethanol was added.

Method 3: Samples were ground and larger glass screw-top test tubes were used (13 x 120 mm)

Method 4: 0.05 g of dried, ground sample was added to 2.5 ml ethanol in 13 x 100 mm glass screw-top test tubes. Instead of boiling, tubes were capped and shaken on their sides for 1 hour on an orbital shaker. Samples were centrifuged and supernatant was removed, dried and reconstituted as in section 2.2.5.3, except the 1.25 ml ethanol rinse was omitted.

Series 2:

Method 5: Same as method 4, with a 1.25 ml ethanol rinse after the removal of the first supernatant following shaking.

Method 6: Same as method 5, and samples were re-shaken on their sides for 1 hour on an orbital shaker after the 1.25 ml ethanol rinse.

Method 7: Same as method 5, except dried extract reconstituted in 0.8 ml of PBSG overnight at 4°C. The following day, tubes were vortexed for 30 seconds and the extracts were removed into 1.5 ml polypropylene eppendorf tubes. The extraction tube was rinsed with a further 0.2 ml of PBSG, vortexed and the extract added to the previous aliquot.

Method 8: Same as method 5, except the extracts were dried to a powdery crust before reconstitution.

2.2.5.4.2.1 Results

Extraction efficiencies were higher for oestradiol and testosterone when smaller volumes of dried dropping and ethanol were used (methods three and four were greater than one and two, One-way ANOVA, with contrasts, $F_{3, 36} = 9.95$, $p = 0.003$, oestradiol; $F_{3, 36} = 59.24$, $p = 0.001$, testosterone; Table 2.6). In the case of oestradiol, an additional comparison was made between smaller samples from the earlier method (2.2.5.3), which were crushed with a spatula and larger samples which were ground. With smaller, crushed samples the mean oestradiol extraction efficiency was 71.99 ± 1.88% (Table 2.3), which was significantly higher than the mean of 47.63 ± 2.51% for oestradiol in treatment 1 (Table 2.6; Independent samples T Test, $t = -7.271$, $df = 22$, $p = 0.001$). The difference between extraction efficiencies was possibly due to either a difference in texture of the sample or the fact that the samples in the trials were different. However, crushing larger samples with a spatula was impractical, so the

possibility that crushed samples yield higher extraction efficiencies was not investigated further. Of the two methods where smaller amounts were used (method three and four), the method where samples were shaken instead of boiled was developed further, due to its less hazardous and less labour intensive nature.

Method 7 (0.05 g sample, 2.5 ml ethanol, shaken, 1.25 ml ethanol rinse, 800 µl PBSG reconstitution, 200 µl rinse) gave a significantly higher mean extraction efficiency for testosterone (One-way ANOVA, with contrasts, $F_{4, 25} = 9.21$, $p = 0.006$). Although this difference was not evident for oestradiol or progesterone, the method from treatment 7 was selected as the most efficient method for extraction of quail faeces for all three hormones in further studies. This method is described in detail in section 3.2.5.

Because extraction efficiency differed between samples of expected high and low steroid concentrations it is suggested that mean extraction efficiency values be calculated for samples grouped by expected hormone concentration. For example, where samples are obtained from four groups of birds (female and male birds held on long days and female and male birds held on short days) four separate mean extraction efficiency values should be calculated. Within treatment 7, the coefficients of variation (CV) for expected high and low oestradiol and testosterone samples were below 10% (Table 2.6). Therefore, in future studies a mean value calculated for each group is acceptable. However, the CV for samples with expected high progesterone concentrations was 18.61%. Extraction efficiency for individual samples should be measured for progesterone.

Table 2.6. Mean quail faecal steroid extraction efficiencies (%) for eight different extraction methods (method 1 & 2, n =14; method 3 – 8, n = 6). Low sample means calculated from samples expected to have low hormone concentrations, high sample means calculated from samples expected to have high hormone concentrations.

Method	1	2	3	4	5	6	7	8
Testosterone								
Mean	48.49	52.67	62.72	65.44	70.49	65.16	74.17	68.61
SE	0.98	1.86	0.77	2.10	1.57	2.53	2.05	0.68
% CV	7.56	12.73	3.01	7.86	5.44	9.52	6.78	2.44
Low sample mean	46.47	49.51	63.89	63.58	69.29	60.75	75.56	67.26
SE	1.03	2.36	1.25	4.11	1.37	3.51	4.28	0.43
% CV	5.88	11.66	3.39	11.19	3.43	10.02	9.80	1.11
High sample mean	50.50	55.38	61.55	67.29	71.69	69.57	72.78	69.96
SE	1.32	2.49	0.22	1.32	2.99	0.55	0.93	0.57
% CV	6.90	11.92	0.61	3.41	7.22	1.36	2.22	1.41

Method	1	2	3	4	5	6	7	8
Oestradiol								
Mean	47.63	53.43	61.87	65.08	61.26	57.56	67.32	55.33
SE	2.51	3.71	5.56	4.25	5.43	4.63	4.99	3.59
% CV	19.71	26.01	22.01	16.01	21.72	19.71	18.16	15.91
Low sample mean	55.52	64.96	73.67	56.47	72.43	67.16	56.85	62.57
SE	1.60	1.53	3.80	2.42	2.28	1.32	3.22	2.70
% CV	7.62	6.24	8.93	7.42	5.45	3.41	9.80	7.49
High sample mean	39.75	41.89	50.07	73.70	50.09	47.96	77.79	48.08
SE	1.99	3.61	0.93	3.21	4.19	3.66	2.14	2.18
% CV	13.28	22.83	3.21	7.55	14.48	13.21	4.77	7.86

Method	1	2	3	4	5	6	7	8
Progesterone								
Mean	35.67	43.82	48.94	49.89	53.21	60.09	65.23	64.12
SE	2.45	4.59	6.41	4.83	6.31	7.71	9.39	7.32
% CV	25.69	39.16	32.07	23.71	29.07	31.41	35.25	27.95
Low sample mean	43.66	58.57	62.42	40.67	39.58	76.65	85.64	79.56
SE	1.67	2.36	4.64	5.35	0.63	3.57	0.76	1.08
% CV	10.12	10.67	12.88	22.77	2.75	8.07	1.54	2.35
High sample mean	27.67	29.08	35.46	59.12	66.84	43.52	44.81	48.68
SE	1.37	3.62	1.45	1.69	3.63	3.12	4.81	5.30
% CV	13.10	32.96	7.10	4.97	9.39	12.41	18.61	18.85

2.2.6 Radioimmunoassay of testosterone

Testosterone concentrations in faecal and plasma extracts were measured by radioimmunoassay (RIA). Samples were thawed and assayed in duplicate. Dilutions of faecal extracts (1:21) were prepared in PBSG. 100 µl of antibody (T3-125, Endocrine Sciences, USA) and 100 µl of tritiated testosterone (~5 000cpm) was added to 100 µl of plasma or diluted faecal extract and standards (9.8 – 5 000 pg/ml) and incubated at 4°C overnight. Bound and free steroid was separated by adding 500 µl of dextran-coated charcoal (2.5 g/l charcoal (Sigma), 0.25 g/l dextran (Dextran T70 Amersham, UK) in PBSG) and incubating for 15 minutes at 4°C. Samples were centrifuged at 2 300 g for 15 minutes at 4°C and the supernatant poured off into a 5ml polypropylene scintillation vial. 3 ml of scintillant was added and the vials counted by the same procedure used for the measurement of extraction efficiencies (2.2.5.3).

The cross-reactivity of the testosterone antibody with other steroids as provided by Endocrine Sciences was: dihydrotestosterone (20%), corticosterone (<0.01%), oestradiol (0.14%), △-1-testosterone (52%), 4-androsten-3β-17β-diol (3%), 5α-androstan-3β-17β-diol (1.8%), △-4-androstenedione (0.5%) and others (<0.5%). The sensitivity of each assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for testosterone was 0.45 ng/ml in plasma and 8.39 ng/g in faeces.

2.2.6.1 Intra- and inter-assay variation

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 658.1 ± 36.5 pg/ml, 179.3 ± 13.7 pg/ml, and 46.7 ± 11.7 pg/ml respectively.

Faecal and plasma samples were randomly assigned between two assays for testosterone. Inter-assay coefficients of variation for low, medium and high quality controls were 19.4%, 15.0% and 14.0% respectively. Intra-assay coefficients of variation were determined by conducting an assay with 20 duplicates of each quality control. Intra-assay coefficients of variation for testosterone for low, medium and high quality controls were 19.3%, 7.8% and 5.5% respectively.

2.2.6.2 Parallelism and hormone recovery

Serial dilutions of extracted quail plasma and faecal samples in assay buffer (PBSG) were parallel to the testosterone standard curve (faecal n=6, plasma n=3; Figure 2.1). The recoveries of testosterone added to two faecal samples were 94.1 ± 5.7% and 93.8 ± 5.5%. The recoveries of testosterone added to three plasma samples were 97.4 ± 1.7%, 99.1 ± 8.1% and 102 ± 2.7%.

2.2.7 Radioimmunoassay of oestradiol

Oestradiol 17-β (oestradiol) concentrations in faecal and plasma extracts were measured by radioimmunoassay, following the same procedures used for testosterone with oestradiol antibody (41-12, Etches, Canada) and tritiated oestradiol. Faecal extracts were used at a 1:3 dilution prepared in PBSG.

The cross-reactivities of the oestradiol antibody with other steroids as provided by Etches *et al.* (1981) were 17α-oestradiol (<1.0%), oestriol (<1.0%) and oestrone (4.3%). The sensitivity of each assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for oestradiol was 105.4 pg/ml in plasma and 4.1 ng/g in faeces.

2.2.7.1 Intra- and inter-assay variation

Solutions of oestradiol 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 2 194.6 ± 253.7 pg/ml, 514.2 ± 70.2 pg/ml, and 119.9 ± 24.6 pg/ml respectively.

Faecal and plasma samples were randomly assigned between four assays for oestradiol. Inter-assay coefficients of variation for low, medium and high quality controls were 16.5%, 14.1% and 14.2% respectively. Intra-assay coefficients of variation were determined by conducting an assay with 20 duplicates of each quality control. Intra-assay coefficients of variation for oestradiol for low, medium and high quality controls were 14.5%, 8.5% and 11.8% respectively.

2.2.7.2 Parallelism and hormone recovery

Serial dilutions of extracted quail plasma and faecal samples in assay buffer (PBSG) were parallel to the oestradiol standard curve (faecal n=6, plasma n=3; Figure 2.1). Recoveries of oestradiol added to two faecal samples were 94.9 ± 8.7% and 105.0 ± 6.7%. Recoveries of oestradiol added to three plasma samples were 90.8 ± 8.8%, 92.9 ± 8.9% and 92.3 ± 13.3%.

2.2.8 Radioimmunoassay of progesterone

Progesterone concentrations in faecal and plasma extracts were measured by radioimmunoassay, following the same procedures used for testosterone, with progesterone antibody (GBTB, Etches, Canada) and tritiated progesterone. Faecal extracts were used at a 1:2 dilution prepared in PBSG.

The cross-reactivities of the progesterone antibody with other steroids as provided by Etches and Croze (1983) were: 5α-pregnane-3,20-dione (15.6%), 5β-pregnane-3,20-dione (6.7%), 11β-hydroxyprogesterone (5.4%), cholesterol, testosterone, 17β-oestradiol, cortisol, corticosterone, deoxycorticosterone, 17α-hydroxyprogesterone,

pregnenalone, 5α-pregnan-3α-ol-20-one, 4-pregnene-20β-ol-3-one, 5β-pregnan-3α-ol-20-one, 5β-pregnan-3β-ol-20-one and 5α-pregnan-3β-ol-20-one (all <1%). The sensitivity of each assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for progesterone was 0.65 ng/ml in plasma and 4.86 ng/g in faeces.

2.2.8.1 Intra- and inter-assay variation

Solutions of progesterone 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 1 734.7 ± 257.3 pg/ml, 490.6 ± 22.8 pg/ml, and 109.1 ± 10.9 pg/ml respectively.

Faecal and plasma samples were randomly assigned between two assays for progesterone. Inter-assay coefficients of variation for low, medium and high quality controls were 20.3%, 17.6% and 12.3% respectively. Intra-assay coefficients of variation were determined by conducting an assay with 20 duplicates of each quality control. Intra-assay coefficients of variation for progesterone for low, medium and high quality controls were 16.3%, 8.8% and 8.4% respectively.

2.2.8.2 Parallelism and hormone recovery

Serial dilutions of extracted quail plasma and faecal samples in assay buffer (PBSG) were parallel to the progesterone standard curve (faecal n=6, plasma n=3; Figure 2.1). Recoveries of progesterone added to two faecal samples were 98.5 ± 12.3% and 110.7 ± 8.7%. Recoveries of progesterone added to three plasma samples were 90.9 ± 3.8%, 93.3 ± 3.6% and 90.6 ± 3.1%.

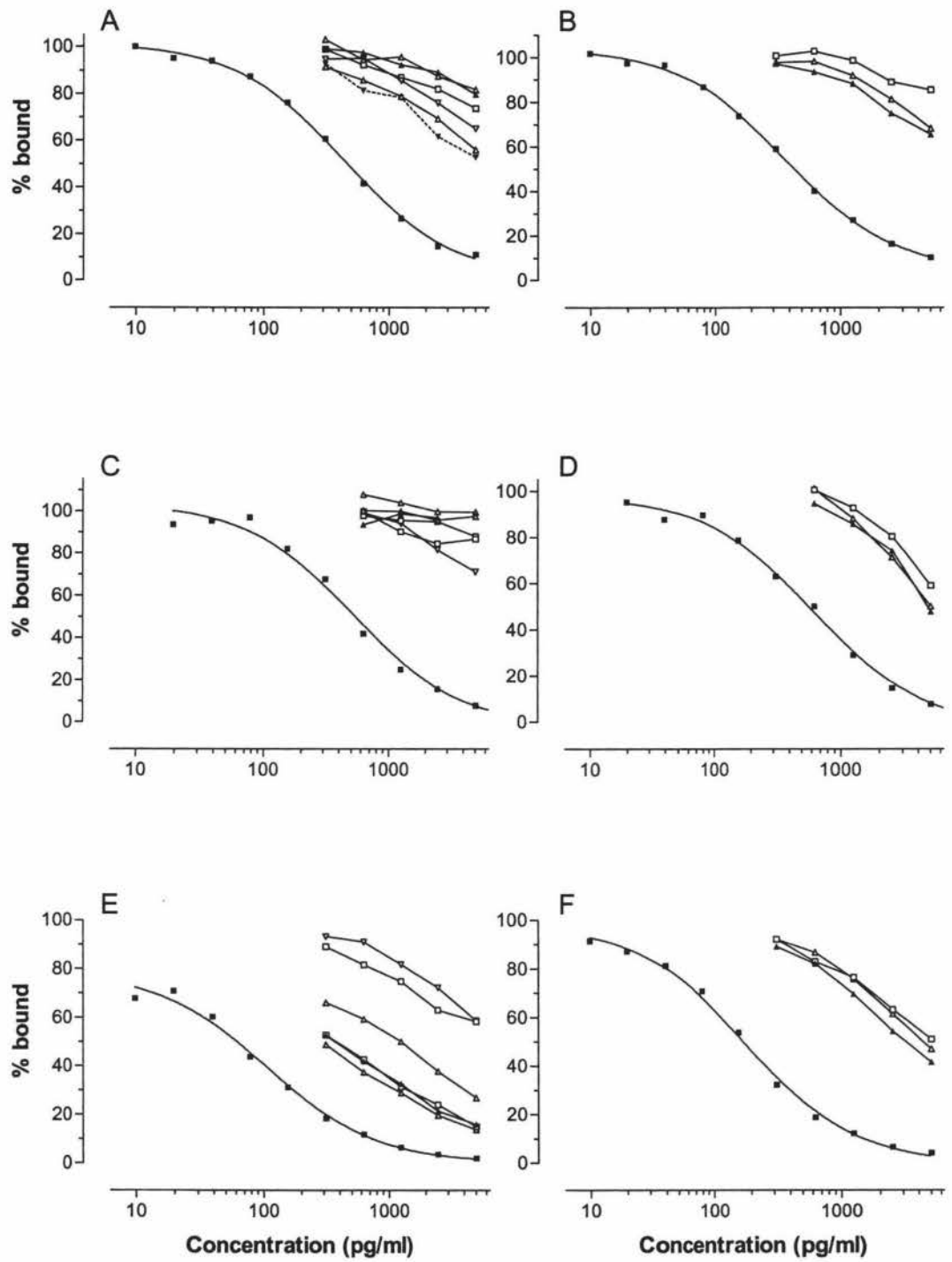

Figure 2.1. Parallelism demonstrated for quail faecal and plasma samples. A: faecal oestradiol, B: plasma oestradiol, C: faecal progesterone, D: plasma progesterone, E: faecal testosterone, F: plasma testosterone. The curves with filled squares are the standard curves and all other curves are samples.

2.2.9 Statistical analyses

Statistical analyses were performed using SPSS version 11.0.0 (2001; SPSS Inc). Where appropriate the following analyses were conducted on normally distributed data with homogeneous variances (as determined by Levene's Test): one-way ANOVA with Bonferroni or Dunnets' post hoc comparisons (Bonferroni where number of groups to compare<5, Dunnets where number of groups >5 and had uneven sample sizes), independent samples t-test, and Pearson correlation. Data were transformed by $\log_{10}$ or square root as appropriate to increase homogeneity for parametric analysis.

2.3 Results

2.3.1 Gonad weight

Gonads were collected from eight females and ten males held on short days and seven females and five males held on long days. Each group initially consisted of ten birds, but some were removed from the experiment due to poor condition. Females on long days had heavier ovaries than females on short days (7.47 ± 1.35 g compared with 1.53 ± 1.36 g; Independent samples T test, $t = 3.077$, $df = 13$, $p = 0.009$). One bird on short days had unusual ovarian growth and one bird on long days showed no ovarian growth (Figure 2.2).

Males held on long days had larger testes than males on short days (paired testis weight 4.91 ± 1.28 g compared with 0.72 ± 0.31 g; Independent samples T test, $t = 4.316$, $df = 13$, $p = 0.001$). One male on long days did not have developed testes.

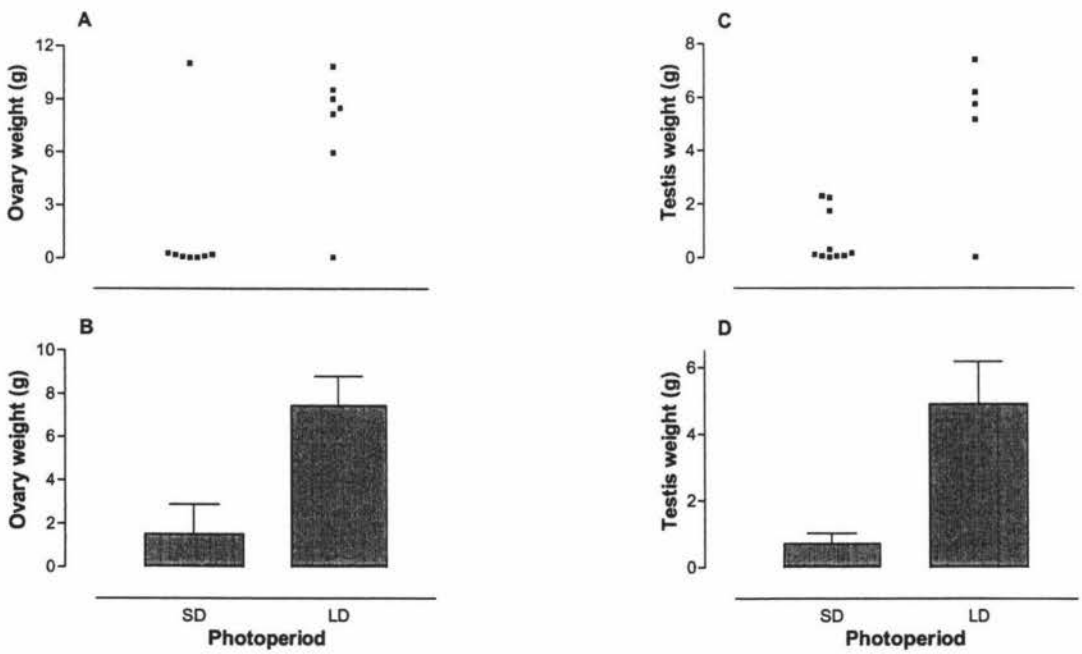

Figure 2.2. Gonad weight in Japanese quail held on different photoperiods (SD = short day, LD = long day). A) Ovary weight for individual females; B) Mean ovary weight for females ± SE; C) Paired testis weight for individual males; D) Mean paired testis weight for males ± SE.

2.3.2 Egg laying and cloacal foam

Females on long days regularly laid eggs, except the individual that showed no ovarian growth. The short day bird with unusual ovarian growth began regularly laying at six weeks of age.

Males on long days produced cloacal foam, except the male that did not have developed testes. Two males on short days produced cloacal foam from six weeks of age.

2.3.3 Dropping weight

Individual dried quail droppings ranged from 0.02 – 0.38 g in weight. Dropping weights differed between groups ($F_{3, 354} = 27.80$, $p = 0.001$) and females held on long days produced significantly heavier droppings than any other group ($p = 0.001$, Figure 2.3). The average amount of water in droppings for each group was 62.52 ± 0.98% of fresh weight for females on short days, 73.33 ± 1.30% for females on long days, 61.13 ± 0.91% for males on short days, and 69.29 ± 0.71% for males on long days.

Both ovary and paired testis weights showed low, but significant correlations with dropping weight (Figure 2.4). For all three hormones, total hormone content in each dropping (ng) increased with increasing dropping weight (Figure 2.5).

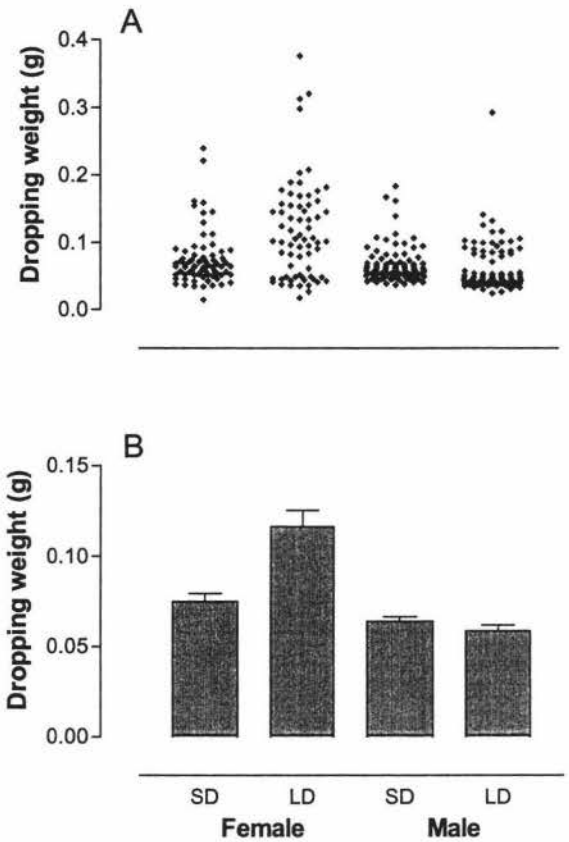

Figure 2.3. Dropping weight in Japanese quail held on different photoperiods (SD = short day, LD = long day). A: weights of all individual droppings (SD female n = 75, LD female n = 68, SD male n = 111, LD male n = 104); B: mean dropping weight ±SE.

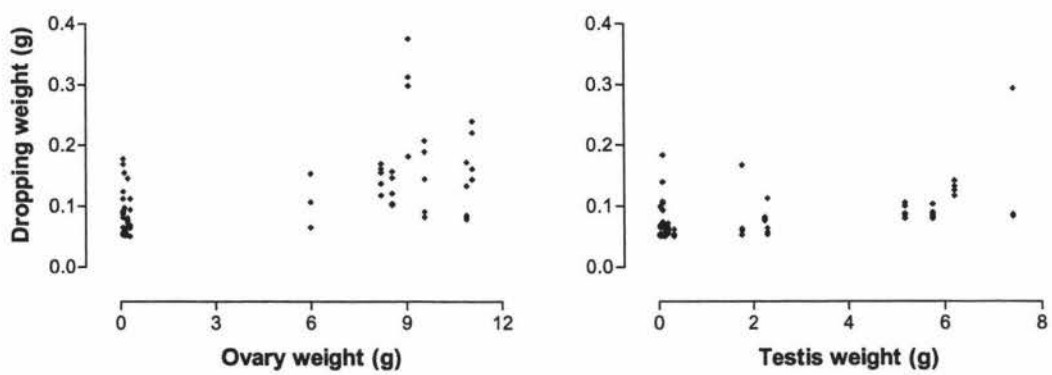

Figure 2.4. Relationships between gonad weight and dropping weight. Correlations between gonad and dropping weight were significant (ovary: r = 0.57, n = 67, p = 0.001; testis: r = 0.48, n = 67, p = 0.001).

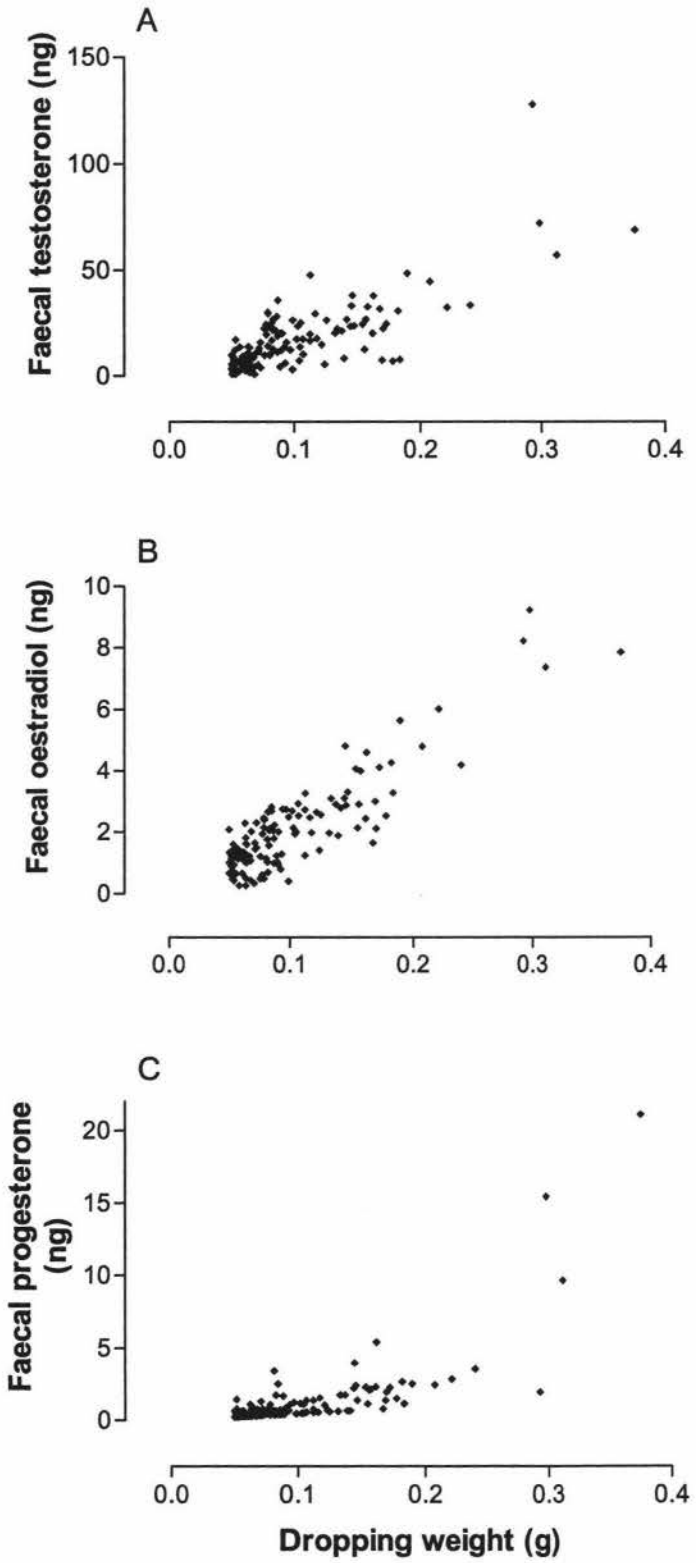

Figure 2.5. Relationship between dropping weight and total faecal hormone content per dropping. A: Testosterone, r = 0.77, n = 134, p = 0.001; B: Oestradiol, r = 0.88, n = 134, p = 0.001; C: Progesterone, r = 0.75, n = 134, p = 0.001.

2.3.4 Plasma testosterone

Plasma testosterone concentrations across all groups ranged from the least detectable dose of 0.45 ng/ml to 6.00 ng/ml (Figure 2.6). There were no significant differences between the groups (Table 2.7).

No significant relationship was found between plasma testosterone concentrations and paired testis weight (Table 2.8).

2.3.5 Faecal testosterone

Faecal testosterone concentrations across all groups ranged from 15.61 to 424.67 ng/g. Within each group there was considerable variation, with concentrations ranging from very low concentrations that were consistent across the groups, to higher concentrations (Figure 2.6). There was a trend towards males on long days having higher faecal testosterone concentrations than the other three groups, although there were no significant differences between the groups (Table 2.7).

A highly significant, positive relationship was found between faecal testosterone and paired testis weight (Table 2.8).

Within all four groups, there were significant differences in faecal testosterone concentration between birds (Figure 2.7, Table 2.9). All short day females had regressed ovaries except bird eight, which had a fully developed ovary. However, faecal testosterone was not significantly different in bird eight when compared to the rest of the birds as a group (Figure 2.7:A; $p>0.05$). All long day females had developed ovaries except bird three. Faecal testosterone was significantly lower in bird three than in the other birds (Figure 2.7:B; $p<0.05$). Similarly, all long day males had developed testes except bird five. Faecal testosterone was significantly lower in bird five than in the other birds (Figure 2.7:D; $p<0.05$).

Table 2.10 highlights the variation in testosterone between birds within each group when considering an individual faecal sample and a mean or pooled faecal sample. The coefficient of variation did not differ greatly between individual or pooled samples.

2.3.6 Relationship between plasma and faecal testosterone

No correlation was found between plasma and faecal testosterone concentrations (Table 2.11).

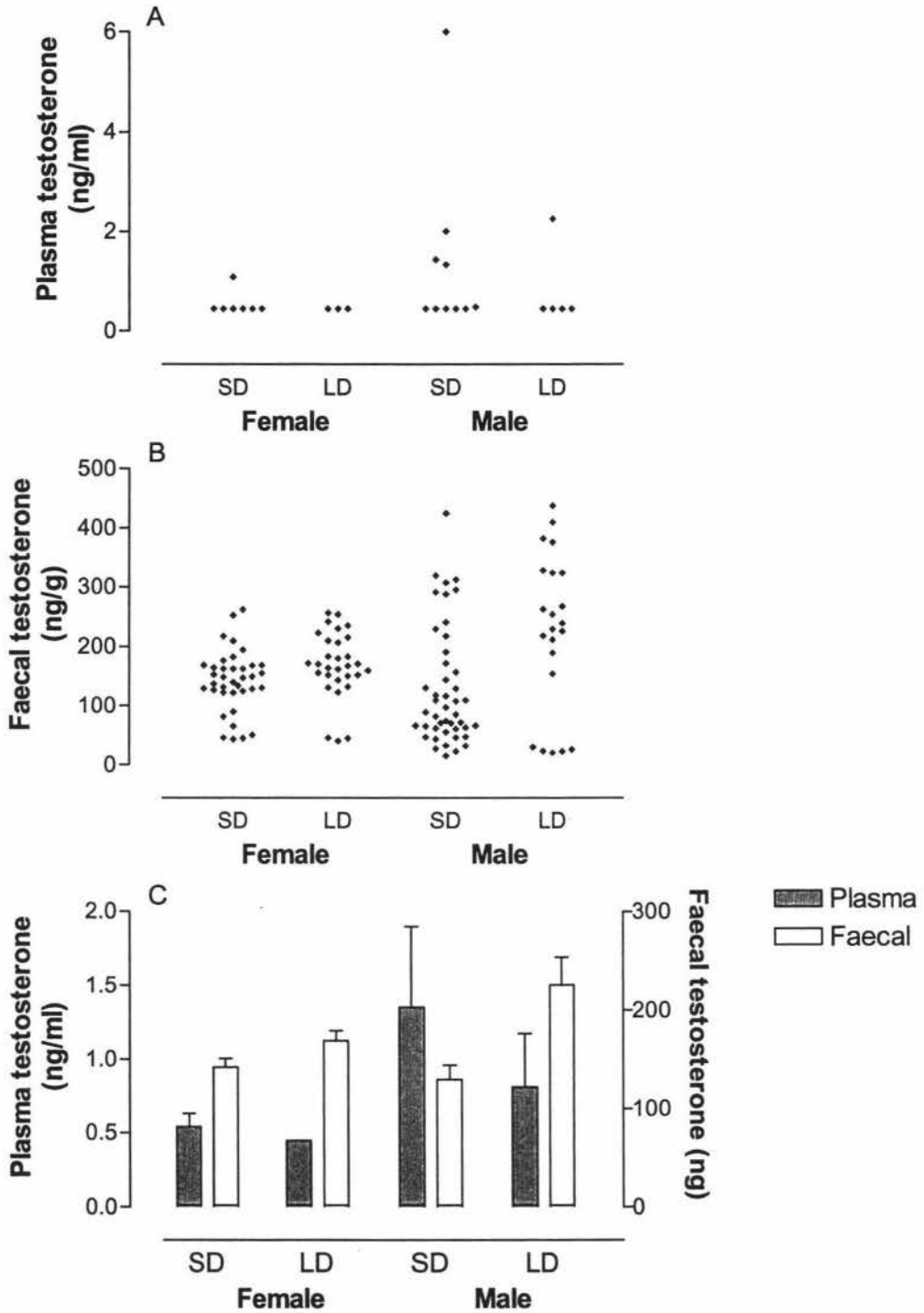

Figure 2.6. Plasma and faecal testosterone concentrations (SD = short day, LD = long day). A) Individual plasma testosterone concentrations for quail held on different photoperiods (note that sample sizes differ from Figure 2.2, due to missing plasma samples for some individuals.) B) Individual faecal testosterone concentrations for quail held on different photoperiods. C) Mean plasma and faecal testosterone concentrations ± SE.

Table 2.7. Summary of one-way ANOVA of plasma and faecal hormone concentrations across birds grouped by photoperiod. Multiple comparisons were made using Bonferroni post-hoc analysis. The mean faecal hormone concentration for all droppings produced by each individual was used for the faecal analysis. SD = short day, LD = long day (note: no comparison between male SD and LD could be made for plasma oestradiol as there was insufficient plasma available for LD birds.)

	Testosterone			Oestradiol			Progesterone		
Effect	**F**	**df**	**P**	**F**	**df**	**P**	**F**	**df**	**P**
Plasma	0.842	3,21	0.486	2.414	2,20	0.115	0.724	3,24	0.548
Comparison between groups									
Female SD vs LD			1.000			0.145			1.000
Male SD vs LD			1.000			-			1.000
Faecal	1.948	3,26	0.147	2.138	3,26	0.120	26.76	3,26	0.001
Comparison between groups									
Female SD vs LD			1.000			1.000			0.309
Male SD vs LD			0.159			1.000			1.000

Table 2.8. Relationships between gonad weight and plasma and faecal hormone concentrations in males (paired testis weight and testosterone) and females (ovary weight and oestradiol or progesterone). n = 15 in all cases except plasma progesterone where n = 13. Significant differences ($p<0.05$) are marked with an asterisk(*), and highly significant ($p<0.01$) differences are marked with a double asterisk (**).

	Plasma		Faecal	
	r	**p**	**r**	**p**
Testosterone	0.10	0.893	0.84	0.001**
Oestradiol	0.42	0.120	0.33	0.234
Progesterone	0.65	0.017*	0.54	0.039*

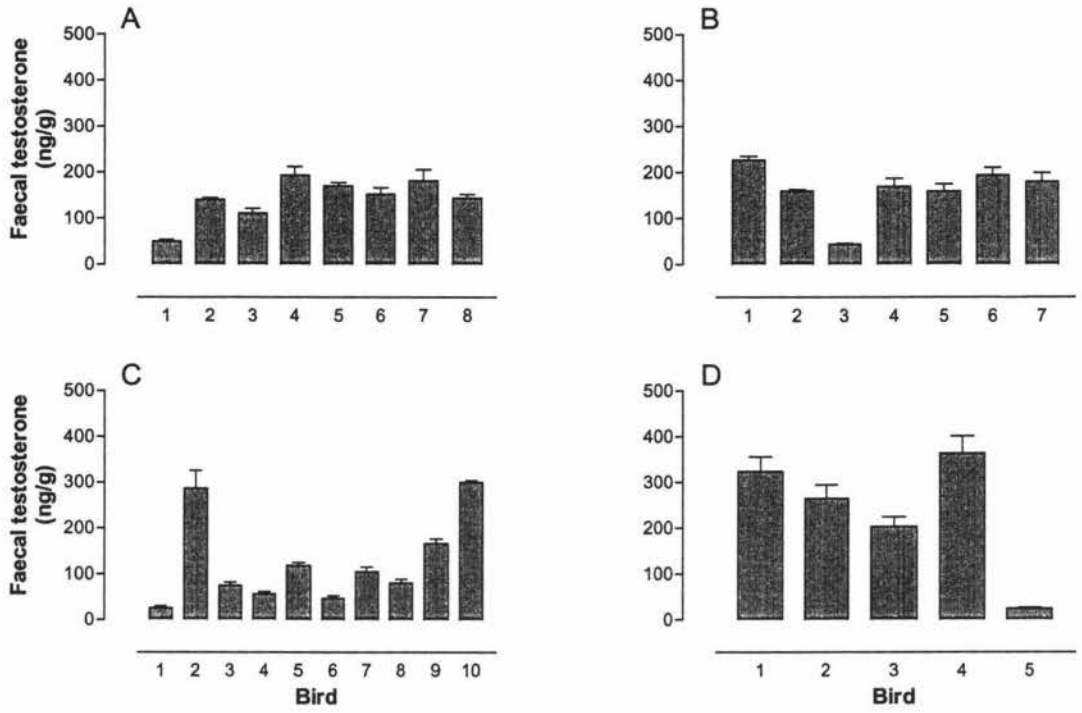

Figure 2.7. Mean faecal testosterone concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) males held on a short day photoperiod, D) males held on a long day photoperiod.

Table 2.9. Summary of one-way ANOVA tests of variation in faecal hormone concentration between birds within each treatment group (SD = short day, LD = long day).

	Testosterone			**Oestradiol**			**Progesterone**		
Group	F	df	P	F	df	P	F	df	P
SD Female	11.662	7,29	0.001	5.208	7,9	0.001	5.699	7,29	0.001
LD Female	11.074	6,23	0.001	4.607	6,23	0.003	3.938	6,23	0.007
SD Male	60.485	9,35	0.001	10.366	9,35	0.001	1.440	9,35	0.209
LD Male	25.413	4,17	0.001	7.832	4,17	0.001	1.219	4,17	0.339

Table 2.10. Variation between birds in individual and mean faecal steroid concentrations. (SD = short day, LD = long day, F = female, M = male).

	Testosterone (ng/g)				Oestradiol (ng/g)				Progesterone (ng/g)			
	SD F	**LD F**	**SD M**	**LD M**	**SD F**	**LD F**	**SD M**	**LD M**	**SD F**	**LD F**	**SD M**	**LD M**
Variation between birds (means of one random sample per bird)												
Mean	141.97	145.85	114.70	230.09	21.60	21.36	15.28	20.48	12.19	17.51	5.12	5.33
SE	17.04	20.17	30.32	60.19	2.11	1.58	2.20	3.18	1.08	3.38	0.18	0.47
CV	33.94	36.60	79.32	58.49	27.64	19.60	43.11	34.75	25.04	51.09	10.55	19.56
n	8	7	10	5	8	7	10	5	8	7	10	5
Variation between birds (means of the individual bird means)												
Mean	142.77	161.74	126.08	235.57	21.51	23.37	16.54	20.75	12.17	18.06	5.34	5.60
SE	16.08	21.59	30.44	59.33	2.08	1.84	2.07	2.44	1.45	3.78	0.16	0.34
CV	31.86	35.31	76.35	56.31	27.40	20.83	39.49	26.30	26.63	55.31	9.64	13.48
n	8	7	10	5	8	7	10	5	8	7	10	5

Table 2.11. Correlations between plasma and mean faecal hormone concentrations.

	r	n	p
Testosterone	0.10	25	0.723
Oestradiol	0.28	23	0.194
Progesterone	0.22	27	0.248

2.3.7 Plasma oestradiol

Plasma oestradiol concentrations across all groups ranged from the least detectable dose of 105.38 pg/ml to 247.06 pg/ml (Figure 2.8). No plasma samples were available from males held on long days. There were no significant differences between groups (Table 2.7).

No significant relationship was found between plasma oestradiol concentrations and ovary weight (Table 2.8).

2.3.8 Faecal oestradiol

Faecal oestradiol ranged from the least detectable dose of 4.13 to 41.33 ng/g. Within each group there was considerable variation (Figure 2.8), and there were no significant differences between any of the groups (Table 2.7).

No significant relationship was found between faecal oestradiol concentrations and ovary weight (Table 2.8).

There were significant differences in faecal oestradiol concentration between individual birds within each group (Figure 2.9, Table 2.9). All short day females had regressed ovaries except bird eight, which had a fully developed ovary. However, faecal oestradiol in bird eight was not significantly different from the other birds (Figure 2.9:A, $p>0.05$). All long day females had developed ovaries except bird three. Faecal oestradiol in bird three was not significantly different from the other birds (Figure 2.9:B, $p>0.05$). Similarly, bird five had an undeveloped testis in comparison to the other

long day males, but it's faecal oestradiol was not significantly different from the other birds (Figure 2.9:D, $p<0.05$).

Table 2.10 highlights the variation in oestradiol between birds within each group when considering an individual faecal sample and a mean or pooled faecal sample. The coefficients of variation did not differ greatly between individual or pooled samples.

2.3.9 Relationship between plasma and faecal oestradiol

No correlation was found between plasma and faecal oestradiol concentrations (Table 2.11).

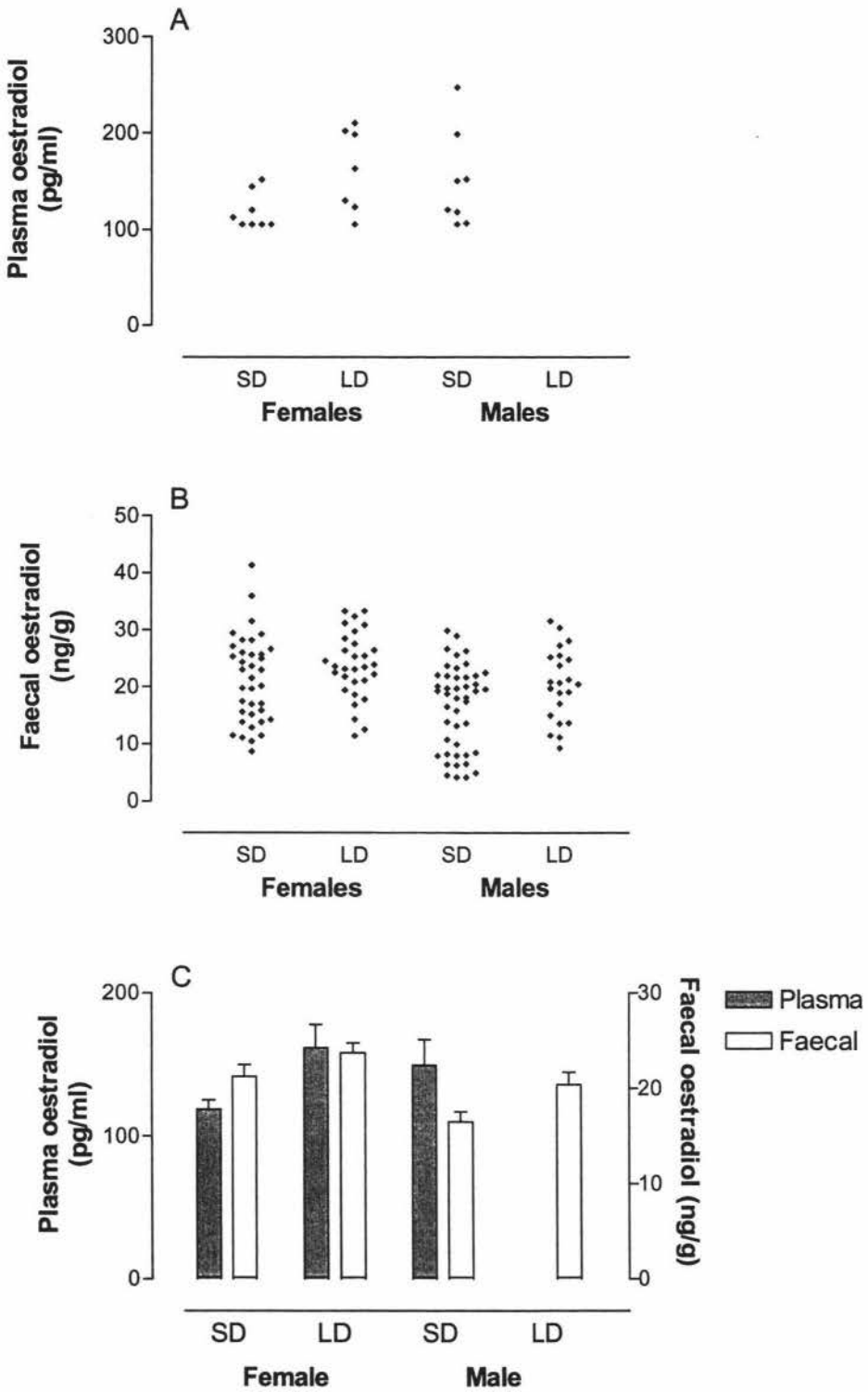

Figure 2.8. Plasma and faecal oestradiol concentrations (SD = short day, LD = long day). A) Individual plasma oestradiol concentrations for quail held on different photoperiods (note that sample sizes differ from Figure 2.2, due to missing plasma samples for some individuals.) B) Individual faecal oestradiol concentrations for quail held on different photoperiods. C) Mean plasma and faecal oestradiol concentrations ± SE.

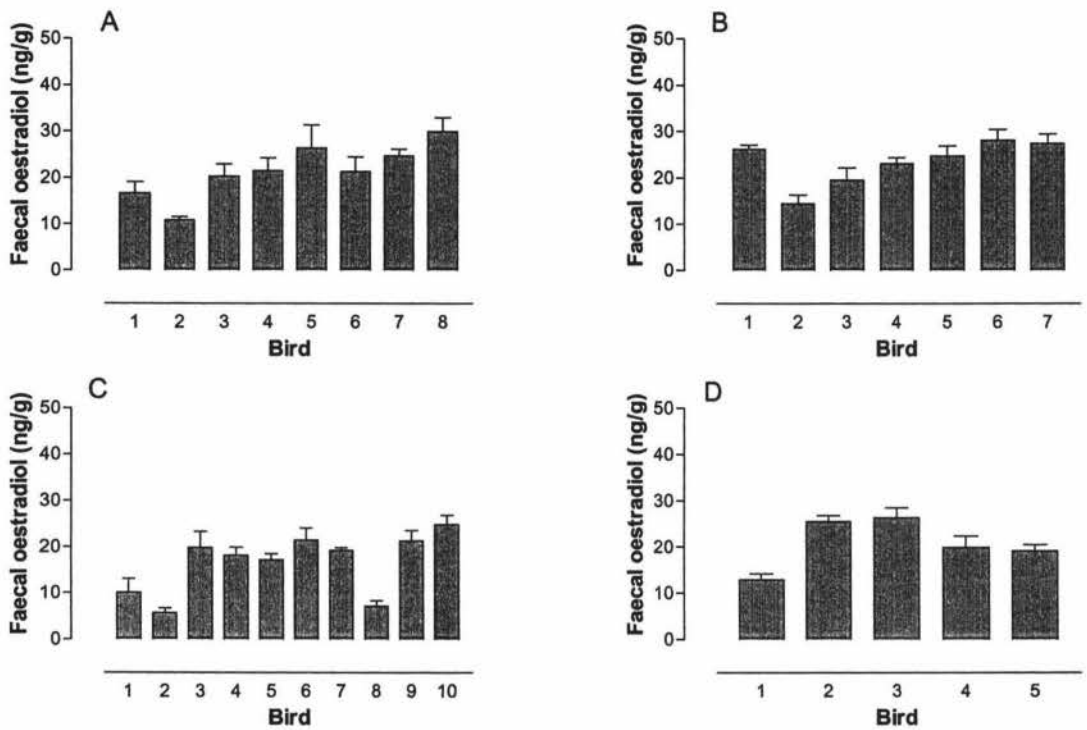

Figure 2.9. Mean faecal oestradiol concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) males held on a short day photoperiod, D) males held on a long day photoperiod.

2.3.10 Plasma progesterone

Plasma progesterone concentrations across all groups ranged from the least detectable dose of 0.65 ng/ml to 5.38 ng/ml (Figure 2.10). There were no significant differences between the groups (Table 2.7).

There was a significant relationship between plasma progesterone concentrations and ovary weight (Table 2.8).

2.3.11 Faecal progesterone

Faecal progesterone ranged from the least detectable dose of 4.86 to 56.10 ng/g. Within the long day female group there was considerable variation and a wide range of progesterone concentrations (Figure 2.10), although there was no significant difference between short and long day birds (Table 2.7). However there was a significant difference between males and females ($p = 0.001$).

A significant relationship was found between faecal progesterone and ovary weight (Table 2.8).

There were significant differences in faecal progesterone concentrations between birds for both the female groups (Figure 2.11, Table 2.9). There were no differences between birds in either of the male groups. All short day females had regressed ovaries except bird eight, which had a fully developed ovary. However, faecal progesterone in bird eight was not significantly different to the other birds (Figure 2.11:A, $p>0.05$). All long day females had developed ovaries except bird three. Faecal progesterone in bird three was not significantly different to the other birds (Figure 2.11:B, $p>0.05$). Similarly, bird five had an undeveloped testis in comparison to the other long day males, but its faecal progesterone did not differ from the other birds in that group (Figure 2.11:D, $p<0.05$).

Table 2.10 highlights the variation in progesterone between birds within each group when considering an individual faecal sample and a mean or pooled faecal sample. The coefficients of variation did not differ greatly between individual or pooled samples.

2.3.12 Relationship between plasma and faecal progesterone

No significant correlation was found between plasma and faecal progesterone (Table 2.11).

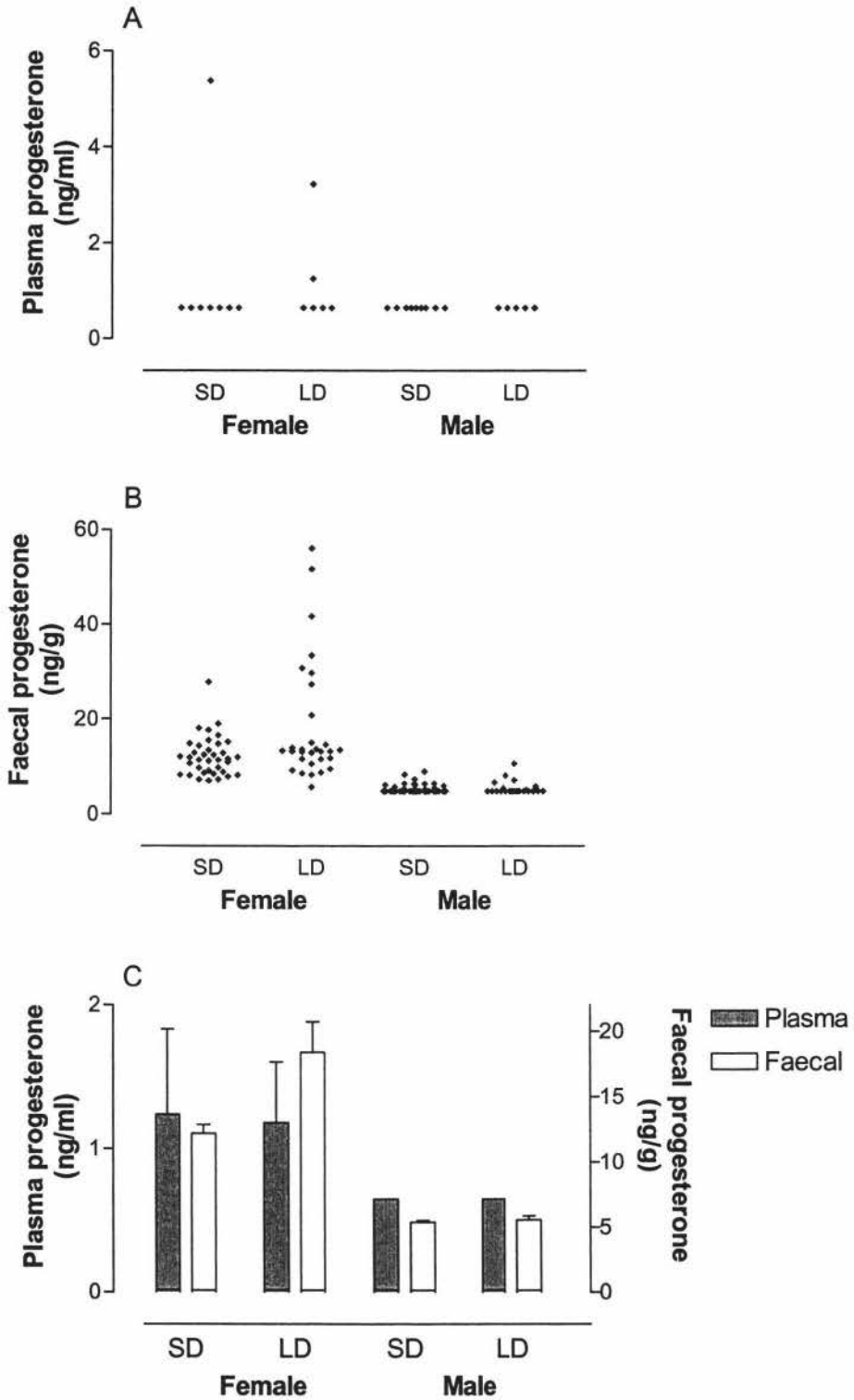

Figure 2.10. Plasma and faecal progesterone concentrations (SD = short day, LD = long day). A) Individual plasma progesterone concentrations for quail held on different photoperiods (note that sample sizes differ from Figure 2.2, due to missing plasma samples for some individuals.) B) Individual faecal progesterone concentrations for quail held on different photoperiods. C) Mean plasma and faecal progesterone concentrations ± SE.

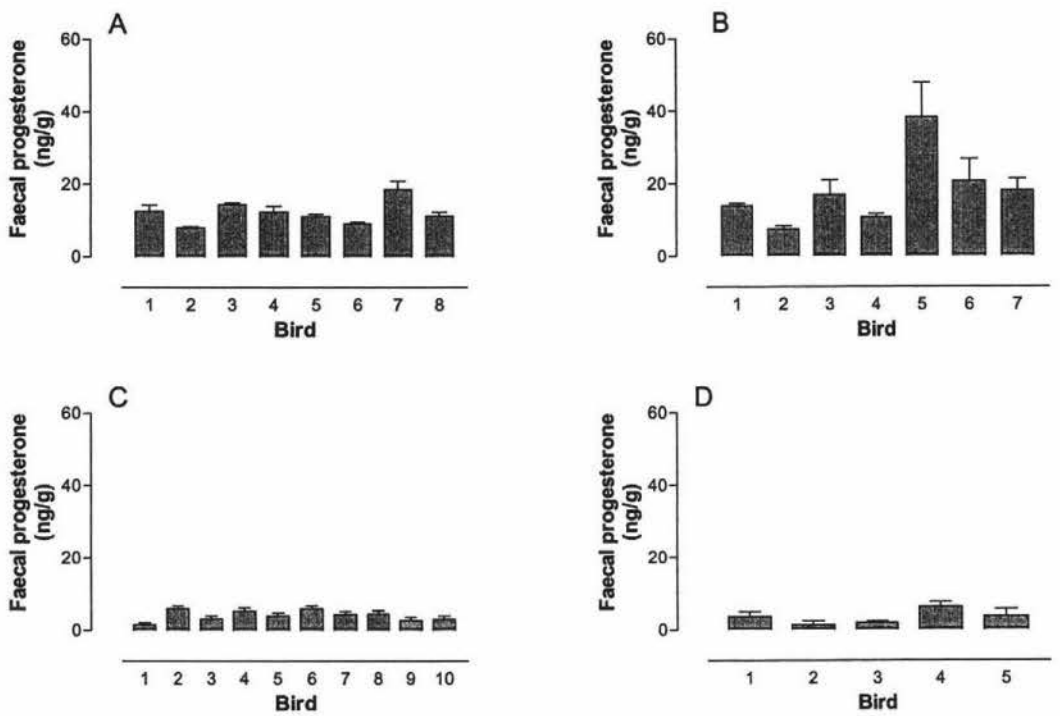

Figure 2.11. Mean faecal progesterone concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) males held on a short day photoperiod, D) males held on a long day photoperiod.

2.4 Discussion

The goals of this study were to establish methods for the extraction of oestradiol, testosterone and progesterone from quail faecal samples and to confirm that faecal concentrations of these hormones would reflect plasma concentrations. A robust extraction method was developed and faecal and plasma steroid concentrations were measured in male and female quail on short and long day lengths. However, marked differences in gonad size between photoperiods were not accompanied by corresponding differences in plasma steroid concentrations. This meant that clear plasma – faecal steroid relationships could not be established across a range of plasma concentrations.

2.4.1 Gonad weight

It is well established that exposure to long day lengths stimulates the hypothalamo-pituitary-gonadal axis, increasing gonadotropin secretion and gonadal growth in Japanese quail (Follett and Sharp, 1969; Follett and Robinson, 1980).

In this study, most birds on long photoperiods had developed gonads in comparison to those held on a short day photoperiod, although one female of seven had an undeveloped ovary and one male of five had an undeveloped testis. Oishi and Konishi (1983), Konishi *et al.* (1988) and Tsuyoshi and Wada (1992) have reported similar occurrences. In the current study, all females on long days regularly laid eggs, except the individual that showed an undeveloped ovary. All males produced cloacal foam, except the bird that showed no testicular development. Neither of these individuals produced eggs or foam at any stage during the study. This suggests that there was an absence of gonadal growth in these two individuals despite long day lengths.

Evidence of similar anomalies has been found in other studies. Honda *et al.* (1987) reported a difference in responsiveness to long day photoperiodic conditions between two lines of Japanese quail. The male birds of one line (KR) responded as expected to long day conditions with an increase in cloacal gland size, whereas males from another line (KN) did not show any response to the conditions. Females from the KR line laid

eggs at a younger age than those from the KN line. In the current study, quail were obtained from a commercial source where birds had generally been selected for increased body weight. This may have led to a reduction in the strength of the photoperiodic response in comparison to quail in other studies where birds were selected for photoperiodic responses (Follett and Sharp, 1969; Follett and Robinson, 1980).

Wada *et al.* (1990) reported that short days did not always suppress LH secretion enough to reduce cloacal activity in the Japanese quail in their laboratory. In addition, both short days and low ambient temperatures (8-10°C) are needed to prevent gonadal growth in some strains of quail in Japan (Oishi and Konishi, 1978; Wada *et al.*, 1990). In the current study, one female on short days had unusual ovarian growth with a fully developed follicular hierarchy. This bird began regularly laying eggs at 6 weeks, despite exposure to short day lengths since the age of 3 weeks. Two males on short days began producing cloacal foam from 6 weeks of age. It has been suggested that there are two types of quail with respect to termination of breeding activity - those that need only short day conditions and those that need both short day conditions and low temperatures. Therefore, in addition to photoperiod, temperature manipulation may be needed to ensure birds with regressed gonads and low reproductive hormone concentrations are produced. Other strains of quail, such as the Follett line of quail, which is currently maintained at the Roslin Institute in Edinburgh, will maintain small gonads on short days without low temperatures. It appears from the current study that the New Zealand strain of quail are similar to those in Japan, where both short days and low temperatures are required to prevent gonadal growth.

2.4.2 Dropping weight

Female quail on long days produced heavier droppings than females on short days or males. All females with developed ovaries on long days were laying eggs daily. Laying birds tended to eat more (pers. obs.) and hence produce larger faeces.

The total faecal hormone content of individual droppings increased, as expected with increasing dropping weight. The effect of dropping size is avoided by expressing the

faecal hormone concentration as the ng steroid/g dried dropping. Diurnal fluctuations in steroid concentrations could pose a problem when faecal steroid analysis is used in diagnostic situations where the precise detection of small changes in hormone concentrations may be required (e.g. detection of ovulation). In this situation the total steroid production over 24 hours may be of greater interest, although it is not always practical to collect faecal samples from individual birds over 24 hours.

2.4.3 Testosterone

Plasma testosterone concentrations did not vary between birds kept on long and short days, and were similar to those recorded previously for quail of similar ages kept on short days (Follett and Maung, 1978; Bishop and Hall, 1991). The exception was one male bird held on short days with a plasma testosterone concentration of 6.0 ng/ml (this concentration was confirmed when the sample was re-assayed). This concentration of hormone is comparable to that for male quail held on long days in other studies (Follett and Maung, 1978; Bishop and Hall, 1991; W.H. Chua, pers. comm.). This bird had undeveloped testes (0.07g) and did not produce cloacal foam at any stage, suggesting that it did not undergo any gonadal development. Boswell *et al.* (1995) reported high concentrations of testosterone in both castrate and intact European quail held on short days, so elevated concentrations in this bird were possibly due to extra-gonadal sources of testosterone.

Four out of five males held on long days had developed testes. However, plasma testosterone concentrations were similar to those seen in males held on short days. Testosterone concentrations can fluctuate with stages of the breeding cycle, even though testes remain large (Wingfield *et al.,* 1987). After the current experiment was completed, further work in the IVABS RIA laboratory showed that plasma testosterone concentrations are not necessarily related to testis size in quail (W.H. Chua, unpubl.). Lower concentrations of testosterone in the long day males could have resulted from an increase in the metabolic clearance rate of testosterone, which can occur without a change in hormone secretion rate. Wingfield (1984) reported such a decline in plasma testosterone despite high circulating LH concentrations in the song sparrow (*Melospiza melodia*). Diurnal variations in testosterone concentrations could affect measured

plasma concentrations. In male Japanese quail held on a similar photoperiod (14 hours light: 10 hours dark, lights on 0600 hours) testosterone production followed cyclic changes with peaks in production at 0500 and again at 1400 hours (Gulati *et al.*, 1981). Similar peaks were identified in nonovulating female quail. Plasma samples in the present study were collected at 1200 hours, which could well have been when concentrations were at a minimum, between peaks.

Females held on long days were expected to have higher plasma testosterone concentrations than females held on short days, since testosterone has a role in ovulation and oviposition (Brain *et al.*, 1988). Although the numbers of plasma samples were small and care should be taken in interpretation of results, there appeared to be no difference in testosterone concentrations between the two groups of female birds.

A clear relationship between steroid hormone concentration and gonadal development has been established in many species of bird, including Japanese quail (Wingfield *et al.*, 1987; Follett and Robinson, 1980). However, in this study the relationship between plasma testosterone and paired testis weight was not significant, most likely due to the fact that plasma testosterone concentrations were low in all groups.

Faecal testosterone concentrations did not vary between groups on long and short day photoperiods, whereas higher concentrations were expected in males on long days. There was a trend towards these males having higher faecal testosterone concentrations than the other three groups, despite no trend in plasma concentrations. Faecal steroid concentrations provide an integrated measure of plasma testosterone over a period of time, whereas plasma concentrations are a measure at a single time point. This may explain why faecal testosterone showed a highly significant, positive relationship with paired testis weight, in contrast to plasma testosterone.

Faecal testosterone concentrations in the two short day groups were up to three-fold higher than those recorded by Bishop and Hall (1991) from quail of a similar age held on short days. This could be due to a difference in non-breeding concentrations of circulating hormone between strains, or a difference in the hormone extraction and assay methods. In their study, male quail held on long days had faecal testosterone

concentrations ranging from approximately 290 – 700 ng/g, in comparison to 150 – 425 ng/g in this study (males with developed testis only). Concentrations in female quail held on long days were similar in the two studies.

Within each group there was considerable variation, with faecal testosterone ranging from very low concentrations found in all groups to higher concentrations. This variation is due to variation between birds. In Figure 2.6:B, the three low points in the LD female group are from the same individual, as are the five low points in the LD male group. Figure 2.7 illustrates the individual variation in faecal testosterone concentrations between birds within each group, and these two individuals are obvious as birds 3 and 5 in graphs B and D respectively. Female 3 and male 5 on long days had undeveloped gonads. These birds also had low plasma testosterone concentrations, which supports the idea that faecal hormone analysis can be used to predict plasma steroid concentrations and gonadal status. Variation between birds was still marked with these birds excluded from analysis.

In Figure 2.6:B, SD male, there are several droppings with relatively high concentrations of faecal testosterone. These are from two individuals, birds 2 and 10 seen in Figure 2.7:C, and both had slightly larger testes than the other males held on short days. Even though these males were not fully reproductively active, they are examples of individuals that may require low temperature as well short day photoperiod to suppress reproductive activity.

This study did not address variation in faecal hormone concentration within a bird over a whole day, therefore it is difficult to say whether an individual dropping is sufficient to accurately measure a birds hormone concentrations. However, the coefficient of variation for random individual droppings was similar to that for pooled samples, indicating that over a sampling period from 0900 – 1200 a single dropping is as accurate as a pooled sample. This is supported by the work of Ishii *et al.* (1994) who successfully compared plasma and faecal steroid concentrations from individual droppings from Japanese quail held under different photoperiodic conditions, Kofuji *et al.* (1993) who described gonadal hormone cycles in the brown dipper (*Cinclus pallasii*) from individual droppings, and Bercovitz *et al.* (1978) showed that sexing cockatiel from

individual droppings was as reliable as from 24-hour collections. Stavy *et al.* (1979) also used single droppings to sex various bird species, but did report one case where a mis-diagnosis would have resulted from one unusually high value due to short-term variations in steroid concentrations. Cockrem and Rounce (1994) measured faecal and plasma oestradiol and testosterone in domestic fowl, and found that variation between samples was not higher for faecal measurements in comparison to plasma. In addition, there was not a great increase in accuracy of predicting plasma concentrations from several faecal samples as opposed to an individual faecal sample. Using individual faecal samples to measure hormone concentrations appears to be adequate for most applications. When precision is needed to detect small differences that may be masked by variation between droppings, a larger sample may be more accurate.

No relationships were found between plasma and faecal testosterone concentrations, probably due to the absence of a wide range of plasma testosterone concentrations across short and long day birds, with low plasma testosterone concentrations associated with a range of faecal testosterone concentrations. Cockrem and Rounce (1994) reported a similar situation in domestic fowl, although the correlation between plasma and faecal testosterone was significant.

2.4.4 Oestradiol

Plasma oestradiol concentrations did not vary between females kept on long and short day photoperiods (no plasma samples were available from males held on long days). There was a trend towards females on long days having higher plasma oestradiol concentrations than females on short days, whereas in other studies females on long days had significantly higher oestradiol concentrations (Bain *et al.*, 1988; Bishop and Hall, 1991; Ishii *et al.*, 1994). Similarly, faecal oestradiol concentrations did not vary between groups held on long and short day photoperiods.

Despite the fact that plasma oestradiol concentrations were comparable to previous reports of oestradiol in female quail during the ovulatory cycle (71-181 pg/ml, Doi *et al.* 1980; and 86-133 pg/ml, Gulati *et al.* 1981), concentrations were lower than those found in other studies conducted in the same laboratory (100 – 400 pg/ml). However, Chua

(pers. comm.) showed that oestradiol decreases with age in this strain of quail (6 – 14 weeks), despite the birds having large ovaries.

Diurnal variations in oestradiol concentrations could be affecting measured plasma concentrations. In ovulating Japanese quail, oestradiol production follows cyclic changes with peaks in production six hours before and three hours after ovulation (Doi *et al.*, 1980; Gulati *et al.*, 1981). Females in the current study laid eggs between 1600 and 1900 hours and are likely to have ovulated between 1500 and 1800 hours. Therefore, a peak in oestradiol concentration would have been expected somewhere between 0900 and 1200 hours and again between 1800 and 2100 hours. In-between the two peaks, minimum concentrations are expected, and a decline is noted in the first three hours post-peak. Plasma samples were collected at 1200 hours, which for some samples could have been when concentrations were beginning to decline or already at a minimum (1200 hours is three hours post-peak, when minimal concentrations were expected, if the peak was early in the time range i.e. 0900 hours). From data from Gulati *et al.* (1981), this minimum concentration would be similar to those measured in short day females in the current study.

Faecal concentrations of oestradiol were low in comparison to those reported previously in quail (up to 180 ng/g; Bishop and Hall, 1991), although in that study oestradiol-3-glucuronide was measured in contrast to free steroids, which are present in lower amounts in droppings than conjugated steroids. Cockrem and Rounce (1994) reported concentrations of faecal oestradiol in domestic fowl that were similar to the present study, ranging from 5 – 89 ng/g.

Within each group there was considerable variation between birds, with faecal hormone concentrations ranging from low to higher concentrations. In the two long day groups there were individuals with undeveloped gonads (Figure 2.9:B and D, birds 3 and 5 respectively), and in the female short day group there was one bird with a developed ovary (Figure 2.9:A, bird 8). In contrast to testosterone, these birds did not have significantly different faecal hormone concentrations from other birds in their groups. The coefficient of variation for random individual droppings was similar to that for

pooled samples, suggesting that a single dropping is adequate for determining oestradiol concentrations during a three-hour sampling period.

Although a clear relationship between steroid hormone concentration and gonadal development has been established in other species, in this study the relationship between plasma and faecal oestradiol and ovary weight was not significant. This was most likely due to the fact that plasma and faecal oestradiol concentrations were similar in all groups. Similarly, no relationship was found between plasma and faecal oestradiol.

2.4.5 Progesterone

Plasma progesterone concentrations did not vary between groups kept on long and short photoperiods. Higher concentrations were expected in female birds held on long days, but only one bird had elevated progesterone. Values were still similar to those recorded in previous experiments from quail of similar ages kept on short and long days (Doi *et al.*, 1980; Gulati *et al.*, 1981; and Bishop and Hall, 1991). One female on short days had a high progesterone concentration (5.4 ng/ml; this concentration was confirmed when the sample was re-assayed), comparable to that reported for female quail held on long days in other studies. This bird had a large ovary (11.06g) and had undergone gonadal growth on short days. It may be that progesterone concentrations are greater in birds with large, developing ovaries than in birds that have been on long days for some time. It is noted that most of the plasma samples had concentrations of progesterone that were below the sensitivity limits of the assay.

Faecal progesterone concentrations varied between males and females, but not between photoperiods. Higher concentrations in females than males, and higher concentrations in females held on long days were expected. Females had significantly higher faecal concentrations than males and there was a trend towards long day females having the highest concentrations of the three groups. That this was found in faecal but not in plasma measurements may be related to the fact that steroids are more concentrated in faeces than in plasma (Peter *et al.*, 1996). Faecal concentrations of progesterone were similar to pregnanediol-3α-glucuronide concentrations reported by Bishop and Hall

(1991), although in that study higher concentrations of pregnanediol-3α-glucuronide were reported in female birds held on long days (up to 204 ng/g). This is consistent with the presence of free steroids in lower amounts in droppings than conjugated steroids.

Within both female groups there was considerable variation between birds. In the female short day group there was one bird with a developed ovary (Figure 2.9:A, bird 8), and in the female long day group there was an individual with an undeveloped ovary (Figure 2.9:B, bird 3). In contrast with testosterone, these birds did not have significantly different faecal hormone concentrations from other birds in their groups. The coefficient of variation for random individual droppings was similar to that for pooled samples, suggesting that a single dropping is adequate for determining progesterone concentrations during a three-hour sampling period.

There were significant relationships between plasma and faecal concentrations of progesterone and ovary weight, although a better relationship would have been expected if there had been a greater range of plasma and faecal concentrations. This limited range of hormone concentrations may also explain the absence of a relationship between plasma and faecal progesterone.

2.5 Conclusion

The aims of this study were to:

- Adapt the faecal extraction method currently used in the laboratory, for use with avian faecal samples.

- Validate this faecal extraction method in Japanese quail for three reproductive steroids (testosterone, oestradiol and progesterone).

- Determine if the relationships between steroid concentration and gonad size are similar for plasma and faecal samples.

- Determine the reliability of individual faecal samples for the assessment of plasma hormone concentrations.

Quail faecal samples are best freeze-dried and ground to an homogenous texture in a centrifugal grinder. In summary, shaking 0.05 g of sample with 2.5 ml of ethanol, followed by a 1.25 ml rinse, reconstituting in 800 µl of PBSG with a 200 µl rinse the following day yielded the highest extraction efficiency. Mean extraction efficiencies can be calculated for groups with different expected hormone concentrations for oestradiol and testosterone, but individual efficiencies are required to be calculated for progesterone due to greater variation between samples.

No relationship was found between plasma and faecal steroid hormone concentrations. The main reason for this was a limited range of plasma steroid concentrations. Therefore, the relationship between faecal steroid measurements and gonadal development and the reliability of individual faecal samples to reflect plasma hormone concentrations remain unclear. These data suggest that the strain of Japanese quail used in this experiment require manipulation of both photoperiod and temperature to ensure birds with regressed gonads and low reproductive hormone concentrations are produced. In addition, diurnal variations in hormone concentrations may have been

influencing steroid measurements. Individual faecal samples appear to be as reliable as pooled 3-hour samples for determining steroid concentration.

The results from this study were used to plan a further study of plasma – faecal steroid relationships using birds held on different photoperiods and temperatures, with more extensive sample collection protocols.

Male Japanese quail, photo: W.H. Chua

3 Plasma and faecal steroids and gonadal function in Japanese quail

3.1 Introduction

The measurement of reproductive steroids in plasma is commonly used in studies of reproductive function in birds. However, the collection of plasma samples, in particular from wild birds, with the frequency and regularity required is often impractical and invasive. The development of faecal steroid analysis has enabled the study of reproductive function in endangered species, which was not considered feasible in the past. In order to explore the reproductive physiology of kakapo (*Strigops habroptilus*) by evaluating annual faecal reproductive steroid cycles in relation to breeding status, faecal steroid measurements must be validated. The validation of faecal steroid measurements is important to ensure that concentrations measured in faeces reflect circulating plasma concentrations.

The previous chapter described a study in which clear plasma – faecal steroid relationships for oestradiol, progesterone and testosterone in Japanese quail (*Coturnix coturnix japonica*) were not established, although a robust extraction method was developed. In the previous study, groups of birds were kept on short and long day photoperiods, at an ambient temperature of 20 - 26°C. Most birds on long photoperiods had developed gonads in comparison to those held on a short day photoperiod and most birds on short photoperiods did not undergo gonadal growth. Marked differences in gonad size between photoperiods were not accompanied by corresponding differences in plasma steroid concentrations. This meant that clear plasma – faecal steroid relationships could not be established across a range of plasma concentrations. To ensure birds with regressed gonads and low reproductive hormone concentrations are produced, both short days and low ambient temperature (8-10°C) may be required. This has been shown in some strains of quail in Japanese laboratories (Oishi and Konishi, 1978; Wada *et al.*, 1990). However, other strains of quail, such as the Follett line of quail, which is currently maintained at the Roslin Institute in Edinburgh, will maintain small gonads on short days without low temperatures.

The results of the previous study were used to plan a second study of the relationships between plasma and faecal steroid concentrations in quail. In the second study, both photoperiod and temperature rather than photoperiod alone were controlled. Other changes from the first study included the collection of blood samples at 0900 rather than 1200 to reduce the possibility that diurnal variation could affect plasma steroid concentrations (Doi *et al.*, 1980; Gulati *et al.*, 1981). In the previous chapter, the total faecal hormone content (ng) of individual droppings increased, as expected, with increasing dropping weight. The effect of dropping size was avoided by expressing the faecal hormone concentration as ng steroid/g dried dropping. In this study, all droppings for 24 hours were collected, so total faecal hormone production over 24 hours (ng) could be calculated in addition to hormone concentrations. Although not always practical in the field situation, expressing total hormone production over 24 hours avoids problems that diurnal fluctuations in steroid concentrations can create. This is especially relevant when faecal steroid analysis is used in diagnostic situations where the precise detection of small changes in hormone concentrations is required (e.g. detection of ovulation).

In the three studies that have successfully validated faecal gonadal steroids against the parent steroid in plasma (Bishop and Hall, 1991; Cockrem and Rounce, 1994; Ishii *et al.*, 1994), the latter two used individual faecal samples as opposed to pooled samples. Cockrem and Rounce (1994) also reported that when a mean faecal hormone concentration was used instead of an individual value, the relationship between plasma and faecal steroid did not improve. In the previous chapter a comparison between steroid concentration of individual dropping and a mean value of the collection period showed that an individual dropping was as reliable as a pooled value for measuring a birds' hormone concentration. In this study a similar comparison was made, although instead of collecting individual droppings over a three-hour period, 24-hour collections over five days were made. In addition, three plasma samples were collected over the period of five days to enable the comparison of faecal : plasma relationships for individual and multiple samples from each bird.

In recent years, the most common reason for using avian faecal gonadal steroid measurement techniques has been to evaluate reproductive function or gonadal activity

in individual birds (for example: Bercovitz *et al.*, 1982; Bishop and Hall, 1991; Kofuji *et al.*, 1993; Cockrem and Rounce, 1994; Ishii *et al.*, 1994; Cockrem and Rounce, 1995; Kikuchi *et al.*, 1996; Hirschenhauser *et al.*, 1999a, 1999b; Sockman and Schwabl, 1999; Hirschenhauser *et al.*, 2000; Kotrschal *et al.*, 2000). It would be useful for free living birds to be able to predict gonad size from faecal hormone concentrations. However, concentrations of one steroid may not be sufficient to distinguish between birds of different gonad size. Therefore a method was developed in the current study to use concentrations of several hormones together to predict gonad size in birds.

The aims of this study were to:

- Validate a faecal extraction method developed in the previous chapter in Japanese quail for testosterone, oestradiol and progesterone.

- Determine if the relationships between steroid concentration and gonad size are similar for faecal and plasma samples.

- Determine if the magnitude of variation within and between birds is similar for faecal and plasma steroid concentrations.

- Determine the reliability of 24-hour faecal samples for estimating plasma hormone concentrations.

- Determine if faecal steroid concentrations can be used to accurately predict reproductive status, and how this compares to the prediction from plasma steroid concentrations.

3.2 Methods

3.2.1 Birds

Male and female Japanese quail were purchased from Rangitikei Game Birds Ltd (Bulls, Manawatu, New Zealand). Birds were housed individually in custom-made cages, in photoperiod and temperature controlled rooms in the Veterinary Science building at Massey University, Palmerston North, New Zealand. Food (quail pellet layer mash, Feed Processing Unit, Massey University) and water were supplied *ad libitum*.

3.2.2 Experimental design

Birds were divided into four groups depending on sex and photoperiod, with ten birds in each group. One group of males and one group of females were purchased at six weeks of age and held on a long day photoperiod (16h light: 8h dark; lights on from 0800 - 1600) at 20°C until 8 weeks of age to allow time for gonadal development in response to photoperiod. The remaining two groups, one of females and one of males were purchased at three weeks of age. The males were held on long days at 20°C, with sampling beginning on the day after arrival to ensure they had undeveloped testes. The females were held on a short day photoperiod (8h light: 16h dark; lights on from 0800 – 0000) at 10°C, until they reached 6.5 weeks of age to ensure undeveloped oviducts and ovaries. 24-hour faecal samples were collected for five days, and three plasma samples were collected over the five-day period. This study was approved by the Massey University Animal Ethics Committee.

3.2.3 Sample collection

3.2.3.1 Faeces

All droppings produced by each bird over a 24-hour period starting at 0900 were collected for five consecutive days. Samples were collected at 1300, 1700 and 0900 the following day. The samples were then pooled to make one 24-hour sample. Droppings were collected from a plastic sheet placed on top of an aluminium tray under each cage. Any droppings that did not fall through the bottom of the cage were pushed through.

Droppings were collected into pre-weighed zip-lock plastic bags, weighed and kept at 4°C until the end of the 24-hour collection period. Samples were then pooled and frozen at -20°C until extraction. Wet weight was measured and recorded for each sample.

3.2.3.2 Plasma samples

Blood samples were collected at 0900 hours - one, three and five days after the commencement of faecal collection. Samples collected after one and three days were taken by puncture of the brachial vein and blood was collected into heparinised capillary tubes. All samples were collected within three minutes from the time the bird was removed from its cage. Terminal samples after the fifth day were collected by decapitation (after stunning) and blood collected into heparinised centrifuge tubes. Blood was expelled from the capillary tubes into heparinised 1 ml polystyrene test tubes (BDH) and kept on ice for up to 1.5 hours until centrifugation. Samples were centrifuged at 1 900 g for 15 minutes (Beckman GS-6R refrigerated centrifuge). Plasma was removed with a glass Hamilton syringe and stored at –70°C in 1 ml polypropylene titre tubes until assay.

3.2.3.3 Reproductive system

Gonads were removed when the birds were killed after the fifth day of sampling. In males individual testis weight was recorded and in females the oviduct and ovary weight and diameter of the five largest ovarian follicles were recorded. Tissue was then stored in formal saline for future reference.

Egg production in females and the presence of cloacal foam under the cages of males was recorded daily from arrival. Birds were weighed and the diameter of the cloaca (females) or the area of the cloacal gland (males) measured on the first and the final days of the sampling period.

3.2.4 Steroid extraction from plasma

Plasma samples were prepared by the same method as previously described in section 2.2.4. Extraction efficiency was determined for testosterone and oestradiol for each of the four experimental groups. The average values for the four groups (short day females, long day immature males*, long day females, long day males) were 98.3 ± 3.7%, 97.8 ± 2.8%, 89. 7 ± 4.1%, and 93.6 ± 3.8% respectively for testosterone (n=10) and 75.9 ± 3.0%, 75.6 ± 4.2%, 60.5 ± 9.5% and 75.0 ± 6.9% respectively for oestradiol. Mean extraction efficiencies were used for the calculation of testosterone and oestradiol concentrations in pooled samples. Progesterone extraction efficiencies were determined for each sample due to high variation between samples (means for each group were 55.1 ± 17.4%, 59.4 ± 8.9%, 61.3 ± 11.6%, and 62.5 ± 12.3%).

3.2.5 Steroid extraction from faecal samples

Faecal samples were prepared using a method developed in the previous chapter (section 2.2.5.4). Frozen faecal samples were lyophilised (Cuddon 0610 Freeze Drier) for 70 hours. Samples were ground to a powder using a centrifugal grinder (RETCH 2M100; 0.25 sieve). All samples from each group were ground in succession, and within each group, samples from each bird were ground in succession. Between birds, the grinder sieve, blade and collection tray were brushed clean and between groups all removable parts were washed and decontaminated with 90% ethanol. 0.05 g of dried sample was added to 2.5 ml of 90% absolute ethanol (AnalaR BDH, Poole, England) in a glass screw-top tube (13 x 100 mm). Tubes were capped, vortexed for 30 seconds and shaken on their side for one hour on an orbital shaker (Chiltern Scientific SS70). Tubes were then removed and refilled to the 2.5 ml level before being capped and centrifuged at 1 000 g (Haraeus Christ 5000S refrigerated centrifuge) for 20 minutes at room temperature. The supernatant was pipetted into a second glass screw-top test tube. The pellet was rinsed with a further 1.25 ml of 90% ethanol, vortexed for 30 seconds and re-centrifuged at 1 000 g for 20 minutes at room temperature. The supernatant was removed and added to the previous aliquot. The faecal pellet was discarded. The ethanol

* These immature males were sampled immediately upon arrival as opposed to the true LD males, which were held for a period of time on long days.

was completely evaporated from the supernatant by placing test tubes in a heating block at 37°C, under a stream of air. The sides of the tubes were rinsed with 90% ethanol twice during this process to ensure that the final level of extract residue was below the level of 1 ml. Extracts were left to cool and reconstituted in 0.8 ml of phosphate buffered saline gel (PBSG; 0.1M, pH 7.0, 0.1% gelatin), gently vortexed for 30 seconds and refrigerated overnight at 4°C. The following day, tubes were vortexed for 30 seconds and the extracts were removed into 1.5 ml polypropylene eppendorf tubes. The extraction tube was rinsed with a further 0.2 ml of PBSG, vortexed and the extract added to the previous aliquot. Extracts were centrifuged for 10 minutes at 14 000 g (IEC Micromax ventilated microcentrifuge), and the supernatant was transferred into a 1.0 ml polypropylene titre tube and stored at –20°C until assay.

Extraction efficiencies were obtained for testosterone, oestradiol and progesterone by the same procedure as described in section 2.2.5.3.

Extraction efficiency was determined for testosterone and oestradiol for each of the four experimental groups. The average values for the four groups (short day females, long day immature males*, long day females, long day males) were 89.8 ± 6.0%, 79.5 ± 7.1%, 69.6 ± 6.9%, and 75.7 ± 7.0% respectively for testosterone (n=10) and 84.0 ± 10.6%, 61.1 ± 10.0%, 53.5 ± 14.7% and 66.4 ± 12.0% respectively for oestradiol. Progesterone extraction efficiencies were determined for each sample due to high variation between samples (means for each group were 67.9 ± 19.5%, 45.1 ± 17.6%, 40.5 ± 14.8%, and 51.5 ± 18.6%).

Each batch of samples extracted also included quality control samples. Two screw-top test tubes containing ethanol but no dried dropping were 'extracted' in exactly the same manner as above. A 100 µl aliquot of the final supernatant was used to determine a total count for the extraction.

* These immature males were sampled immediately upon arrival as opposed to the true LD males, which were held for a period of time on long days.

3.2.6 Radioimmunoassay of testosterone

Testosterone concentrations in faecal and plasma extracts were measured by radioimmunoassay, as described in section 2.2.6. Faecal extracts were used at a 1:30 dilution prepared in PBSG.

The sensitivity of each assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for testosterone was 0.36 ng/ml in plasma and 9.27 ng/g in faeces.

3.2.6.1 Intra- and inter-assay variation

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 509.7 ± 17.8 pg/ml, 136.8 ± 5.4 pg/ml, and 36.9 ± 2.2 pg/ml respectively.

Faecal and plasma samples were randomly assigned between five assays for testosterone. Inter-assay coefficients of variation for high, medium and low quality controls were 11.0%, 11.0% and 14.0% respectively. Intra-assay coefficients of variation were determined by conducting an assay with 20 duplicates of each quality control. Intra-assay coefficients of variation for testosterone for low, medium and high quality controls were 21.1%, 12.5% and 8.4% respectively.

3.2.6.2 Parallelism and hormone recovery

Serial dilutions of extracted quail plasma and faecal samples in assay buffer (PBSG) were parallel to the testosterone standard curve (Figure 3.1). The recoveries of testosterone added to two faecal samples were 98.4 ± 4.5% and 101.1 ± 3.3%. The recoveries of testosterone added to three plasma samples were 97.4 ± 1.7%, 99.1 ± 8.1% and 102 ± 2.7%.

3.2.7 Radioimmunoassay of oestradiol

Oestradiol 17-β (oestradiol) concentrations in faecal and plasma extracts were measured by radioimmunoassay, as described in section 2.2.7. Faecal extracts were used at a 1:4 dilution prepared in PBSG.

The sensitivity of each assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for oestradiol was 63.0 pg/ml in plasma and 2.96 ng/g in faeces.

3.2.7.1 Intra- and inter-assay variation

Solutions of oestradiol 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 2292.6 ± 108.1 pg/ml, 549.3 ± 25.2 pg/ml and 139.7 ± 7.7 pg/ml respectively.

Faecal and plasma samples were randomly assigned between five assays for oestradiol. Inter-assay coefficients of variation for low, medium and high quality controls were 19.0%, 10.2% and 13.2% respectively. Intra-assay coefficients of variation were determined by conducting an assay with 20 duplicates of each quality control. Intra-assay coefficients of variation for oestradiol for low, medium and high quality controls were 29.4%, 9.5% and 11.2% respectively.

3.2.7.2 Parallelism and hormone recovery

Serial dilutions of extracted quail plasma and faecal samples in assay buffer (PBSG) were parallel to the oestradiol standard curve (Figure 3.1). Recovery of oestradiol added to one faecal sample was 95.5 ± 4.8%. The recoveries of oestradiol added to three plasma samples were 90.8 ± 8.8%, 92.9 ± 8.9% and 92.3 ± 13.3%.

3.2.8 Radioimmunoassay of progesterone

Progesterone concentrations in faecal and plasma extracts were measured by radioimmunoassay, as described in section 2.2.8. Faecal extracts were used at a 1:2 dilution prepared in PBSG.

The sensitivity of each assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for progesterone was 0.48 ng/ml in plasma and 4.14 ng/g in faeces.

3.2.8.1 Intra- and inter-assay variation

Solutions of progesterone 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 2085.8 ± 143.9 pg/ml, 572.6 ± 54.3 pg/ml and 156.9 ± 20.8 pg/ml respectively.

Faecal and plasma samples were randomly assigned between five assays for progesterone. Inter-assay coefficients of variation for low, medium and high quality controls were 13.1%, 17.3% and 17.5% respectively. Intra-assay coefficients of variation were determined by conducting an assay with 20 duplicates of each quality control. Intra-assay coefficients of variation for progesterone for low, medium and high quality controls were 12.8%, 5.6% and 7.6% respectively.

3.2.8.2 Parallelism and hormone recovery

Serial dilutions of extracted quail plasma and faecal samples in assay buffer (PBSG) were parallel to the progesterone standard curve (Figure 3.1). The recoveries of progesterone added to two faecal samples were 100.6 ± 6.4% and 103.6 ± 13.0%. The recoveries of progesterone added to three plasma samples were 90.9 ± 3.8%, 93.3 ± 3.6% and 90.6 ± 3.1%.

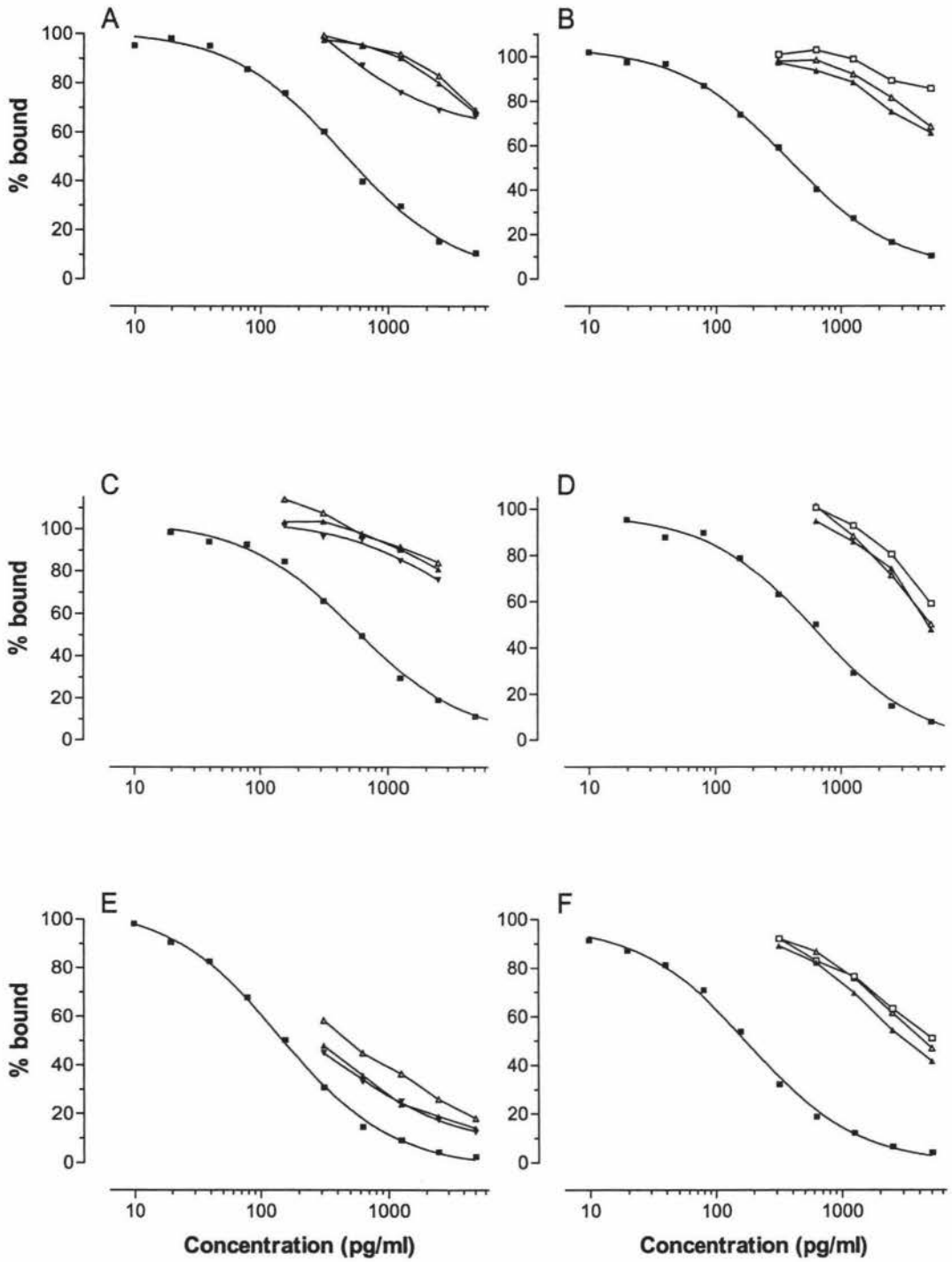

Figure 3.1. Parallelism demonstrated for quail faecal and plasma samples. A: faecal oestradiol, B: plasma oestradiol, C: faecal progesterone, D: plasma progesterone, E: faecal testosterone, F: plasma testosterone. The curves with filled squares are the standard curves and all other curves are samples.

3.2.9 Statistical analyses

Statistical analyses were performed using SPSS version 11.0.0 (2001; SPSS Inc). Where appropriate the following analyses were conducted on normally distributed data with homogeneous variances (as determined by Levene's Test or F-max for repeat measures tests): independent samples t-test, paired T-test, one way ANOVA with Bonferroni post hoc comparisons, and Pearson correlation. Data were transformed by $\log_{10}$ or square-root as appropriate to increase homogeneity for parametric analysis. Where parametric tests were not suitable, Kruskal-Wallis, Friedman's, or Mann-Whitney U test non-parametric analyses were performed. Data are presented as individual values or as mean ± S.E.

Canonical discriminant analysis (CDA) is a multivariate statistical technique that identifies and summarises important differences among groups. It enables the identification of complex relationships among many variables (Cruz-Castillo *et al.*, 1994). Dimensionality of the data set (number of variables being considered) is reduced by linear functions that are derived from the original data which separate the groups, while maintaining the group structure and keeping the variation within the groups small (Hair *et al.*, 1987). The linear functions are of the form:

$$z = ax_1 + bx_2 + cx_3.... \qquad \text{(Equation 3.1)}$$

Where a, b and c are standardised canonical coefficients (SCC) whose sign and magnitude reflect the contribution of the original variables (x_1, x_2 and x_3) to the discriminating function (z). CDA is appropriate when it is important to separate known groups or *a priori* groupings (Cruz-Castillo *et al.*, 1994), as in this study.

Once developed, CDA functions can also be used for prediction of group membership for samples not in the original data set. Prediction of group membership is based on the probability of a new sample falling within classification boundaries. Boundaries for group classification are developed on the variation in the discriminant values of the samples within the *a priori* group. Cross validation determines the predictive capability of the discriminant functions. Cross validation was achieved by predicting group

membership of each sample individually from the functions. The number of correctly classified samples was expressed as a proportion of the total number of samples to give the prediction accuracy.

CDA was used to explore relationships between faecal or plasma testosterone, oestradiol and progesterone concentrations and create a predictive model for reproductive status. Data were analysed and cross validated using the discriminant procedure of SPSS.

3.3 Results

3.3.1 Gonad weight

Gonads were collected from nine females and nine males held on long days, ten females held on short days and ten immature males held on long days. Each group was initially made up of ten birds, but two birds were withdrawn due to fatality or poor condition. Females held on long days had a mean ovary weight of 9.26 ± 0.59 g which was significantly heavier than the mean ovary weight of 0.11 ± 0.01 g from females held on short days (Independent samples T test, $t = 15.470$, $df = 17$, $p = 0.001$). Females held on long days had a mean oviduct weight of 9.53 ± 0.44 g which was significantly heavier than the mean oviduct weight of 0.03 ± 0.00 g from females held on short days (Independent samples T test, $t = 21.412$, $df = 17$, $p = 0.001$). All females held on long days had developed ovaries and oviducts and all females held on short days had undeveloped ovaries and oviducts (Figure 3.2). Ovaries from females held on long days showed follicular hierarchies, with mean diameters of 20.20 cm, 18.21 cm, 15.21 cm, 11.38 cm, and 7.63 cm for the five largest yellow follicles.

Males held on long days had a mean paired testis weight of 6.22 ±0.27 g which was significantly heavier than a mean paired testis weight of 0.48 ±0.09 g from males held on short days (Independent samples T test, $t = 21.320$, $df = 17$, $p = 0.001$). All mature males held on long days had developed testes and all immature males had undeveloped testes (Figure 3.2).

3.3.2 Egg laying, cloacal foam and cloacal measurements

Females on long days regularly laid eggs whereas females on short days did not produce any eggs. Females on long days had significantly larger cloacal diameters than females on short days (Paired samples T test, $t = -7.938$, $df = 17$, $p = 0.001$). Over the course of the sampling period, cloacal diameter in females on short days increased (Paired samples T test, $t = 3.870$, $df = 9$, $p = 0.004$; Table 3.1), but body weight did not ($t = 1.339$, $df = 9$, $p = 0.214$). Both cloacal diameter and body weight increased in females on long days ($t = 3.231$, $df = 8$, $p = 0.012$; $t = 3.877$, $df = 8$, $p = 0.005$, respectively).

Mature males on long days had significantly larger cloacal glands than immature males on long days (Paired samples T test, $t = -11.670$, $df = 17$, $p = 0.001$). Males on long days produced cloacal foam each day. One immature male on long days (bird #39) produced cloacal foam on the final three days of its sampling period. Over the course of the sampling period, body weight in both immature and mature males on long days increased (Paired samples T test, $t = 6.725$, $df = 9$, $p = 0.001$; $t = 6.374$, $df = 8$, $p = 0.001$, respectively; Table 3.1), but cloacal gland area did not ($t = 1.586$, $df = 9$, $p = 0.147$; $t = 2.290$, $df = 8$, $p = 0.051$, respectively).

3.3.3 Faecal production

24 hour pooled dried quail droppings ranged from 5.25 g – 17.25 g in weight. Dropping production over 24 hours (g/24hrs) did not differ between females on short and long days and between immature and mature males on long days ($p > 0.05$ in both cases). However, there was an overall difference between groups ($F_{3, 186} = 66.35$, $p = 0.001$) and females produced a greater amount of droppings than males ($p = 0.001$, Figure 3.3). The average amount of water in droppings for each group was 59.89 ± 0.60% of fresh weight for females on short days, 73.54 ± 1.17% for females on long days, 61.30 ± 1.58% for immature males on long days, and 68.56 ± 1.34% for mature males on long days.

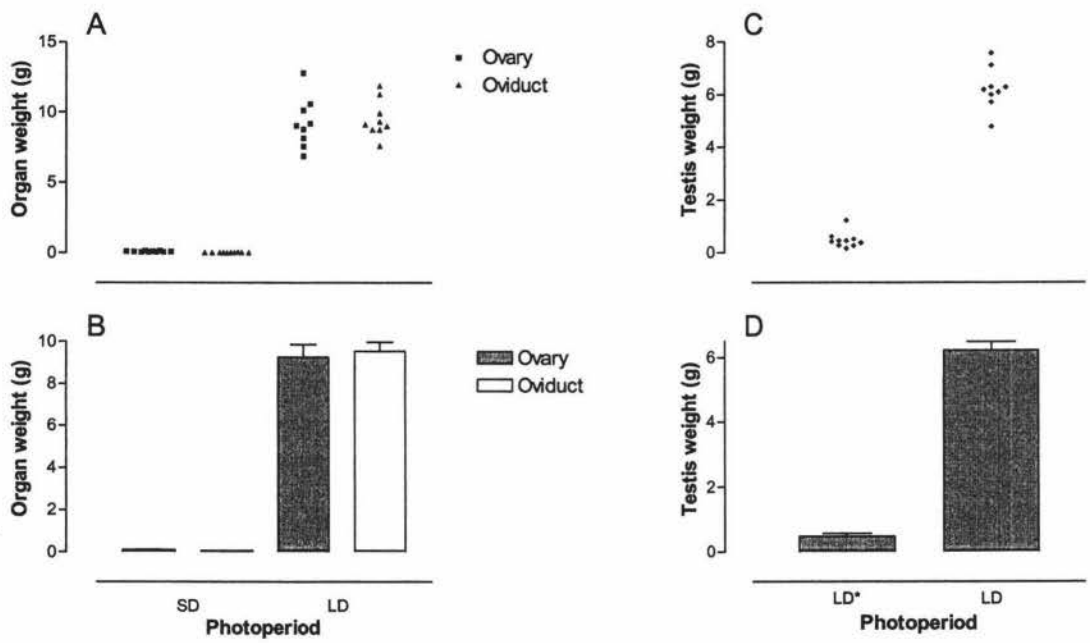

Figure 3.2. Gonad weight in Japanese quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males). A) Ovary and oviduct weight for individual females; B) Mean ovary and oviduct weight for females ± SE; C) Paired testis weight for individual males; D) Mean paired testis weight for males ± SE.

Table 3.1. Body weight and cloacal diameter (females) or cloacal area (males) at the start and at the end of the sampling period. (Mean ± SE; SD = short day, LD = long day, LD* = immature males).

	First sampling day	Final sampling day
SD Female		
Body weight (g)	212.54 ± 6.50	214.71 ± 6.82
Cloacal diameter (cm)	5.30 ± 0.42	6.69 ± 0.41
LD Female		
Body weight (g)	250.82 ± 5.61	260.63 ± 5.85
Cloacal diameter (cm)	10.84 ± 0.60	12.58 ± 0.63
LD* Male		
Body weight (g)	135.54 ± 2.67	164.03 ± 3.51
Cloacal area (cm^2)	64.62 ± 2.24	81.38 ± 8.72
LD Male		
Body weight (g)	218.08 ± 6.90	223.76 ± 7.52
Cloacal area (cm^2)	207.10 ± 8.54	224.67 ± 8.58

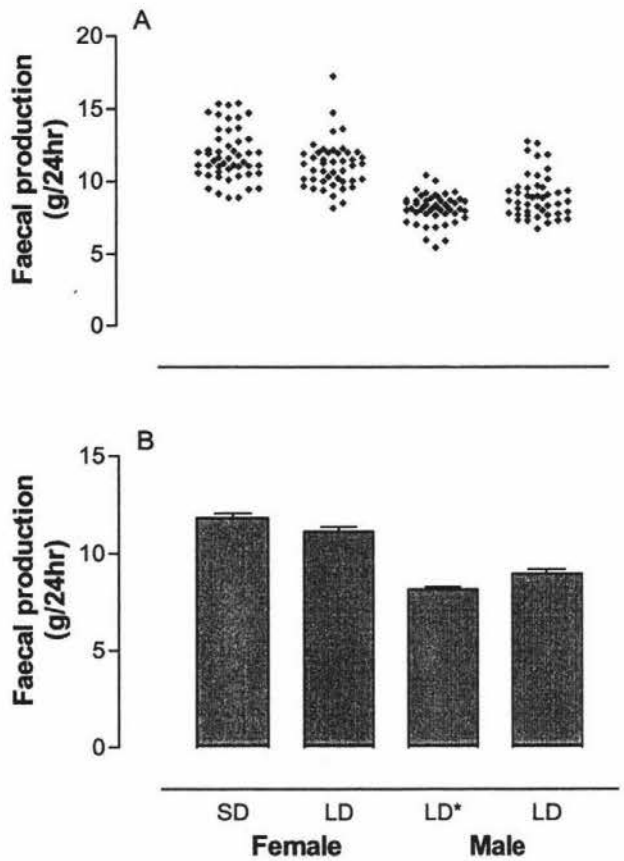

Figure 3.3. Faecal production (g/24hr) in Japanese quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males). A: daily faecal production for individual birds; B: mean faecal production weight ±SE.

3.3.4 Plasma testosterone

Plasma testosterone concentrations across all groups ranged from the least detectable dose of 0.36 ng/ml to 7.95 ng/ml. The ranges of concentrations were much greater for birds on long days than for females on short days and for immature males (Figure 3.4). Plasma testosterone was significantly higher in females on long days than in females on short days, and significantly higher in mature males on long days than in immature males on long days, both for three samples collected from each bird (Figure 3.4) and for single samples (Figure 3.5; see Table 3.2 for statistics). Data from the first plasma sample collected 1 day after the start of the faecal collection period were used for the single day comparisons.

A highly significant relationship was found between plasma testosterone concentrations and paired testis weight over the sampling period of five days. A significant relationship was found for a single day (Figure 3.6 and Table 3.3).

There were no significant differences in plasma testosterone concentration between birds in each group (Figure 3.7, Table 3.4). No significant changes in plasma testosterone were found for any of the groups over the sampling period (Friedman's' non-parametric analyses were inappropriate due to missing values so paired T-tests were conducted for each pairing of days within a group. $p>0.05$ in all cases, Figure 3.8 - Figure 3.12). The coefficients of variation in plasma testosterone concentration for individual birds were 14.5 ± 6.6% in females on short days, 45.3 ± 8.3 % in females on long days, 27.3 ± 4.9 % in immature males on long days and 49.4 ± 11.7 % in mature males on long days (Figure 3.8 - Figure 3.11). Variation in plasma testosterone concentrations between birds was examined using the first plasma sample for each bird and also using the mean of the three samples for each bird as a data point (Table 3.5). The coefficients of variation between birds did not differ greatly between individual or combined values for females or mature males on long days or females on short days. The coefficient of variation was considerably higher for a pooled five-day sample for immature males on long days.

3.3.5 Faecal testosterone

Faecal testosterone concentrations across all groups ranged from the least detectable dose of 9.27 to 380.60 ng/g (Figure 3.4). Total faecal testosterone production over 24 hours ranged from 95.81 ng to 4 535.00 ng (Figure 3.13). Faecal testosterone concentrations were significantly higher in females on long days than in females on short days, and significantly higher in mature males on long days than in immature males on long days, across the sampling period of five days and for a single day (Table 3.2). Faecal testosterone production followed a similar pattern (Figure 3.14 and Figure 3.13). Data from the second faecal sample were used for the single day comparisons.

Highly significant relationships were found between faecal testosterone concentrations and paired testis weight over the sampling period of five days and for a single day (Figure 3.15 and Table 3.3).

There were significant differences in faecal testosterone concentration between birds in each group (Figure 3.16, Table 3.6). No significant changes in faecal testosterone were found for females or mature males on long days over the sampling period (Figure 3.18, Figure 3.20 and Figure 3.21; Friedman's' non-parametric analyses, $t = 7.022$, $p = 0.135$; $t = 2.044$, $p = 0.728$ respectively). However, a slight increase from the beginning of the sampling period to the end was significant for females on short days and a more pronounced increase was significant for immature males on long days (Figure 3.17, Figure 3.19 and Figure 3.21; Mann – Whitney tests, $U = 20.000$, $p = 0.023$; $U = 7.000$, $p = 0.001$ respectively; Figure 3.17 - Figure 3.21). The coefficients of variation in faecal testosterone concentration for individual birds were 36.3 ± 5.9% in females on short days, 13.8 ± 1.2% in females on long days, 30.7 ± 3.4% in immature males on long days and 9.8 ± 1.2% in mature males on long days (Figure 3.17 - Figure 3.20). The coefficients of variation between birds were slightly lower for five-day pooled than for individual faecal samples (Table 3.7).

3.3.6 Relationship between plasma and faecal testosterone

Highly significant correlations were found between plasma and faecal testosterone over the sampling period of five days and for a single day, for both faecal concentration and production (Figure 3.22, Table 3.8). The correlations were lower for data from a single day compared with data from five days. The single day correlation was conducted for plasma samples collected one day after the start of the faecal collection, and faecal samples from the second day. Table 3.8 summarises the relationships found within each group between plasma and faecal concentration and the relationships between plasma concentrations and faecal production for five days and for a single day. All relationships within each group were insignificant, except between plasma and faecal testosterone concentration in mature long day males for a single day ($r = 0.6688$, $n = 10$, $p = 0.0345$).

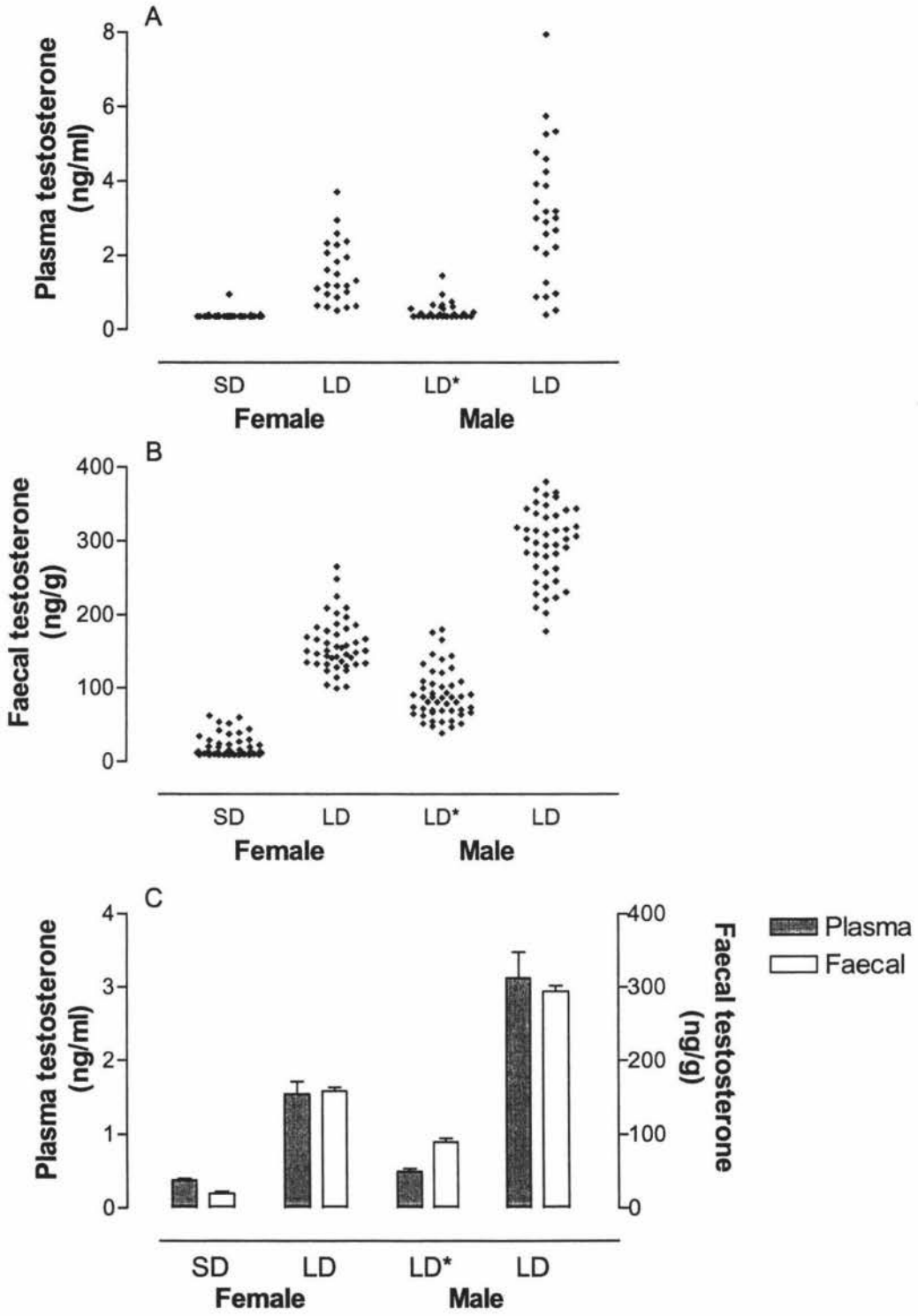

Figure 3.4. Plasma and faecal testosterone concentrations (SD = short day, LD = long day, LD* = immature males). A) Individual plasma testosterone concentrations for quail held on different photoperiods (n = 29, 24, 29 and 26 for each group respectively). B) Individual faecal testosterone concentrations for quail held on different photoperiods (n = 50, 45, 50 and 45 for each group respectively). C) Mean plasma and faecal testosterone concentrations ± SE.

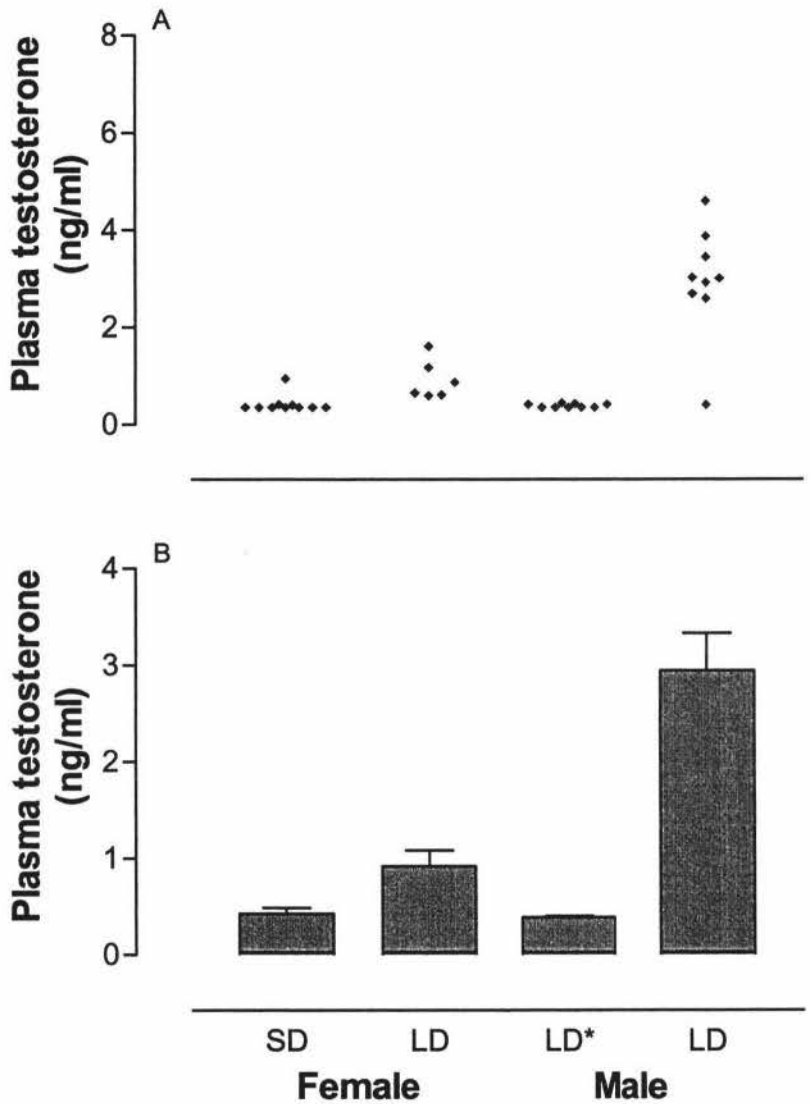

Figure 3.5. Plasma testosterone concentrations on one day (first plasma sample, SD = short day, LD = long day, LD* = immature males). A) Individual plasma testosterone concentrations for quail held on different photoperiods (n = 10, 6, 9 and 9 for each group respectively). B) Mean plasma testosterone concentrations ± SE.

Table 3.2. Summary of one-way ANOVA of plasma and faecal hormone concentrations across birds grouped by photoperiod. Multiple comparisons were made using Bonferroni post-hoc analysis (SD = short day, LD = long day, LD* = immature males).

	Testosterone			Oestradiol			Progesterone		
Effect	**F**	**df**	**P**	**F**	**df**	**P**	**F**	**df**	**P**
Plasma 5 days	32.436	3,34	0.001	30.952	3,34	0.001	43.614	3,34	0.001
Comparison between groups									
Female SD vs LD			0.002			0.001			0.001
Male LD* vs LD			0.001			1.000			1.000
Plasma 1 day	36.923	3,30	0.001	10.405	3,25	0.001	15.567	3,25	0.001
Comparison between groups									
Female SD vs LD			0.015			0.001			0.001
Male LD* vs LD			0.001			1.000			1.000
Faecal 5 days	151.652	3,34	0.001	189.478	3,34	0.001	42.968	3,34	0.001
Comparison between groups									
Female SD vs LD			0.001			0.001			0.001
Male LD* vs LD			0.001			0.746			0.085
Faecal 1 day	160.995	3,34	0.001	51.235	3,34	0.001	23.251	3,34	0.001
Comparison between groups									
Female SD vs LD			0.001			0.001			0.001
Male LD* vs LD			0.001			0.022			0.017
Faecal production/24hr 5 days	104.013	3,34	0.001	81.113	3,34	0.001	24.655	3,34	0.001
Comparison between groups									
Female SD vs LD			0.001			0.001			0.001
Male LD* vs LD			0.001			0.128			1.000

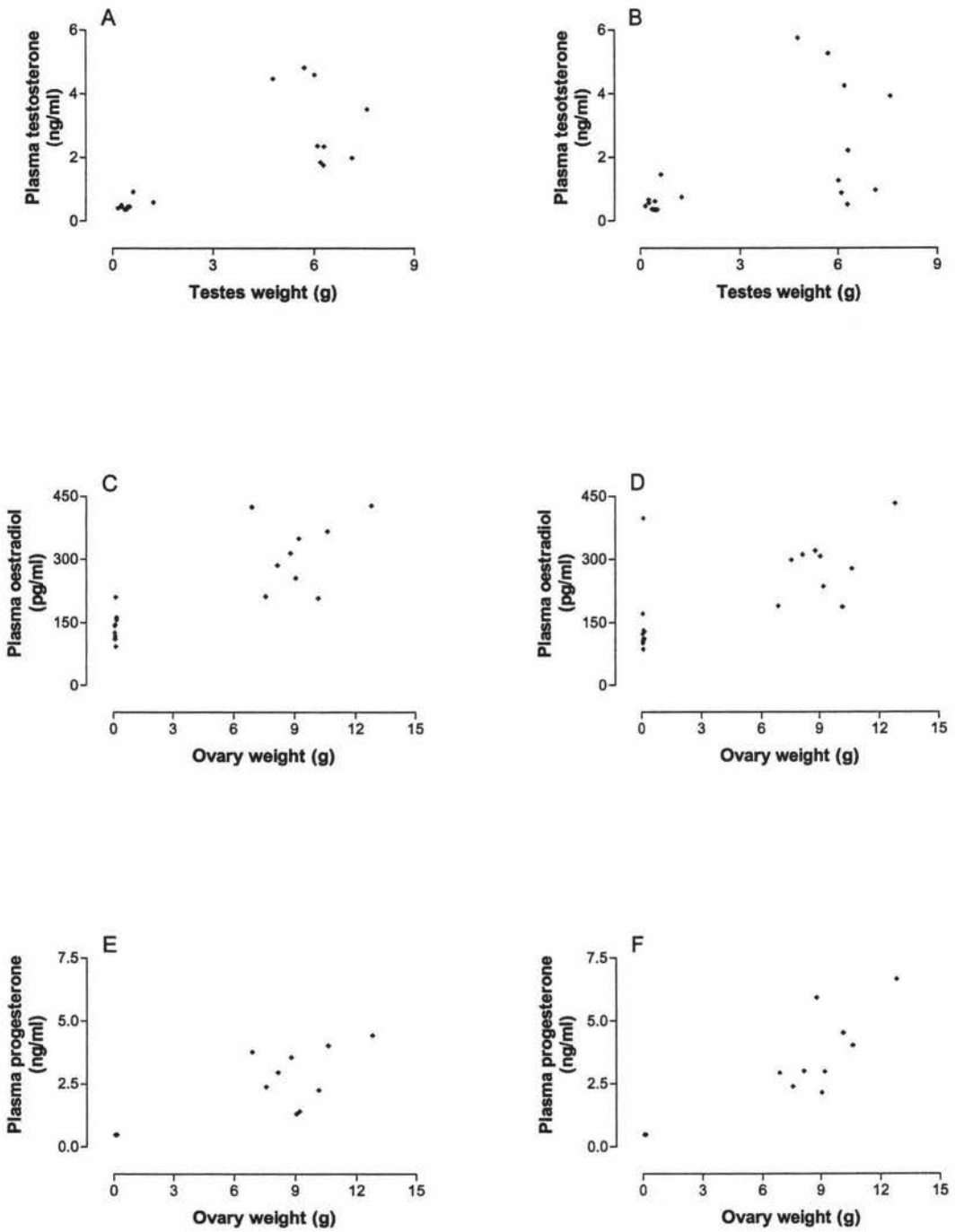

Figure 3.6. Relationships between gonad weight and plasma hormone concentrations. A) Paired testis weight versus mean plasma testosterone over five days. B) Paired testis weight versus mean plasma testosterone for one day. C) Ovary weight versus mean plasma oestradiol over five days. D) Ovary weight versus mean plasma oestradiol for one day. E) Ovary weight versus mean plasma progesterone over five days. F) Ovary weight versus mean plasma progesterone for one day. The mean of three plasma samples for each bird was used for the correlation over five days, n = 19 in all cases, correlation coefficients are summarised in Table 3.3.

Table 3.3. Relationships between gonad weight and plasma and faecal hormone concentrations in males (paired testis weight and testosterone) and females (ovary weight and oestradiol or progesterone). The means of three plasma and five faecal samples for each bird were used for the correlation over five days (n = 19 in all cases). All differences were highly significant ($p<0.01$), except one significant difference ($p<0.05$), marked with an asterisk (*).

	Plasma r		Faecal r	
	5 days	1 day	5 days	1 day
Testosterone	0.78	0.57*	0.95	0.90
Oestradiol	0.84	0.69	0.96	0.91
Progesterone	0.87	0.92	0.97	0.86

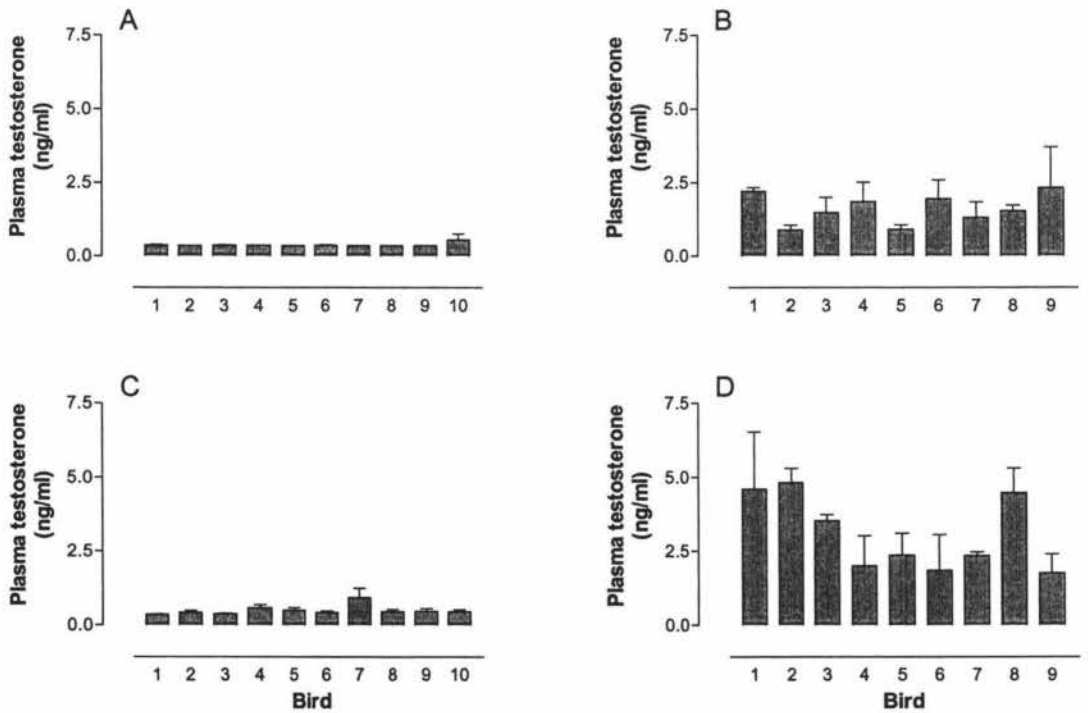

Figure 3.7. Mean plasma testosterone concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) immature males held on a long day photoperiod, D) males held on long day photoperiod.

Table 3.4. Summary of one-way ANOVA for comparisons of plasma hormone concentrations between birds in each treatment group (SD = short day, LD = long day, LD* = immature males sampled on arrival). All plasma progesterone concentrations for SD female were at the least detectable dose.

	Testosterone			Oestradiol			Progesterone		
Group	**F**	**df**	**P**	**F**	**df**	**P**	**F**	**df**	**P**
SD Female	0.882	9,19	0.557	0.815	9,17	0.610			
LD Female	0.845	8,15	0.579	1.471	8,13	0.258	0.832	8,9	0.597
LD* Male	1.695	9,19	0.159	0.739	9,12	0.670	0.645	9,14	0.743
LD Male	1.795	8,17	0.148	0.838	8,6	0.603	0.647	8,14	0.728

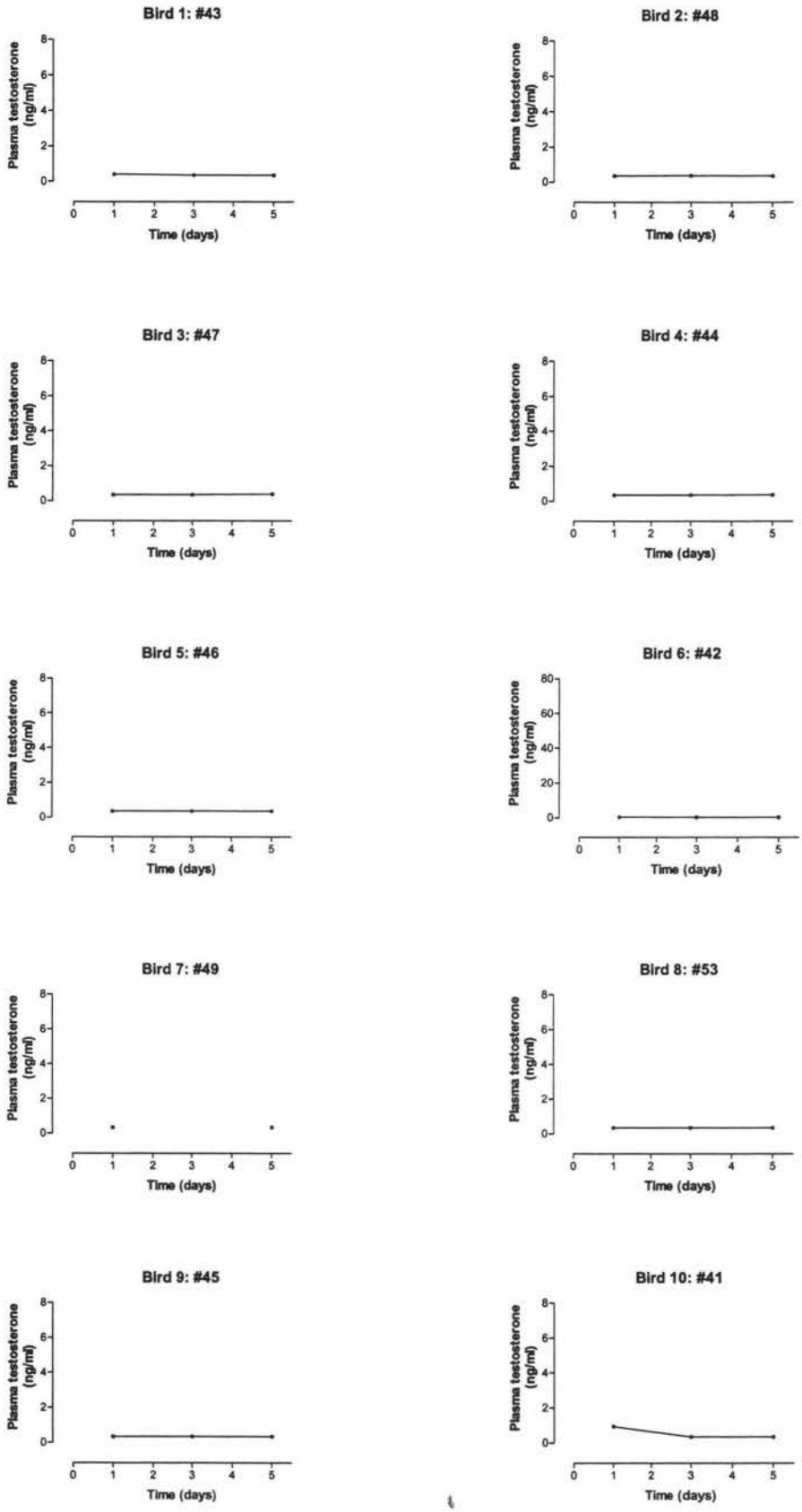

Figure 3.8. Changes in plasma testosterone concentrations over time in individual females on short days. Samples were collected one, three and five days after the start of the faecal collections.

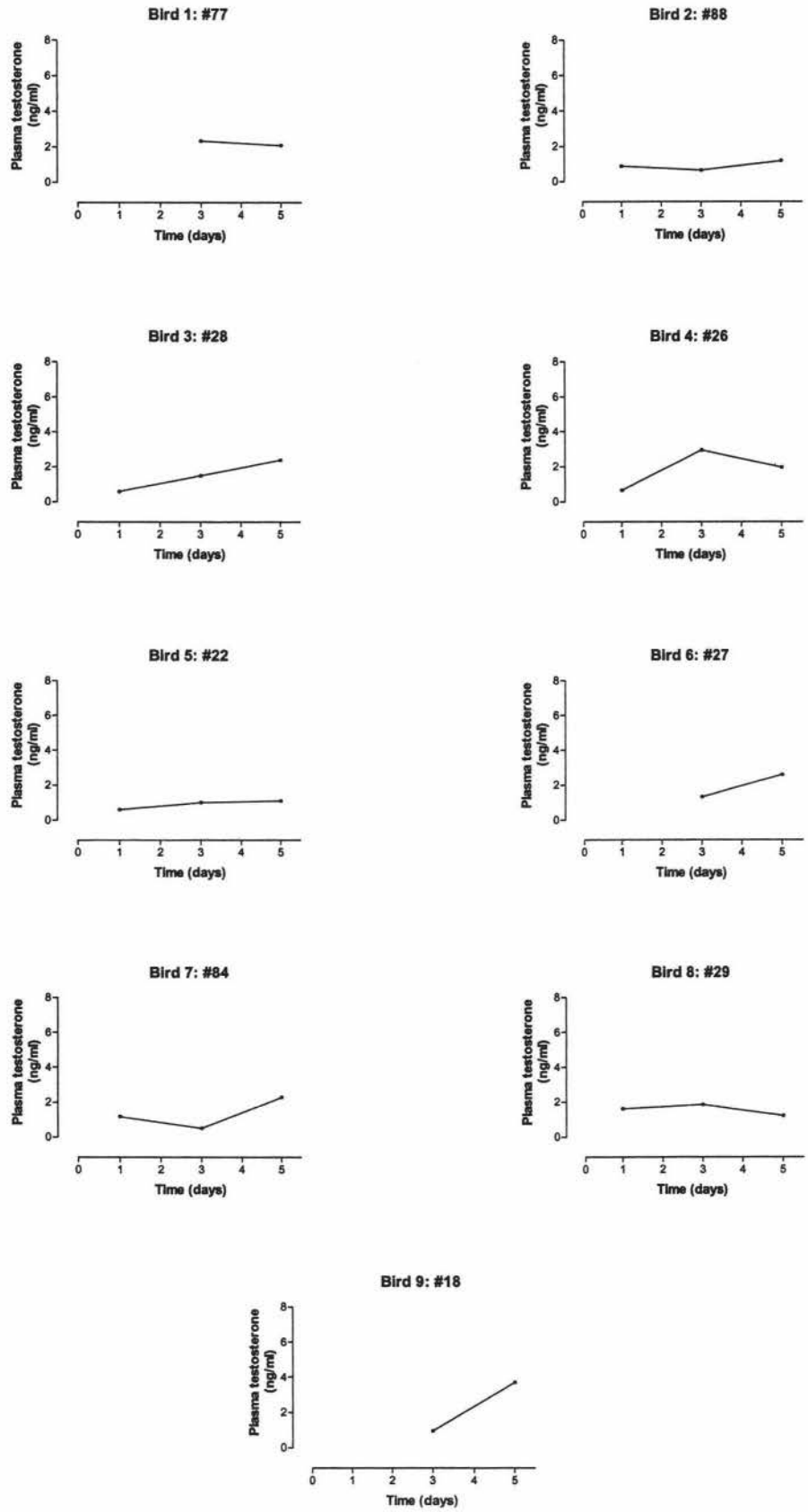

Figure 3.9. Changes in plasma testosterone concentrations over time in individual females on long days. Samples were collected one, three and five days after the start of the faecal collections.

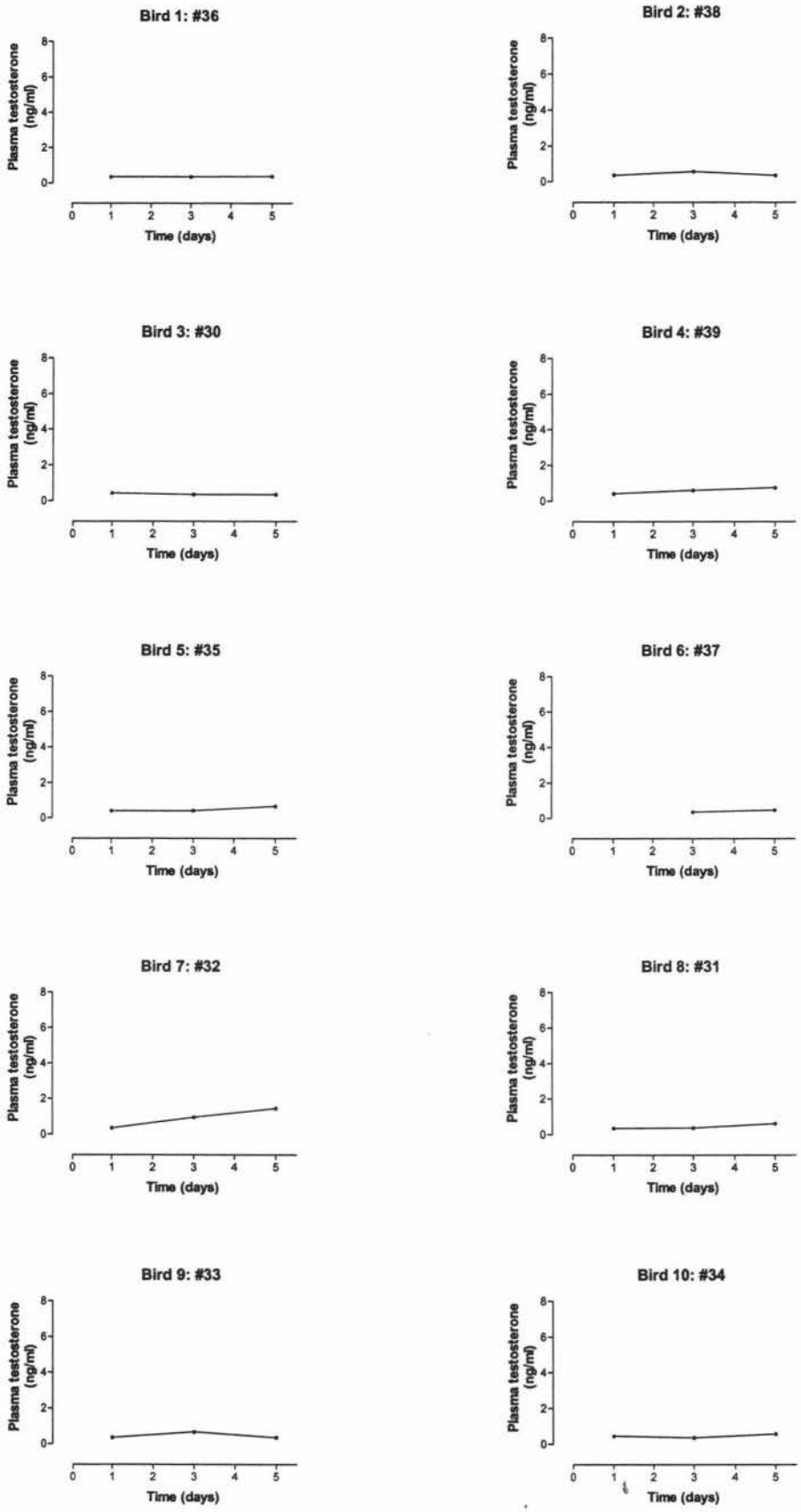

Figure 3.10. Changes in plasma testosterone concentrations over time in individual immature males on long days. Samples were collected one, three and five days after the start of the faecal collections.

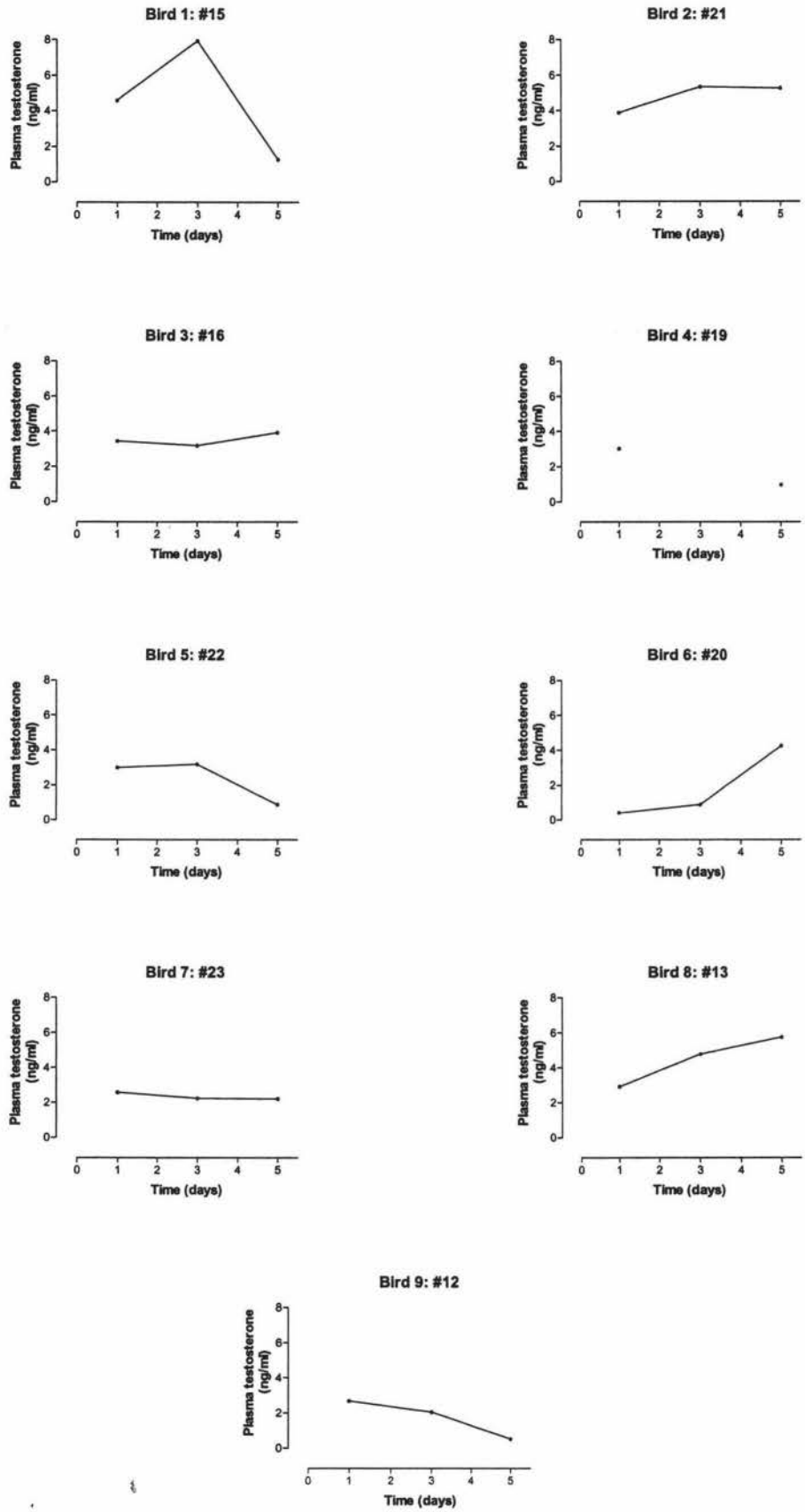

Figure 3.11. Changes in plasma testosterone concentrations over time in individual males on long days. Samples were collected one, three and five days after the start of the faecal collections.

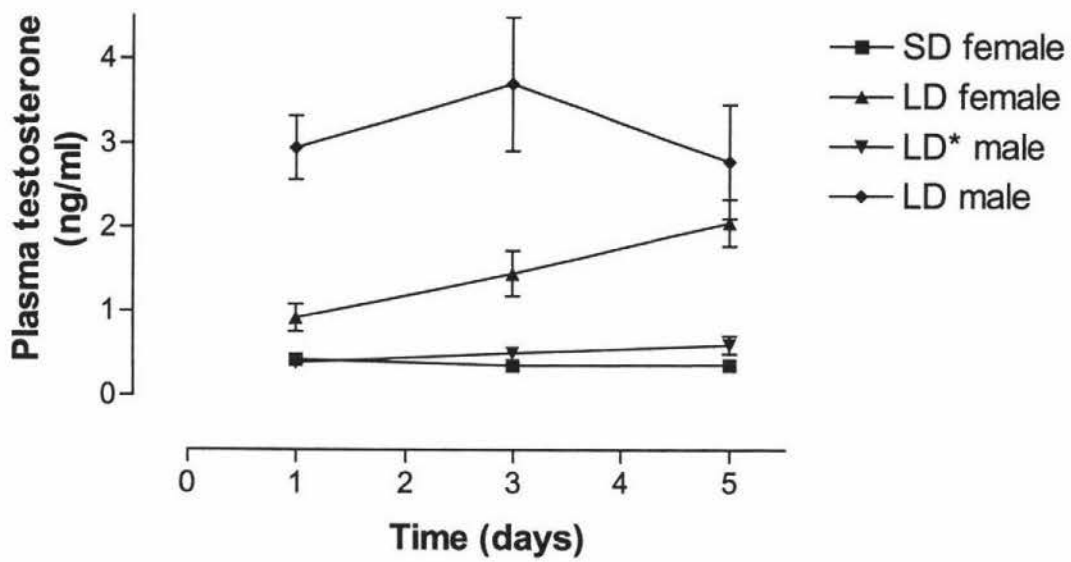

Figure 3.12. Change in mean plasma testosterone concentrations over time in quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males). Samples were collected one, three and five days after the start of the faecal collections.

Table 3.5. Variation between birds in plasma steroid concentrations. (SD = short day, LD = long day, F = female, M = male, * = immature).

	Testosterone (ng/ml)				Oestradiol (pg/ml)				Progesterone (ng/ml)			
	SD F	**LD F**	**LD* M**	**LD M**	**SD F**	**LD F**	**LD* M**	**LD M**	**SD F**	**LD F**	**LD* M**	**LD M**
One sample per bird collected after first day												
Mean	0.37	0.78	0.39	2.98	134.36	290.19	188.20	169.30	0.48	2.08	0.48	0.48
SE	0.01	0.11	0.01	0.43	18.61	24.62	24.22	19.94	0.00	0.63	0.00	0.00
CV	6.09	31.82	9.56	40.92	41.56	25.45	31.52	26.33	0.00	73.81	0.00	0.00
n	10	6	9	9	9	9	6	5	9	6	6	8
Mean of three samples per bird used as data point for each bird												
Mean	0.36	1.52	0.50	3.25	137.75	302.30	155.34	152.22	0.48	2.76	0.51	0.49
SE	0	0.19	0.05	0.44	10.68	20.28	5.22	15.89	0.00	0.34	0.02	0.01
CV	1.99	33.66	32.62	38.70	24.51	20.13	10.64	31.32	0.00	37.03	12.67	3.26
n	10	9	10	9	10	9	10	9	10	9	10	9

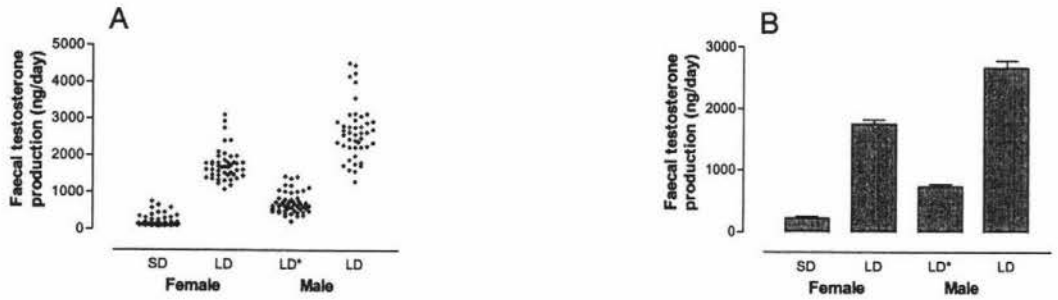

Figure 3.13. Daily faecal testosterone production (SD = short day, LD = long day, LD* = immature males). A) Individual faecal testosterone production for quail held on different photoperiods. B) Mean faecal testosterone production ± SE.

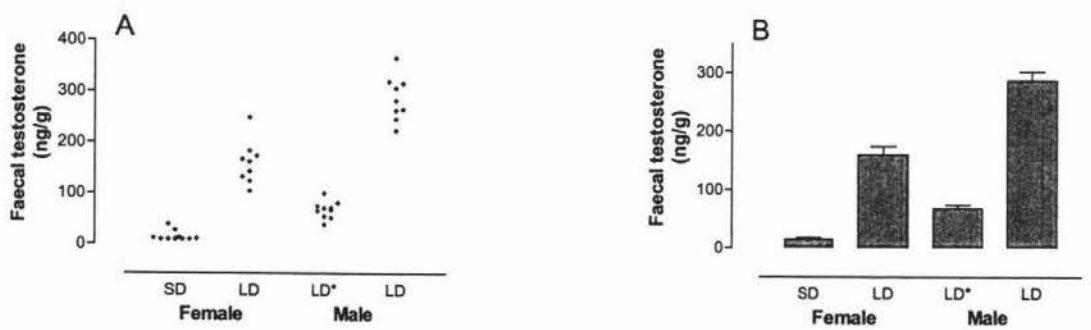

Figure 3.14. Faecal testosterone concentrations for one day (second faecal sample, day two; SD = short day, LD = long day, LD* = immature males). A) Individual faecal testosterone concentrations for quail held on different photoperiods. B) Mean faecal testosterone concentrations ± SE.

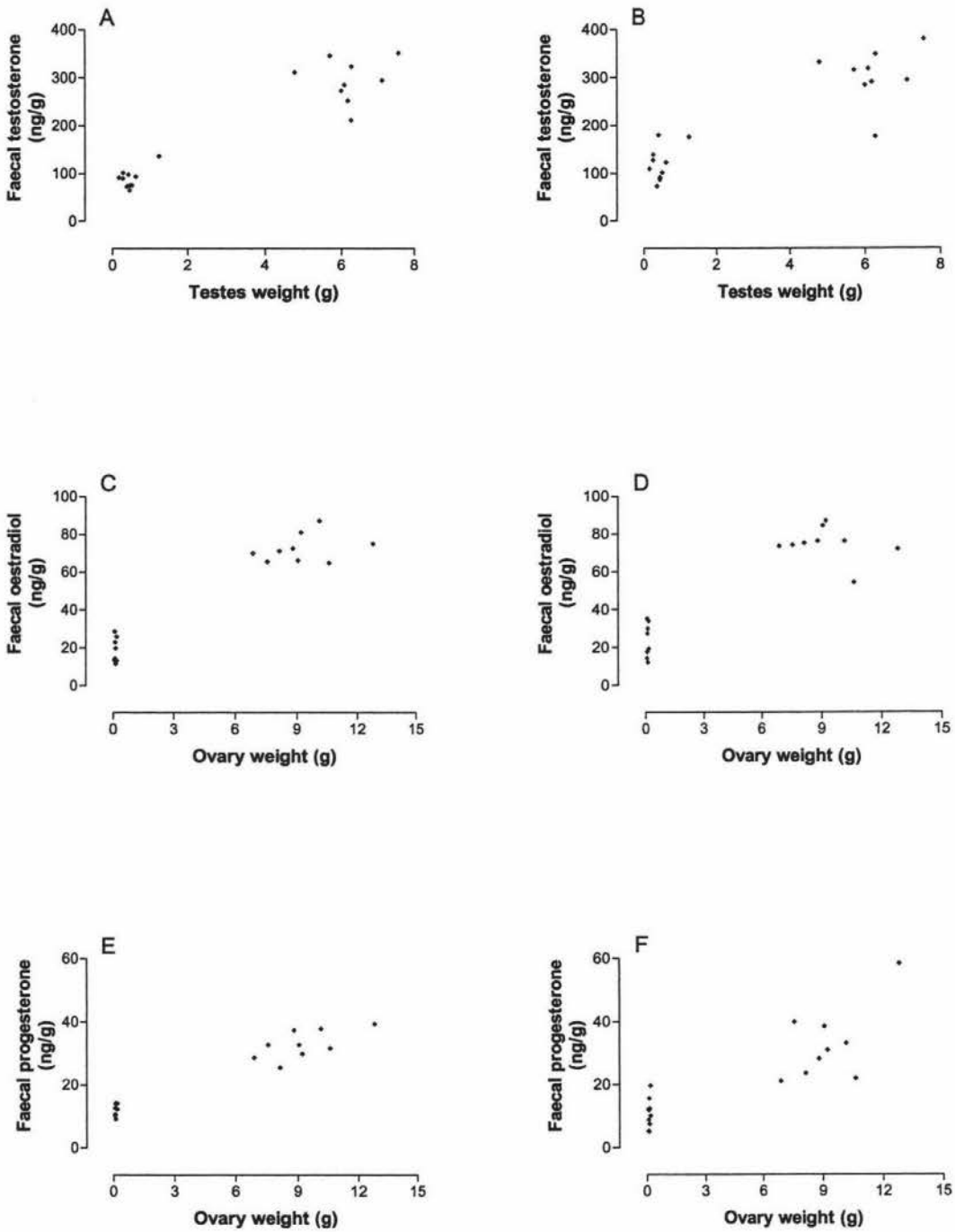

Figure 3.15. Relationships between gonad weight and faecal hormone concentrations. A) Paired testis weight versus mean faecal testosterone over five days. B) Paired testis weight versus mean faecal testosterone for one day. C) Ovary weight versus mean faecal oestradiol over five days. D) Ovary weight versus mean faecal oestradiol for one day. E) Ovary weight versus mean faecal progesterone over five days. F) Ovary weight versus mean faecal progesterone for one day. The mean of five faecal samples for each bird was used for the correlation over five days, n = 19 in all cases, correlation coefficients are summarised in Table 3.3

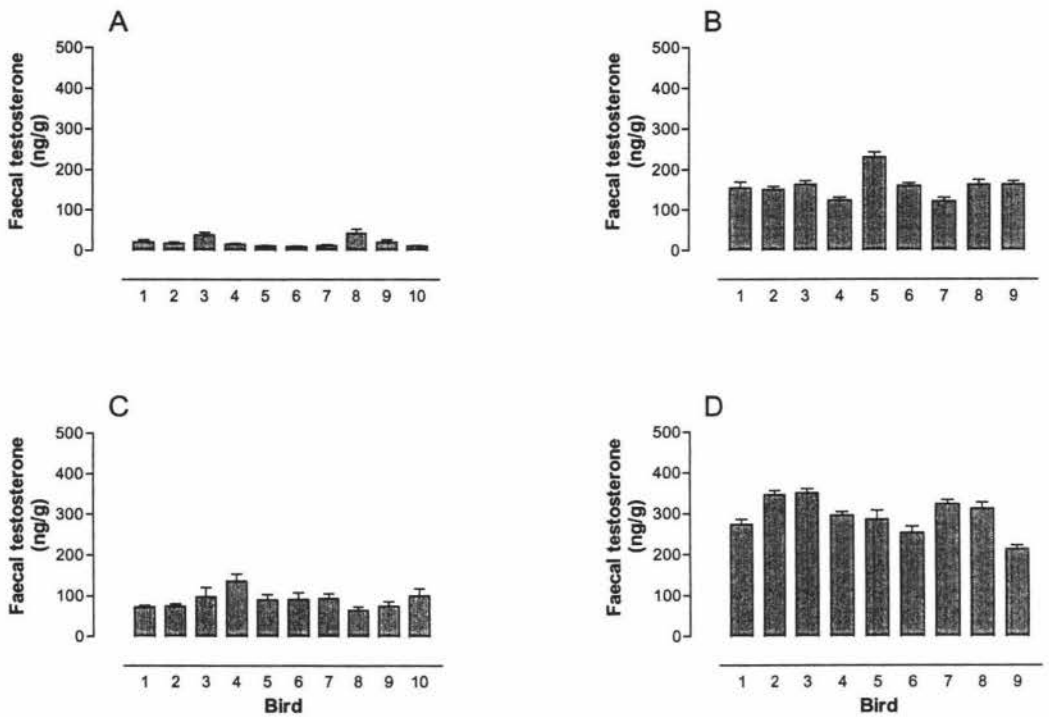

Figure 3.16. Mean faecal testosterone concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) immature males held on a long day photoperiod, D) males held on a long day photoperiod.

Table 3.6. Summary of one-way ANOVA for comparisons of faecal hormone concentration between birds within each treatment group.

	Testosterone			Oestradiol			Progesterone		
Group	**F**	**df**	**P**	**F**	**df**	**P**	**F**	**df**	**P**
SD Female	5.640	9,40	0.001	4.767	9,40	0.001	0.923	9,40	0.516
LD Female	9.820	8,36	0.001	1.795	8,36	0.110	1.834	8,36	0.103
LD* Male	2.187	9,40	0.044	1.226	9,40	0.307	1.863	9,40	0.087
LD Male	11.503	8,36	0.001	2.039	8,36	0.069	4.004	8,36	0.002

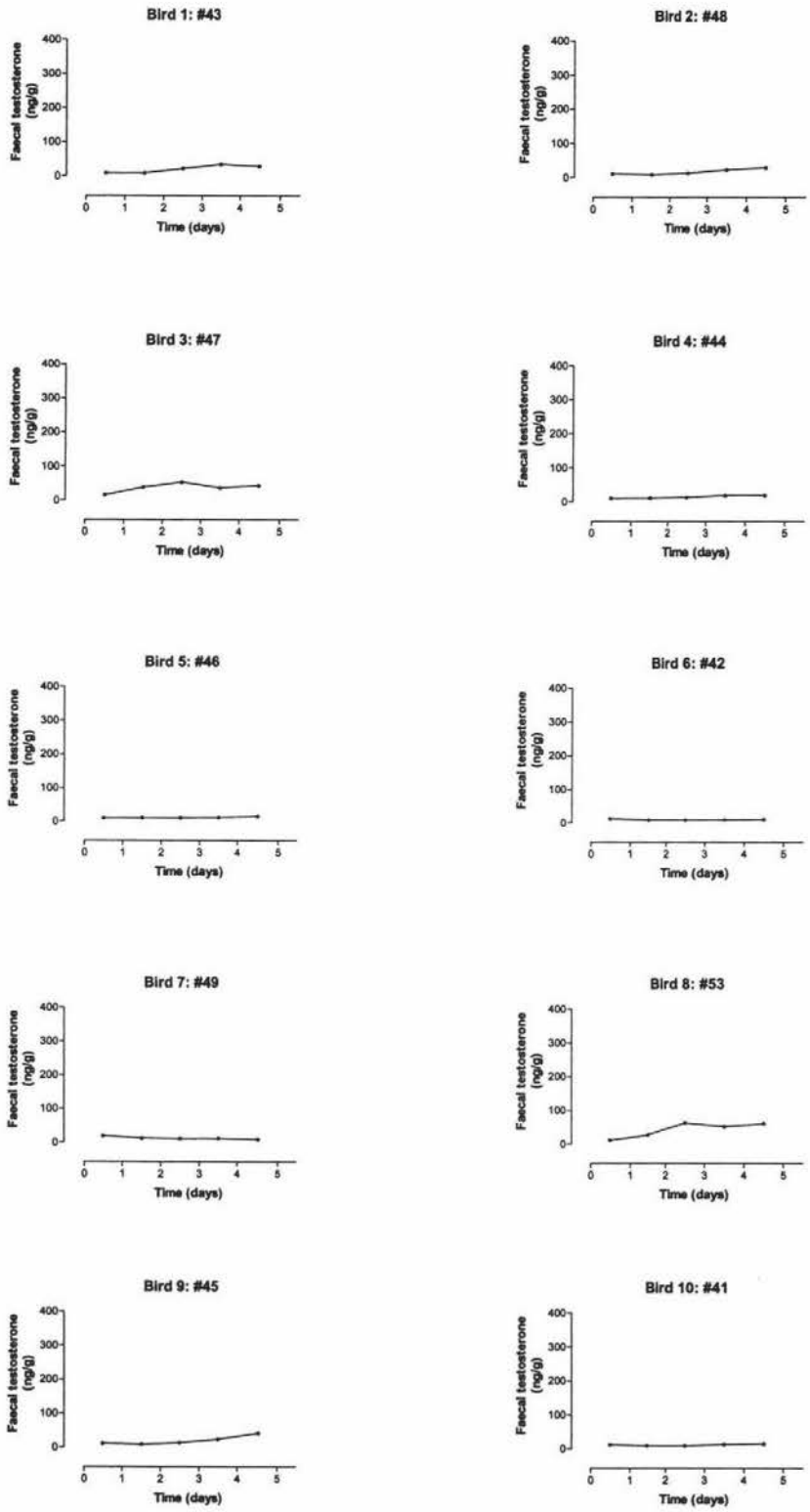

Figure 3.17. Changes in faecal testosterone concentrations over time in individual females on short days.

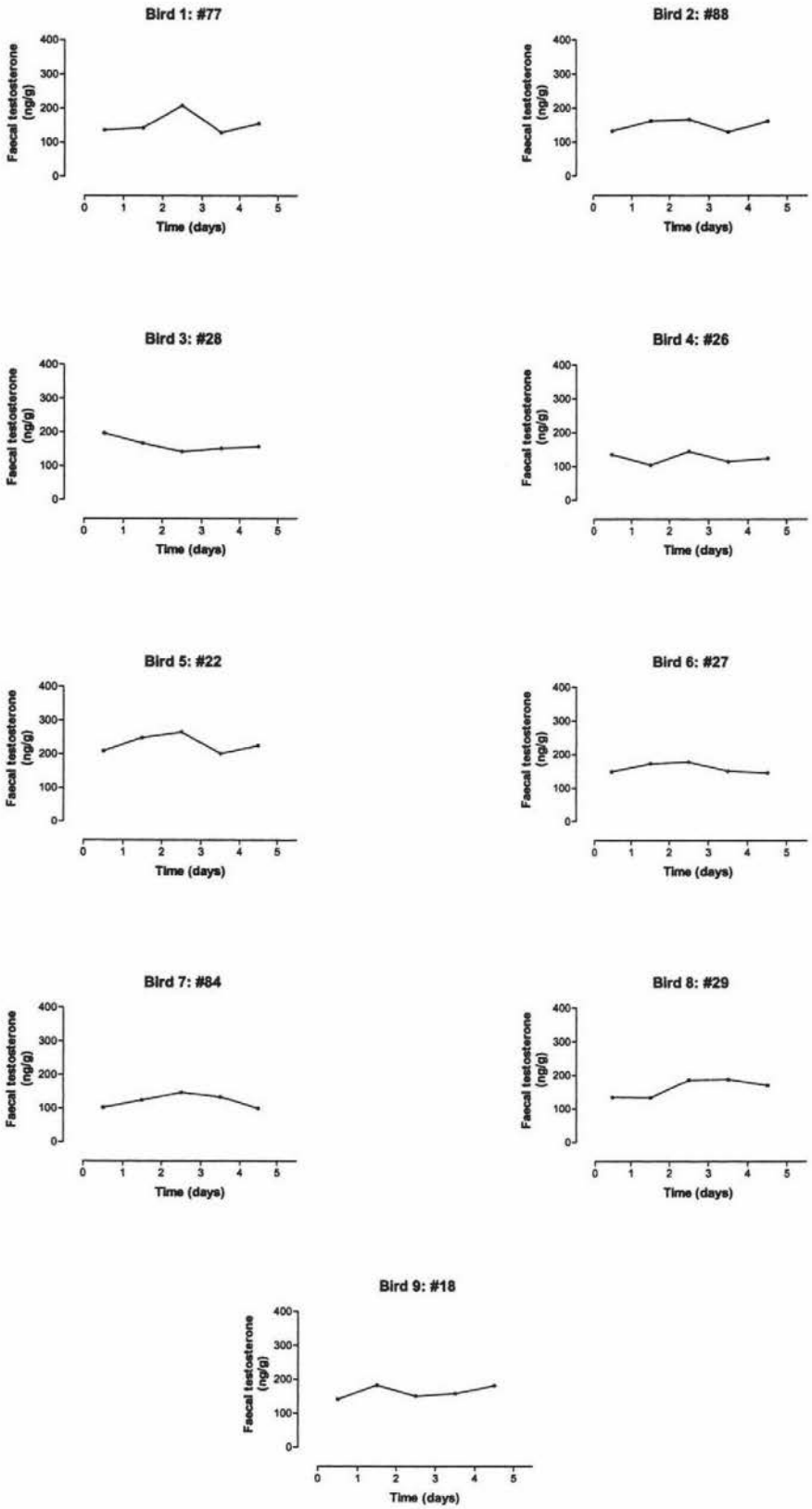

Figure 3.18. Changes in faecal testosterone concentrations over time in individual females on long days.

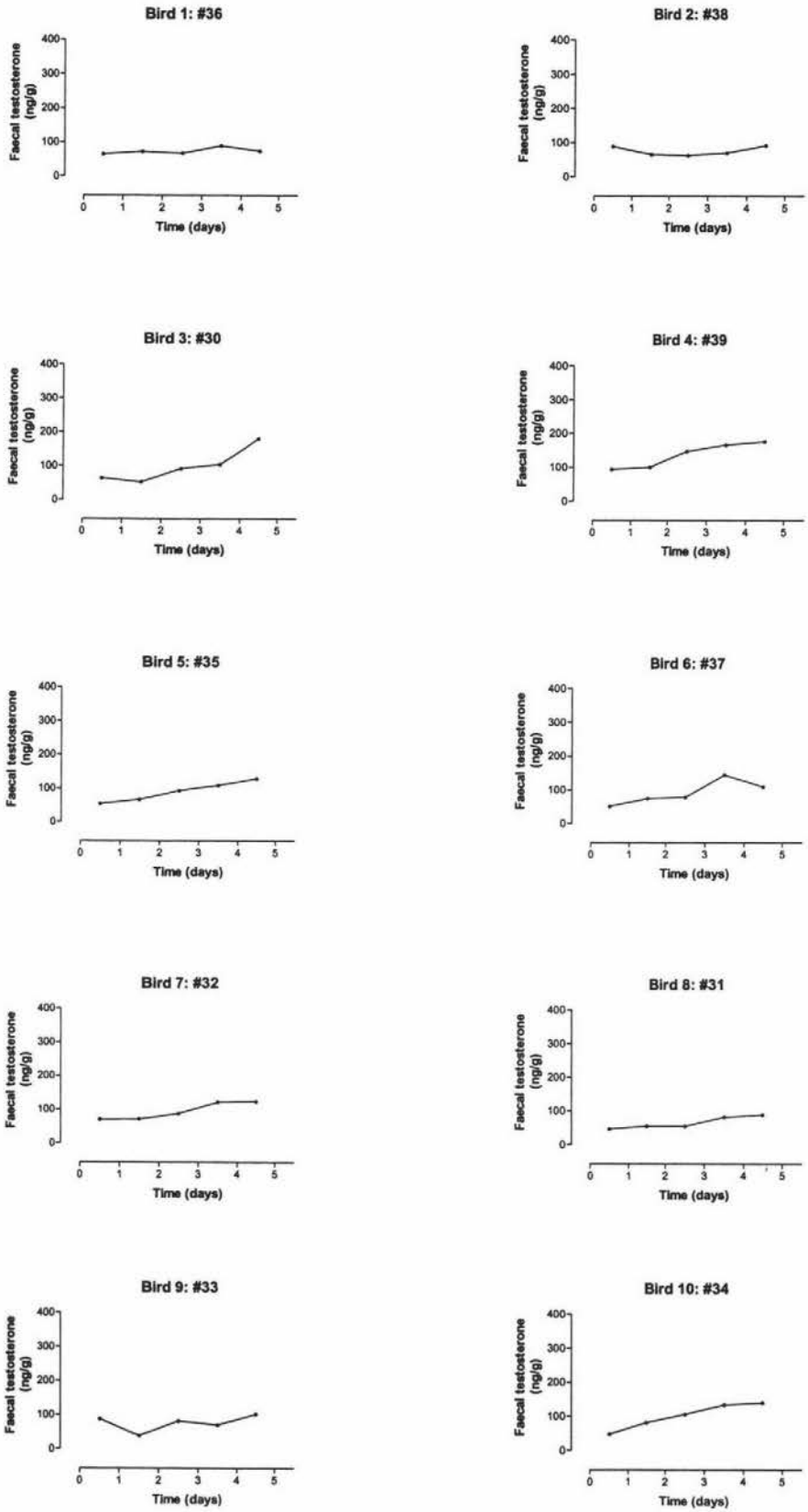

Figure 3.19. Changes in faecal testosterone concentrations over time in individual immature males on long days.

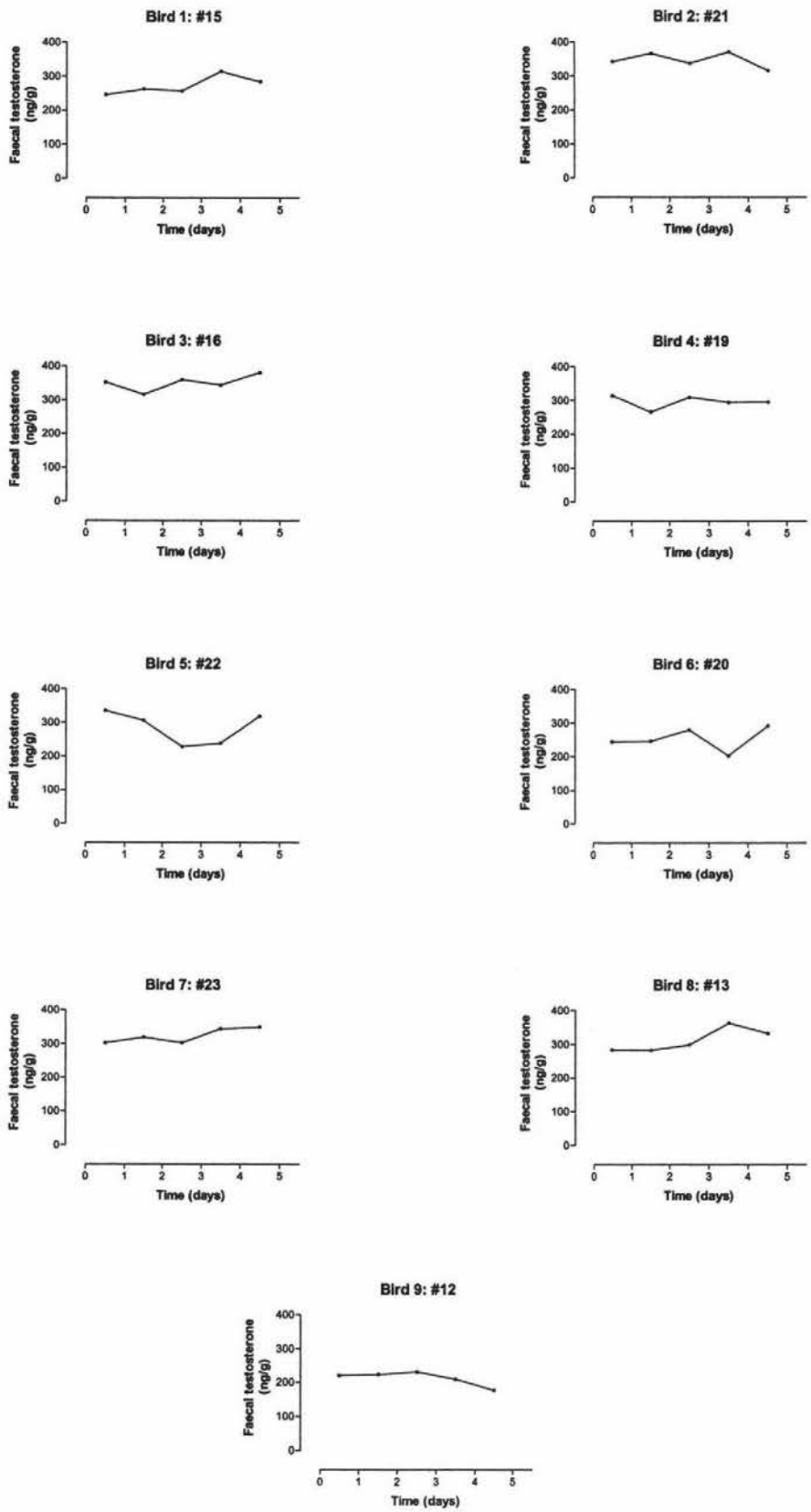

Figure 3.20. Changes in faecal testosterone concentrations over time in individual males on long days.

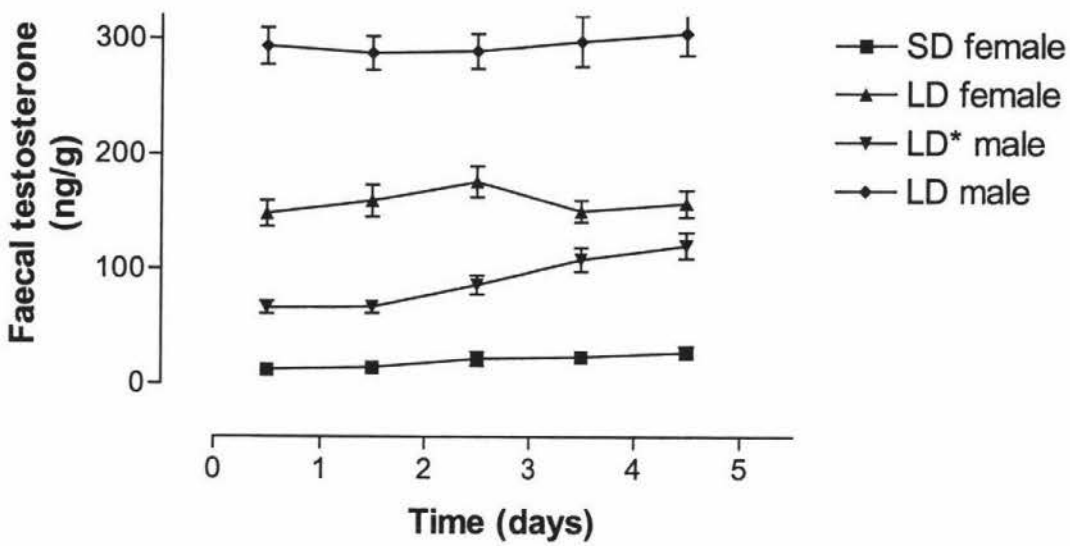

Figure 3.21. Change in mean faecal testosterone concentrations over time in quail held on different photoperiod regimes (SD = short day, LD = long day, LD* = immature males).

Table 3.7. Variation between birds in faecal steroid concentrations (SD = short day, LD = long day, F = female, M = male, * = immature).

	Testosterone (ng/g)				Oestradiol (ng/g)				Progesterone (ng/g)			
	SD F	**LD F**	**LD* M**	**LD M**	**SD F**	**LD F**	**LD* M**	**LD M**	**SD F**	**LD F**	**LD* M**	**LD M**
One sample per bird collected on second day												
Mean	16.12	160.24	67.46	295.62	14.96	70.66	17.32	26.16	13.43	32.42	23.95	15.93
SE	3.89	17.49	5.33	13.83	2.22	5.37	1.75	1.82	1.37	2.17	1.76	1.75
CV	68.20	28.87	24.99	13.23	46.93	22.81	32.04	20.89	32.19	20.04	23.20	33.00
n	10	9	10	9	10	9	10	9	10	9	10	9
Mean of five samples per bird used as data point for each bird												
Mean	21.24	157.50	90.01	304.94	17.82	72.63	21.93	26.01	12.41	32.96	23.72	18.92
SE	4.37	13.62	6.43	12.28	1.92	2.51	1.14	1.57	0.56	1.68	1.31	1.68
CV	58.15	22.88	22.57	11.39	34.12	10.37	16.43	18.16	14.20	15.26	17.52	26.59
n	10	9	10	9	10	9	10	9	10	9	10	9

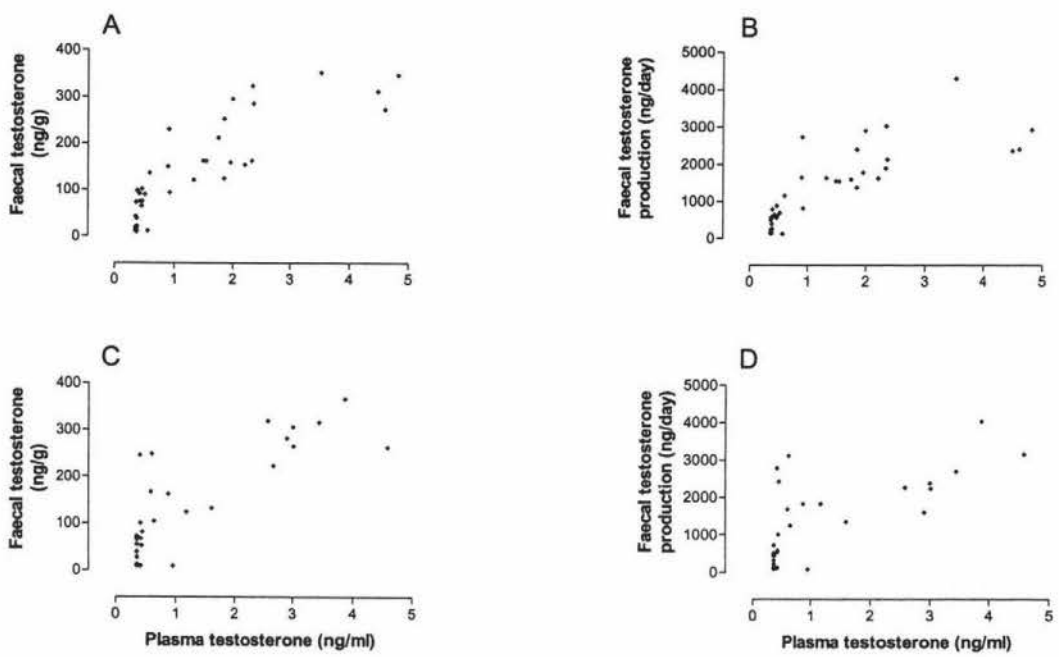

Figure 3.22. Correlations between plasma concentrations and faecal testosterone concentrations or production. Correlations are between: A mean plasma and faecal concentrations over five-days; B: mean plasma concentration and mean faecal production over five days; C: mean plasma and faecal concentrations for one day; D: mean plasma concentration and mean faecal production for one day.

Table 3.8. Correlations between plasma and faecal testosterone concentrations and production (SD = short day, LD = long day, LD* = immature males). Significant differences ($p<0.05$) are marked with an asterisk (*), and highly significant ($p<0.01$) differences are marked with a double asterisk (**).

	Five days			One day		
	r	**n**	**p**	**r**	**n**	**p**
Plasma vs faecal concentration (ng/g)	0.8550	38	0.001**	0.8304	34	0.001**
SD Female	0.2569	10	0.4738	0.2236	10	0.5348
LD Female	0.3192	9	0.4025	0.4165	6	0.4114
LD* Male	0.3205	10	0.3667	0.2650	8	0.5260
LD Male	0.5485	9	0.1263	0.6688	10	0.0345*
LD* & LD Male	0.8836	19	0.001**	0.8774	18	0.001**
Plasma vs faecal production (ng)	0.8031	38	0.001**	0.7183	33	0.001**
SD Female	0.2823	10	0.4294	0.2891	10	0.4177
LD Female	0.3608	9	0.3401	0.3865	6	0.4491
LD* Male	0.4129	10	0.2356	0.6063	8	0.1111
LD Male	0.2540	9	0.5095	0.3157	10	0.4079
LD* & LD Male	0.8087	19	0.001**	0.7748	18	0.001**

3.3.7 Plasma oestradiol

Plasma oestradiol concentrations across all groups ranged from 49.0 pg/ml to 489.2 pg/ml. Plasma oestradiol was significantly higher in females on long days than in females on short days, but there were no significant differences between mature males on long days and immature males on long days, across either the sampling period of five days or for a single day (Table 3.2, Figure 3.23 and Figure 3.24). Data from the first plasma sample collected one day after the start of the faecal collection period were used for the single day comparisons.

Highly significant relationships were found between plasma oestradiol concentrations and ovary weight over the sampling period of five days and for a single day (Figure 3.6 and Table 3.3).

There were no significant differences in plasma oestradiol concentration between birds in each group (Figure 3.25, Table 3.4). No significant changes in plasma oestradiol were found for any of the groups over the sampling period (Friedman's' non-parametric analyses were inappropriate due to missing values so paired T-tests were conducted for each pairing of days within a group; $p>0.05$ in all cases, Figure 3.26 - Figure 3.30). The coefficients of variation in plasma oestradiol concentration for individual birds were $32.4 \pm 6.5\%$ in females on short days, $18.5 \pm 5.4\%$ in females on long days, $32.1 \pm 7.4\%$ in immature males on long days and $30.9 \pm 4.0\%$ in mature males on long days (Figure 3.26 - Figure 3.29). Variation in plasma oestradiol concentrations between birds was examined using the first plasma sample for each bird and also using the mean of the three samples for each bird as a data point (Table 3.5). The coefficients of variation between birds did not differ greatly between individual or combined values for females or mature males on long days. The coefficient of variation was considerably higher for a single day sample for immature males on long days and females on short days.

3.3.8 Faecal oestradiol

Faecal oestradiol concentrations across all groups ranged from 7.24 to 113.30 ng/g (Figure 3.23). Total faecal oestradiol production over 24 hours ranged from 80.8 to 1 300.0 ng (Figure 3.31). Faecal oestradiol concentrations were significantly higher in females on long days than in females on short days across the sampling period of five days and for a single day (Table 3.2). Faecal oestradiol production was also significantly higher in females in long days than in females on short days. There were no significant differences in faecal oestradiol concentration or production between mature and immature males on long days across the sampling period of five days. However, for a single day, mature males on long days had significantly higher faecal oestradiol concentrations than immature males on long days (Table 3.2, Figure 3.32). Data from the second faecal sample were used for the single day comparisons.

Highly significant relationships were found between faecal oestradiol concentrations and ovary weight over the sampling period of five days and for a single day (Figure 3.15 and Table 3.3).

Short day females, were the only group to show significant differences in faecal oestradiol concentration between birds (Figure 3.33, Table 3.6). No significant changes in faecal oestradiol were found for females or mature and immature males on long days over the sampling period (Friedman's' non-parametric analyses, $t = 6.400$, $p = 0.171$; $t = 8.800$, $p = 0.066$; and $t = 7.111$, $p = 0.130$ respectively). However, a slight increase from the beginning of the sampling period to the end was significant for females on short days (Mann – Whitney test, $U = 15.000$, $p = 0.008$; Figure 3.34 - Figure 3.38). The coefficients of variation in faecal oestradiol concentration for individual birds were 29.8 ± 4.3% in females on short days, 16.6 ± 1.6% in females on long days, 31.3 ± 3.2% in immature males on long days and 26.4 ± 2.6% in mature males on long days (Figure 3.34 - Figure 3.37). The coefficients of variation between birds were lower for five-day pooled than for individual faecal samples for three of the groups of birds (Table 3.7).

3.3.9 Relationship between plasma and faecal oestradiol

Highly significant correlations were found between plasma and faecal oestradiol over the sampling period of five days and for a single day, for both faecal concentration and production (Figure 3.39, Table 3.9). The correlations were lower for data from a single day compared with data from five days. The single day correlation was conducted between plasma samples collected one day after the start of the faecal collection, and faecal samples from the second day. Table 3.9 also summarises the relationships found within each group between plasma and faecal concentration and production for five days and a single day. All relationships within each group were insignificant ($p>0.05$ in all cases).

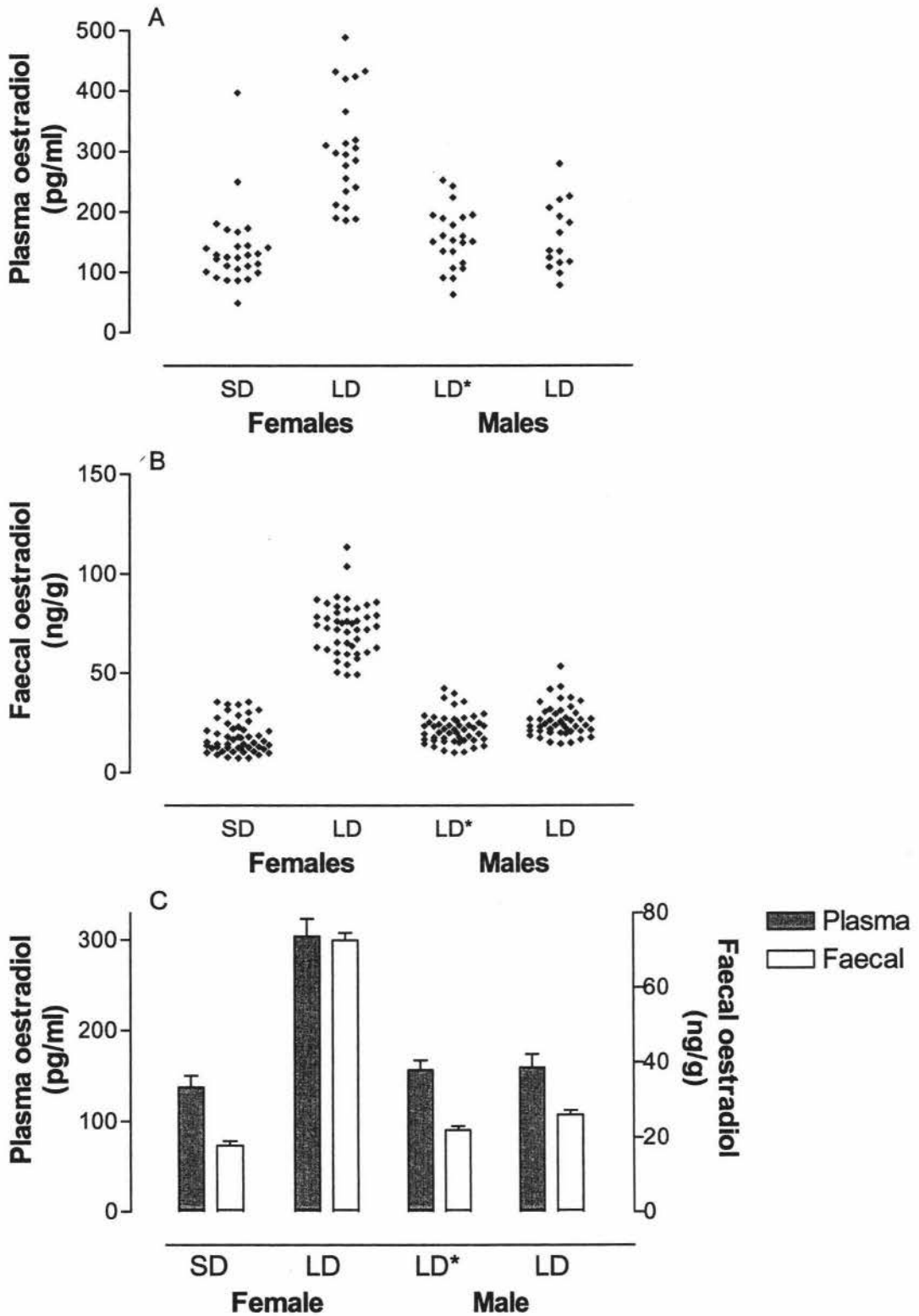

Figure 3.23. Plasma and faecal oestradiol concentrations (SD = short day, LD = long day, LD* = immature males). A) Individual plasma oestradiol concentrations for quail held on different photoperiods (n = 27, 22, 22 and 15 for each group respectively). B) Individual faecal oestradiol concentrations for quail held on different photoperiods (n = 50, 45, 50 and 45 for each group respectively). C) Mean plasma and faecal oestradiol concentrations ± SE.

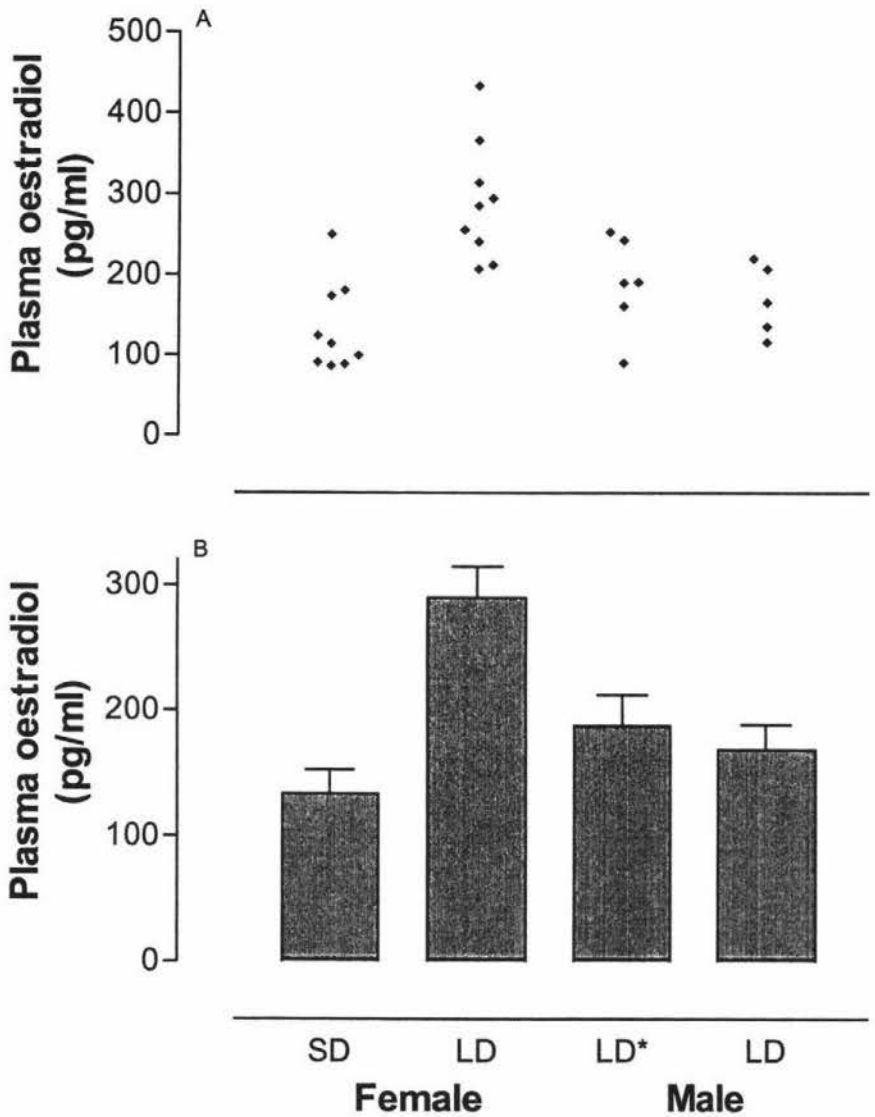

Figure 3.24. Plasma oestradiol concentrations for one day (first plasma sample, SD = short day, LD = long day, LD* = immature males). A) Individual plasma oestradiol concentrations for quail held on different photoperiods (n = 9, 9, 6 and 5 for each group respectively). B) Mean plasma oestradiol concentrations ± SE.

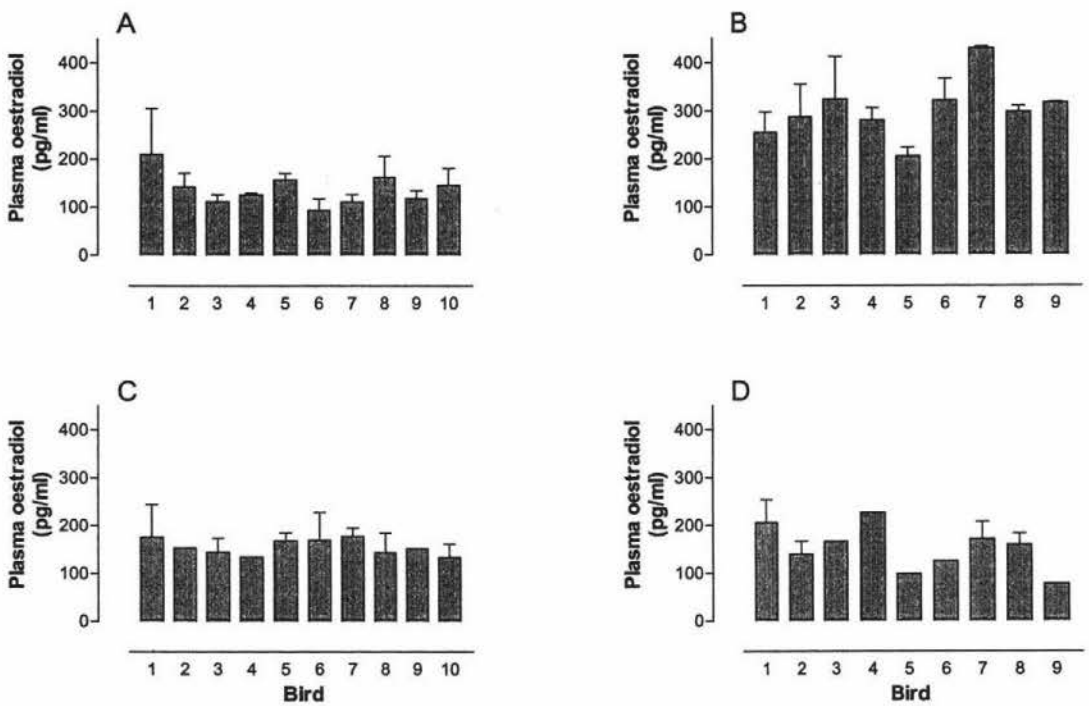

Figure 3.25. Mean plasma oestradiol concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) immature males held on a long day photoperiod, D) males held on long day photoperiod.

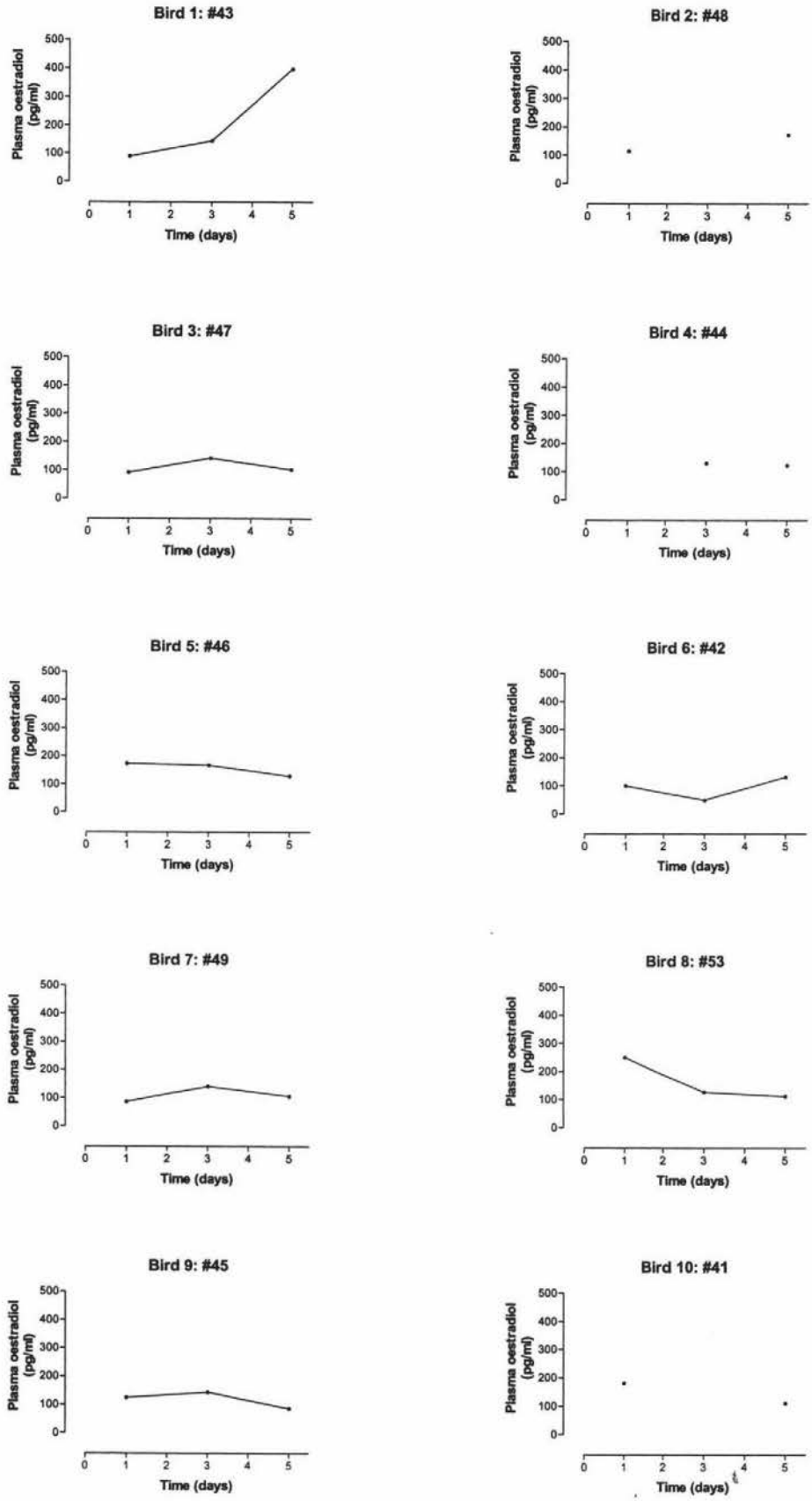

Figure 3.26. Changes in plasma oestradiol concentrations over time in individual females on short days. Samples were collected one, three and five days after the start of the faecal collections.

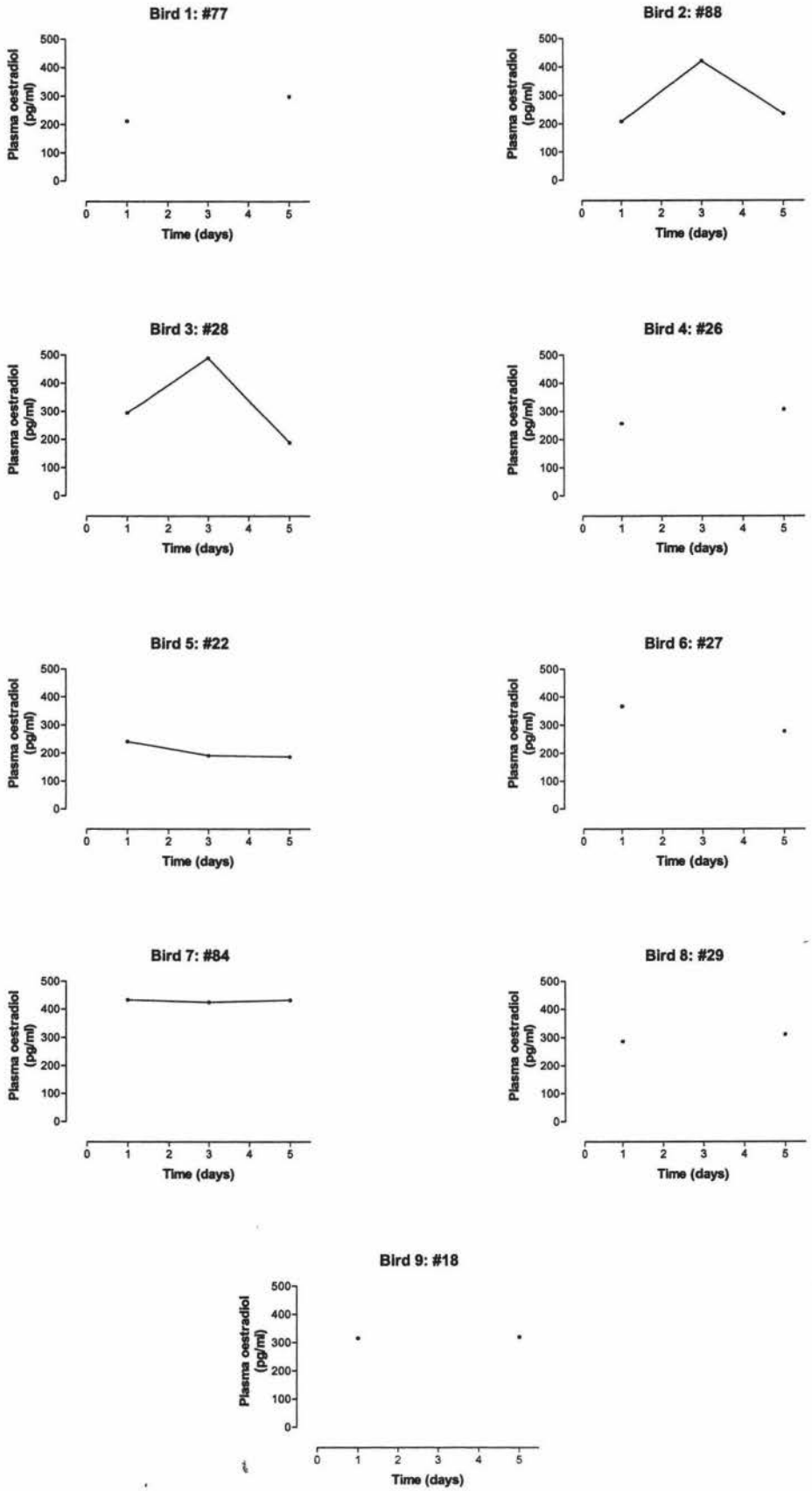

Figure 3.27. Changes in plasma oestradiol concentrations over time in individual females on long days. Samples were collected one, three and five days after the start of the faecal collections.

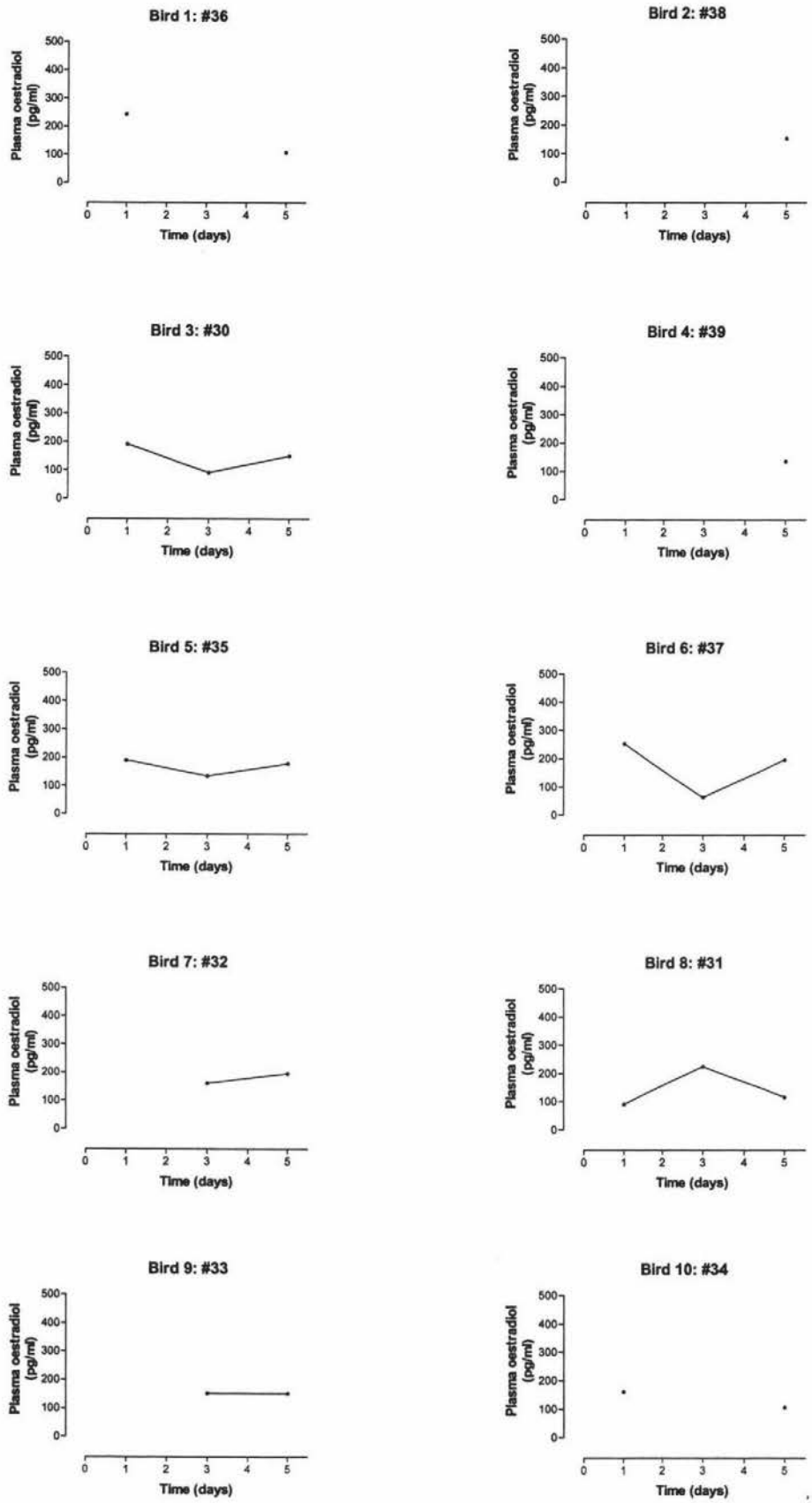

Figure 3.28. Changes in plasma oestradiol concentrations over time in individual immature males on long days. Samples were collected one, three and five days after the start of the faecal collections.

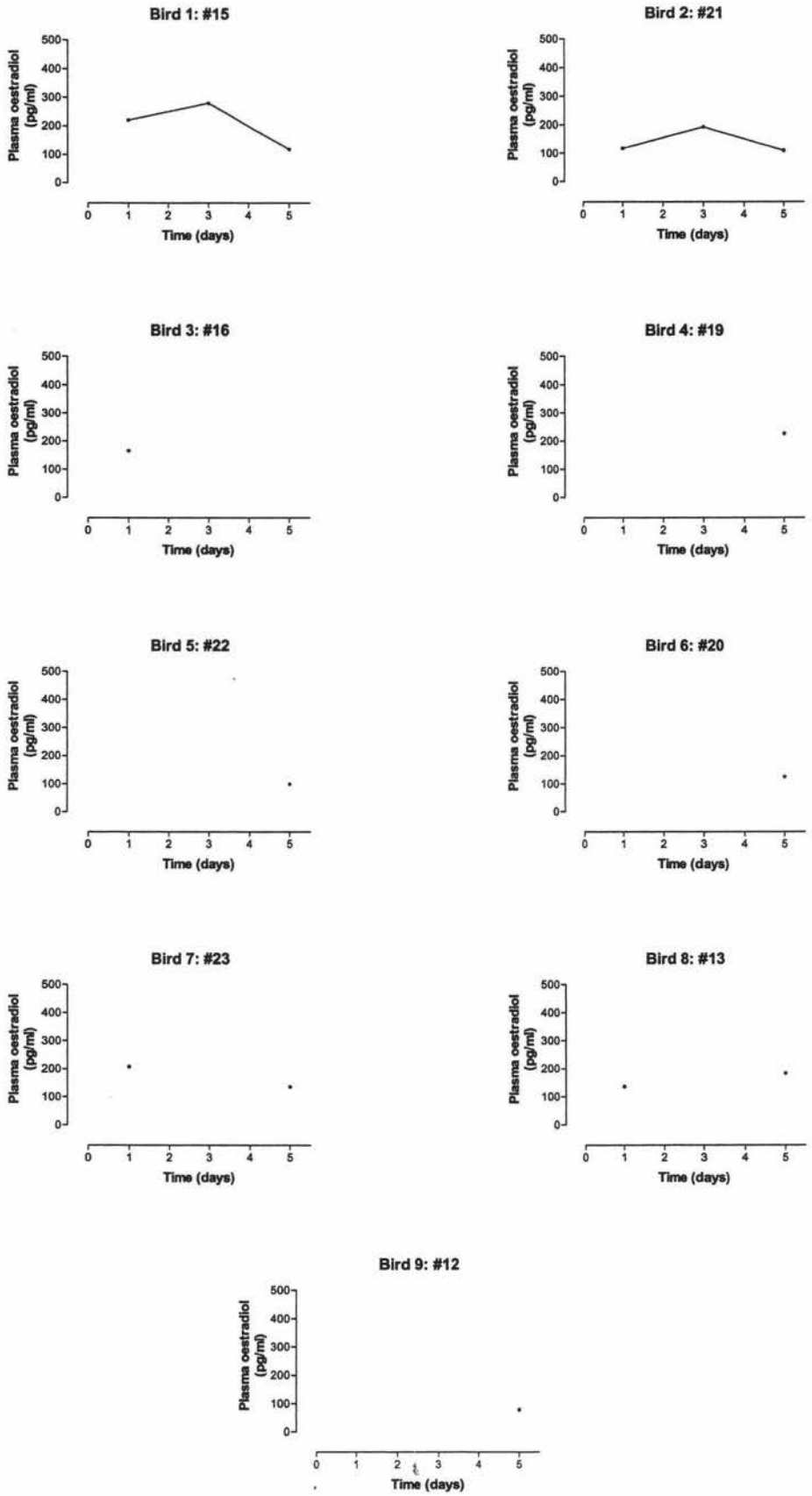

Figure 3.29. Changes in plasma oestradiol concentrations over time in individual males on long days. Samples were collected one, three and five days after the start of the faecal collections.

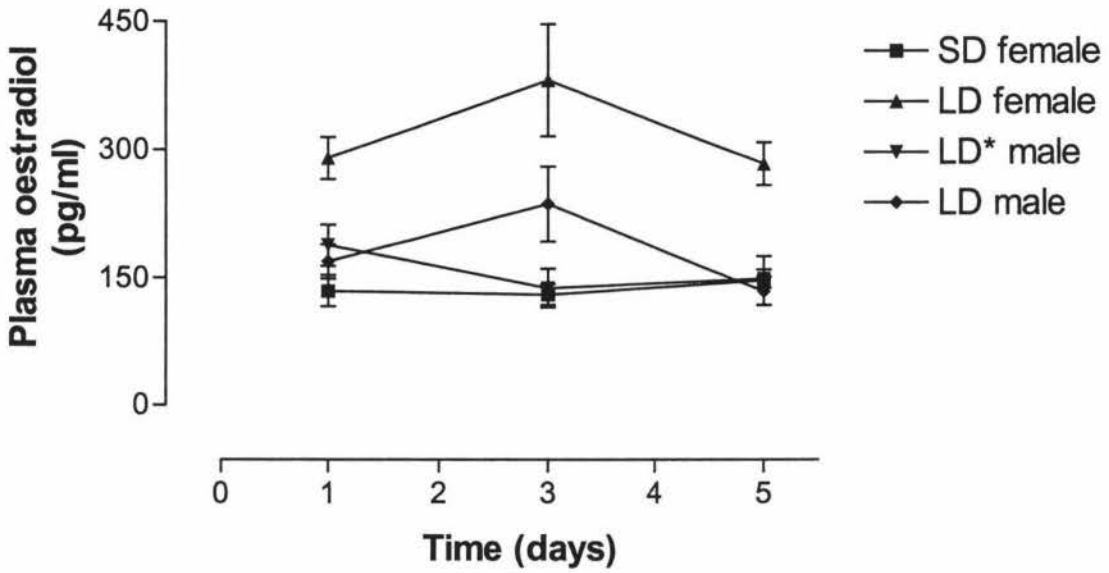

Figure 3.30. Change in mean plasma oestradiol concentrations over time in quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males). Samples were collected one, three and five days after the start of the faecal collections.

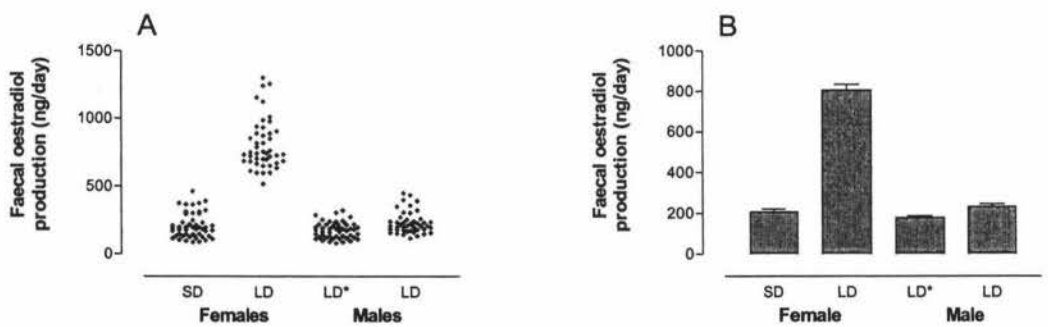

Figure 3.31. Daily faecal oestradiol production (SD = short day, LD = long day, LD* = immature males). A) Individual faecal oestradiol production for quail held on different photoperiods. B) Mean faecal oestradiol production ± SE.

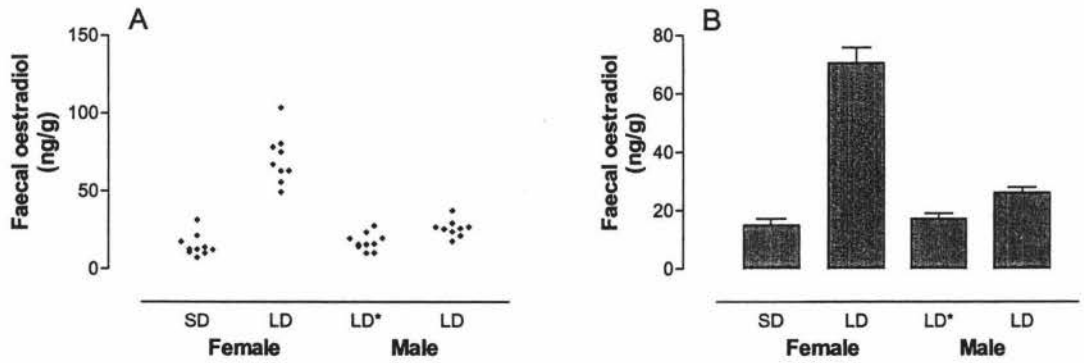

Figure 3.32. Faecal oestradiol concentrations for one day (second faecal sample, day two; SD = short day, LD = long day, LD* = immature males). A) Individual faecal oestradiol concentrations for quail held on different photoperiods. B) Mean faecal oestradiol concentrations ± SE.

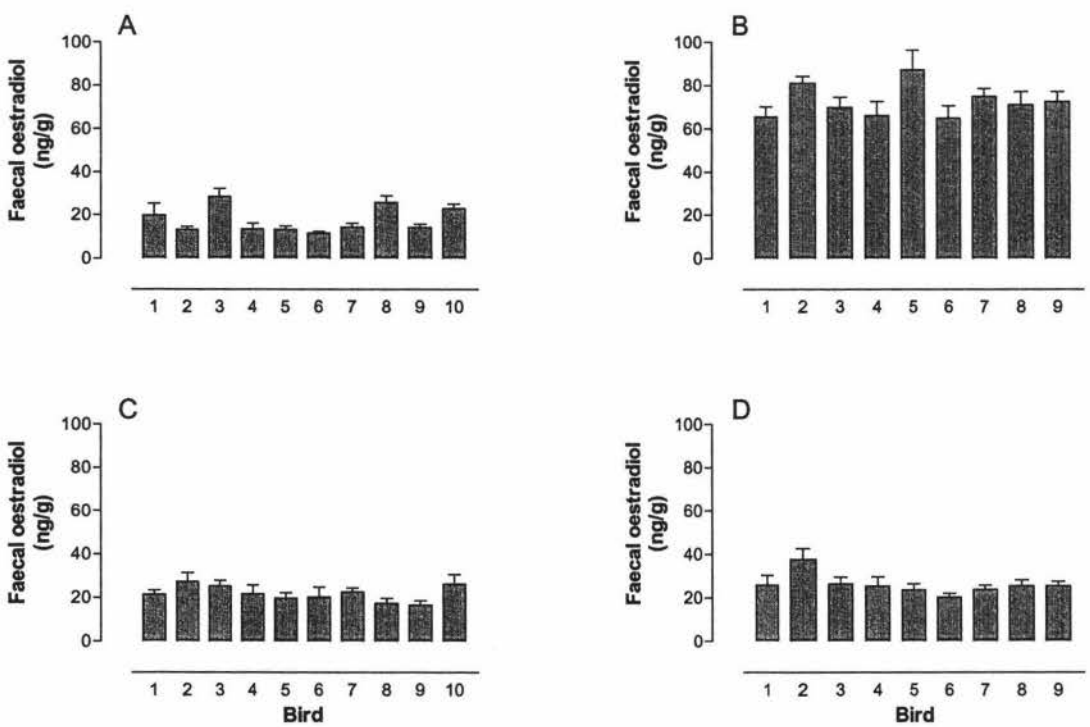

Figure 3.33. Mean faecal oestradiol concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) immature males held on a long day photoperiod, D) males held on a long day photoperiod.

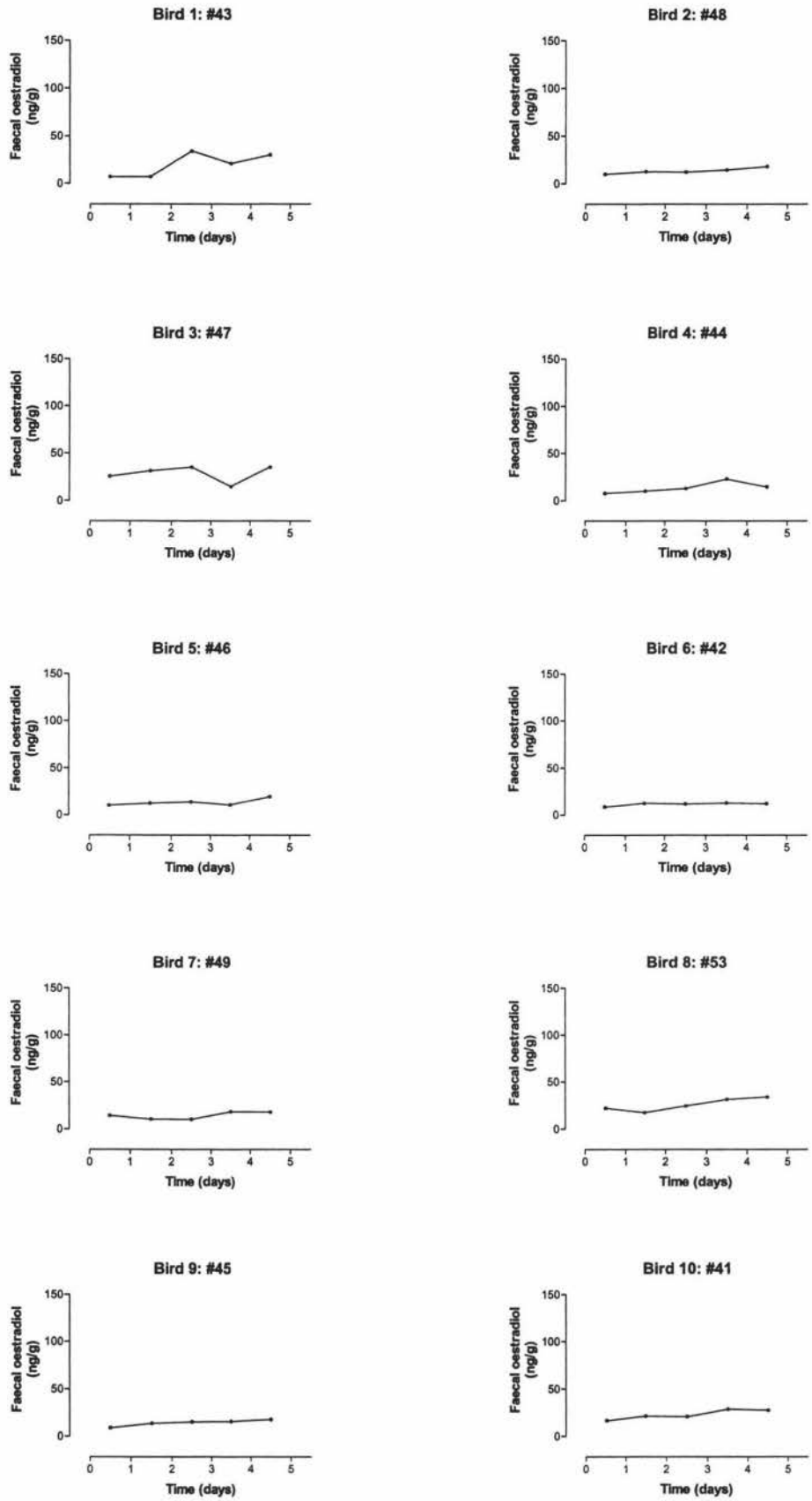

Figure 3.34. Changes in faecal oestradiol concentrations over time in individual females on short days.

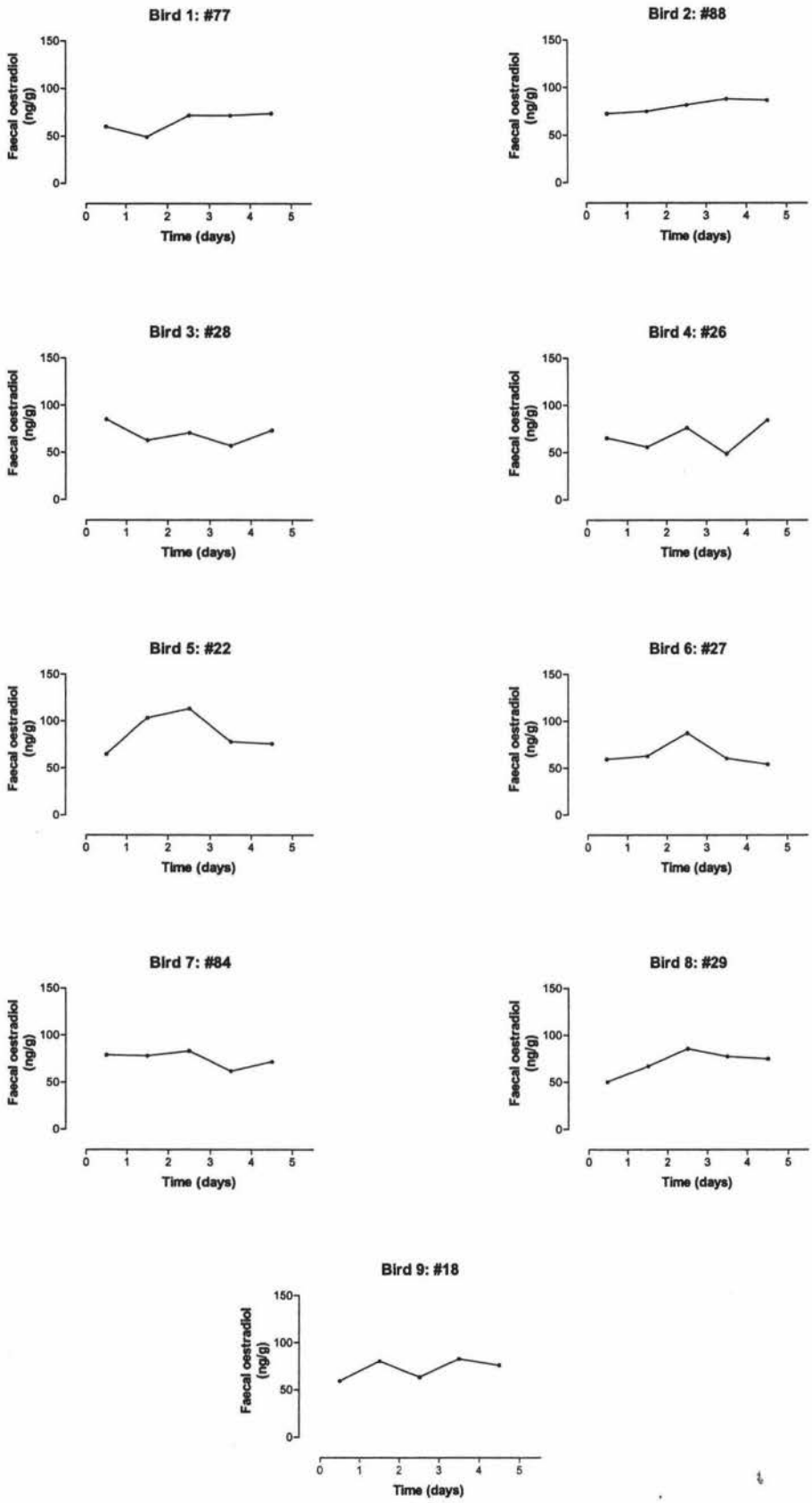

Figure 3.35. Changes in faecal oestradiol concentrations over time in individual females on long days.

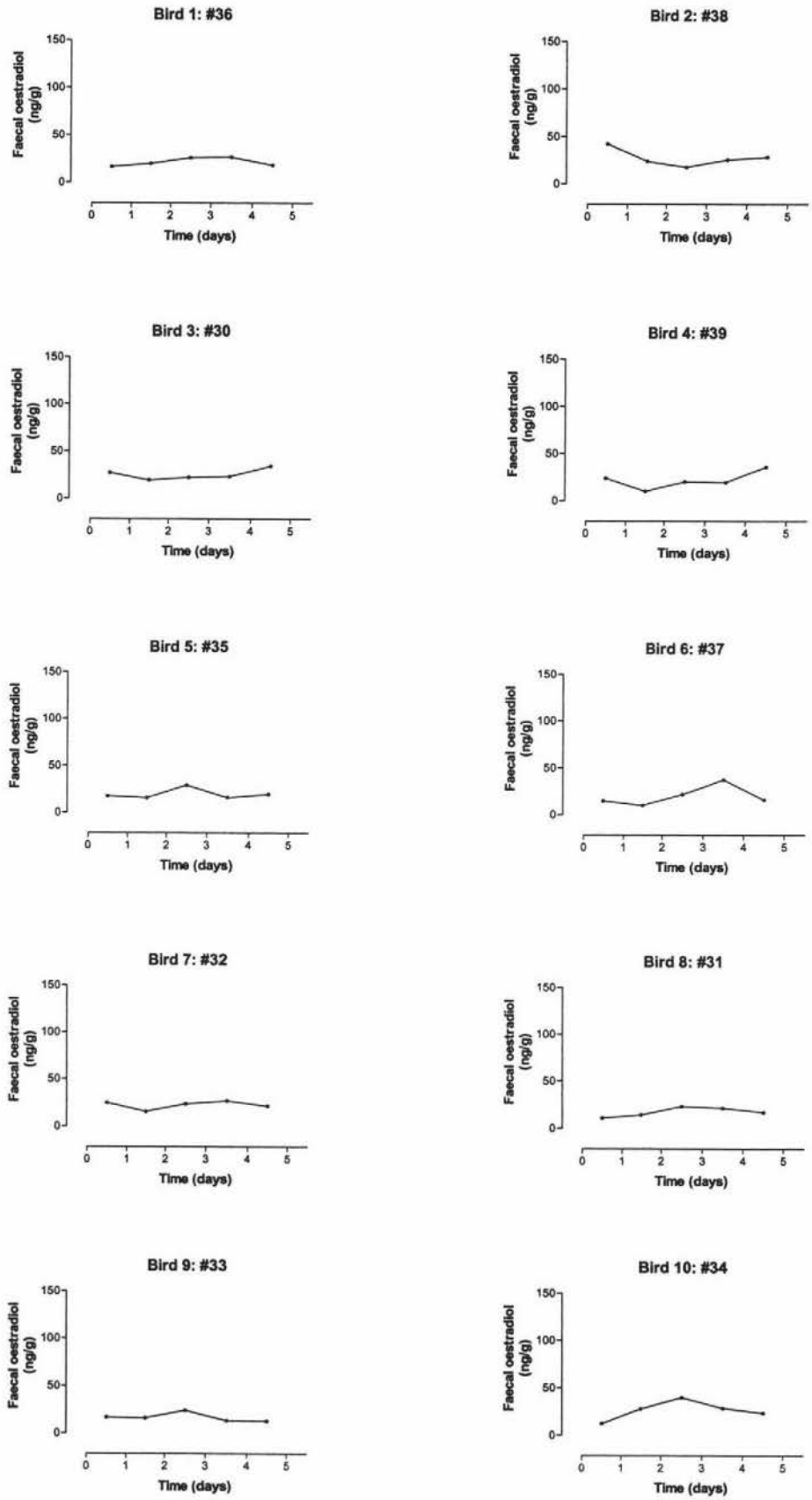

Figure 3.36. Changes in faecal oestradiol concentrations over time in individual immature males on long days.

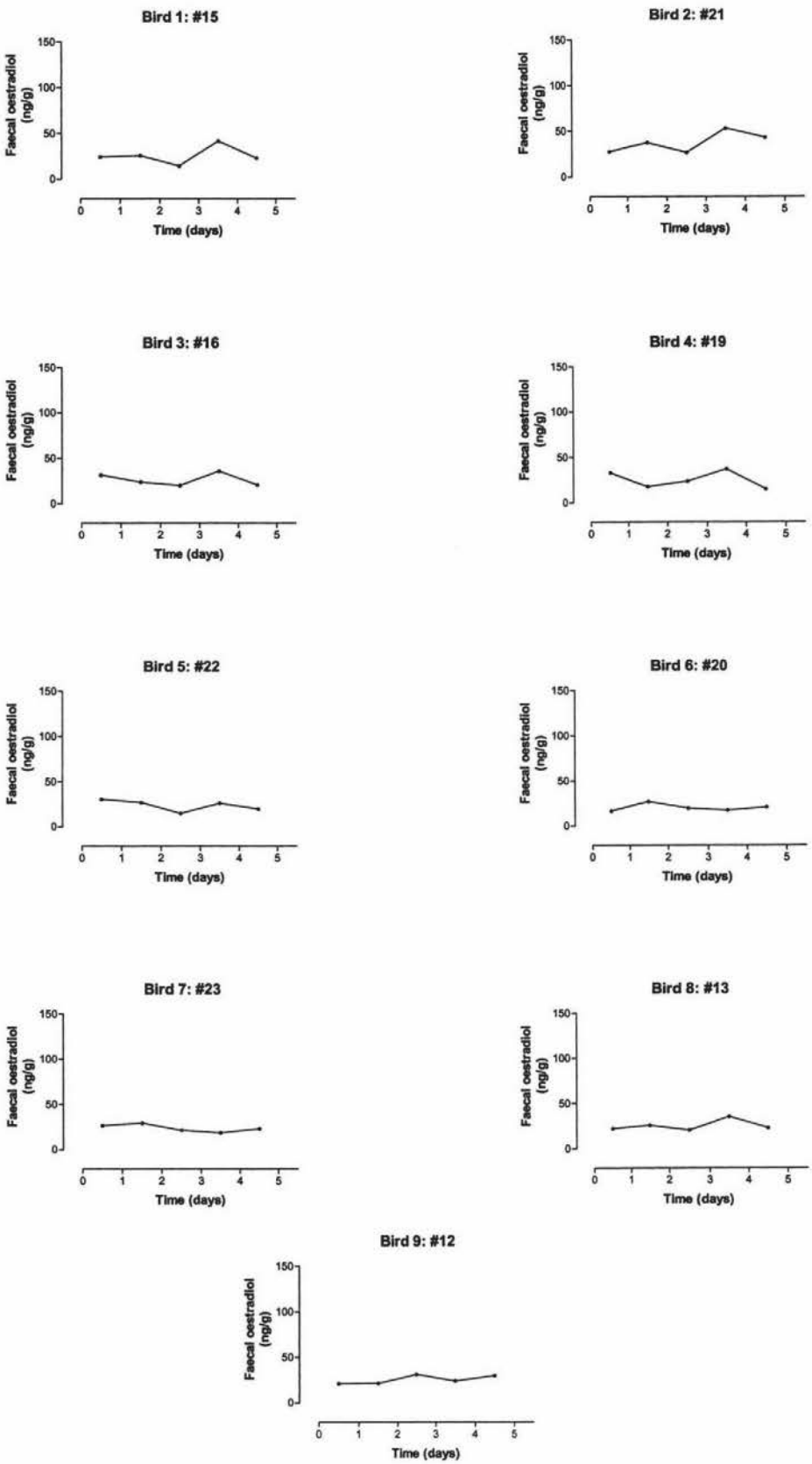

Figure 3.37. Changes in faecal oestradiol concentrations over time in individual males on long days.

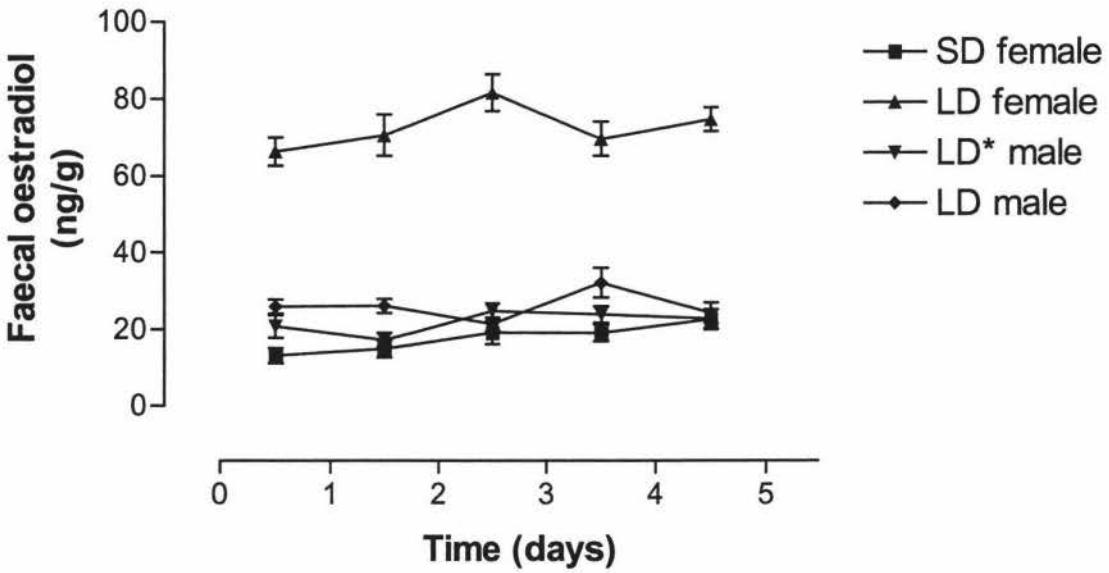

Figure 3.38. Change in mean faecal oestradiol concentrations over time in quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males).

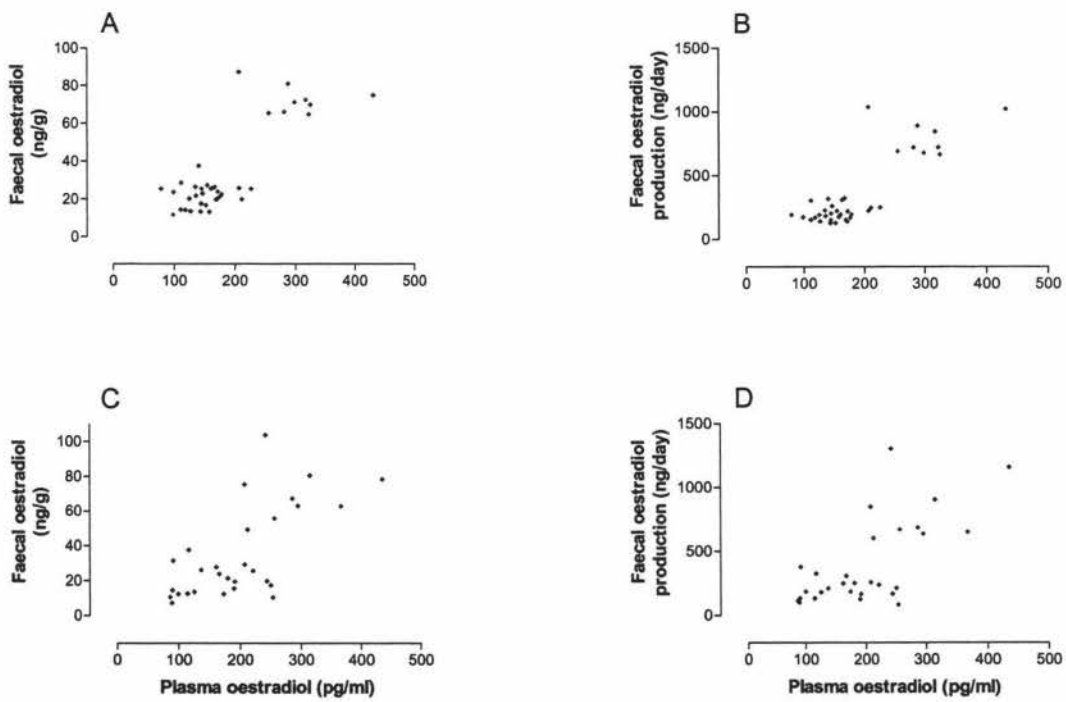

Figure 3.39. Correlations between plasma concentrations and faecal oestradiol concentrations or production. Correlations are between: A mean plasma and faecal concentrations over five-days; B: mean plasma concentration and mean faecal production over five days; C: mean plasma and faecal concentrations for one day; D: mean plasma concentration and mean faecal production for one day.

Table 3.9. Correlations between plasma and faecal oestradiol concentrations and production (SD = short day, LD = long day, LD* = immature males). Significant differences ($p<0.05$) are marked with an asterisk (*), and highly significant ($p<0.01$) differences are marked with a double asterisk (**).

	Five days			One day		
	r	**n**	**p**	**r**	**n**	**P**
Plasma vs faecal concentration (ng/g)	0.8199	38	0.001**	0.6713	29	0.001**
SD Female	0.2538	10	0.4795	0.1487	9	0.7027
LD Female	0.2636	9	0.4932	0.0954	9	0.8067
SD & LD Female	0.8405	19	0.001**	0.7468	18	0.001**
LD* Male	0.2177	10	0.5459	0.1758	6	0.7391
LD Male	0.1292	9	0.9010	0.5183	5	0.3710
Plasma vs faecal production (ng)	0.8345	38	0.001**	0.6693	29	0.001**
SD Female	0.3350	10	0.3442	0.1634	9	0.6747
LD Female	0.1068	9	0.7845	0.2267	9	0.5576
SD & LD Female	0.8495	19	0.001**	0.7463	18	0.001**
LD* Male	0.3242	10	0.3609	0.2737	6	0.5997
LD Male	0.3746	9	0.3206	0.3842	5	0.5231

3.3.10 Plasma progesterone

Plasma progesterone concentrations were low in groups except long day females, which had a range from the least detectable dose of 0.48 ng/ml to 6.66 ng/ml (Figure 3.40). Plasma progesterone was significantly higher in females on long days than in females on short days, but there were no significant differences between mature males on long days and immature males on long days, across the sampling period of five days and for a single day (Table 3.2, Figure 3.40 and Figure 3.41). Data from the first plasma sample collected one day after the start of the faecal collection period were used for the single day comparisons.

Highly significant relationships were found between plasma progesterone concentrations and ovary weight over the sampling period of five days and for a single day (Figure 3.6 and Table 3.3).

There were no significant differences in plasma progesterone concentration between birds in each group (Figure 3.42, Table 3.4). All plasma progesterone concentrations for short day females were measured at the least detectable dose, and only one bird in each of the male groups had plasma concentrations measured above the least detectable dose. No significant changes in plasma progesterone were found for any of the groups over the sampling period (Friedmans' non-parametric analyses were inappropriate due to missing values so paired T-tests were conducted for each pairing of days within a group; $p>0.05$ in all cases, Figure 3.43 - Figure 3.47), except for long day females. A significant increase in plasma progesterone was found between the second and final plasma sample ($t = -5.746$, $df = 2$, $p = 0.029$). There was no significant difference between the first and final plasma sample ($t = -1.833$, $df = 5$, $p = 0.126$). The coefficients of variation in plasma progesterone concentration for individual birds were $71.2 \pm 10.2\%$ in females on long days, $30.8 \pm 18.5\%$ in immature males on long days and $12.1 \pm 0.1\%$ in mature males on long days. Since all plasma progesterone concentrations for short day females were measured at the least detectable dose, the coefficient of variation was 0% for this group (Figure 3.43 - Figure 3.46). The coefficient of variation was considerably higher for a single day sample for females on long days (Table 3.5).

3.3.11 Faecal progesterone

Faecal progesterone concentrations across all groups ranged from 5.20 to 58.39 ng/g (Figure 3.40). Total faecal progesterone production over 24 hours ranged from 46.29 to 1 007.00 ng (Figure 3.48). Faecal progesterone concentrations were significantly higher in females on long days than in females on short days across the sampling period of five days and for a single day (Table 3.2). Faecal progesterone production was also significantly higher in females on long days than in females on short days. There were no significant differences in faecal progesterone concentration or production between mature males on long days and immature males on long days across the sampling period of five days. However, for a single day, immature males on long days had significantly higher faecal progesterone concentrations than mature males on long days (Table 3.2, Figure 3.49). Data from the second faecal sample were used for the single day comparisons.

Highly significant relationships were found between faecal progesterone concentrations and ovary weight over the sampling period of five days and for a single day (Figure 3.15 and Table 3.3).

There were significant differences in faecal progesterone concentration between birds for long day males but not for other groups (Figure 3.50, Table 3.6). No significant changes in faecal progesterone were found for any of the groups over the sampling period (Friedmans' non-parametric analyses, short day female: $t = 5.840$, $p = 0.211$; long day female: $t = 2.667$, $p = 0.615$; immature long day male: $t = 3.200$, $p = 0.525$; long day male: $t = 6.667$, $p = 0.155$; Figure 3.51 - Figure 3.55). The coefficients of variation in faecal progesterone concentration for individual birds were 33.1 ± 3.2% in females on short days, 21.6 ± 2.3% in females on long days, 27.5 ± 3.7% in immature males on long days and 27.2 ± 3.9% in mature males on long days (Figure 3.51 - Figure 3.54). The coefficient of variation did not differ greatly between individual or pooled samples for birds on long days. However, in females on short days the coefficient of variation was lower for pooled samples (Table 3.7).

3.3.12 Relationship between plasma and faecal progesterone

Highly significant correlations were found between plasma and faecal progesterone over the sampling period of five days for both faecal concentration and production. Significant correlations were found between plasma and faecal progesterone for a single day for both faecal concentration and production (Figure 3.56, Table 3.10). The correlations were much lower for data from a single day compared with data from five days. The single day correlation was conducted between plasma samples collected one day after the start of the faecal collection, and faecal samples from the second day. Table 3.10 also summarises the relationships found within each group between plasma and faecal concentration and production for five days and a single day. All relationships within each group were insignificant ($p>0.05$ in all cases). No correlation was performed where all plasma samples were at the least detectable dose.

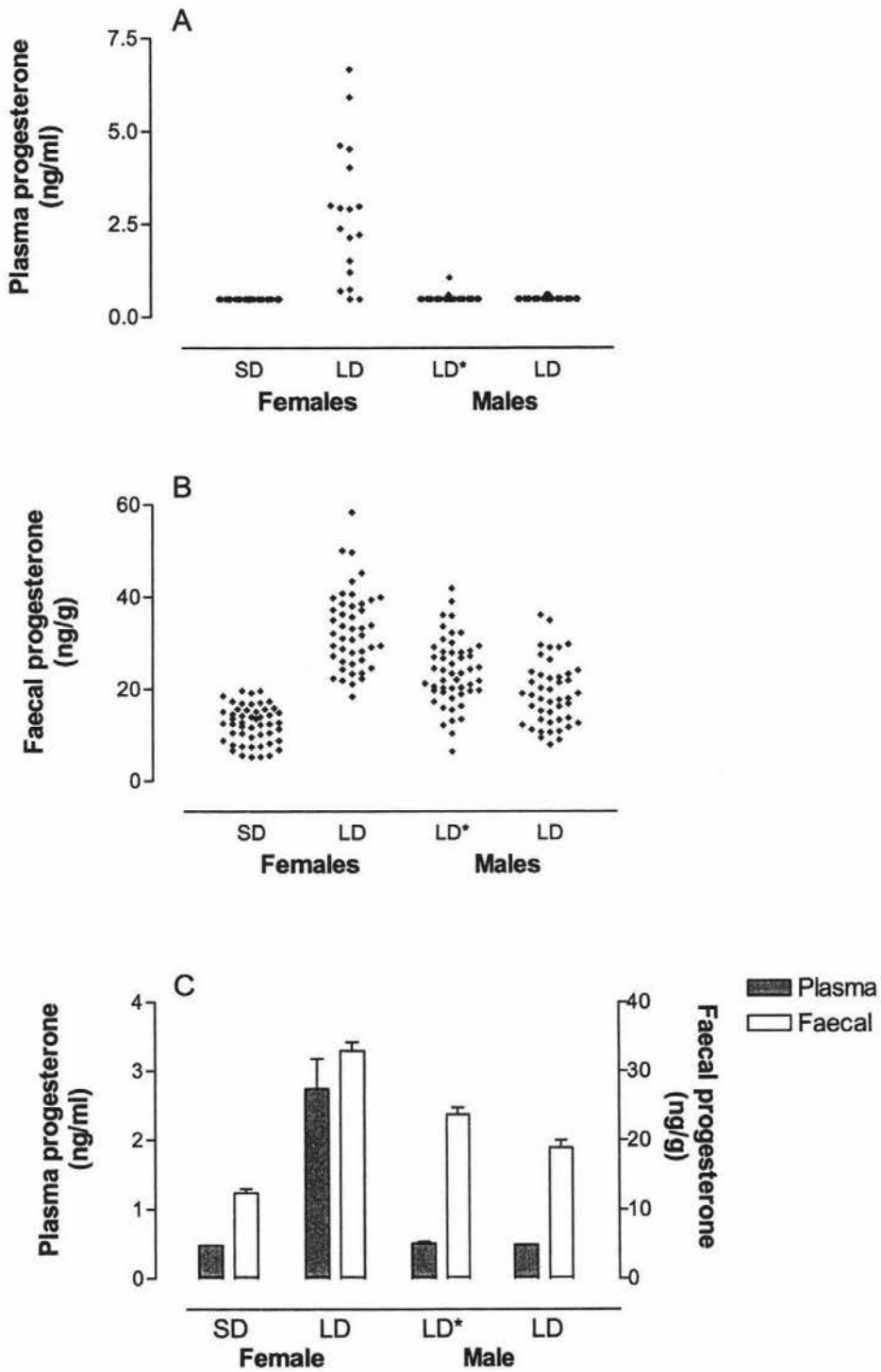

Figure 3.40. Plasma and faecal progesterone concentrations (SD = short day, LD = long day, LD* = immature males). A) Individual plasma progesterone concentrations for quail held on different photoperiods (n = 28, 18, 24 and 23 for each group respectively). B) Individual faecal progesterone concentrations for quail held on different photoperiods (n = 50, 45, 50 and 45 for each group respectively). C) Mean plasma and faecal progesterone concentrations ± SE.

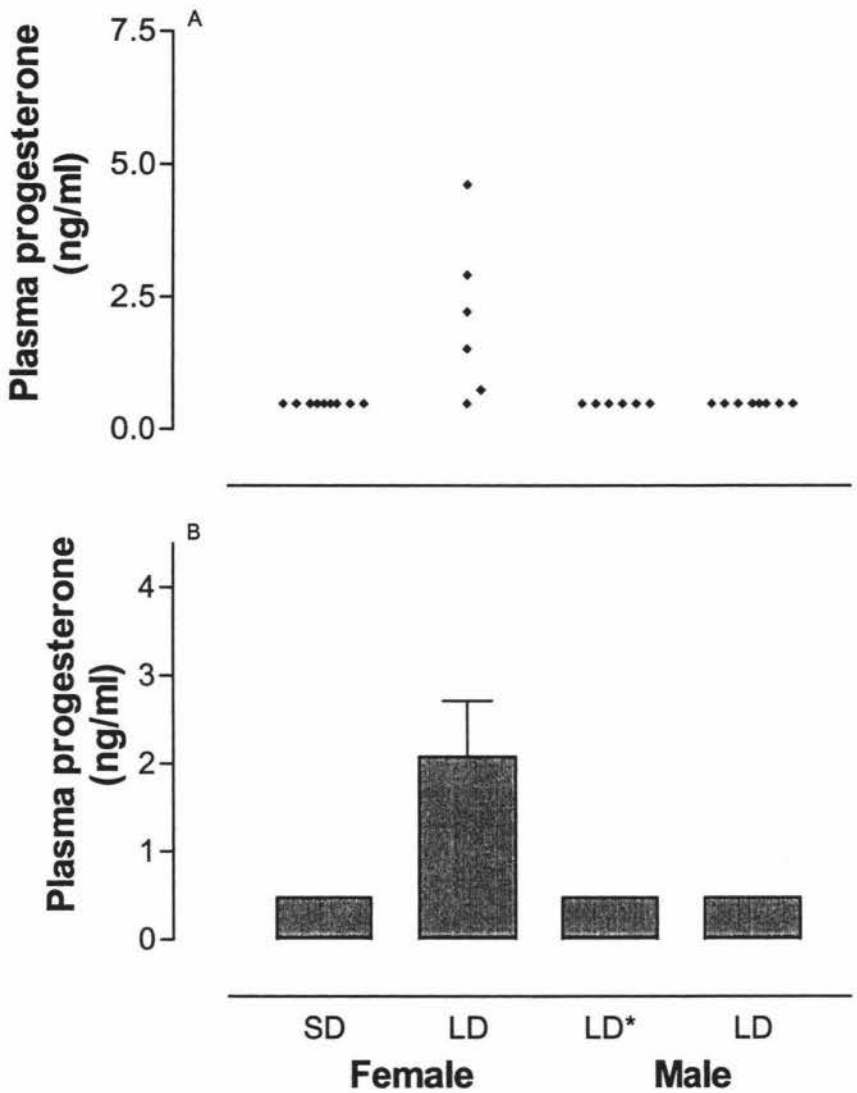

Figure 3.41. Plasma progesterone concentrations for one day (first plasma sample, SD = short day, LD = long day, LD* = immature males). A) Individual plasma progesterone concentrations for quail held on different photoperiods (n = 9, 6, 6 and 8 for each group respectively). B) Mean plasma progesterone concentrations ± SE.

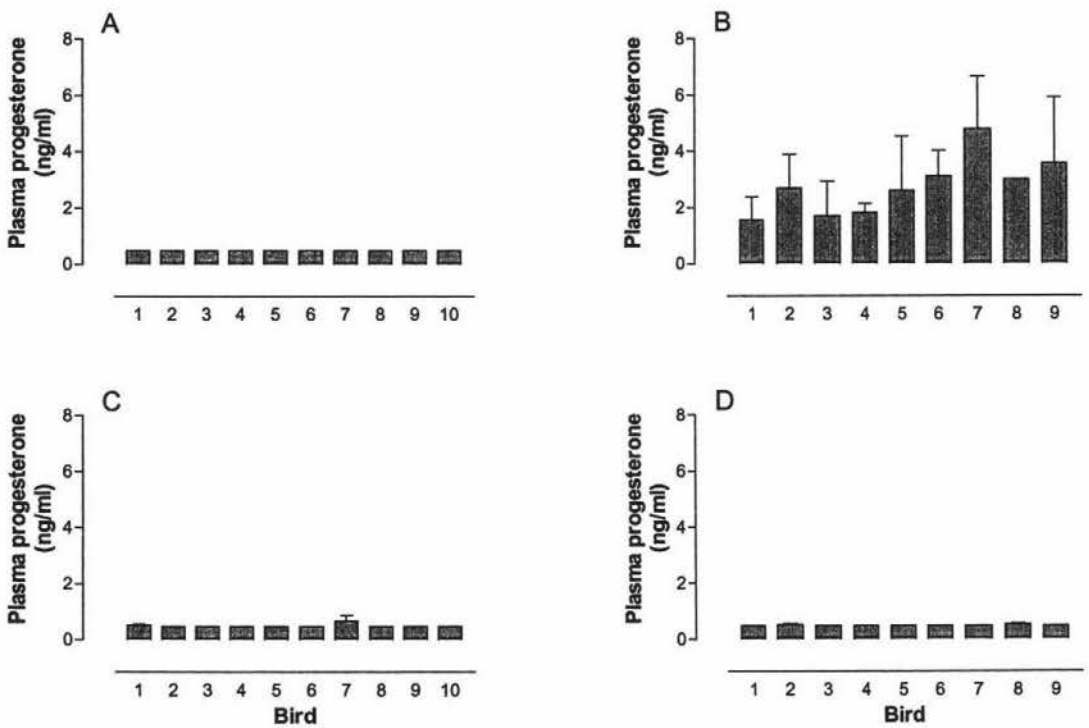

Figure 3.42. Mean plasma progesterone concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) immature males held on a long day photoperiod, D) males held on long day photoperiod.

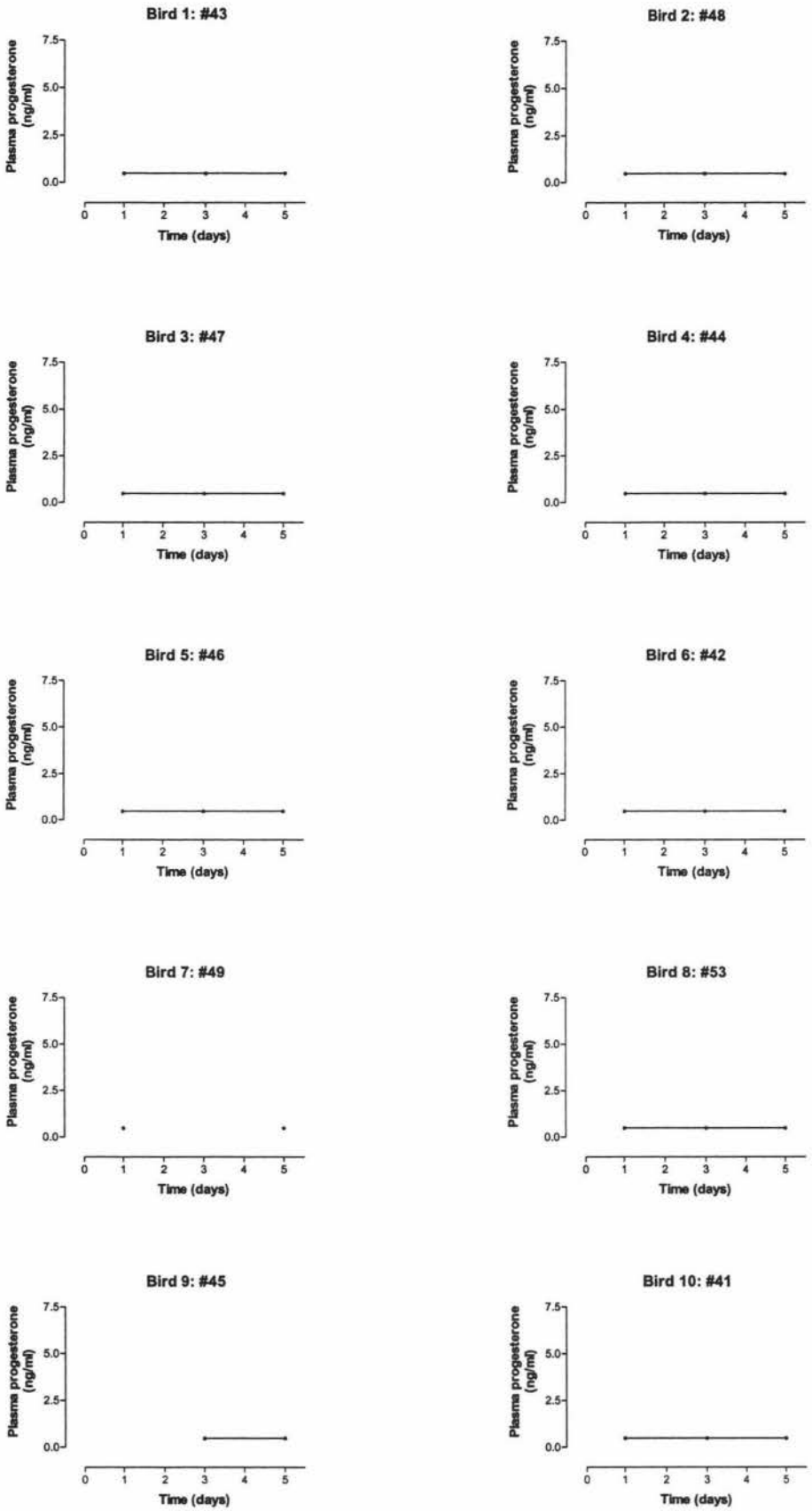

Figure 3.43. Changes in plasma progesterone concentrations over time in individual females on short days. Samples were collected one, three and five days after the start of the faecal collections.

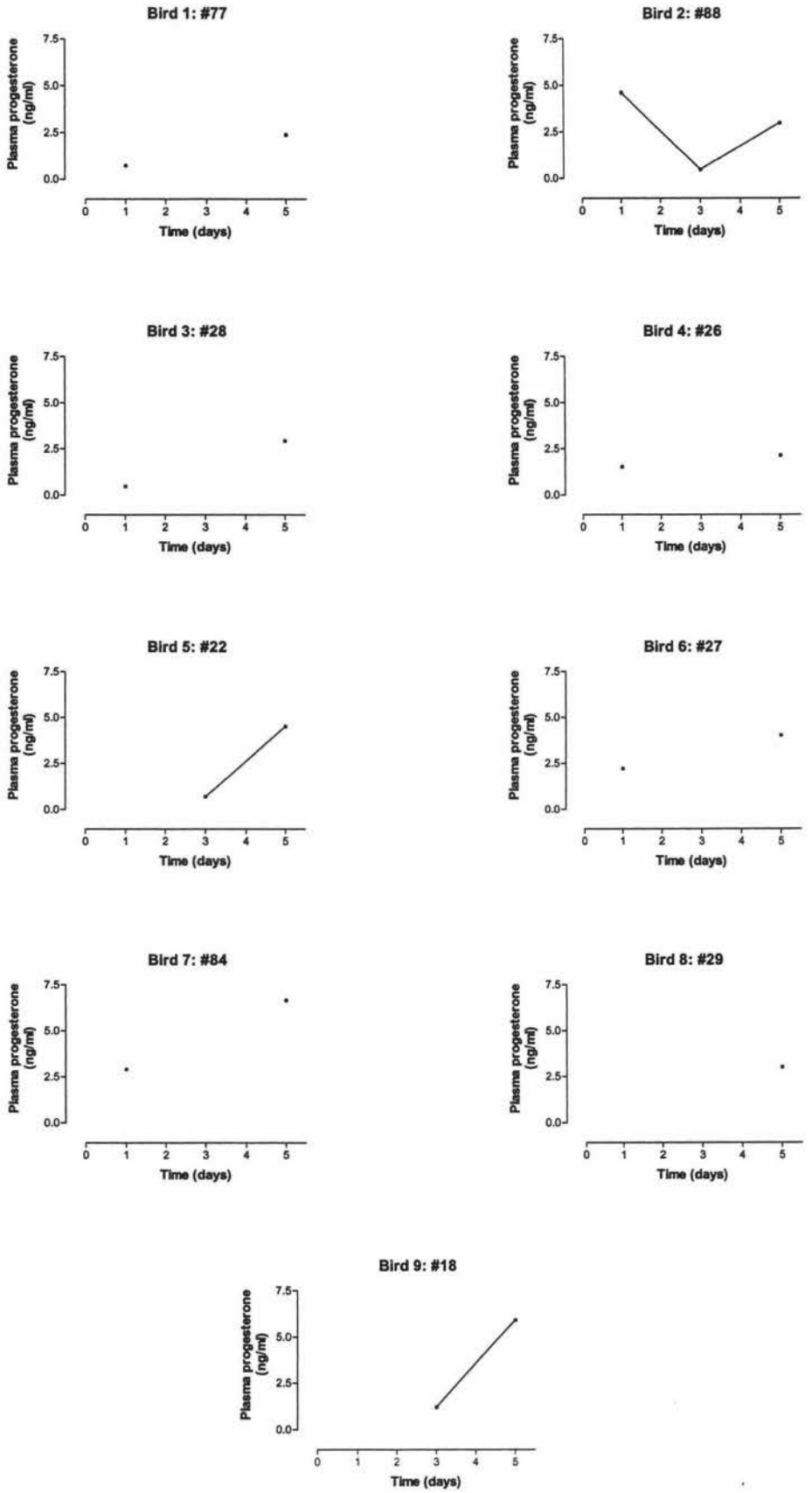

Figure 3.44. Changes in plasma progesterone concentrations over time in individual females on long days. Samples were collected one, three and five days after the start of the faecal collections.

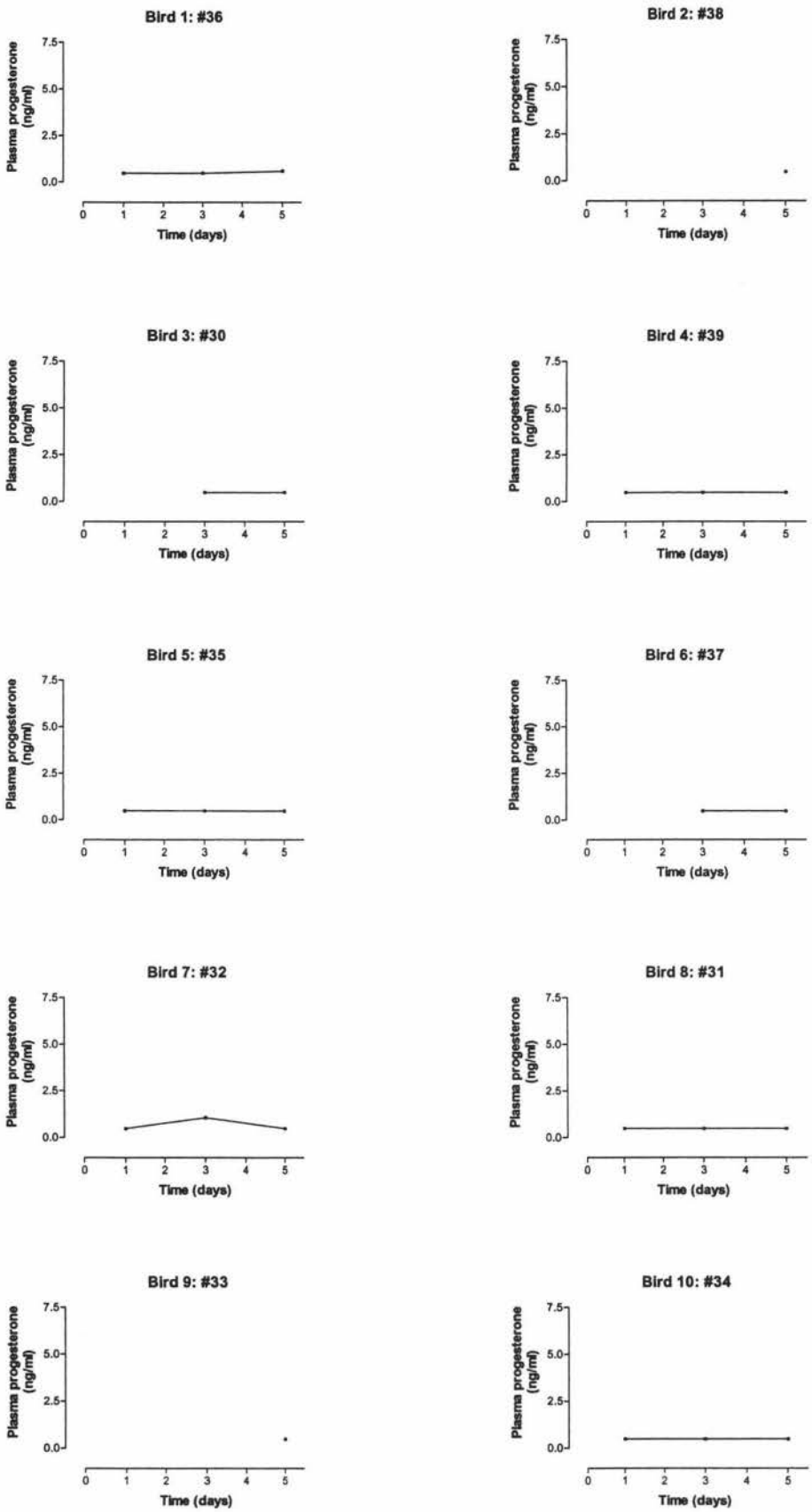

Figure 3.45. Changes in plasma progesterone concentrations over time in individual immature males on long days. Samples were collected one, three and five days after the start of the faecal collections.

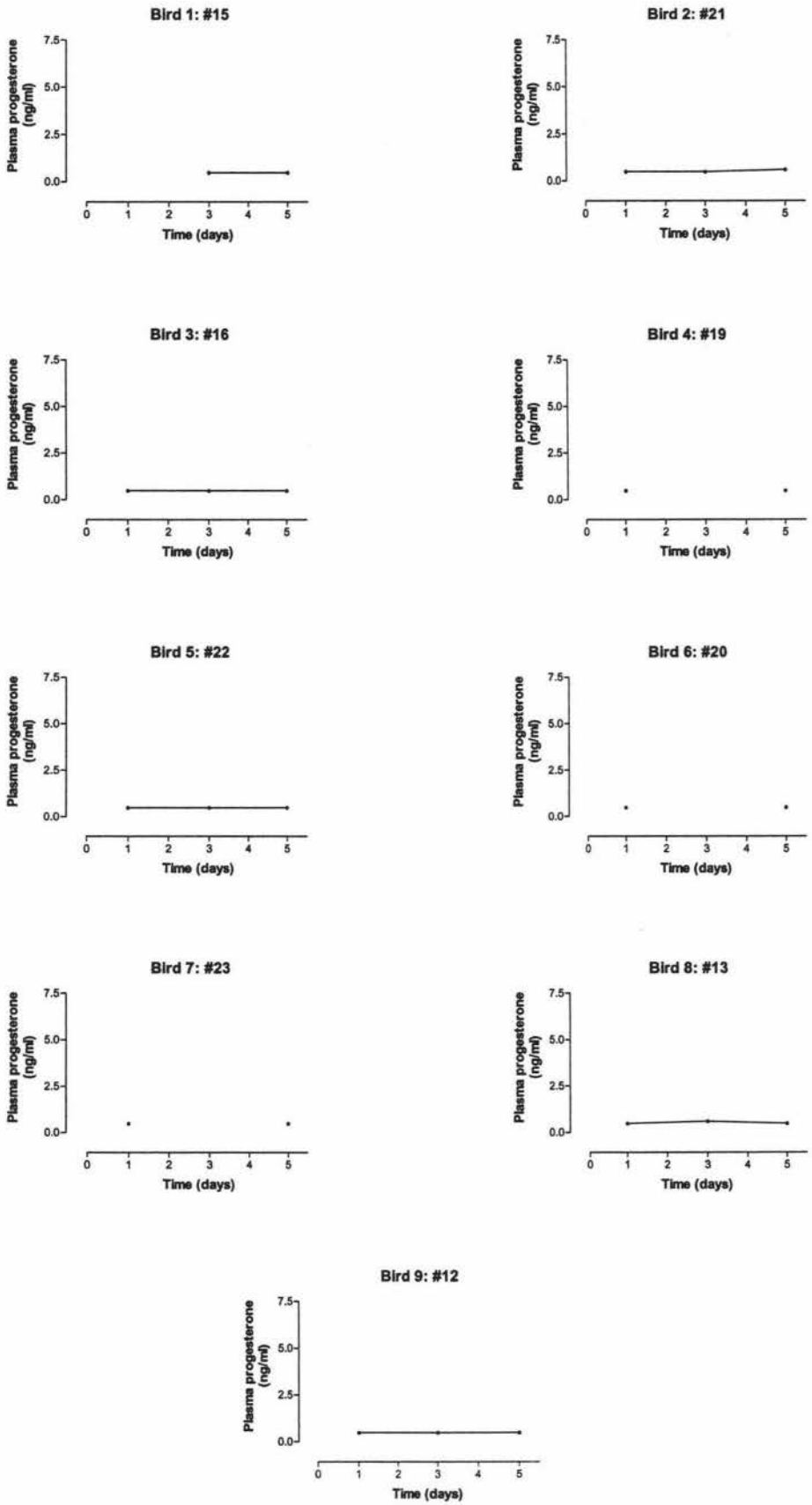

Figure 3.46. Changes in plasma progesterone concentrations over time in individual males on long days. Samples were collected one, three and five days after the start of the faecal collections.

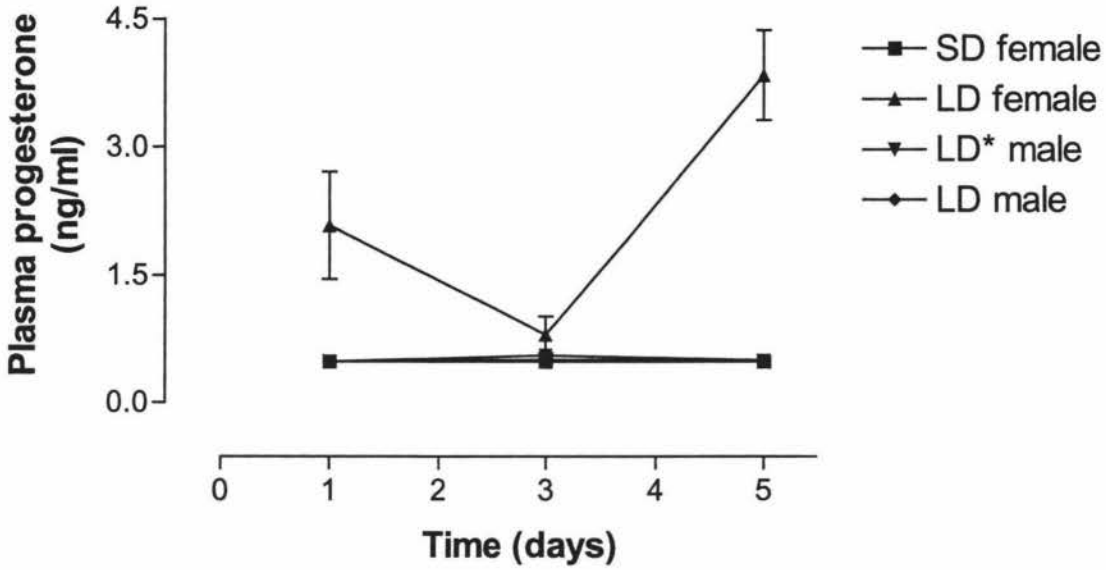

Figure 3.47. Change in mean plasma progesterone concentrations over time in quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males). Samples were collected one, three and five days after the start of the faecal collections.

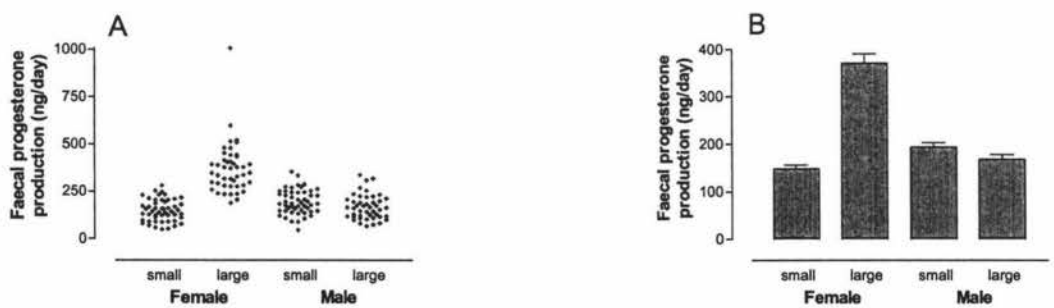

Figure 3.48. Daily faecal progesterone production (SD = short day, LD = long day, LD* = immature males). A) Individual faecal progesterone production for quail held on different photoperiods. B) Mean faecal progesterone production ± SE.

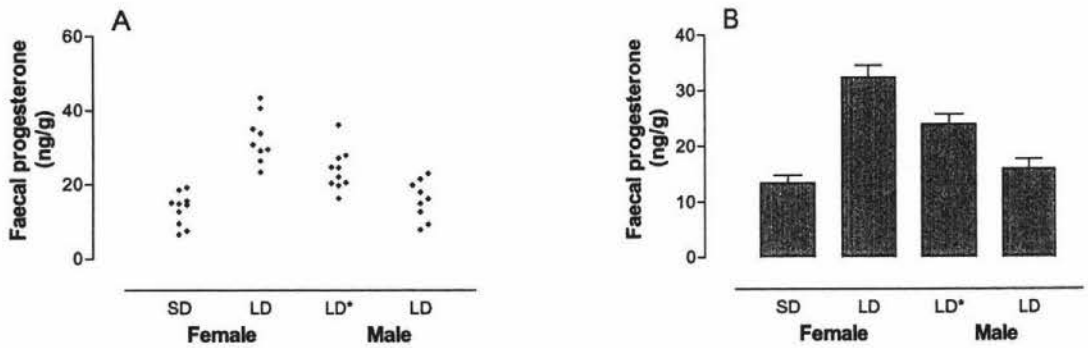

Figure 3.49. Faecal progesterone concentrations for one day (second faecal sample, day two; SD = short day, LD = long day, LD* = immature males). A) Individual faecal progesterone concentrations for quail held on different photoperiods. B) Mean faecal progesterone concentrations ± SE.

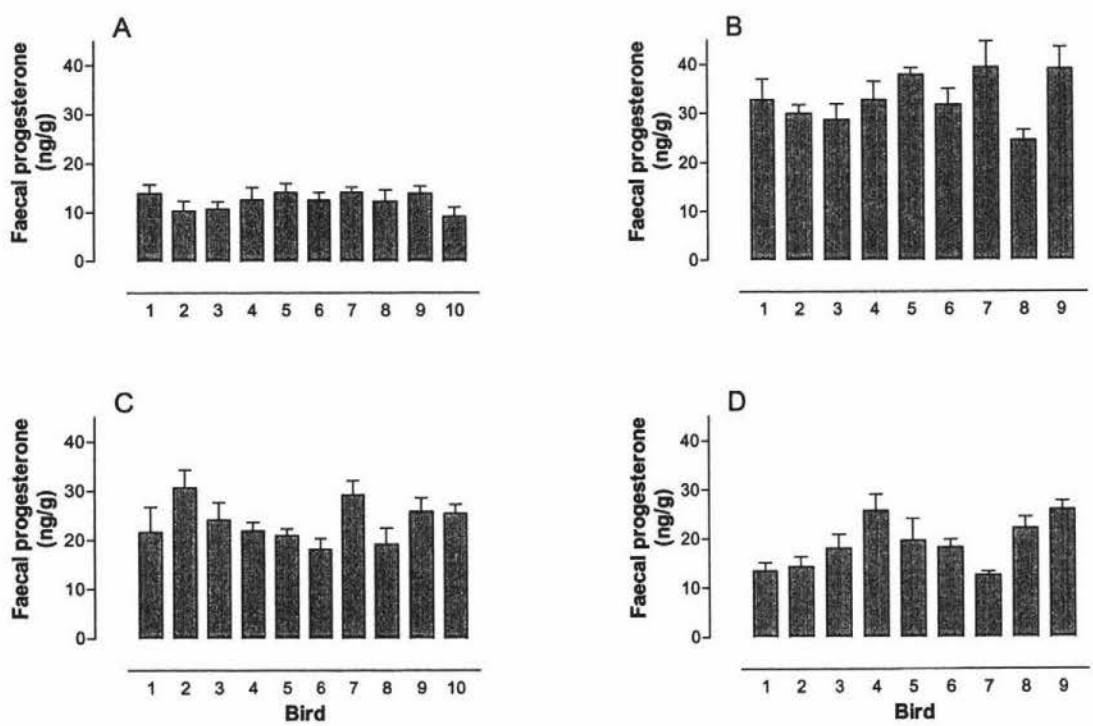

Figure 3.50. Mean faecal progesterone concentrations (± SE) for individual birds. A) females held on a short day photoperiod, B) females held on a long day photoperiod, C) immature males held on a long day photoperiod, D) males held on a long day photoperiod.

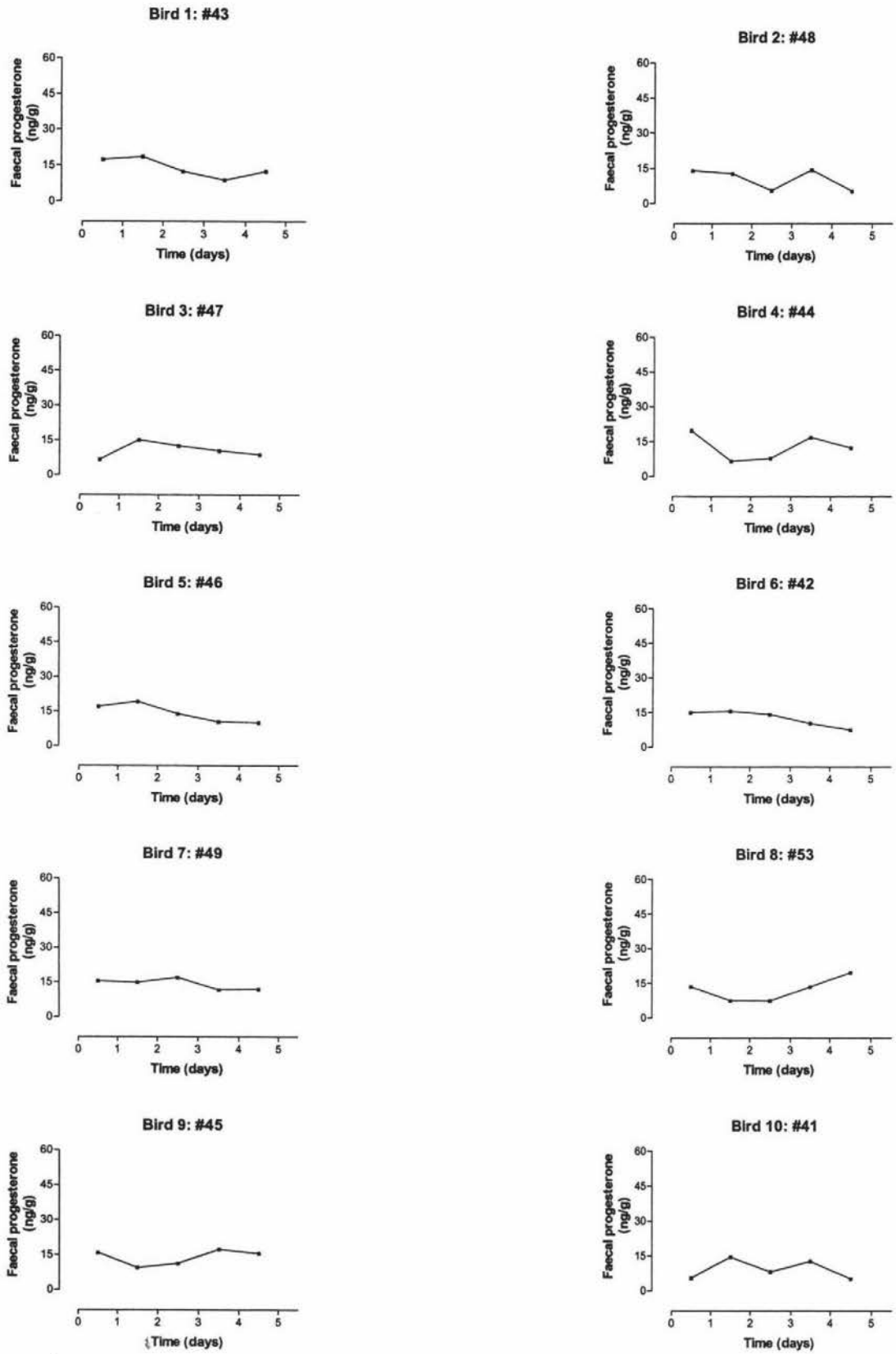

Figure 3.51. Changes in faecal progesterone concentrations over time in individual females on short days.

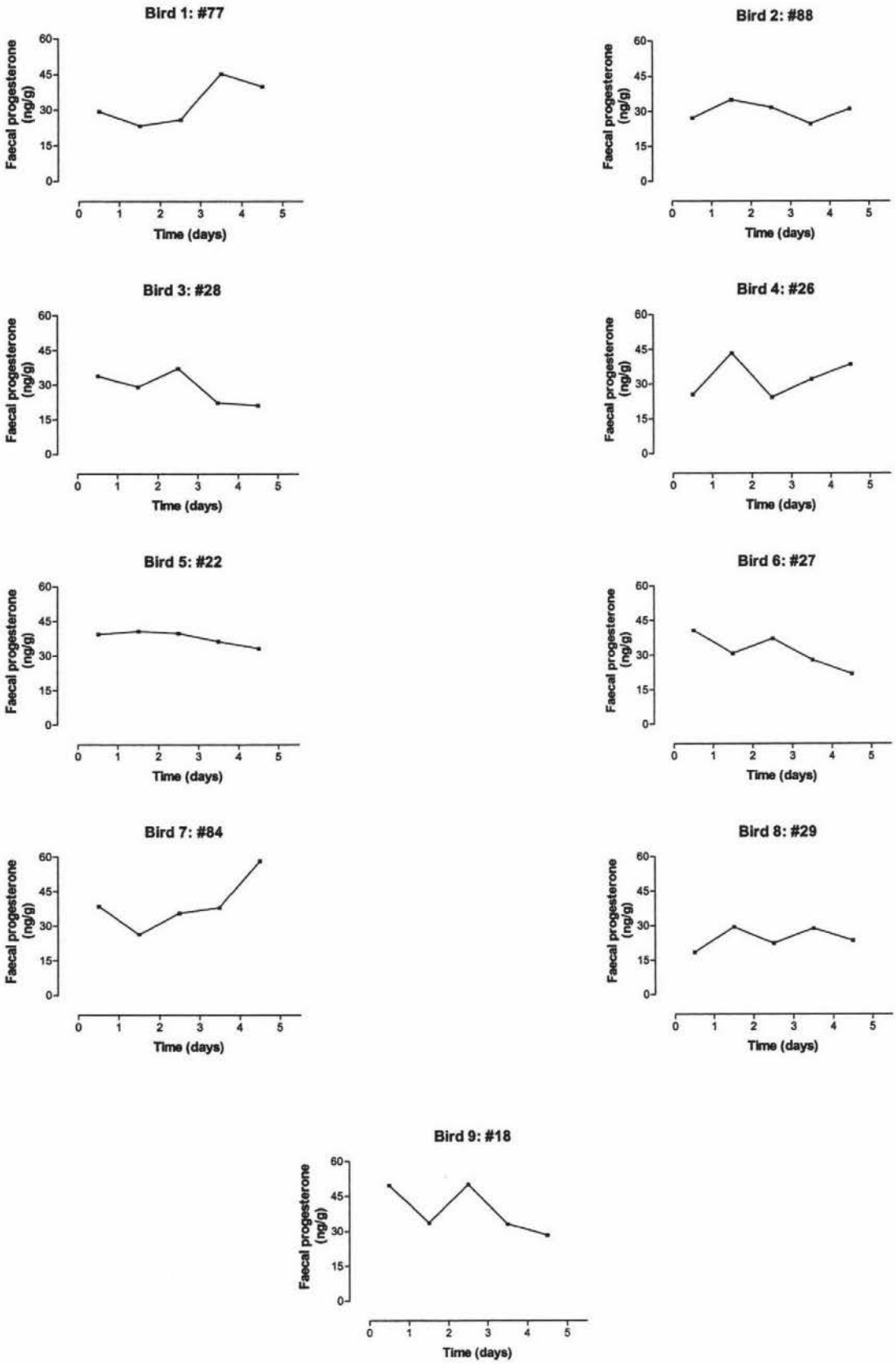

Figure 3.52. Changes in faecal progesterone concentrations over time in individual females on long days.

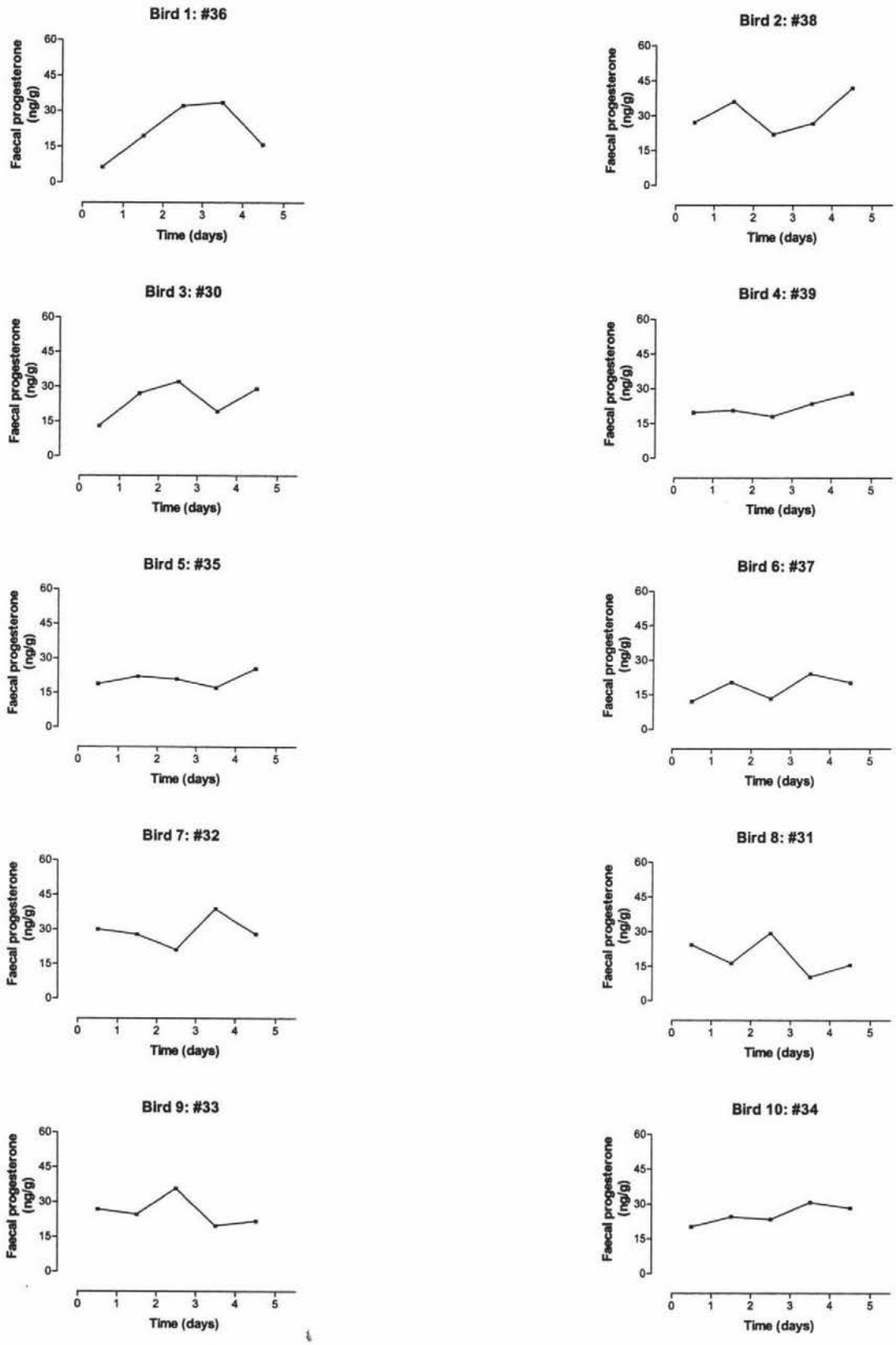

Figure 3.53. Changes in faecal progesterone concentrations over time in individual immature males on long days.

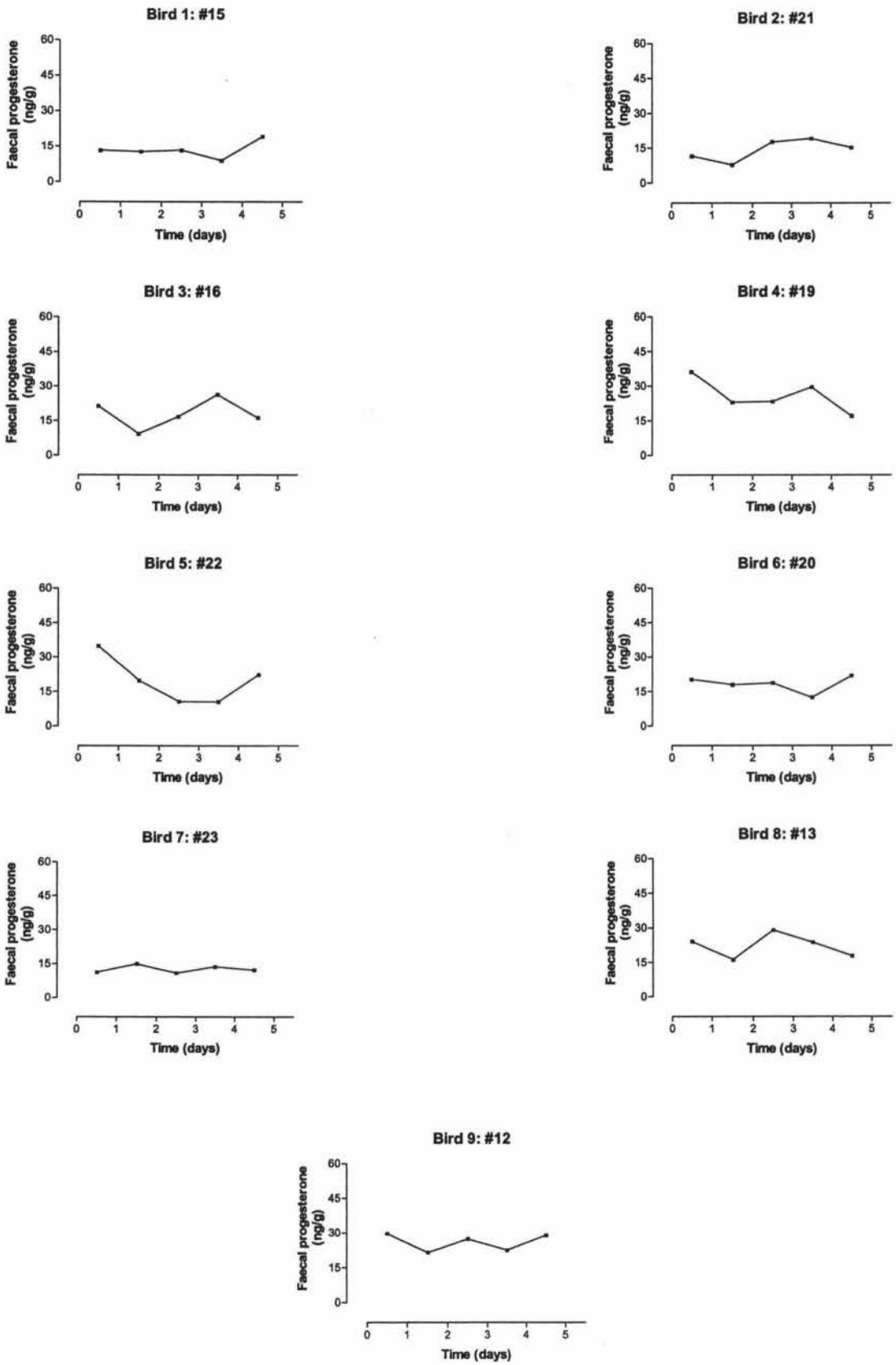

Figure 3.54. Changes in faecal progesterone concentrations over time in individual males on long days.

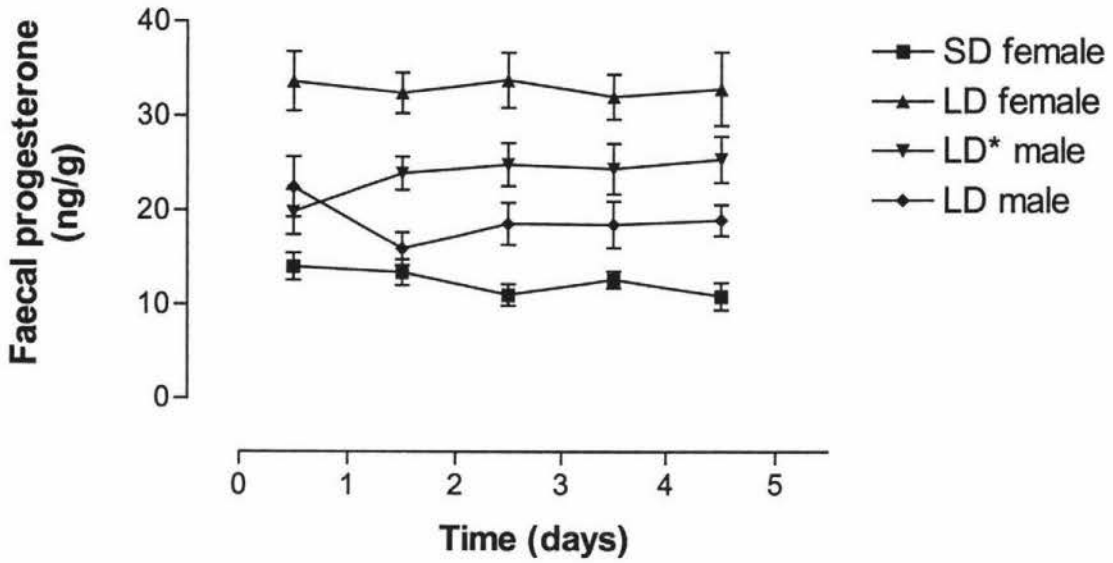

Figure 3.55. Change in mean faecal progesterone concentrations over time in quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males).

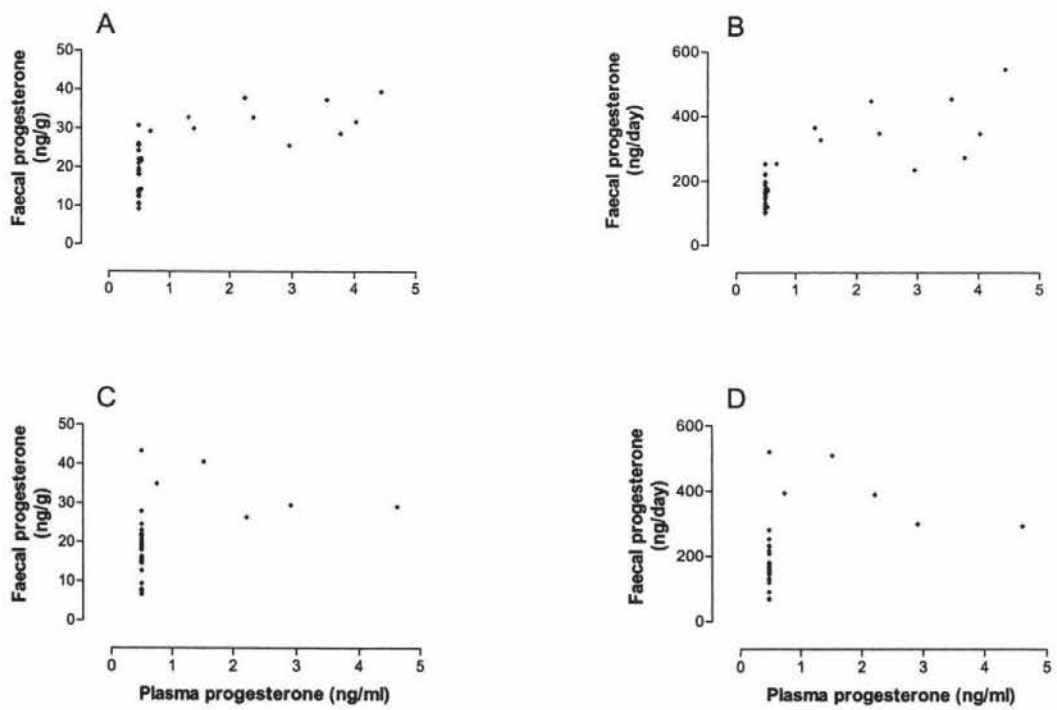

Figure 3.56. Correlations between plasma concentrations and faecal progesterone concentrations or production. Correlations are between: A mean plasma and faecal concentrations over five-days; B: mean plasma concentration and mean faecal production over five days; C: mean plasma and faecal concentrations for one day; D: mean plasma concentration and mean faecal production for one day.

Table 3.10. Correlations between plasma and faecal progesterone concentrations and production (SD = short day, LD = long day, LD* = immature males). Significant differences ($p<0.05$) are marked with an asterisk (*), and highly significant ($p<0.01$) differences are marked with a double asterisk (**). No correlation was performed where all plasma samples were at the least detectable dose.

	Five days			One day		
	r	**n**	**p**	**r**	**n**	**p**
Plasma vs faecal concentration (ng/g)	0.6909	38	0.001**	0.3961	29	0.0334*
SD Female	-	10	-	-	9	-
LD Female	0.2166	9	0.5757	0.7070	6	0.1162
SD & LD Female	0.8456	19	0.001**	0.3985	15	
LD* Male	0.4367	10	0.2070	-	6	-
LD Male	0.0735	9	0.8511	-	8	-
Plasma vs faecal production (ng)	0.7928	38	0.001**	0.3864	29	0.0384*
SD Female	-	10	-	-	9	-
LD Female	0.2735	9	0.4764	0.7996	6	0.0562
SD & LD Female	0.8002	19	0.001**	0.2933	15	
LD* Male	0.4993	10	0.1417	-	6	-
LD Male	0.2766	9	0.4713	-	8	-

3.3.13 Hormone concentration interactions in plasma

Two canonical discriminant functions (CDFs) were developed from the testosterone, oestradiol and progesterone concentrations measured in plasma samples from the four groups of birds. These functions were able to separate the four groups of birds ($p<0.001$; Figure 3.57). The first CDF (CDF_1) accounted for 67% of the variation in the data and the second, CDF_2 accounted for 33% of the variation among the four groups. CDF_1 separated the long day females and long day males from the remaining birds on the basis of progesterone and testosterone concentrations (Largest positive and largest negative SCC, Table 3.11). Long day females had high progesterone concentrations and low testosterone concentrations. In contrast, long day males had high testosterone and lower progesterone concentrations (Figure 3.57). Long day male birds were further separated from the remaining birds by testosterone concentration (CDF_2, largest positive SCC, Table 3.11). Long day males had high plasma testosterone concentrations (Figure 3.57). Immature long day male and short day females had similar hormone concentrations and were not able to be separated by either CDF.

The two CDFs correctly classified the bird groups based on hormone concentrations with 70% accuracy under cross validation.

Table 3.11. Standardised canonical coefficients (SCC) from the first two canonical discriminant functions (CDFs) developed from plasma hormone concentrations on four groups of birds

Variable	CDF_1	CDF_2
Testosterone (ng/ml)	-0.349	0.987
Oestradiol (pg/ml)	0.495	0.015
Progesterone (ng/ml)	0.835	0.037

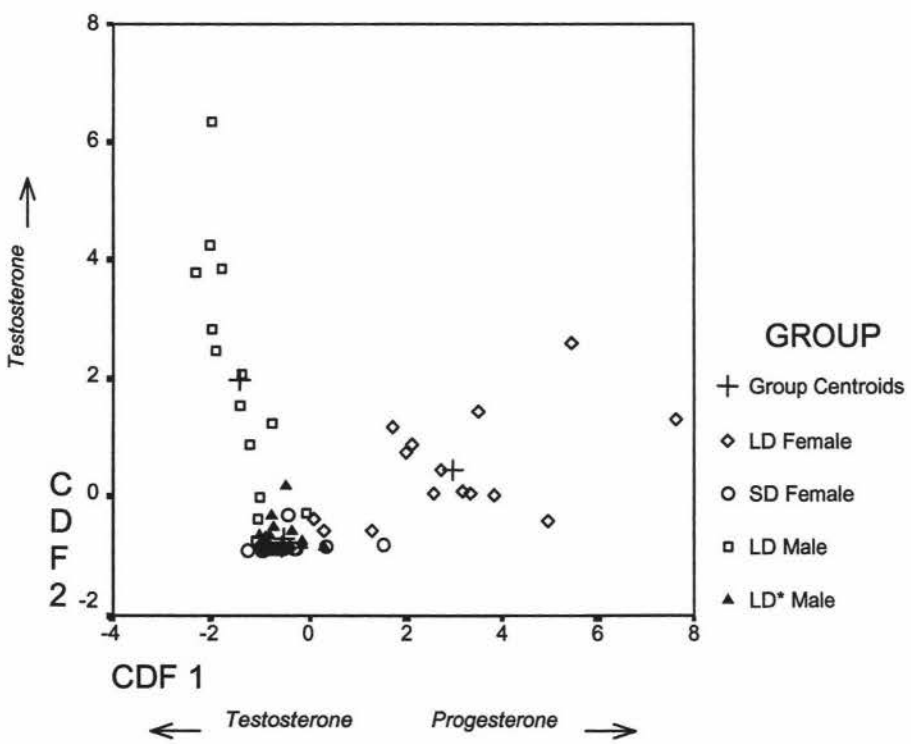

Figure 3.57. Canonical scores for the first two canonical discriminant functions (CDFs) of plasma hormone concentrations in four groups of quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males held on long days).

3.3.14 Hormone concentration interactions in faecal samples

Two canonical functions (CDFs) were developed from the testosterone, oestradiol and progesterone concentrations measured in faecal samples from the four groups of birds. These functions were able to separate the four groups of birds ($p<0.001$; Figure 3.58). The first CDF (CDF_1) accounted for 63% of the variation in the data and the second, CDF_2 accounted for 36% of the variation among the four groups (CDF_3 only accounted for the remaining 1% of the variation). CDF_1 separated the long day males from the remaining birds, and the immature long day males from the short day females on the basis of testosterone concentration (largest positive SCC, Table 3.12). Long day males had high testosterone concentrations, immature long day males had medium-level testosterone concentrations and short day females had low testosterone concentrations. (Figure 3.58). Long day females were separated from short day females and immature long day males by oestradiol concentration (CDF_2, largest positive SCC, Table 3.12). Long day females had high faecal oestradiol concentrations (Figure 3.58).

The two CDFs correctly classified the bird groups based on hormone concentrations with 97% accuracy under cross validation.

Table 3.12. Standardised canonical coefficients (SCC) from the first two canonical discriminant functions (CDFs) developed from faecal hormone concentrations on four groups of birds

Variable	CDF_1	CDF_2
Testosterone (ng/ml)	1.104	0.185
Oestradiol (pg/ml)	-0.669	0.821
Progesterone (ng/ml)	0.062	0.284

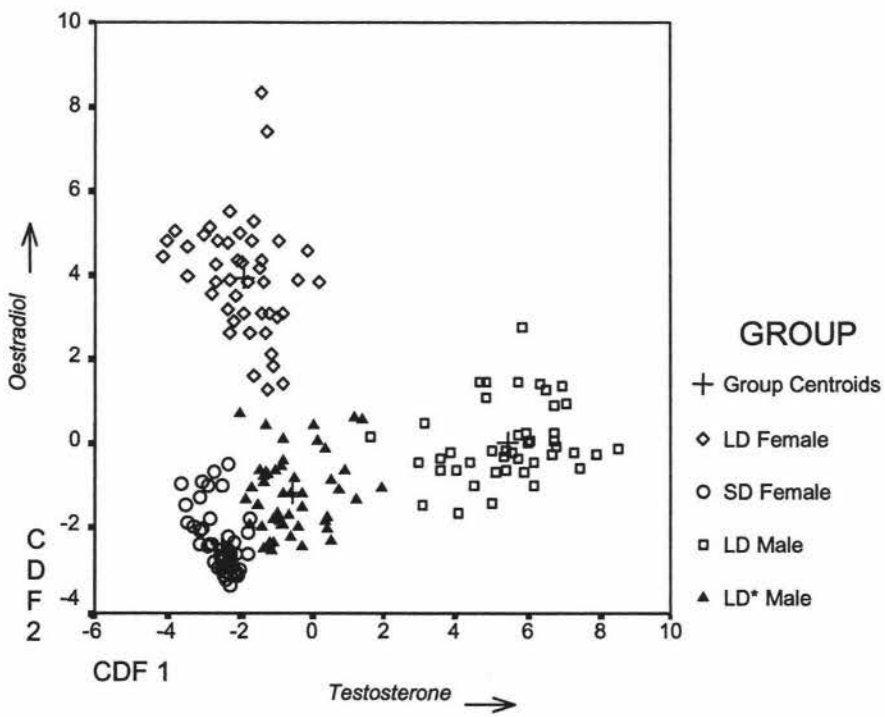

Figure 3.58. Canonical scores for the first two canonical discriminant functions (CDFs) of faecal hormone concentrations in four groups of quail held on different photoperiods (SD = short day, LD = long day, LD* = immature males held on long days).

3.4 Discussion

The goals of this study were to validate the extraction method developed in the previous chapter and to confirm that faecal concentrations of testosterone, oestradiol and progesterone reflect plasma concentrations. Faecal and plasma steroid concentrations were measured in male and female quail on short and long day lengths. Marked differences in gonad size between photoperiods were accompanied by corresponding differences in plasma and faecal steroid concentrations and clear plasma – faecal steroid relationships were established.

3.4.1 Gonad weight

It is well established that exposure to long daylengths stimulates the hypothalamo-pituitary-gonadal axis, increasing gonadotropin secretion and gonadal growth in Japanese quail (Follett and Sharp, 1969; Follett and Robinson, 1980).

In this study, all eight-week-old males on long photoperiods had developed testes and larger cloacal glands in comparison to the immature three-week-old males. The cloacal gland of male quail is androgen dependent and has been shown to be a reliable indicator of testis size and activity (Siopes and Wilson, 1975; Follett and Maung, 1978). The production of cloacal foam is also associated with reproductively active male quail (Siopes and Wilson, 1975). All eight-week-old males on long days produced cloacal foam and one immature male on long days (bird #39) produced cloacal foam on the final three days of its sampling period. There is wide variation in the number of days required for sexual maturation in wild quail (Kawahara, 1973 in Wada *et al.* 1990). One of the immature males had larger testes and higher plasma testosterone than the other males. This bird had started gonadal growth earlier than usual and presumably plasma testosterone had increased sufficiently to stimulate cloacal gland growth and function.

It is well established that cloacal gland area and testes weight increase in birds on long days (Sachs, 1967; Follett and Sharp, 1969; Follett and Maung, 1978; Follett and Robinson, 1980). Honda *et al.* (1987) reported increases in cloacal gland size in male birds from one line (KR) of Japanese quail under long days. An early study with male

Japanese quail by Oishi and Konishi (1978) investigated the size of the cloacal gland in response to various combinations of photoperiod and temperature. Low temperatures (10°C) and short day lengths were required before cloacal gland regression was significant. Wada *et al.* (1990) and Tsuyoshi and Wada (1992) showed that both low ambient temperature and a change in photoperiod from long to short days were required to suppress LH secretion and cause regression of the cloacal gland in male Japanese quail. Control birds on long days and 19°C maintained cloacal gland size and LH secretion in both studies. The New Zealand strain of Japanese quail used in the current study also requires short days and low temperatures to prevent gonadal growth from three weeks of age.

All females on long photoperiods had developed ovaries, oviducts and larger cloacal diameters in comparison to those held on a short day photoperiod and 10°C. All females on long days regularly laid eggs and showed an increase in cloacal diameter and body weight over the course of the study period. Females on short days and 10°C showed an increase in cloacal diameter, but no increase in body weight. Similar differences in ovarian and oviduct weight and egg laying behaviour between birds held on long and short days were reported by Brain *et al.* (1988), although in that study birds on short days were not kept at lower temperatures.

In the previous chapter, groups of birds were kept on short and long day photoperiods, at an ambient temperature of 20 - 26°C. Most birds on long photoperiods had developed gonads in comparison to those held on a short day photoperiod, although one female of seven had an undeveloped ovary and one male of five had an undeveloped testis. One female on short days had unusual ovarian growth with a fully developed follicular hierarchy. This bird began regularly laying eggs at 6 weeks, despite exposure to short day lengths from the age of 3 weeks. Two males on short days began producing cloacal foam from 6 weeks of age. Results from the current study support the idea that there are two types of quail with respect to termination or suppression of breeding activity - those that need only short days and those that need both short days and low temperatures. It appears from the current study that the New Zealand strain of quail require both short days and low temperatures to prevent gonadal growth.

3.4.2 Faecal production

Female quail produced a greater amount of droppings compared to males. All females on long days were laying eggs daily. Laying females tended to eat more than males (pers. obs.) and hence produce larger faeces. Females on short days and 10°C. had increased energy requirements due to the low ambient temperature and also tended to eat more than the males on long days (pers. obs.).

In the study described in the previous chapter, individual droppings were collected over a period of three hours. Females on long days produced significantly heavier droppings than any other group. The total faecal hormone content of individual droppings increased as expected with increasing dropping weight, so faecal hormone concentrations were expressed as the ng steroid/g dried dropping to avoid the effect of dropping size. In this study, all droppings for 24 hours were collected, so total faecal hormone production over 24 hours (ng) could be calculated in addition to faecal hormone concentration.

3.4.3 Testosterone

Plasma and faecal testosterone concentrations were higher in birds on long days than in birds on short days (or LD* males in the case of males), and were similar to those recorded previously for quail kept on the respective photoperiods (Follett and Maung, 1978; Doi *et al.*, 1980; Bishop and Hall, 1991; Ishii *et al.*, 1994) and for domestic fowl (Cockrem and Rounce, 1994). Not all birds in the other studies were of comparable age, but reproductive status was similar. In the study described in the previous chapter, faecal testosterone concentrations in the two short day groups were up to three-fold higher than those reported in this study. This could be due to a difference in the hormone extraction, or in the age of the birds. Immature males on long days and females on short days in this study were of three and 6.5 weeks of age respectively, whereas both groups of short day birds in the previous study were 11 weeks of age.

Data were analysed across the sampling period of five days and for a single day. Faecal testosterone concentrations may represent plasma testosterone concentrations

approximately one to four hours prior to their collection (Tell, 1997). Therefore, data from the first plasma sample collected one day after the start of the faecal collection period and from the second faecal collection were used for the single day comparisons to ensure samples representing the same time period were being compared. Differences between groups of birds in plasma and faecal testosterone concentrations were apparent using single or multiple samples from each bird. This is consistent with the study in the previous chapter, where individual droppings were found to be as accurate for measuring the hormone concentration in a bird as a pooled faecal sample. Although no other studies have investigated the relationship between individual and pooled samples over 24-hour periods as in this study, individual droppings have been used to compare plasma and faecal hormone concentrations (Cockrem and Rounce, 1994; and Ishii *et al.*, 1994), describe gonadal cycles (Kofuji *et al.*, 1993) and sex various bird species (Bercovitz *et al.*, 1978; Stavy *et al.*, 1979).

There was a slight increase in faecal testosterone from the beginning to the end of the sampling period for females on short days and a greater increase for immature males. These results may indicate a minor increase in ovarian steroidogenic activity in female quail on short days from 6.5 – 7.5 weeks of age. They also reflect an expected increase in testicular steroidogenesis in male quail on long days from three to four weeks of age.

Total daily faecal testosterone production showed the same pattern as plasma and faecal concentrations. Measurement of the total faecal hormone production over 24 hours avoids possible diurnal fluctuations in steroid concentrations in individual samples, and would be useful in diagnostic situations where precise detection of small changes in hormone concentrations may be required (e.g. detection of ovulation). However, when faecal samples cannot be collected from individual birds over 24 hours, faecal steroids should be reported as hormone concentration per gram of dried dropping.

Plasma testosterone in long day males and females varied over wider ranges than faecal testosterone for these groups. The correlations between hormone concentrations and gonad size were also greater for faecal than for plasma samples. The faecal samples reflected the mean faecal hormone concentration over a 24-hour collection period, whereas the plasma samples were single determinations of plasma hormone

concentration at one time. Variation between individual faecal samples can be greater than between individual plasma samples (Ishii *et al.*, 1994; Hirschenhauser *et al.*, 2000), but the integrated measure of hormone production provided by 24-hour faecal samples may in fact provide a better indication of plasma steroid concentrations in a bird than a single plasma sample.

In immature males on long days, the coefficient of variation between birds for a pooled five-day plasma sample was considerably higher than for a single day sample, whereas in the other groups they were similar. The single day sample was the first plasma sample collected one day after the start of the faecal collection period. As these immature males were expected to undergo some gonadal growth over the time period of collection, the higher coefficient of variation for a pooled sample was not unexpected. Since faecal concentrations of testosterone increased over the five days in this group, an increased coefficient of variation would be expected in the pooled five-day faecal sample as well. However, the coefficients for pooled and single faecal samples were similar for all four groups of birds. Therefore, as found in the previous chapter and by Cockrem and Rounce (1994), a single sample does not appear to result in greater variation between birds than pooled samples.

The variation in testosterone concentrations was greater in plasma samples than in faecal samples within an individual for the groups where high testosterone concentrations were measured. In the other two groups, plasma testosterone was below or near the least detectable dose, so variation was low. Mean coefficients of variation within an individual were 14.5 ± 6.6% in plasma and 36.3 ± 5.9% in faeces in short day females, 45.3 ± 8.3% and 13.8 ± 1.2% in long day females, 27.3 ± 4.9% and 30.7 ± 3.4% in immature long day males, and 49.4 ± 11.7% and 9.8 ± 1.2% in long day males. This was expected as the faecal steroid concentrations reflect plasma concentrations over 24 hours in comparison to the single time point plasma measurement.

A clear relationship between steroid hormone concentration and gonadal development has been established in many species of bird, including Japanese quail (Follett and Maung, 1978; Follett and Robinson, 1980). As expected, relationships between both plasma and faecal testosterone and paired testes weight were evident over five days and

one day of sampling. The significance level was lower for a single day plasma sample. This suggests that although a single sample is as accurate as an average value taken over five days to measure testosterone concentration, an average value is more valuable when relationships between concentration and gonad size are being evaluated. The relationship between paired testis weight and faecal testosterone was stronger than the relationship with plasma testosterone. A similar result was reported in the previous chapter (r for faecal testosterone = 0.84, no correlation for plasma).

The correlations between plasma and faecal testosterone concentrations for single and multiple samples (r = 0.83 and 0.86) were markedly higher than previously reported for birds. Faecal testosterone has been validated in three previous studies (Bishop and Hall, 1991; Cockrem and Rounce, 1994; and Ishii *et al.*, 1994), but correlation coefficients have only been reported in two of these. Cockrem and Rounce (1994) reported r = 0.51 and 0.49 for single and multiple samples from chickens and Bishop and Hall (1991) reported r = 0.59 for multiple samples from male quail. In this study an r-value of 0.88 was found for samples from males. In both the Cockrem and Rounce and Bishop and Hall studies, individual droppings were collected, whereas in the current study, 24-hour samples were collected. This may have contributed to the higher correlations between faecal and plasma testosterone concentrations found in this study. Hirschenhauser et al., (2000) measured plasma and faecal androgens in geese, but did not find any significant correlations between them. In the previous chapter, demonstration of the relationship between plasma and faecal testosterone was attempted with individual droppings. This was unsuccessful (r = 0.10) due to a lack in ranges of plasma steroid concentrations.

No relationships were found between plasma and faecal testosterone concentration or production within each group of birds. It has been clearly demonstrated that large differences between birds in plasma testosterone will be reflected by differences in faecal testosterone. However, smaller differences in plasma testosterone, as seen within each group are not reflected by differences in faecal testosterone due to a lower resolution of the faecal method.

3.4.4 Oestradiol

Plasma and faecal oestradiol concentrations were higher in females on long days than in females on short days. However, no differences in plasma oestradiol concentrations were found between mature and immature males kept on long days. Similar plasma measurements have been reported previously for male and female quail under the same photoperiods (W.H. Chua, pers. comm.) and female domestic fowl kept on long days (mean of 380 pg/ml; Cockrem and Rounce, 1994). Doi *et al.* (1980) and Gulati *et al.* (1981) reported plasma oestradiol concentrations during the ovulatory cycle in laying female quail on 14 hours of light which were approximately two-fold lower than in this study (ranging from 71–181 pg/ml and 86-133 pg/ml respectively compared to 190-490 pg/ml). Differences may be due to assay methods, age or strain of quail. In those studies, quail were 10-13 weeks of age compared to 8 weeks in this study. Chua (pers. comm.) has shown that oestradiol can decrease with age (6 – 14 weeks) in the strain of quail used in this study despite the birds having large ovaries. Comparable faecal oestradiol concentrations have also been previously reported in domestic fowl (Cockrem and Rounce, 1994) and in both male and female quail on similar photoperiods (Bishop and Hall, 1991), although in that study oestradiol-3-glucuronide was measured in contrast to free steroids. In the study described in the previous chapter, plasma and faecal oestradiol concentrations in the long day female group were lower than those reported in this study. This is most likely due to a difference in the hormone extraction, or in the age of the birds. Females on long days in this study were of eight weeks of age, whereas in the previous study they were of 11 weeks of age, at which time oestradiol concentrations may have decreased (W.H. Chua, pers. comm.).

The relative differences in oestradiol concentrations between groups of birds were similar for single and multiple samples from each bird, as found for testosterone.

There was a slight increase in faecal oestradiol from the beginning to the end of the sampling period for females on short days, as found for testosterone. This might reflect increased steroidogenesis in the females over this period.

Total daily faecal oestradiol production showed the same pattern as plasma and faecal concentrations over five days. Production was higher in females kept on long days than in females kept on short days and there were no differences between the two groups of males.

In three of the four groups of birds no variation in plasma or faecal oestradiol concentrations between birds was found. The exception was the short day females. In this group there was variation in faecal concentrations of oestradiol between birds. Only three or two plasma samples per bird (in a few cases only one) were used to evaluate the variation between birds compared to five faecal samples. Therefore, sample size may well be affecting this outcome.

In females on short days and immature males on long days, the coefficient of variation for a pooled five-day plasma sample was considerably lower than for a single sample. In the remaining two long day groups they were similar. The coefficients for pooled faecal samples were also lower in females on short days and immature males on long days, as well as in the long day female group. Therefore, in contrast to testosterone, the previous chapter and Cockrem and Rounce (1994), there was greater variation between birds in oestradiol concentrations for single compared with multiple samples.

Variability in oestradiol concentration was very similar within an individual for plasma and faecal samples for all groups. Mean coefficients of variation within an individual were 32.4 ± 6.5% in plasma and 29.8 ± 4.3% in faeces in short day females, 18.5 ± 5.4% and 16.6 ± 1.6% in long day females, 32.1 ± 7.4% and 31.3 ± 3.2% in immature long day males, and 30.9 ± 4.0% and 26.4 ± 2.6% in long day males. Variability in plasma samples within an individual were expected to be higher than faecal samples, as in the case of testosterone, as faecal steroid concentrations reflect plasma concentrations over 24 hours in comparison to the single time point plasma measurement.

There were strong correlations between plasma and faecal oestradiol and ovary weight in this study, with higher correlations when multiple rather than single samples were used. Although a single day sample may be as accurate as an average value taken over several days to measure oestradiol concentration, an average value is more valuable

when relationships between concentration and gonad size are being evaluated. In the previous chapter correlations between plasma and faecal oestradiol and ovary weight were insignificant.

A correlation between plasma and faecal oestradiol concentration has only been reported previously for one avian species. Cockrem and Rounce (1994) reported r-values of 0.59 for multiple samples and 0.62 for single samples in chickens. In the current study, correlation coefficients of $r = 0.82$ for multiple samples and $r = 0.67$ for single samples were found. It is important to note that in the Cockrem and Rounce study, individual droppings were collected and pooled, whereas in the current study, 24-hour samples were collected and pooled. An r-value of 0.85 was found for samples from females. In the previous chapter, a demonstration of the relationship between plasma and faecal oestradiol was attempted with individual droppings. This was unsuccessful ($r = 0.28$) due to a lack in ranges of plasma and faecal steroid concentrations across the groups of birds.

No relationships were found between plasma and faecal oestradiol concentration or production within each group. Therefore, over a small range of concentrations there is no relationship between plasma and faecal oestradiol.

3.4.5 Progesterone

Plasma and faecal progesterone concentrations were higher in females on long days than in females on short days. However, no differences in plasma progesterone concentrations were found between mature and immature males kept on long days. Similar plasma measurements have been reported previously for male and female quail under similar photoperiods (Doi *et al.*, 1980; Gulati *et al.*, 1981; Bishop and Hall, 1991; W.H. Chua, pers. comm.). Faecal concentrations of progesterone were similar to pregnanediol-3α-glucuronide concentrations reported by Bishop and Hall (1991), although in that study higher concentrations of pregnanediol-3α-glucuronide were reported in female birds held on long days (up to 204 ng/g). This is consistent with the presence of free steroids in lower amounts in droppings than conjugated steroids. Plasma and faecal progesterone concentrations are similar to those reported in the study

in the previous chapter, although slightly higher faecal progesterone concentrations were measured in both male groups in this study.

The relative differences in progesterone concentrations between groups of bird were similar for single and multiple plasma samples. There was a small difference in faecal progesterone between immature and mature males for single but not multiple samples, indicating the value of greater sample sizes when using faecal analysis.

In the long day female group, an increase in plasma progesterone between the second and final plasma sample was found. However, only three plasma samples were collected from individual birds on the second plasma sampling day (Figure 3.44), so this result is likely to be a consequence of the small sample size.

Total daily faecal progesterone production showed the same pattern as plasma and faecal concentrations over five days. Production was higher in females kept on long days than in females kept on short days and there were no differences between the two groups of males.

The coefficient of variation between birds was twice as high for single compared with multiple plasma samples for long day females. Plasma concentrations for the other groups were all at the least detectable dose for the assay. Coefficients were also higher for single than multiple faecal samples from all groups of birds. These results indicate that variation between birds in progesterone concentrations is lower for multiple than single plasma and faecal samples, as was found for oestradiol.

Variability in progesterone concentration within an individual was similar for faecal and plasma samples in the immature long day male birds. The comparison could not be made for short day females, as all plasma samples were measured below the least detectable dose. In long day females, variability within an individual was higher in plasma samples, and the reverse was true for long day males. This again, was confounded by most samples measuring below the least detectable dose. Mean coefficients of variation within an individual were 71.3 ± 10.2% in plasma and 21.7 ± 2.3% in faeces in long day females, 30.8 ± 18.5% and 27.5 ± 3.7% in immature long

day males, and 12.1 ± 0.1% and 27.2 ± 3.9% in long day males. Since all plasma progesterone concentrations for short day females were measured at the least detectable dose, the coefficient of variation was 0% in plasma and 33.1 ± 3.2% in faeces this group. Variability in plasma samples within an individual were expected to be higher than faecal samples, as in the case of testosterone and oestradiol, as faecal steroid concentrations reflect plasma concentrations over 24 hours in comparison to the single time point plasma measurement.

There were strong correlations between plasma and faecal progesterone and ovary weight in this study, with a higher correlation when multiple rather than single faecal samples were used. Correlations with multiple and single plasma samples were similar. As with testosterone and oestradiol, an average value may be more valuable than a single sample when relationships between concentration and gonad size are being evaluated.

Correlations between plasma and faecal progesterone concentrations were markedly lower for single than for multiple samples ($r = 0.40$ compared to $r = 0.69$) and were lower than for the other steroids. The lower correlations may be associated with many of the plasma concentrations being at the least detectable dose for the assay. There has only been one previous report of a correlation between plasma and faecal progesterone in birds ($r = 0.79$ for female quail; faecal pregnanediol-3α-glucuronide measured; Bishop and Hall, 1991). In this study a correlation coefficient of $r = 0.85$ was found for samples from females. Non-significant correlations were found for male quail due to low faecal and plasma progesterone concentrations. In the previous chapter, a demonstration of the relationship between plasma and faecal progesterone was attempted with individual droppings. This was unsuccessful ($r = 0.22$) due to a lack in ranges of plasma and faecal steroid concentrations across the groups of birds.

No relationships were found between plasma and faecal progesterone concentration or production within each group. Therefore, like testosterone and oestradiol, over a small range of concentrations there is no relationship between plasma and faecal progesterone.

3.4.6 Hormone concentration interactions in plasma and faeces

Canonical discriminant analysis was able to predict which group individual birds belonged to from plasma testosterone, oestradiol and progesterone concentrations with 70% accuracy. Separation was mainly based on plasma testosterone and progesterone concentrations. The CDFs determined that long day females had high plasma progesterone concentrations and low plasma testosterone concentrations and long day males had high plasma testosterone and lower plasma progesterone concentrations. Immature long day males and short day females could not be separated based on plasma hormone concentrations.

From faecal testosterone, oestradiol and progesterone, canonical discriminant analysis was able to correctly predict group to 97% accuracy. Separation was based mainly on faecal testosterone and oestradiol concentrations and all groups were separated from each other. Therefore, predicting which group a bird belonged to was more accurate with faecal than plasma steroid measurements. Faecal steroid measurements are used for free living birds to provide an indication of gonad size without the need to capture birds. As demonstrated in this study, information about the concentration of one hormone is not always sufficient to determine the gonadal status or sex of a bird. For example, plasma and faecal oestradiol and progesterone concentrations were similar between both male groups over five days (Table 3.2) and plasma testosterone concentrations could not distinguish short day females and immature long day males. The collection of plasma samples from free-ranging individuals is often impractical and invasive, so the ability to predict reproductive status from faecal steroid measurements with greater accuracy than from plasma measurements is a great advantage. In a study on breeding cycles and faecal gonadal steroids in wild Brown Dippers (*Cinclus pallasii*), only a proportion of the faeces were identified to individuals of known sex (Kofuji *et al.*, 1993). Concentrations of faecal oestradiol and testosterone in these droppings were used to estimate the gender from which the remaining droppings came from. High concentrations of testosterone and low concentrations of oestradiol were assigned as male droppings, and those with high concentrations of oestradiol were classified as droppings from females. However, the sex of droppings with low oestradiol and testosterone could not be distinguished, so these were randomly assigned between male and females for statistical analysis. The use of a model such as the canonical model in

situations like this, may allow for greater accuracy when distinguishing between birds. It is important to note that in a biological system, 70% accuracy is still extremely high. This canonical model could be developed for use in other species, such as the kakapo. In addition to faecal hormone concentrations, other information about individual droppings could be included, such as physical characteristics and extraction efficiency. In this study, hormone concentrations from individual plasma and 24 hour faecal samples were used in the analysis. 24-hour faecal samples are often impractical to collect in a field environment, so the prediction accuracy may decrease when using individual droppings, since variation between birds is greater for a single day sample compared to a five-day sample.

3.5 Conclusions

The aims of this study were to:

- Validate a faecal extraction method developed in the previous chapter in Japanese quail for testosterone, oestradiol and progesterone.

- Determine if the relationships between steroid concentration and gonad size are similar for faecal and plasma samples.

- Determine if the magnitude of variation within and between birds is similar for faecal and plasma steroid concentrations.

- Determine the reliability of 24-hour faecal samples for estimating plasma hormone concentrations.

- Determine if faecal steroid concentrations can be used to accurately predict reproductive status, and how this compares to the prediction from plasma steroid concentrations.

Clear relationships were found between plasma and faecal testosterone, oestradiol and progesterone concentrations. Stronger correlations were found for multiple compared

with individual samples from each bird. Over a small range in plasma and faecal values, no correlations were apparent. Clear relationships were also found between hormone concentration and gonadal development, and these relationships were stronger for faecal concentrations than plasma. Pooled five-day samples tended to give stronger correlations than single day samples.

Variation between birds tended to be greater for faecal steroid concentrations than for plasma concentrations. Variation within a bird was also greater for plasma steroid concentrations.

24-hour faecal samples were found to reflect plasma hormone concentrations with similar reliability to pooled five-day samples. However, when demonstrating relationships between hormone concentration and other variables, for example, gonad size or plasma – faecal relationships, an average value over five days gave better results. In the previous chapter, demonstration of the relationship between plasma and faecal steroids was attempted with individual droppings. This was unsuccessful due to a lack in ranges of plasma steroid concentrations. Therefore a comparison between the reliability of individual droppings, pooled individual droppings, 24-hour samples and pooled 24-hour samples for reflecting plasma hormone concentrations is not possible from the present data. A positive relationship between faecal and plasma steroid measurements has been demonstrated in this study with pooled 24-hour faecal samples, indicating that the reliability of faecal steroid measurements for assessing plasma hormone concentrations is increased when multiple samples can be collected from individual birds.

Faecal steroid concentrations were used to predict reproductive status of quail with greater accuracy than plasma steroid concentrations.

This is the first study in which relationships between plasma and faecal steroid concentrations have been reported for testosterone, oestradiol and progesterone together in one species and the first to clearly demonstrate the value of collecting multiple faecal samples. This is also the first study to report relationships between faecal steroid concentrations and gonad size in birds. The knowledge of relationships between plasma

and faecal hormone concentrations and gonad size gained in the quail provides a sound foundation for studies of faecal steroids in free living birds such as the kakapo.

Female kakapo and chick, photo: Department of Conservation, New Zealand.

4 Faecal reproductive steroids in Kakapo

4.1 Introduction

The kakapo (*Strigops habroptilus*) is an endangered parrot endemic to New Zealand. It is nocturnal, herbivorous and flightless, and is the world's largest parrot with males weighing up to four kilograms. The majority of the adult kakapo are relatively old, but successful breeding attempts in the last several years have brought renewed hope to the conservation programme. At the outset of this study, the population stood at 21 females and 36 males. Seven of these females and two of these males were juvenile birds, that have hatched since 1991. Aspects of kakapo breeding behaviour were noted over 100 years ago (reviewed in Merton *et al.*, 1984), but a more detailed understanding has only been obtained in the last 25 years. The kakapo is a solitary species, with home ranges from 15 to 50 ha in area (Merton *et al.*, 1984; Moorhouse and Powlesland, 1991) and a lek mating system. In the breeding season, males gather and excavate track and bowl systems, usually on a ridge or hilltop and perform a visual and vocal display to attract females for mating. After mating, females nest and provide sole care for the altricial chicks until fledging. Kakapo generally bred at intervals of three to four years that appear to coincide with fruit availability, although Cockrem (1989) suggested that kakapo were capable of breeding annually. This was confirmed when breeding occurred in successive years on Little Barrier Island in 1990 and 1991.

Little is known of kakapo reproductive physiology. The only study to date is that of Cockrem and Rounce (1995) who investigated the annual gonadal cycle in kakapo using faecal steroid analysis. Faecal collections were made on an opportunistic basis from the forest floor over a period of 16 months. Faecal oestradiol and testosterone concentrations were measured and related to events of the breeding cycle. They reported a clear annual cycle of testosterone in males, but only a trend towards an annual cycle of oestradiol in females. The study was conducted on Little Barrier Island where there was approximately double the number of male to female birds, and droppings could not be identified to individual birds. Therefore, most droppings were probably from males

rather than females. The changes in testosterone and oestradiol concentrations coincided with events of the breeding season such as booming and egg laying, suggesting that faecal steroid concentrations reflected patterns of gonadal growth and regression.

Like most species of birds, kakapo are seasonal breeders and breed when specific environmental conditions are appropriate. It has been suggested that kakapo become photosensitive in the autumn, with testicular growth occurring in the spring (September – November) and ovarian growth occurring during late spring and early summer (October – December). Photorefractoriness and gonadal regression were postulated to occur in the late summer, at the end of the breeding season. (Cockrem, 1995; Cockrem and Rounce, 1995). The timing of avian breeding seasons is regulated by environmental factors termed proximate factors, as discussed in section 1.2. Of these, essential supplementary information stimulates the final stages of gonadal development, nest building and egg laying. Supplementary information for the kakapo might include the presence of rimu *(Dacrydium cupressinum)* fruit, *Pinus radiata* foliage or supplementary food (Merton and Clout, 1998). The timing of the kakapo breeding season differs between years and between islands. In 1993, males on Little Barrier Island boomed from late October to mid-April, but males on Maud Island did not start booming until a month later and stopped mid-March (J.F. Cockrem, pers. comm.). Egg-laying in females can occur anytime between late January and mid-March. It tends to be highly synchronised, suggesting that supplementary information is very important for breeding in the kakapo. The extent to which this type of information is influencing reproductive development each season is of great interest. A difference in the temporal pattern of gonadal steroid concentrations between breeding and non-breeding years would indicate a difference in the supplementary information perceived between seasons and might provide valuable information on the amount of gonadal development occurring each year.

Conventionally, endocrine data has been obtained through the analysis of plasma samples. However this method requires the capture and handling of the bird, which in the case of kakapo is not ideal. Repeated sampling is needed to evaluate changes in gonadal steroids during a season and the Department of Conservation, in their role of protecting the population can not allow regular capture of kakapo for the collection of

blood samples. In the wild situation, capture and handling is very labour intensive. Each bird must be located using radio-telemetry equipment, caught and a blood sample taken. Capture and handling can cause stress, which may alter the production of hormones being monitored. There are also weather restrictions on capturing kakapo. During periods when the undergrowth is wet, birds are not disturbed in order to avoid the birds becoming excessively wet if they try to escape. Once collected, plasma samples must be transported and stored in specific conditions that are often impractical in the field situation. Therefore, faecal steroid analysis has been utilised in this study because of its non-invasive and practical nature.

Faecal gonadal steroids have been investigated in a wide variety of avian species. In the 1970's many avian captive breeding programmes were encountering problems identifying the sex of individuals in order to pair birds for mating. Early studies therefore investigated alternative methods of sexing monomorphic bird species (Czekala and Lasley, 1977; Bercovitz *et al.*, 1978; Stavy *et al.*, 1979; Erb and Bercovitz, 1980, Tell and Lasley, 1991). These investigations were the first to explore the potential of faecal steroid analysis techniques. In addition to gender identification, oestrogen: testosterone ratios were used for assessing gonadal status in individual captive zoo birds (Bercovitz *et al.*, 1978). In recent years, faecal steroid analysis has been used to evaluate breeding cycles and seasonal changes in gonadal steroid concentrations. Changes in hormone production relative to gonadal activity and the onset of incubation have been measured in individual bald eagles (*Haliaeetus leucocephalus*, Bercovitz *et al.*, 1982) and canaries (*Serinus canaria*, Sockman and Schwabl, 1999) respectively. Identifying faecal samples to individual birds is relatively simple in the captive situation, especially when birds are housed in separate cages. Various other researchers have utilised the convenience of captive birds to validate the relationship between faecal and plasma steroid concentrations (Bishop and Hall, 1991; Cockrem and Rounce, 1994; Ishii *et al.*, 1994).

Faecal steroid analysis techniques have also been successfully applied to several species of free-living birds (brown dipper, *Cinclus pallasii*, Kofuji et al., 1993; kakapo, Cockrem and Rounce, 1995; rock ptarmigan, *Lagopus mutus*, Kikuchi et al., 1996; greylag geese, *Anser anser*, Hirschenhauser et al., 1999a, 1999b). In all cases except

kakapo, individual faecal samples were generally collected soon after excretion and could be identified to an individual bird through observation. Kakapo faecal samples were collected on an opportunistic basis and could not be identified to an individual bird. Clear seasonal patterns of faecal steroid hormones that related to behavioural changes of the breeding season were found in all four species. In the brown dipper, profiles of oestradiol and testosterone were measured for individual birds, which gave valuable information on the breeding condition of each dipper. Information of this quality would be of great value for the kakapo and could provide an insight into the level of gonadal development that is occurring each year in individual birds. In addition, the use of faecal steroid analysis for the evaluation of reproductive status or sexual maturity of individual birds would be a valuable tool in conservation management practices. Presently, birds are translocated between islands for mating based on a limited knowledge of breeding history. Accurate diagnosis of reproductive status would optimise the benefits of translocations.

The aims of this study in the kakapo were to:

- Determine the annual pattern of steroid hormone concentrations.
- Verify the annual cycle of testosterone reported by Cockrem and Rounce (1995).
- Determine the feasibility of collecting droppings identified to individual birds.
- Compare reproductive hormone cycles between successive years.
- Compare reproductive hormone profiles between reproductively active and inactive birds.
- Determine the reliability of individual faecal samples for estimating steroid concentrations.
- Evaluate the potential of faecal steroid analysis as a diagnostic tool for kakapo.

4.2 Methods

4.2.1 Bird information and habitat

The kakapo population on Whenua Hou, New Zealand was used in this study. Whenua Hou is a 1 396 ha island located off the northwest coast of Stewart Island and is a wildlife reserve managed by the Department of Conservation (DoC). During the time of sample collection the population varied due to bird-relocation practices, but approximately 17 male and 14 female birds inhabited the island. Faecal samples were collected from all birds by DoC staff during the period of study, with samples from nine females and 17 males used for faecal steroid measurements. The ages of most birds were unknown, but probably ranged from 1-20+ years. The female birds Zephyr and Hoki fledged during the 1980-81 and 1991-92 breeding seasons respectively. Sirocco, a male bird fledged during the 1996-97 breeding season.

During 1998, all kakapo were transferred to Pearl Island to allow for rat eradication on Whenua Hou. Pearl Island is a 500 ha island situated in Port Pegasus, Stewart Island and is also a wildlife reserve managed by DoC. Birds were transferred back to Whenua Hou once the 1998-99 breeding season had ended.

4.2.2 Sample collection

Droppings were collected from the forest floor from January 1996 – January 1999. Department of Conservation staff collected droppings during routine procedures. Only droppings that could be attributed to an individual kakapo based on knowledge of home ranges, use of feeding stations and roost sites were collected. The date of collection and a qualitative measure of condition (old or fresh) were recorded. Only droppings considered to be fresh were used in this study. When more than one fresh sample was collected from an individual on the same day, samples were pooled. However, in ten situations (nine for progesterone) where at least two fresh samples were collected on the same day, hormone concentrations were measured in individual samples to investigate the variation between droppings. The mean of the concentrations in individual samples collected on one day were used as the data points for that day for the annual cycle and individual profile analyses.

In January and February 1998 specific female birds and roost sites were located and droppings collected by the author. Droppings were either collected the day the female was located or on the following day to minimise disturbance to the birds. For example, if the roost site was on a tree branch, droppings on the ground were collected. If the roost site was on the ground, the site was marked and returned to the next day, once the bird had moved on. The period between defaecation and sample collection was therefore up to 30 hours. Samples were collected into zip-lock plastic bags of various sizes, returned to the field centre and frozen at -20°C.

4.2.3 Steroid extraction from faecal samples

Samples were transported on ice to Massey University, Palmerston North, where they were weighed and scored on the physical characteristics in Table 4.1.

Table 4.1. Fresh kakapo dropping characteristic grading scheme.

Characteristic	Score					
	1	2	3	4	5	6
Odour	light	mildly pungent	sweet	strongly sweet	strong, pungent	
Colour	tan	brown	black	green	bright green	red
Texture	powdery	grainy powder	crumbly	fibrous	very solid, fibrous	
Vegetation	none	fine	small pieces	large pieces	large, coarse	
Liquid	yes	no				
Uric acid	yes	no				

Faecal samples were prepared by the method developed for Japanese quail (described in chapter three), except the amount of dried sample was doubled to increase the concentration of hormone in the PBSG extract. Frozen faecal samples were lyophilised (Cuddon 0610 Freeze Drier) for 70 hours. Samples were ground to a powder using a centrifugal grinder (RETCH 2M100; 0.25 sieve). All samples from each bird were ground in chronological succession. The grinder sieve, blade and collection tray were brushed clean after each sample and all removable parts were washed in water and decontaminated with 90% ethanol between birds. 0.1 g of dried sample was added to 2.5 ml of 90% absolute ethanol (AnalaR BDH, Poole, England) in a glass screw-top tube (13 x 100 mm). Tubes were capped, vortexed for 30 seconds and suspension shaken on their side for one hour on an orbital shaker (Chiltern Scientific SS70). Tubes were then removed and refilled to the 2.5 ml level before being capped and centrifuged at 1 000 g (Haraeus Christ 5000S refrigerated centrifuge) for 20 minutes at room temperature. The supernatant was pipetted into a second glass screw-top test tube. The pellet was rinsed with a further 1.25 ml of 90% ethanol, vortexed for 30 seconds and re-centrifuged at 1 000 g for 20 minutes at room temperature. The supernatant was removed and added to the previous aliquot. The faecal pellet was discarded. The ethanol was completely evaporated from the supernatant by placing test tubes in a heating block at 37°C, under a stream of air. The sides of the tubes were rinsed with 90% ethanol twice during this process to ensure that the final level of extract residue was below the level of 1 ml. Extracts were left to cool and reconstituted in 0.8 ml of phosphate buffered saline gel (PBSG; 0.1M, pH 7.0, 0.1% gelatin), gently vortexed for 30 seconds and refrigerated overnight at 4°C. The following day, tubes were vortexed for 30 seconds and the extracts were removed into 1.5 ml polypropylene eppendorf tubes. The extraction tube was rinsed with a further 0.2 ml of PBSG, vortexed and the extract added to the previous aliquot. Extracts were centrifuged for 10 minutes at 14 000 g (IEC Micromax ventilated microcentrifuge), and the supernatant was transferred into a 1.0 ml polypropylene titre tube and stored at –20°C until assay.

Extraction efficiencies were obtained for testosterone, oestradiol and progesterone by adding 20 µl (~ 5 000 cpm for testosterone and oestradiol and 15 000 cpm for progesterone) of the respective tritiated hormone (^{3}H-testosterone, TRK.406 Amersham, UK; ^{3}H-oestradiol, TRK.332 Amersham, UK; ^{3}H-progesterone, TRK.413, Amersham,

UK) to samples before shaking. Tritiated hormone was also added to four scintillation vials (polypropylene, 5 ml) for total counts. After extraction, a 20 µl aliquot of the reconstituted extract containing tritiated hormone was used to determine extraction efficiency.

Extraction efficiency was determined for testosterone, oestradiol and progesterone for each sample due to high variation between samples (means were 38.81 ± 45.02%, 33.22 ± 36.92, and 15.08 ± 55.98% respectively).

Each batch of samples extracted also included quality control samples. Two screw-top test tubes containing ethanol but no dried dropping were 'extracted' in exactly the same manner as above. A 20 µl aliquot of the final supernatant was used to determine a total count for the extraction.

4.2.4 Radioimmunoassay of testosterone

Testosterone concentrations in faecal extracts were measured with an ICN Biomedicals ImmunoChem Double Antibody Testosterone ^{125}I RIA Kit. Samples were thawed and assayed in duplicate. 20 µl of sex binding globulin inhibitor solution (SGBI, Cat # 07-166812), 100 µl of iodinated testosterone (Cat # 07-189121, ~5 000cpm) and 100 µl of antibody (anti-testosterone Cat # 07-189113) were added to 10 µl of undiluted faecal extract and standards (9.8 – 5 000 pg/ml) and incubated at 37°C for 120 minutes. After incubation, 20 µl of the second antibody (Cat # 07-166602) was added to all tubes and the tubes were incubated at 37°C for a further 60 minutes. Samples were centrifuged at 2 300 g for 15 minutes at 4°C and the supernatant removed by aspiration. The precipitates were counted in a gamma counter (LKB Wallac 1261 Multigamma Gamma Counter).

The cross-reactivity of the testosterone antibody with other steroids was reported by ICN Biomedicals Inc. as: 5α-dihydrotestosterone (3.4%), 5α-androstane-3β, 17β-diol (2.2%), 11-oxotestosterone (2.0%), 6β-hydroxytestosterone (0.95%), 5β-androsten-3β, 17β-diol (0.71%), 5β-dihydrotestosterone (0.63%), androstenedione (0.56%),

epiandrosterone (0.20%), and others (<0.01%). The sensitivity of the assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for faecal testosterone was 0.01 ng/g.

4.2.4.1 Intra- and inter-assay validation

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 the 20%, 50% and 80% standards were 2210.2 ± 202.7 pg/ml, 471.1 ± 43.2 pg/ml and 84.2 ± 11.8 pg/ml respectively.

Faecal samples were randomly assigned between three assays for testosterone. Inter-assay coefficients of variation for low, medium and high quality controls were 24.4%, 1.0% and 4.2% respectively. Intra-assay coefficients of variation are yet to be determined by conducting an assay with 20 duplicates of each quality control.

4.2.4.2 Parallelism and hormone recovery

Serial dilutions of extracted kakapo faecal samples in assay buffer (PBSG) were parallel to the testosterone standard curve (n=3; Figure 4.1). The recovery of testosterone added to one faecal sample was 103.5 ± 2.6%.

4.2.5 Radioimmunoassay of oestradiol

Oestradiol 17-β (oestradiol) concentrations in faecal extracts were measured by radioimmunoassay with an ICN Biomedicals ImmunoChem Double Antibody 17-β Estradiol ^{125}I RIA Kit. 100 µl of iodinated oestradiol (Cat # 07-138121, ~5 000cpm) and 100 µl of antibody (anti-17-β-oestradiol Cat # 07-138113) were added to 10 µl of undiluted faecal extract and standards (9.8 – 5 000 pg/ml) and incubated at 37°C for 90

minutes. After incubation, 100 µl of the precipitant solution (Cat # 07-166624) was added to all tubes and mixed thoroughly. Samples were centrifuged at 2 300 g for 15 minutes at 4°C and the supernatant removed by aspiration. The precipitates were counted in a gamma counter.

The cross-reactivities of the oestradiol antibody with other steroids were reported by ICN Biomedicals Inc. as: 17α-oestradiol (0.68%), oestriol (1.51%), oestrone (20%) and others (<0.01%). The sensitivity of the assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for faecal oestradiol was 0.05 ng/g.

4.2.5.1 Intra- and inter-assay validation

Solutions of oestradiol 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 2 077.2 ± 169.8 pg/ml, 353.8 ± 46.5 pg/ml, and 82.1 ± 14.2 pg/ml respectively.

Faecal samples were randomly assigned between three assays for oestradiol. Inter-assay coefficients of variation for low, medium and high quality controls were 26.0%, 18.7% and 3.7% respectively. Intra-assay coefficients of variation are yet to be determined by conducting an assay with 20 duplicates of each quality control.

4.2.5.2 Parallelism and hormone recovery

Serial dilutions of extracted kakapo faecal samples in assay buffer (PBSG) were parallel to the oestradiol standard curve (n=3; Figure 4.1). Recoveries of oestradiol added to two faecal samples were 109.6 ± 8.0% and 93.9 ± 5.7 %.

4.2.6 Radioimmunoassay of progesterone

Progesterone concentrations in faecal extracts were measured by radioimmunoassay with an ICN Biomedicals ImmunoChem Double Antibody Progesterone ^{125}I RIA Kit. 50 µl of antibody (anti-progesterone Cat # 07-170113) and 20 µl of iodinated progesterone (Cat # 07-170121, ~5 000cpm) were added to 10 µl of undiluted faecal extract and standards (19.5 – 10 000 pg/ml) and incubated at 37°C for 60 minutes. After incubation, 50 µl of the precipitant solution (Cat # 07-166624) was added to all tubes and mixed thoroughly. Samples were centrifuged at 2 300 g for 15 minutes at 4°C and the supernatant removed by aspiration. The precipitates were counted in a gamma counter.

The cross-reactivities of the progesterone antibody with other steroids were reported by ICN Biomedicals Inc. as: 20α-dihydroprogesterone (5.41%), desoxycorticosterone (3.8%), corticosterone (0.7%), 17α-hydroxyprogesterone (0.67%), pregnenolone (0.41%), androstenedione (0.23%), testosterone (0.16%), and others (<0.01%). The sensitivity of the assay was the minimum hormone concentration that could consistently be distinguished from zero. It was determined as the hormone concentration at the mean minus two standard deviations from the zero hormone point on the standard curves. Assay sensitivity for faecal progesterone was 0.075 ng/g.

4.2.6.1 Intra- and inter-assay validation

Solutions of progesterone 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 the 20%, 50% and 80% standards were 8 945.6 2 ± 603.7 pg/ml, 125.5 ± 143.4 pg/ml and 348.7 ± 53.2 pg/ml respectively.

Faecal samples were randomly assigned between three assays for oestradiol. Inter-assay coefficients of variation for low, medium and high quality controls were 11.2%, 13.1% and 15.9% respectively. Intra-assay coefficients of variation are yet to be determined by conducting an assay with 20 duplicates of each quality control.

4.2.6.2 Parallelism and hormone recovery

Serial dilutions of extracted kakapo faecal samples in assay buffer (PBSG) were parallel to the progesterone standard curve (n=3; Figure 4.1). Recoveries of progesterone added to two faecal samples were 104.2 ± 6.5% and 102.6 ± 9.9 %.

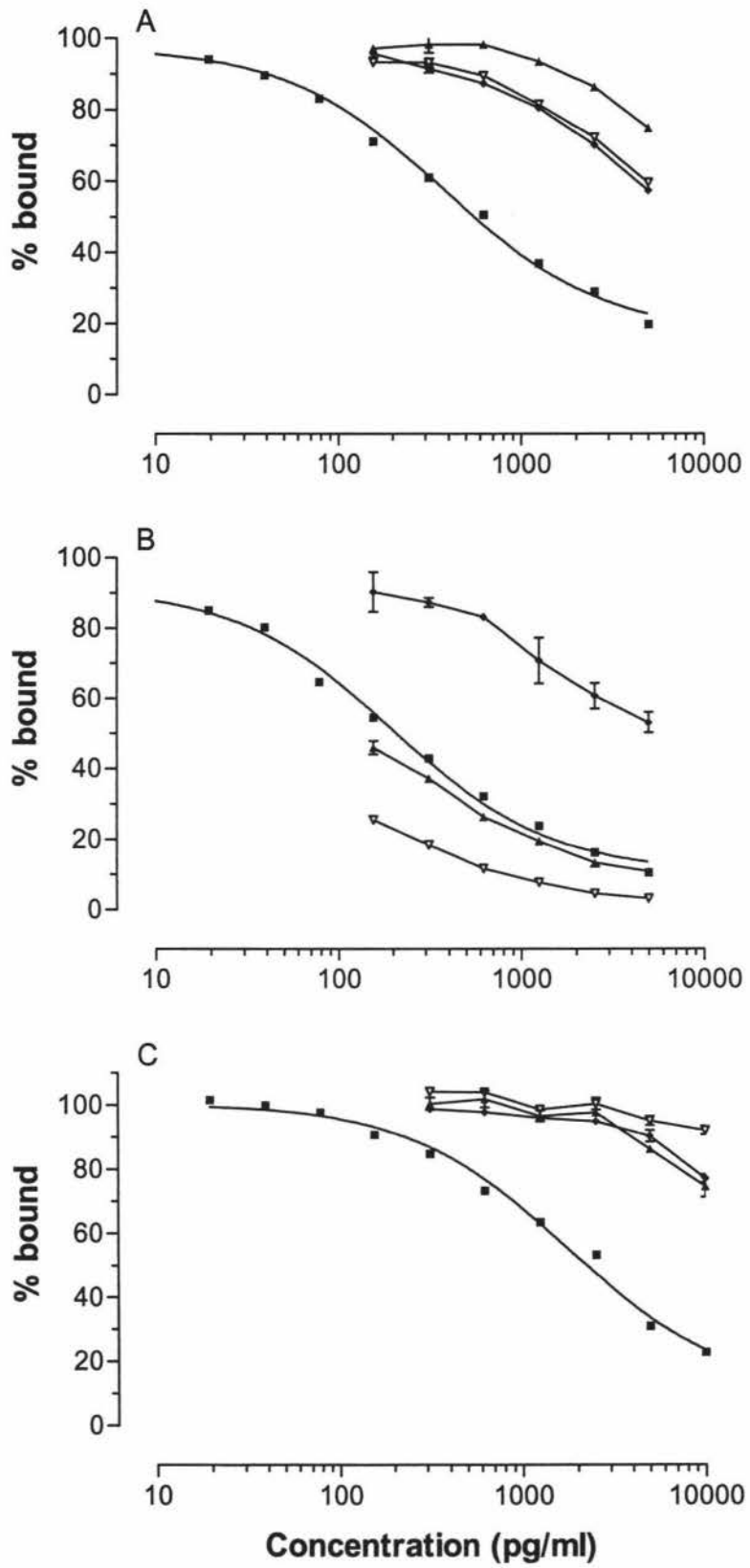

Figure 4.1. Parallelism demonstrated for kakapo faecal samples. A: faecal testosterone, B: faecal oestradiol, C: faecal progesterone. The curves with filled squares are the standard curves and all other curves are samples.

4.2.7 Statistical analyses

Statistical analyses were performed using SPSS version 11.0.0 (2001; SPSS Inc). Where appropriate the following analyses were conducted on normally distributed data with homogeneous variances (as determined by Levene's Test): independent samples t-test, one-way ANOVA with Bonferroni post hoc comparisons and Pearson correlation.

In the case of comparing testosterone profiles between years, a polynomial curve of the form:

$$y = a + bx + cx^2 + dx^3 \qquad \text{(Equation 4.1)}$$

was fitted to the data from each year from July – June (where complete data sets were collected). In the case of comparing oestradiol and progesterone profiles between years, polynomial curves of the form:

$$y = a + bx + cx^2 \qquad \text{(Equation 4.2)}$$

were fitted to the data from each year from July – June (where complete data sets were collected). Parameters a, b, c and d in the case of testosterone, of the curves were then compared by paired samples t-test using the standard construction of a t-test:

$$t = \frac{parameter_1 - parameter_2}{\sqrt{SE_1 + SE_2}} \qquad \text{(Equation 4.3)}$$

where SE1 and SE2 are the SE of the parameters of the regressions of the two years.

To analyse annual cycles of hormone concentration (over all years), data were combined into bimonthly groups (July/August to May/June) because of low sample sizes. Standard data transformations did not achieve homogeneity, so bimonthly data were analysed by one-way ANOVA with weighted analysis using the inverse of the SE

(Myers, 1990). Post hoc comparison of means was completed using Least Significant Difference (LSD).

Confidence intervals (95%) were calculated for each hormone based on the standard deviation of data from individual droppings collected in months when hormone concentrations were low. These periods were March to June for testosterone, March to October for oestradiol and May to October for progesterone (all years combined). Confidence intervals were used to give an indication of the occurrence of elevated hormone concentrations in individual bird profiles.

Variation between faecal steroid concentrations of individual droppings collected from a range of roost sites was analysed by one-way ANOVA with weighted analysis using the inverse of the SE. This analysis was used because of heterogeneity in the variances of the individual roost sites. In addition, for each hormone a 95% confidence interval of the mean hormone concentration was calculated from the pooled SE.

4.3 Results

4.3.1 Breeding activity

Low intensity booming by male kakapo occurred from late 1995 to early 1996, although exact dates were unknown. The identity of individuals that were booming was uncertain, although booming from Lionel, Nog and Felix was confirmed. No breeding occurred in female birds during this season. Booming was recorded from 9th December 1996 to 15th March 1997 from Basil, Ben, Felix, Gunner, Lee, Lionel, Merv, Nog, Ralph, Sass, Waynebo and Whiskas. Matings were recorded for Ben, Nog, Sass, Whiskas, and Felix. Six females produced eggs early in 1997 (Table 4.2). No reproductive activity was recorded during the 1997 – 98 breeding season in female birds and only very light activity was recorded at bowls. Nog was recorded booming on the 22nd February 1998. Intense booming was recorded on Pearl Island from 12th December 1998 to 12th March 1999 from Basil, Felix, Gumboots, Gunner, Lee, Merv, Nog, Ralph, Waynebo and Whiskas. Matings were recorded for Felix, Gumboots, Nog, Ralph and Waynebo. Five females laid eggs (Table 4.2) and all were collected for hand rearing to prevent loss to weka (*Gallirallus australis*), kiore (*Rattus exulans*) and ship rats (*Rattus rattus*).

Table 4.2. Egg-laying by female kakapo on Whenua Hou, during the 1996-1997 season and on Pearl Island, 1998-1999 season.

Female	Season	Eggs	Hatched	Fledged
Alice	1996-97	3	1	1
	1998-99 Clutch 1	3	2	0
	1998-99 Clutch 2	2	0	0
Cyndy	1996-97	3	2	0
	1998-99	2	0	0
Sandra	1996-97	2	0	0
	1998-99	2	1	1
Sara	1996-97	1	0	0
Sue	1996-97	1	0	0
Zephyr	1996-97	2	2	2
	1998-99	2	2	2
Suzanne	1998-99 Clutch 1	2	0	0
	1998-99 Clutch 2	1	0	0
TOTALS	1996-97	12	5	3
	1998-99	14	5	3

4.3.2 Faecal testosterone

Faecal testosterone concentrations ranged from the least detectable dose of 0.01 ng/g to 35.77 ng/g (Figure 4.2 A) in droppings collected from July 1996 to January 1999. A wide range in concentration was common in many months during this period.

The cubic polynomial fitted to data from the 1996-97 season was a poor fit. In contrast, the cubic polynomial fitted to data from the 1997-98 season was highly significant (Table 4.3). There were no differences between the parameters of the cubic polynomials from the two seasons (Table 4.3; paired samples T-test, t = 0.70, 0.90, 1.07, 1.20 for parameters *a*, *b*, *c* and *d* respectively; df = 14, $p>0.05$ in all cases; Figure 4.2).

There was an annual cycle of faecal testosterone (Figure 4.3) and concentrations varied significantly between bimonthly periods ($F_{5, 95} = 3.653$, $p = 0.005$). A clear peak was not distinguished although mean faecal testosterone concentrations were elevated from July/August to November/December. Concentrations were declining through January/February to lowest concentrations in March/April (LSD = 6.71, df = 95, $p < 0.01$ in all cases) and remained at lower concentrations through May/June.

The standard deviation of data for all individual droppings collected in the months from March to June was 4.28 ng/g. The 95% confidence interval was 8.4 ng/g. Therefore a difference between individual droppings in faecal testosterone concentration of more than 16.8 ng/g is likely to indicate different testosterone concentrations.

Testosterone concentration varied between situations of multiple dropping collection (Figure 4.4, $F_{9, 27} = 27.418$, $p < 0.001$). The SE of a pooled sample (mean of individual droppings from one roost site) was 1.89 ng/g. This equates to a 95% confidence interval of 3.70 ng/g. Across the ten situations where multiple fresh droppings were collected there was a mean coefficient of variation of 38.4 ± 4.3%.

Insufficient droppings were collected to give clear testosterone profiles for individual males (Figure 4.5). However, the relative size of the 95% confidence interval developed from data on individual variation in droppings collected from March to June (Figure 4.4), in comparison to single data points on the profiles for individual birds (Figure 4.5) indicates that elevated testosterone concentrations may have been detected in February 1997 and 1998 in the profile from Ken, August 1998 from Merv, December 1996 and August 1998 from Nog and in November 1996 in the profile from Whiskas.

The extraction efficiency of faecal testosterone was lower and more variable than extraction efficiencies for quail faecal samples using the same extraction method in the previous chapter (Table 4.4). There was a low, negative relationship between extraction efficiency and faecal testosterone concentration (Figure 4.6). Testosterone extraction efficiency did not vary with size of vegetation in individual droppings, dropping texture, or with the presence of liquid (Figure 4.7, Table 4.5). However, extraction efficiency did vary between samples with different odours, colours or with the presence of uric

acid. Multiple comparisons were made using Bonferroni post-hoc analysis. Samples that had a strong pungent odour had significantly lower extraction efficiencies than samples with either a light or mildly pungent odour ($p = 0.017$ and 0.002 respectively). Tan coloured samples had significantly lower extraction efficiencies than black samples ($p = 0.029$), and black samples had lower extraction efficiencies than brown, black or red samples ($p = 0.001$, 0.001 and 0.020 respectively). Samples without uric acid had significantly higher extraction efficiencies than samples with uric acid (Table 4.5). Testosterone concentration did not differ across any of the physical characteristics (Figure 4.8, Table 4.5).

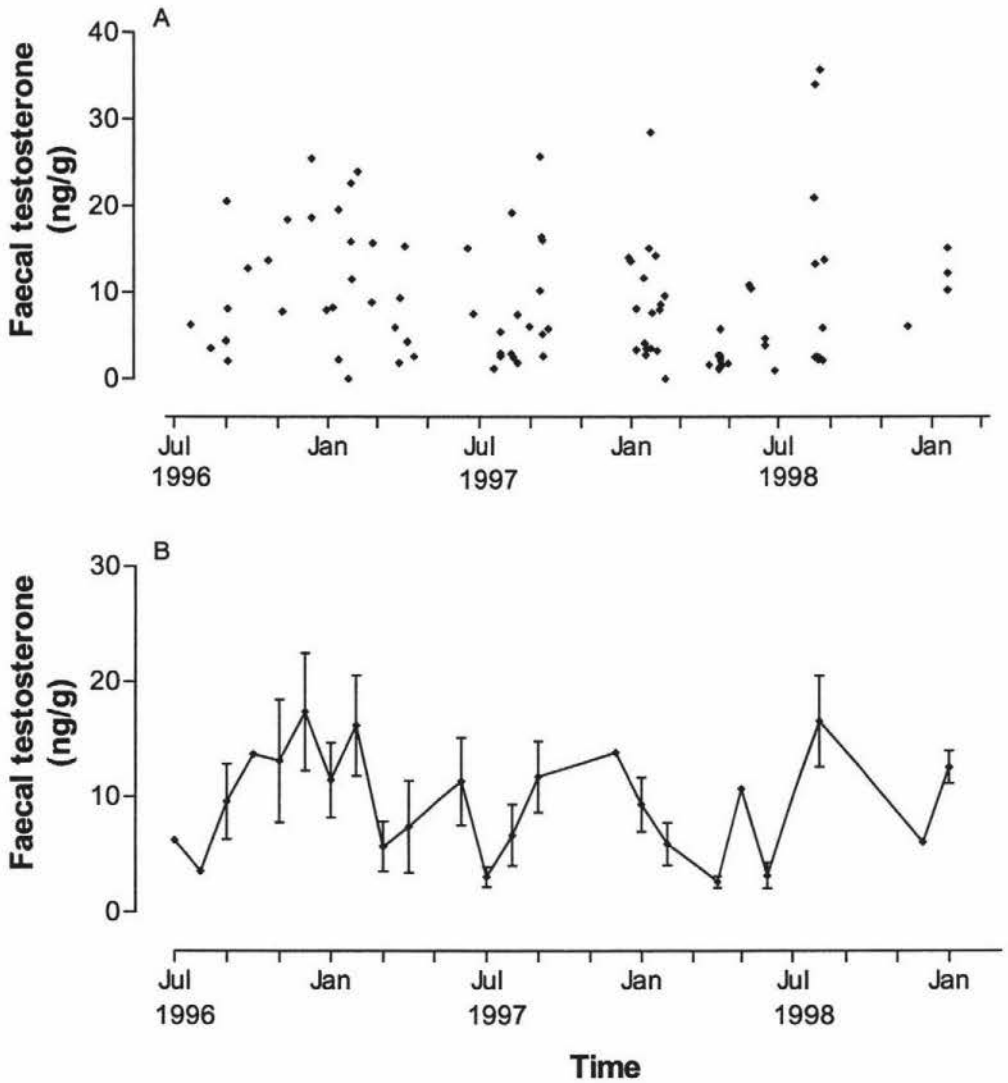

Figure 4.2. Faecal testosterone concentrations in male kakapo. A) All individual faecal samples collected over sampling period, plotted on day of collection. B) Mean faecal concentration for each month over the sampling period ± SE.

Table 4.3. Parameter estimates of cubic equation (Equation 4.1) fitted to mean testosterone concentrations by month for two seasons, 1996-97 and 1997-98.

Variable	Parameter estimate	SE	p
Season 1996-97 ($R^2 = 0.56$)			0.302
Intercept			
A	-1.71	7.80	0.847
B	5.84	5.60	0.356
C	-0.68	0.99	0.531
D	0.02	0.05	0.670
Season 1997-98 ($R^2 = 0.93$)			0.010
Intercept			
A	-7.52	2.86	0.058
B	11.19	2.06	0.006
C	-1.80	0.36	0.008
D	0.08	0.02	0.012

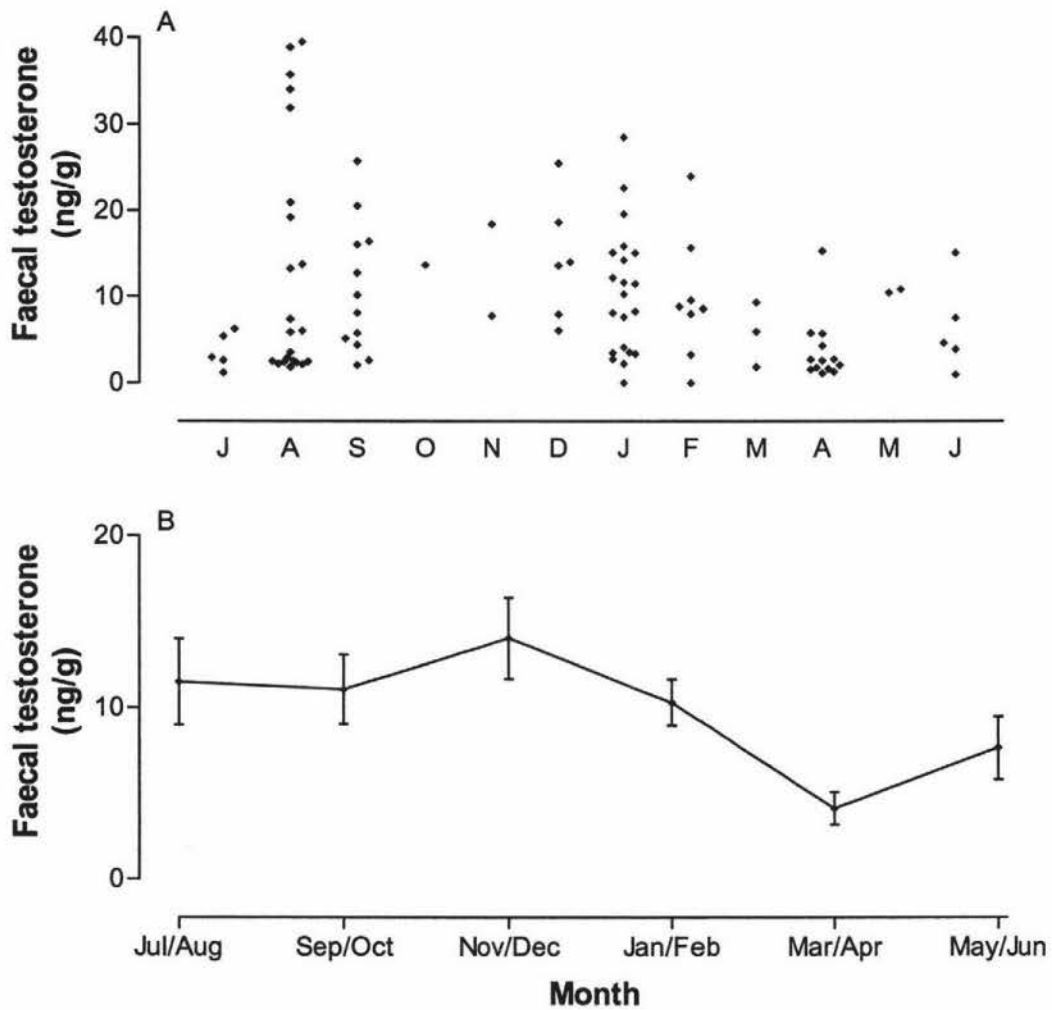

Figure 4.3. Faecal testosterone concentrations in male kakapo. A) All individual faecal samples grouped by month. B) Mean faecal concentration for bi-monthly groupings over all years combined ± SE.

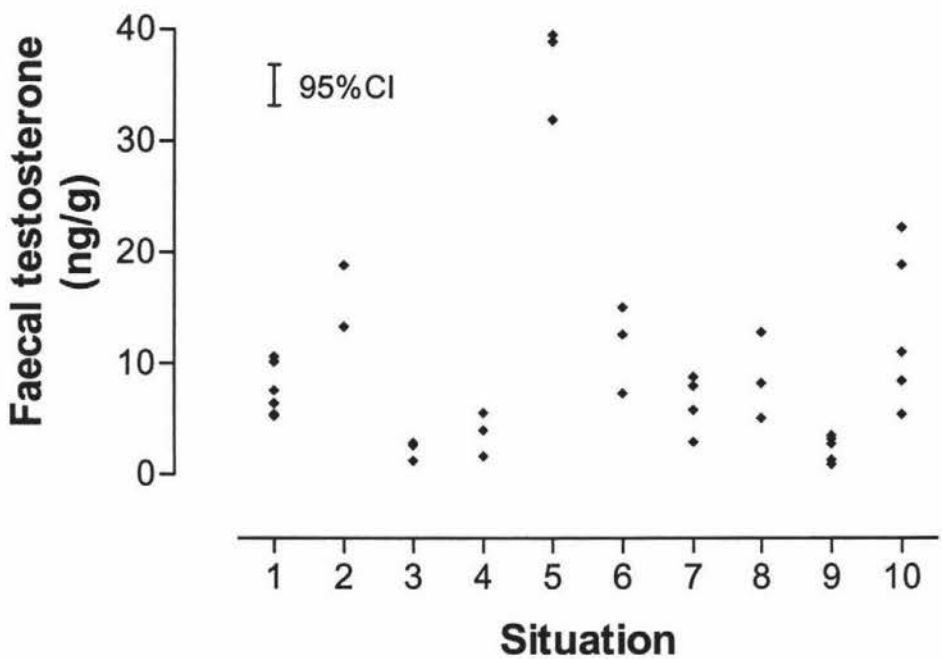

Figure 4.4. Variation in faecal testosterone concentration between fresh droppings from individual birds from the same day or roost site. 95% confidence interval of the mean is indicated.

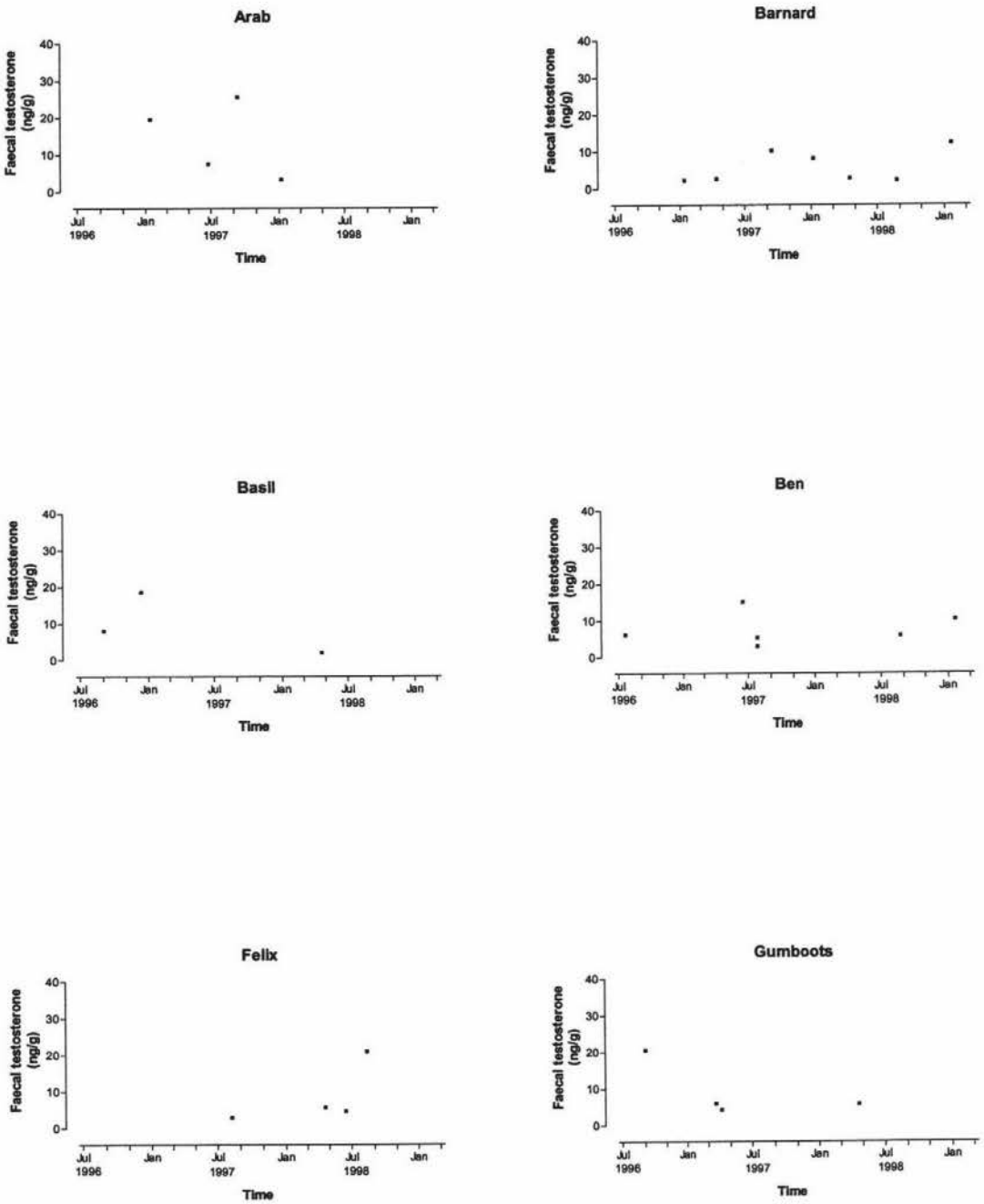

Figure 4.5.A Faecal testosterone profiles for individual male kakapo.

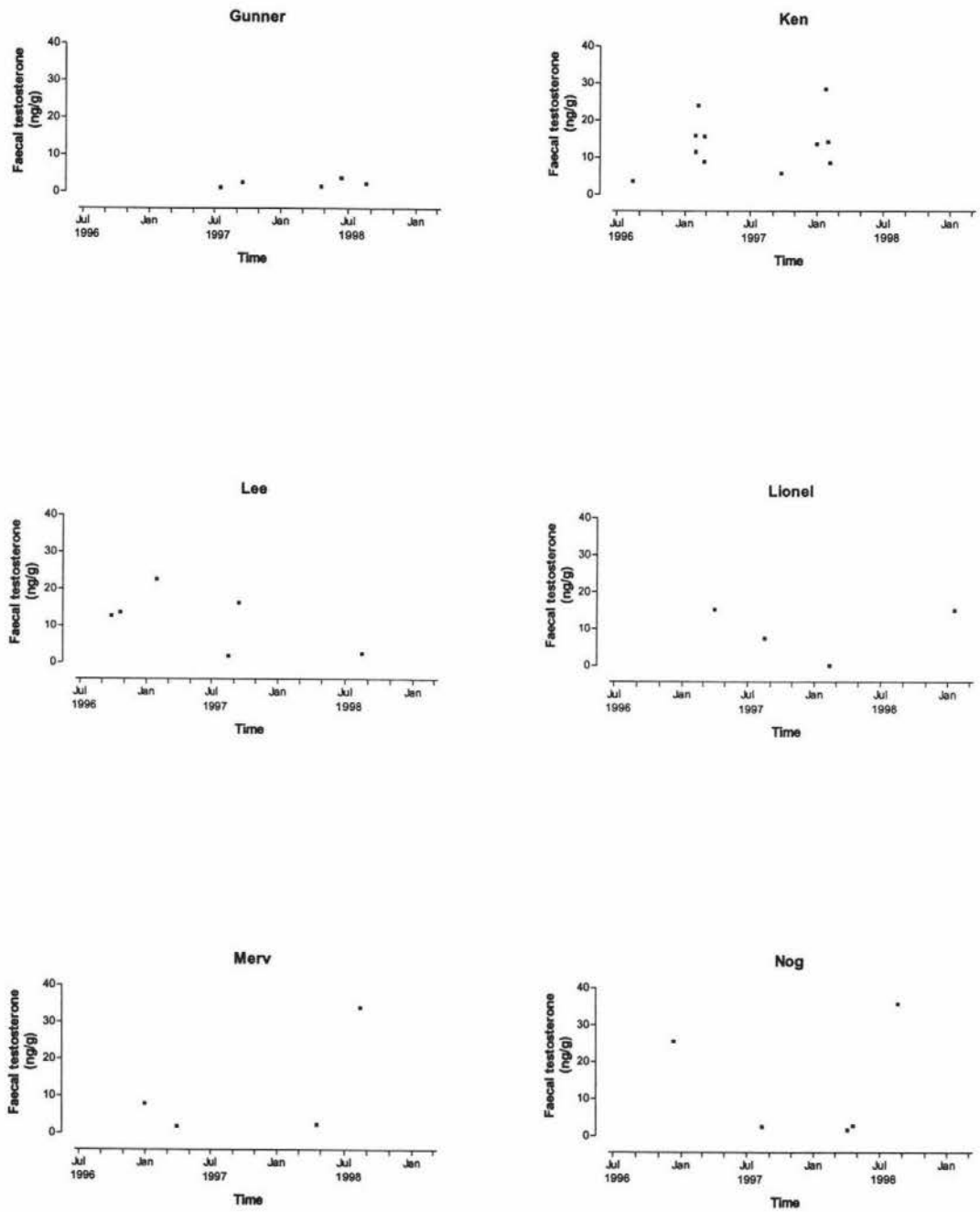

Figure 4.5 B Faecal testosterone profiles for individual male kakapo.

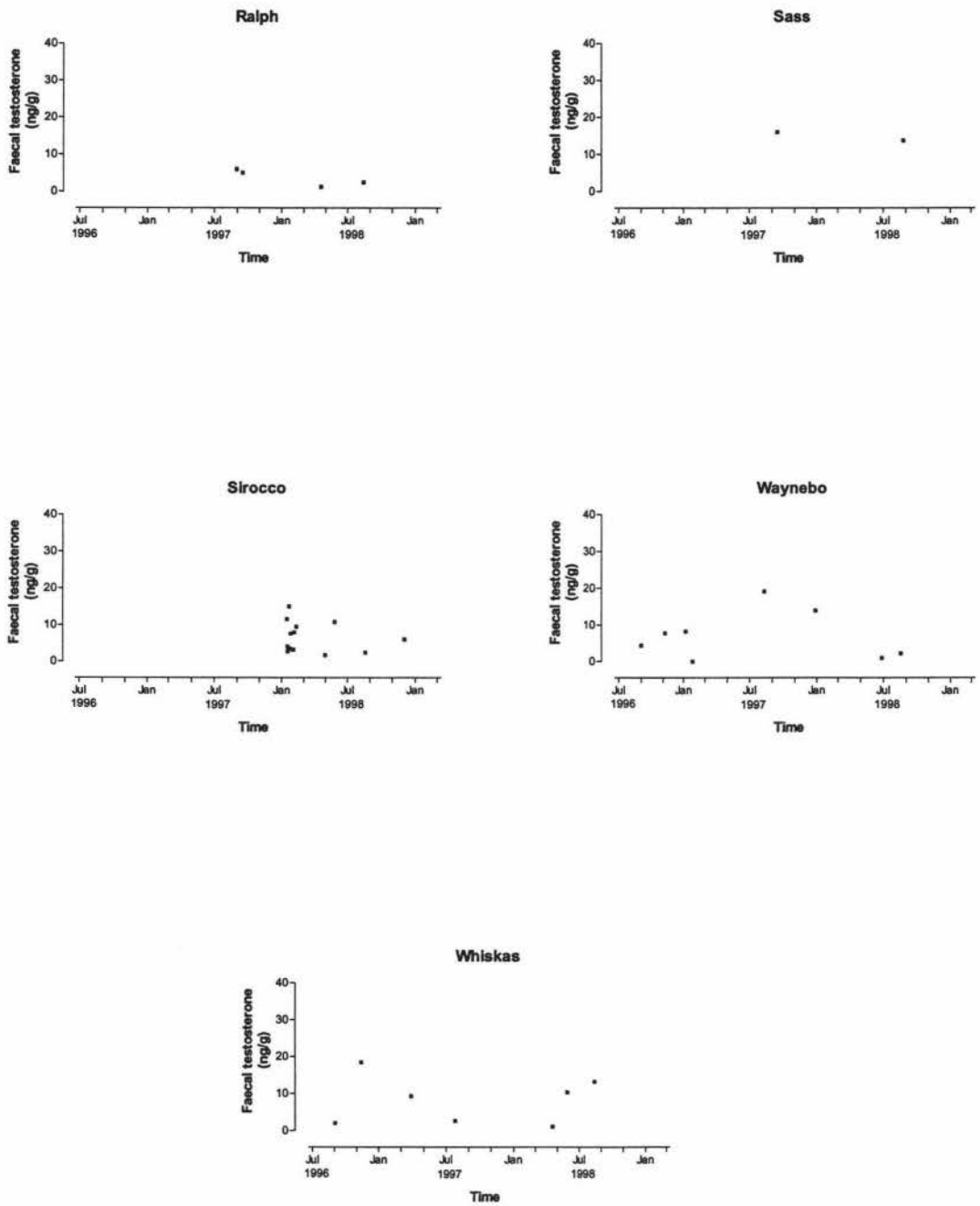

Figure 4.5 C Faecal testosterone profiles for individual male kakapo.

Table 4.4. Extraction efficiencies for testosterone, oestradiol and progesterone in quail and kakapo (using the same extraction method). Efficiencies are shown as mean ± SE.

Hormone	Quail	Kakapo
Testosterone	69.6 ± 6.9% – 89.8 ± 6.0%	38.8 ± 45.0%
Oestradiol	53.5 ± 14.7% – 84.0 ± 10.6%	33.2 ± 36.9%
Progesterone	40.5 ± 14.8% – 67.9 ± 19.5%	15.1 ± 56.0%

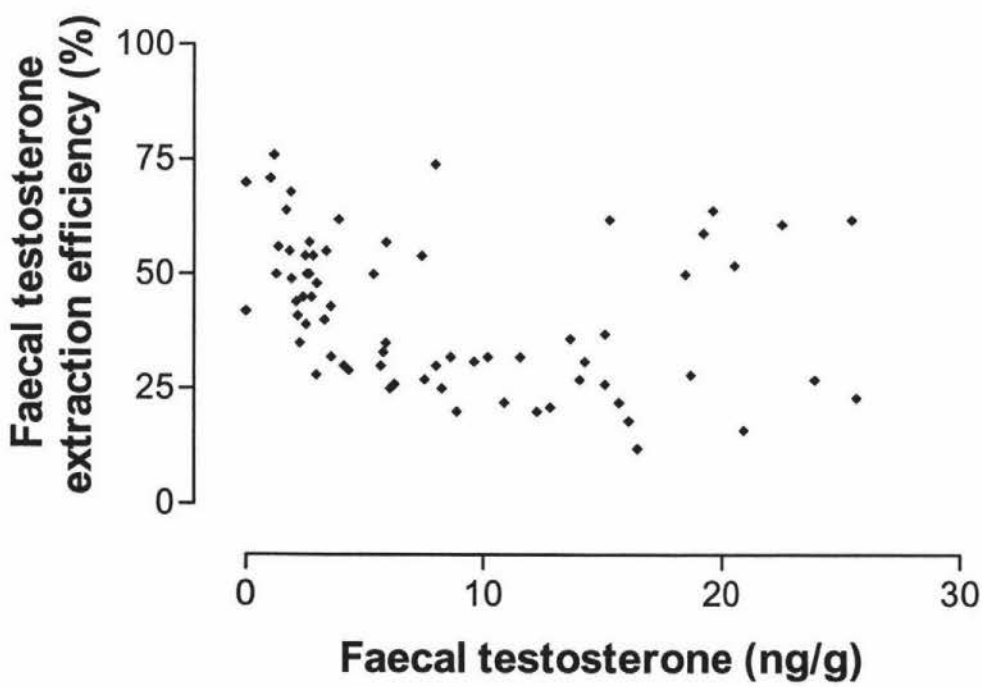

Figure 4.6. Relationship between faecal testosterone concentration and faecal testosterone extraction efficiency. Correlation coefficient r = 0.30, p = 0.013.

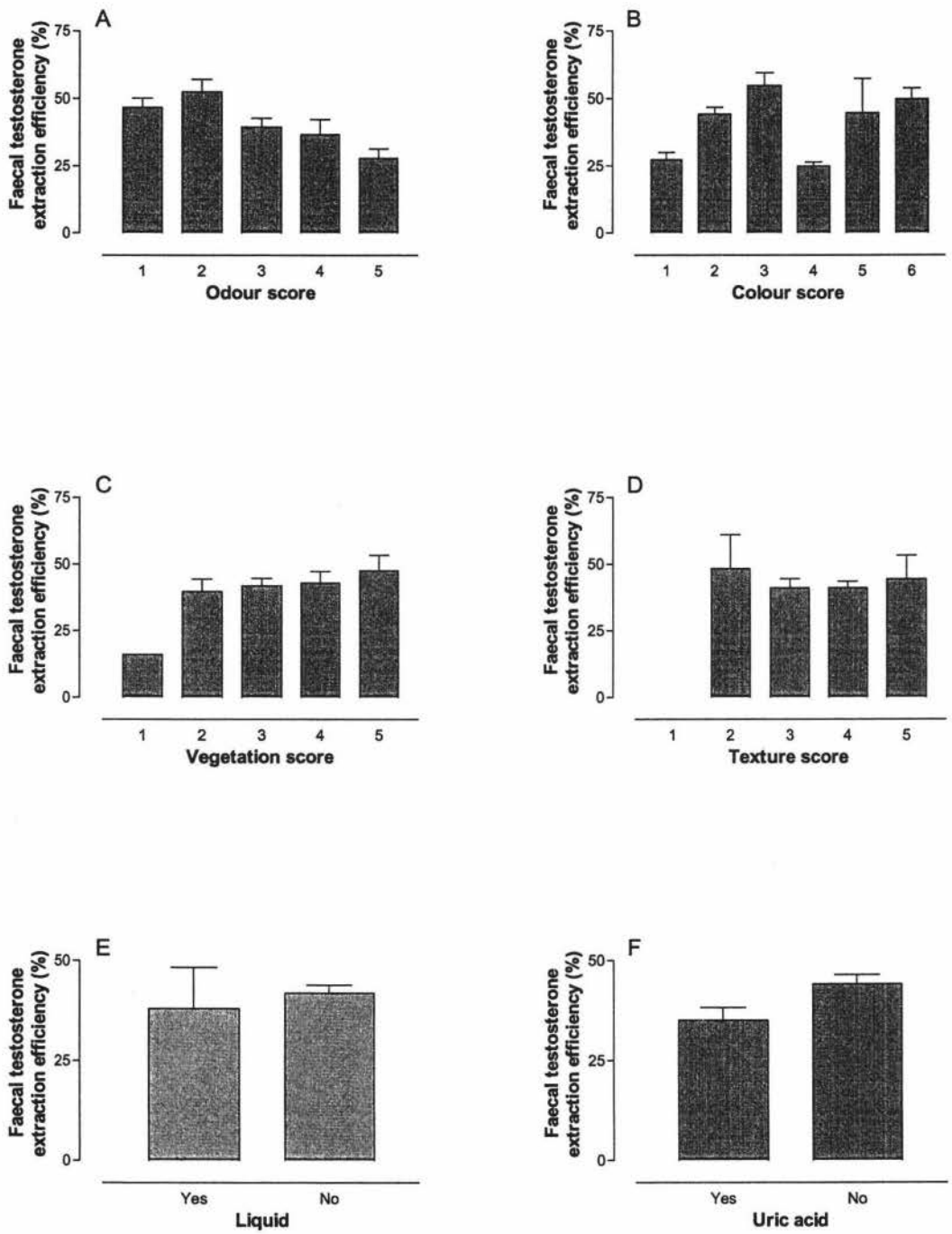

Figure 4.7. Faecal testosterone extraction efficiencies for different scores of physical characteristics of individual droppings (mean % ± SE). A) Odour; B) Colour; C) Size of vegetation; D) Texture; E) Presence of liquid; F) presence of uric acid. Details of the scores for characteristics can be found in Table 4.1.

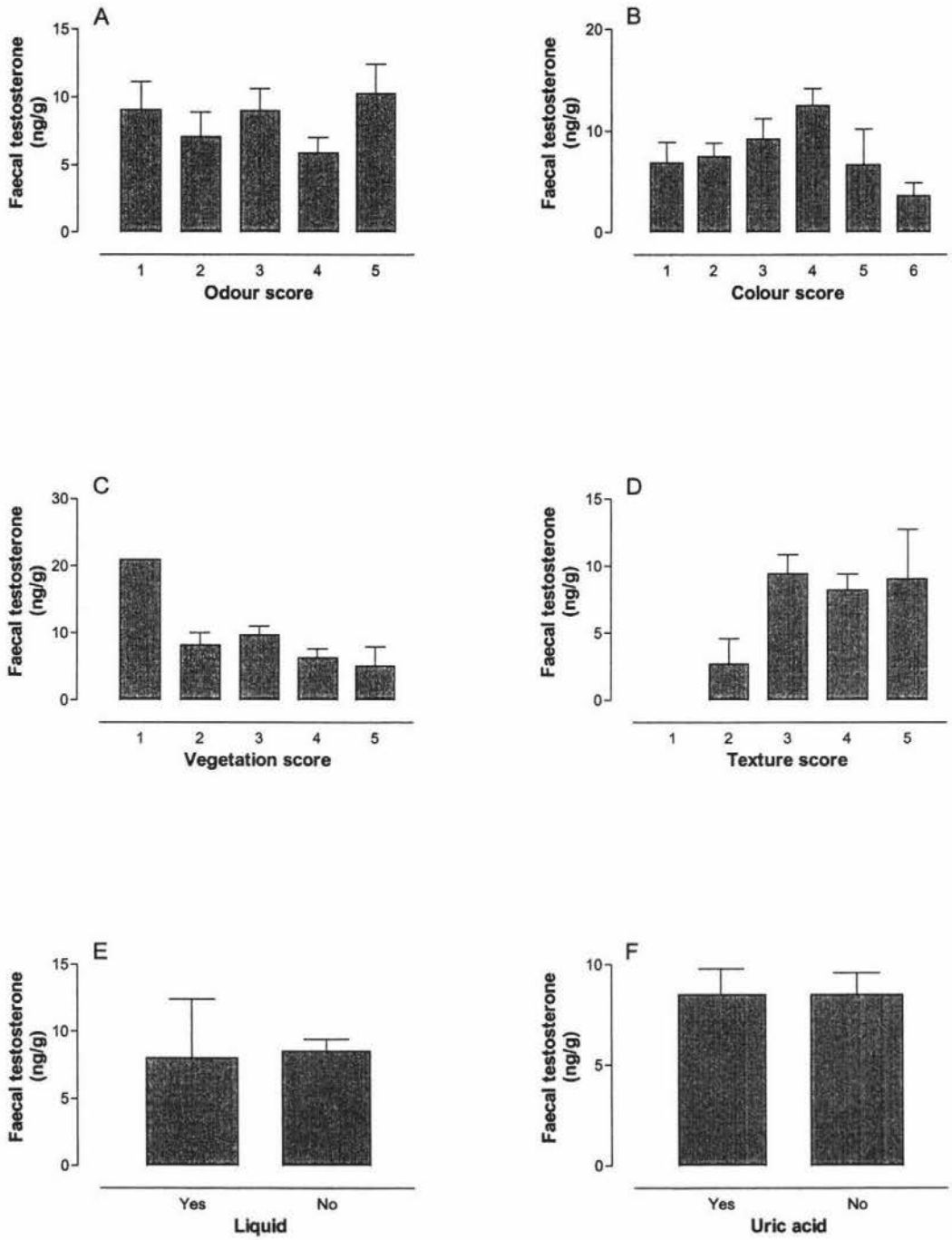

Figure 4.8. Faecal testosterone concentrations for different scores of physical characteristics of individual droppings (mean ± SE). A) Odour; B) Colour; C) Size of vegetation; D) Texture; E) Presence of liquid; F) presence of uric acid. Details of the scores for characteristics can be found in Table 4.1.

Table 4.5. Summary of one-way ANOVA and t-tests of faecal testosterone concentrations and extraction efficiencies across different scores of physical characteristics of individual droppings. Categories with fewer than two cases were excluded from the analysis.

	Testosterone concentration				Extraction efficiency			
	F	t	df	p	F	t	df	p
Odour	0.586		4, 64	0.674	4.784		4, 64	0.002
Colour	1.578		5, 63	0.179	8.176		5, 63	0.001
Vegetation	1.178		3, 64	0.325	0.261		3, 64	0.853
Texture	0.839		3, 65	0.477	0.248		3, 65	0.862
Liquid		-0.135	67	0.893		-0.447	67	0.657
Uric acid		-0.001	67	0.999		-2.146	67	0.036

4.3.3 Faecal oestradiol

Faecal oestradiol concentrations ranged from 1.07 ng/g to 28.88 ng/g (Figure 4.9 A) in droppings collected from January 1996 to January 1999. The cubic polynomials fitted to data from the 1996-97 and 1997-98 seasons were significant (Table 4.6). There were no differences between the parameters of the cubic polynomials from the two seasons (Table 4.6; paired samples T-test, t = 0.94, 0.70 and 0.60 for parameters *a*, *b*, and *c* respectively; df = 17, p>0.05 in all cases; Figure 4.9).

There was an annual cycle of faecal oestradiol (Figure 4.10) and concentrations varied significantly between bimonthly periods ($F_{5, 112} = 9.866$, $p < 0.0001$). Faecal oestradiol concentrations in females rose from September/October and remained elevated between November/December and January/February (LSD = 2.6, df = 112, $p < 0.0001$ in both cases), before declining to lower concentrations in March/April.

The standard deviation of data for all individual droppings collected in the months from March to October was 2.24 ng/g. The 95% confidence interval was 4.39 ng/g. Therefore a difference between individual droppings in faecal oestradiol concentration of more than 8.78 ng/g is likely to indicate different oestradiol concentrations.

Oestradiol concentration varied between situations of multiple dropping collection (Figure 4.11, $F_{9, 39} = 19.058$, $p < 0.001$). The SE of a pooled sample (mean of individual droppings from one roost site) was 0.62 ng/g. This equates to a 95% confidence interval of 1.22 ng/g. Across the ten situations where multiple fresh droppings were collected there was a mean coefficient of variation of 26.1 ± 2.9%.

Insufficient droppings were collected to give clear oestradiol profiles for individual females (Figure 4.12). However, the relative size of the 95% confidence interval developed from data on individual variation in droppings collected from March to October (Figure 4.10), in comparison to single data points on the profiles for individual birds (Figure 4.12) indicates that elevated oestradiol concentrations may have been detected in December 1996 and January 1997 in the profiles from Alice, Sandra and Suzanne.

The extraction efficiency of faecal oestradiol was lower and more varied than extraction efficiencies for quail faecal samples using the same extraction method in the previous chapter (Table 4.4). Extraction efficiency did not have a significant relationship with faecal oestradiol concentration (Figure 4.13). Oestradiol concentration in individual droppings did not vary with odour, colour, size of vegetation or dropping texture, or with the presence of liquid or uric acid (Figure 4.15, Table 4.7). However, extraction efficiency did vary between samples with different sized vegetation (Figure 4.14).

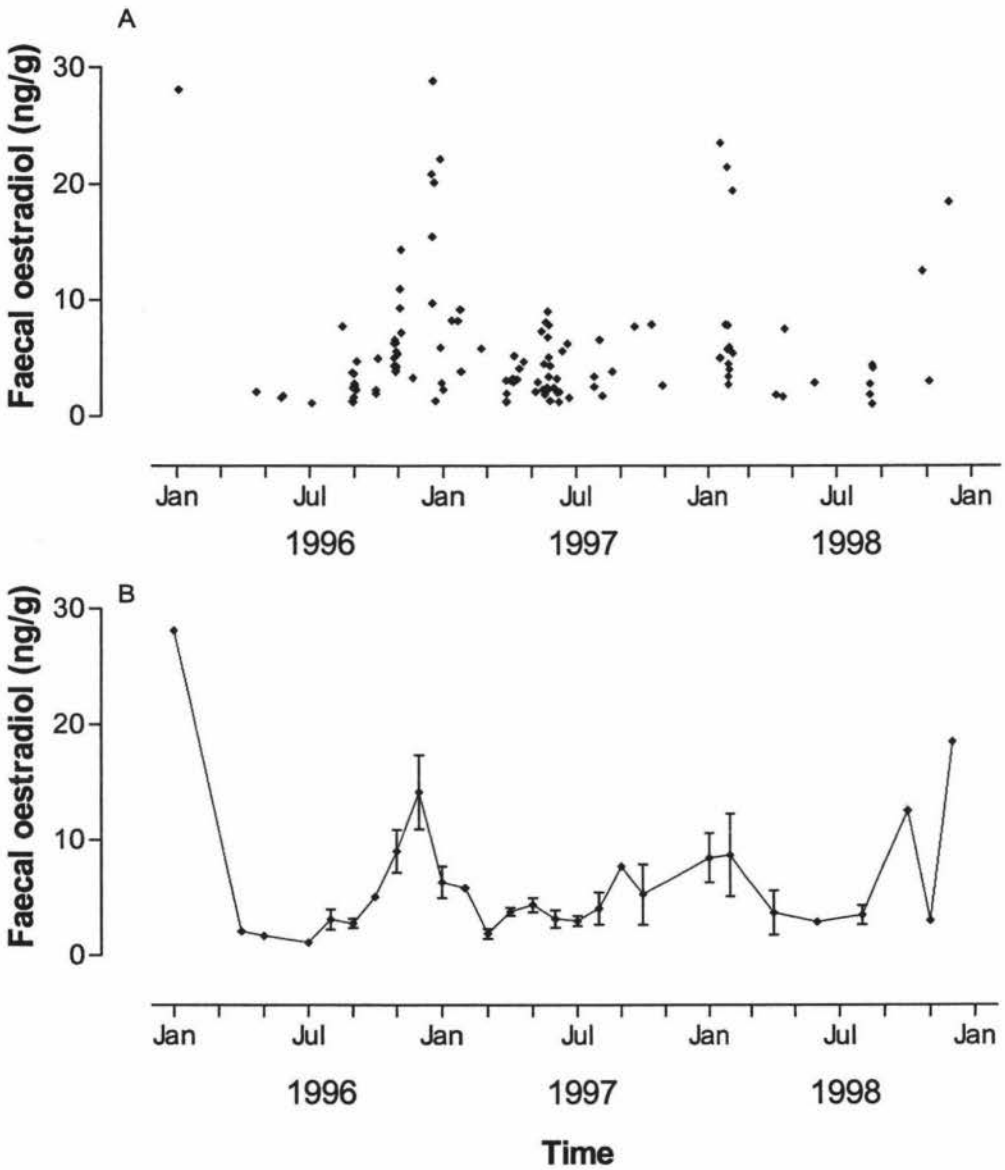

Figure 4.9. Faecal oestradiol concentrations in female kakapo. A) All individual faecal samples collected over sampling period, plotted on day of collection. B) Mean faecal concentration for each month over the sampling period ± SE.

Table 4.6. Parameter estimates of quadratic equation (Equation 4.2) fitted to mean oestradiol concentrations by month for two seasons, 1996-97 and 1997-98.

Variable	Parameter estimate	SE	p
Season 1996-97 ($R^2 = 0.54$)			0.044
Intercept			
A	-2.28	2.86	0.448
B	3.13	1.02	0.015
C	-0.23	0.08	0.016
Season 1997-98 ($R^2 = 0.72$)			0.042
Intercept			
A	0.84	1.67	0.636
B	2.27	0.66	0.018
C	-0.18	0.05	0.016

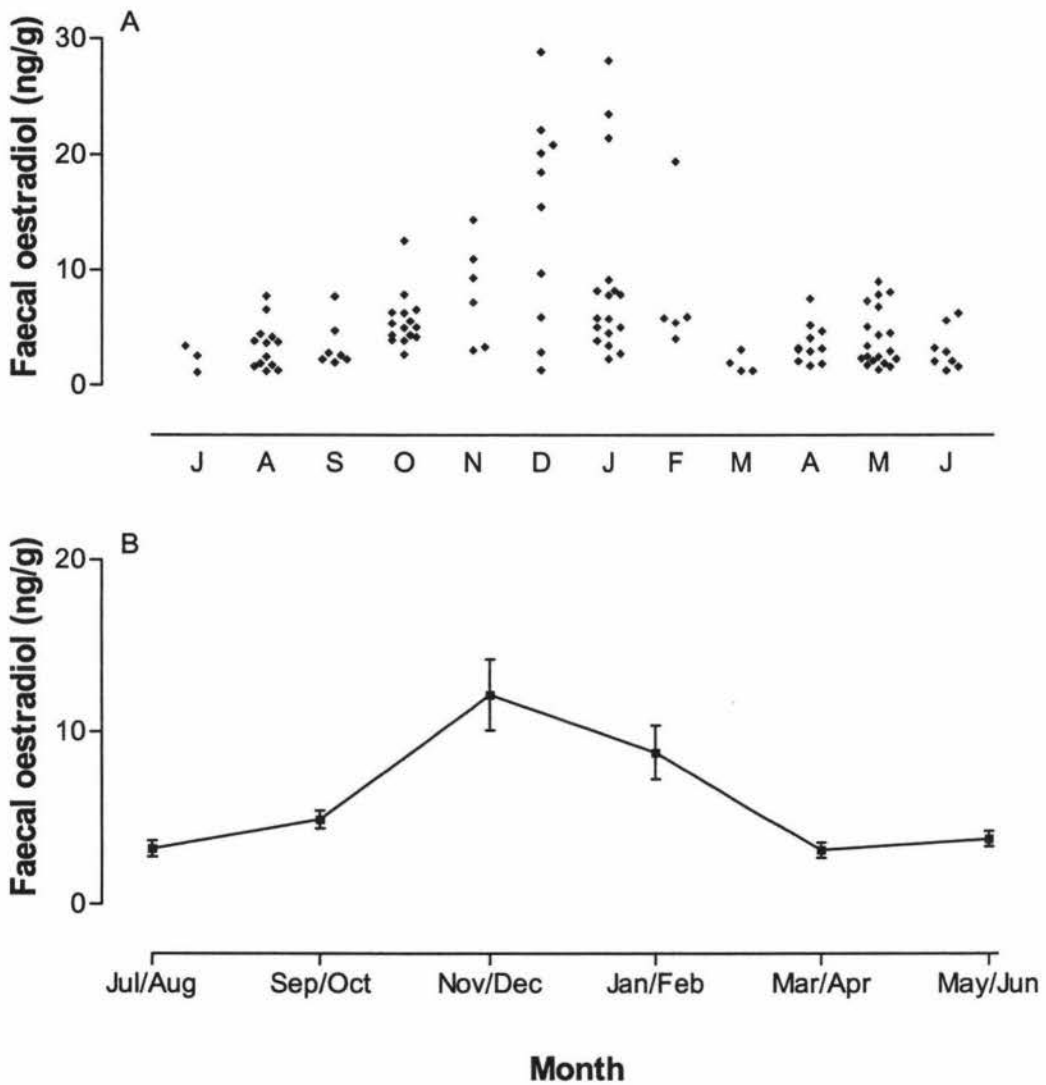

Figure 4.10. Faecal oestradiol concentrations in female kakapo. A) All individual faecal samples grouped by month. B) Mean faecal concentration for bi-monthly groupings over all years combined ± SE.

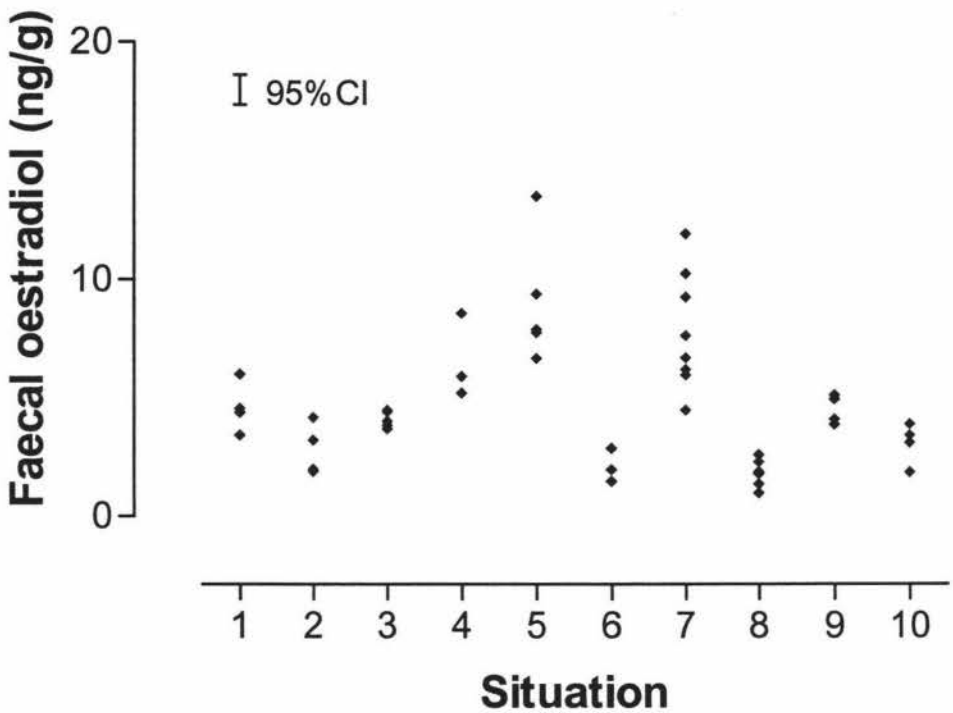

Figure 4.11. Variation in faecal oestradiol concentration between fresh droppings from individual birds from the same day or roost site. 95% confidence interval of the mean is indicated.

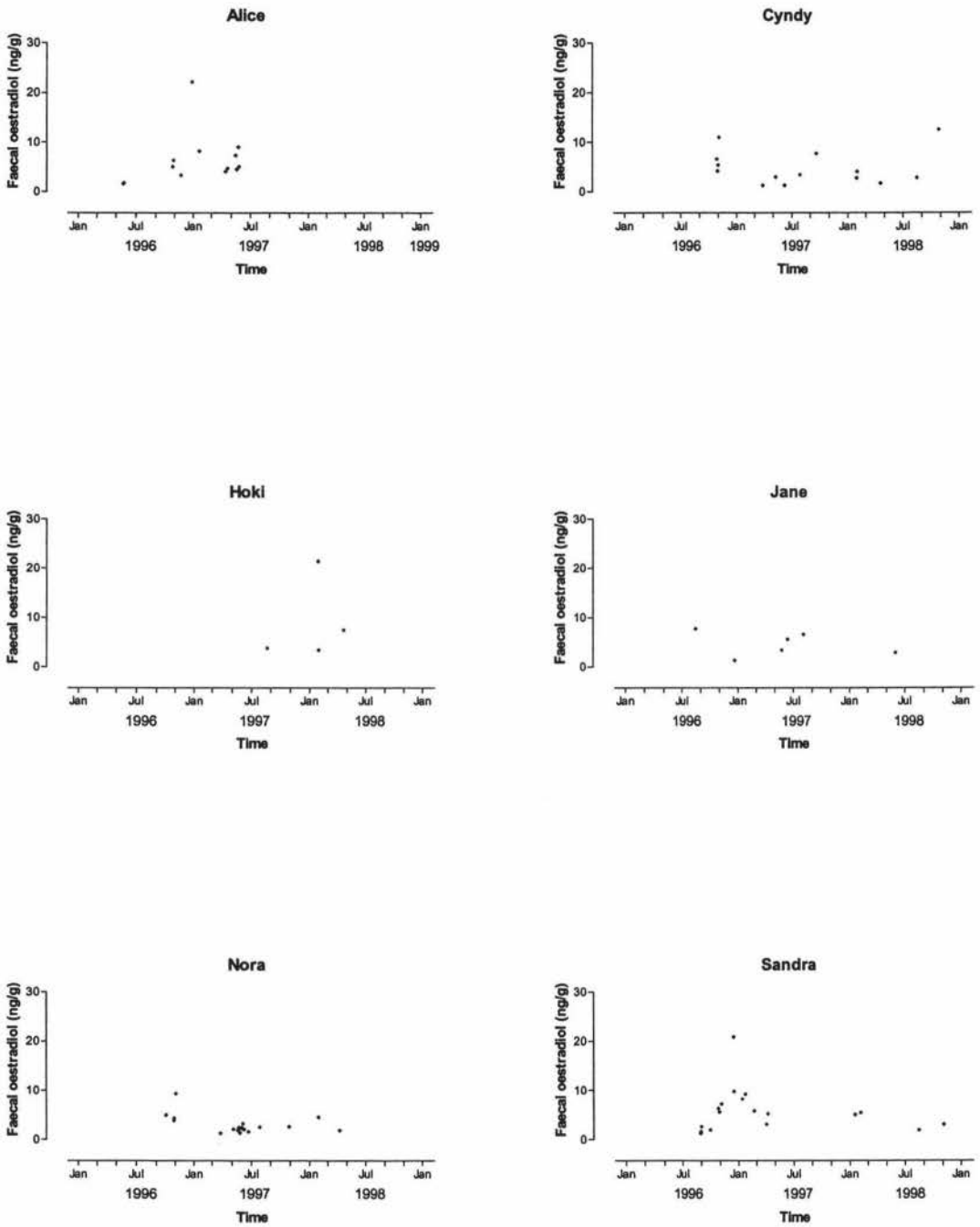

Figure 4.12.A Faecal oestradiol profiles for individual female kakapo.

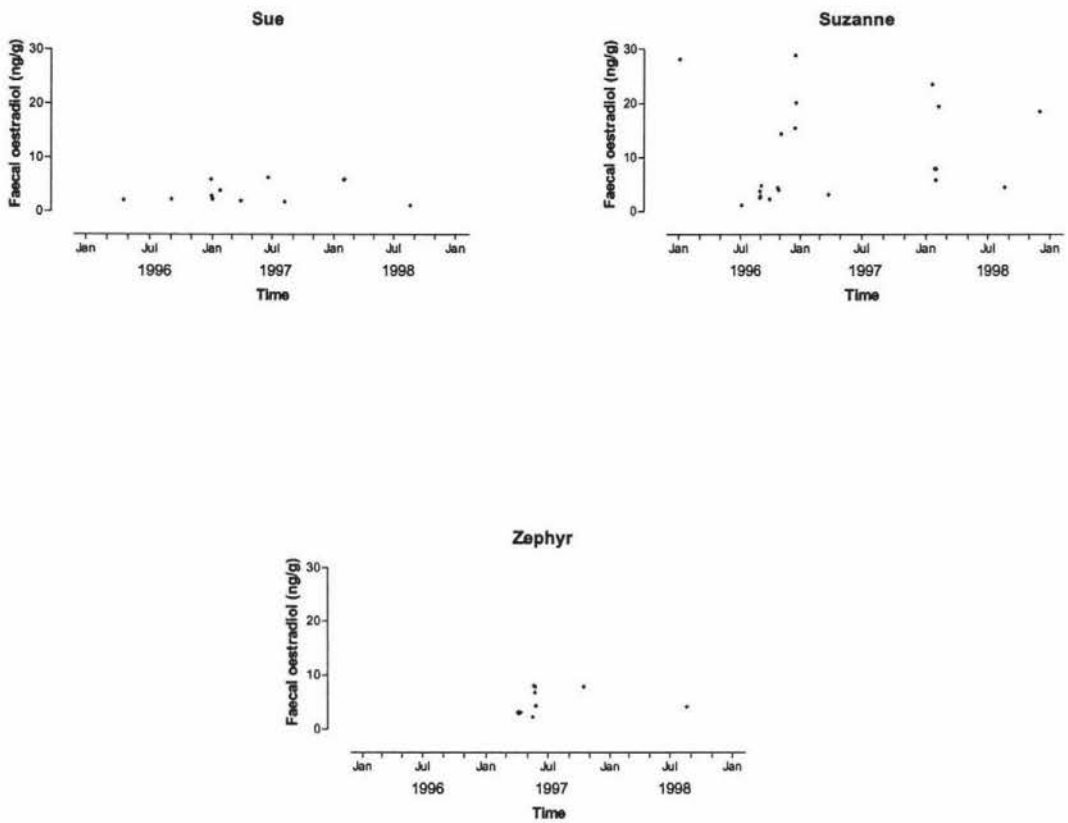

Figure 4.12 B Faecal oestradiol profiles for individual female kakapo.

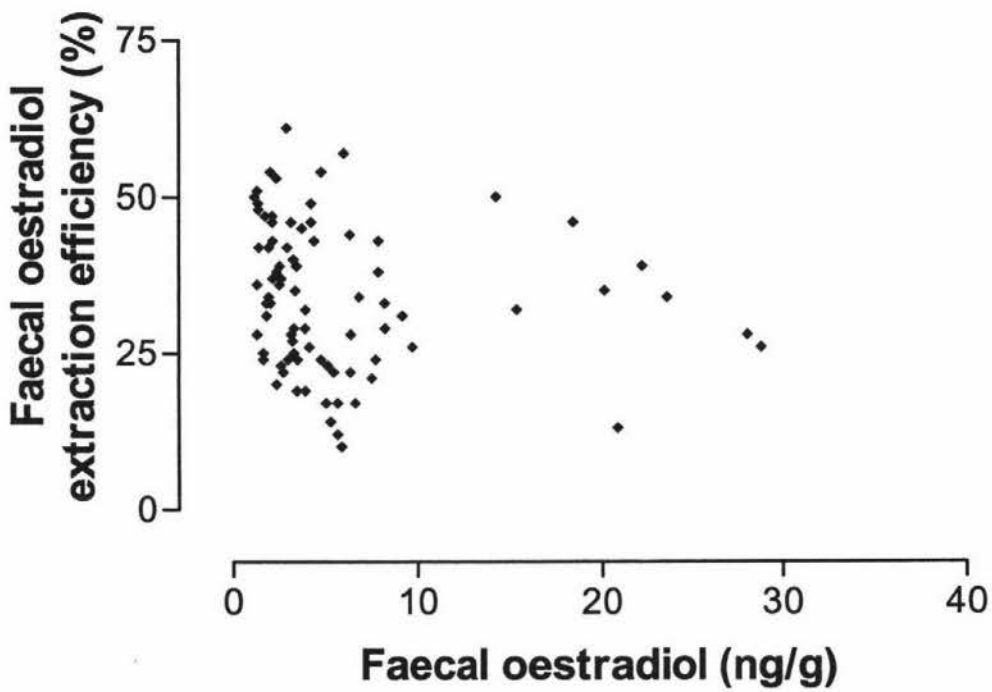

Figure 4.13. Relationship between faecal oestradiol concentration and faecal oestradiol extraction efficiency. Correlation coefficient $r = 0.14$, $p = 0.196$.

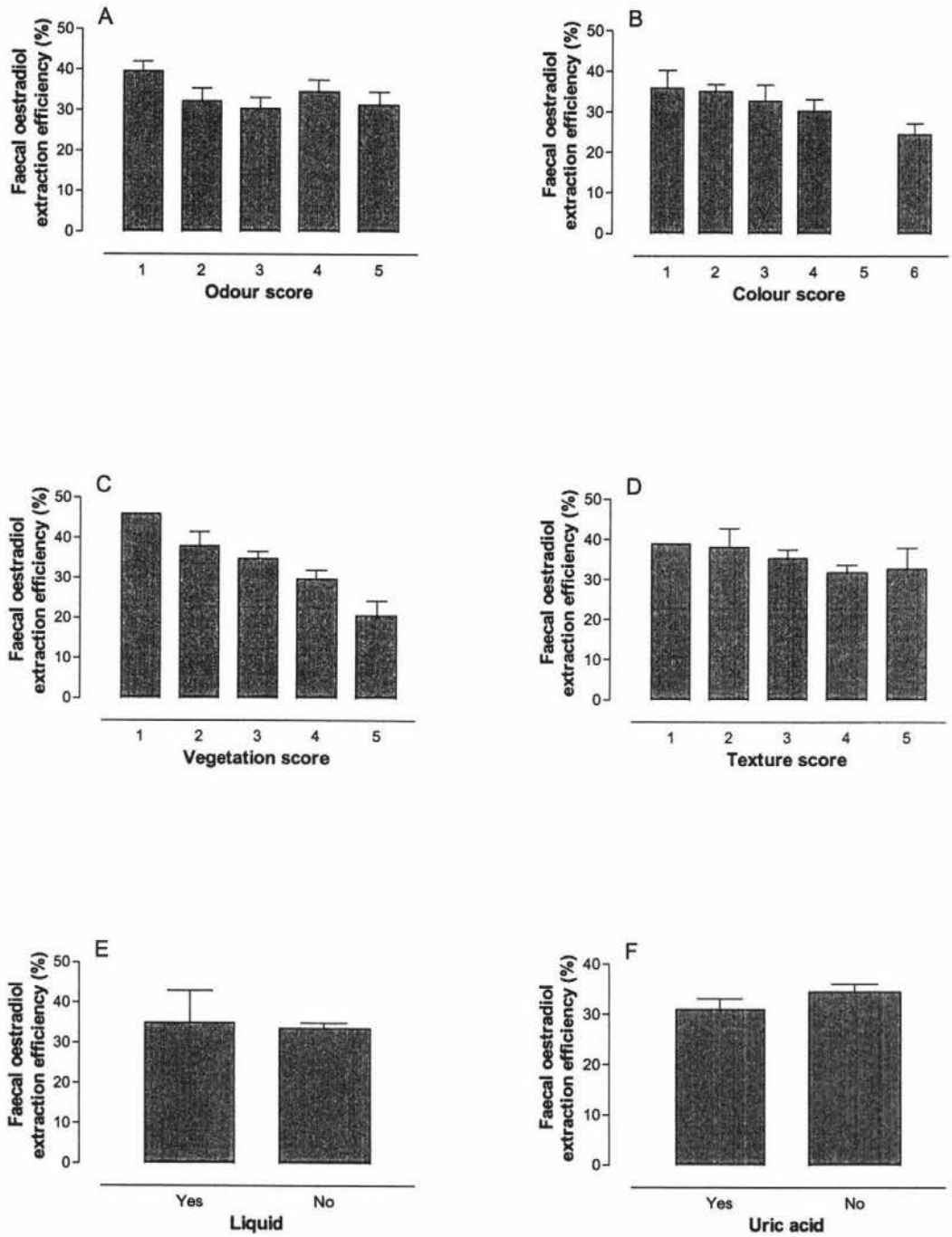

Figure 4.14. Faecal oestradiol extraction efficiencies for different scores of physical characteristics of individual droppings (mean % ± SE). A) Odour; B) Colour; C) Size of vegetation; D) Texture; E) Presence of liquid; F) presence of uric acid. Details of the scores for characteristics can be found in Table 4.1.

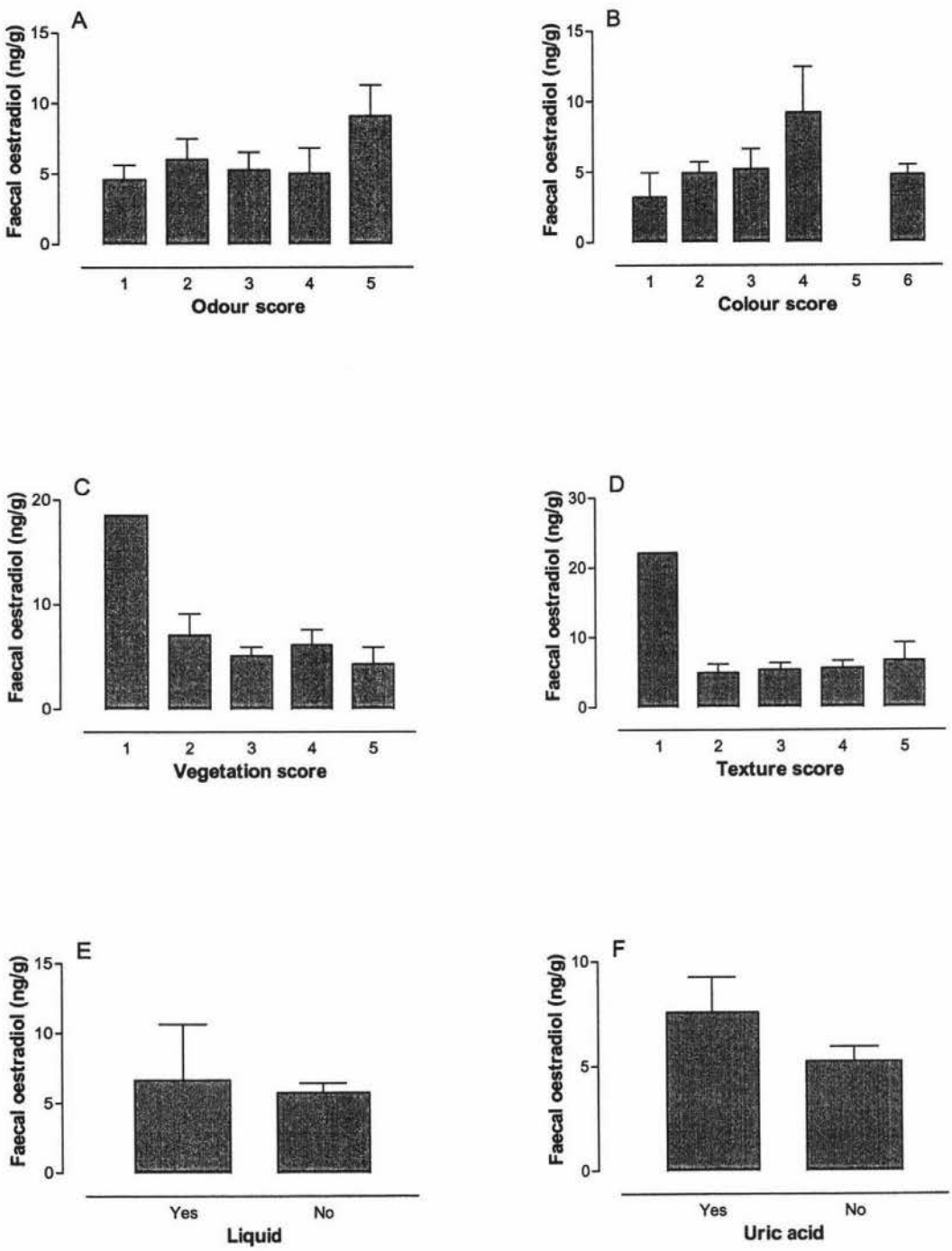

Figure 4.15. Faecal oestradiol concentrations for different scores of physical characteristics of individual droppings (mean ± SE). A) Odour; B) Colour; C) Size of vegetation; D) Texture; E) Presence of liquid; F) presence of uric acid. Details of the scores for characteristics can be found in Table 4.1.

Table 4.7. Summary of one-way ANOVA and t-test of faecal oestradiol concentrations and extraction efficiencies across different scores of physical characteristics of individual droppings. Categories with fewer than two cases were excluded from the analysis.

	Oestradiol concentration				Extraction efficiency			
	F	t	df	p	F	t	df	p
Odour	1.175		4, 77	0.329	1.806		4, 77	0.136
Colour	1.371		4, 77	0.252	1.096		4, 77	0.365
Vegetation	0.465		3, 77	0.707	2.897		3, 77	0.040
Texture	0.146		3, 77	0.932	0.737		3, 77	0.533
Liquid		0.287	80	0.775		0.247	80	0.805
Uric acid		1.508	80	0.135		-1.161	80	0.249

4.3.4 Faecal progesterone

Faecal progesterone concentrations ranged from the least detectable dose of 0.075 ng/g to 347.70 ng/g (Figure 4.16 A) in droppings collected from January 1996 to January 1999. The cubic polynomial fitted to data from the 1996-97 was significant, however that for the 1997-98 seasons was marginal (Table 4.8). There were no differences between the parameters of the cubic polynomials from the two seasons (Table 4.8; paired samples T-test, t = 0.12, 0.60 and 0.81 for parameters *a*, *b*, and *c* respectively; df = 17, p>0.05 in all cases; Figure 4.16).

There was an annual cycle of faecal progesterone (Figure 4.17) and concentrations varied significantly between bimonthly periods ($F_{5,\ 112}$ = 4.361, p = 0.001). Faecal progesterone concentrations in females rose from September/October and remained elevated between November/December and March/April (LSD = 48.1, df = 112, p < 0.02 in all cases), before declining to lower concentrations in May/June.

The standard deviation of data for all individual droppings collected in the months from May to October was 69.63 ng/g. The 95% confidence interval was 136.47 ng/g. Therefore a difference between individual droppings in faecal progesterone

concentration of more than 272.94 ng/g is likely to indicate different progesterone concentrations.

Progesterone concentration varied between situations of multiple dropping collection (Figure 4.18, $F_{8, 33} = 7.453$, $p < 0.001$). The SE of a pooled sample (mean of individual droppings from one roost site) was 32.64 ng/g. This equates to a 95% confidence interval of 63.97 ng/g. Across the ten situations where multiple fresh droppings were collected there was a mean coefficient of variation of 47.03 ± 7.7%.

Insufficient droppings were collected to give clear progesterone profiles for individual females (Figure 4.19). However, the relative size of the 95% confidence interval developed from data on individual variation in droppings collected from May to October (Figure 4.17), in comparison to single data points on the profiles for individual birds (Figure 4.19) indicates that elevated progesterone concentrations may have been detected in December 1997 and January 1998 in the profile for Sandra and in January 1998 in the profile from Nora.

Extraction efficiency of faecal progesterone was lower and more varied than extraction efficiencies for quail faecal samples using the same extraction method in the previous chapter (Table 4.4). Extraction efficiency did not have a significant relationship with faecal progesterone concentration (Figure 4.20). Progesterone concentration in individual droppings did not vary with a range in odour, colour, size of vegetation, dropping texture, or with the presence of liquid (Figure 4.22, Table 4.9). However, progesterone concentration was significantly higher in samples that had uric acid on the surface. Extraction efficiency varied between samples with different sized vegetation (Figure 4.21). Multiple comparisons were made using Bonferroni post-hoc analysis. Samples that had no remnants of vegetation in them had significantly higher extraction efficiencies than samples with vegetation ($p = 0.001$).

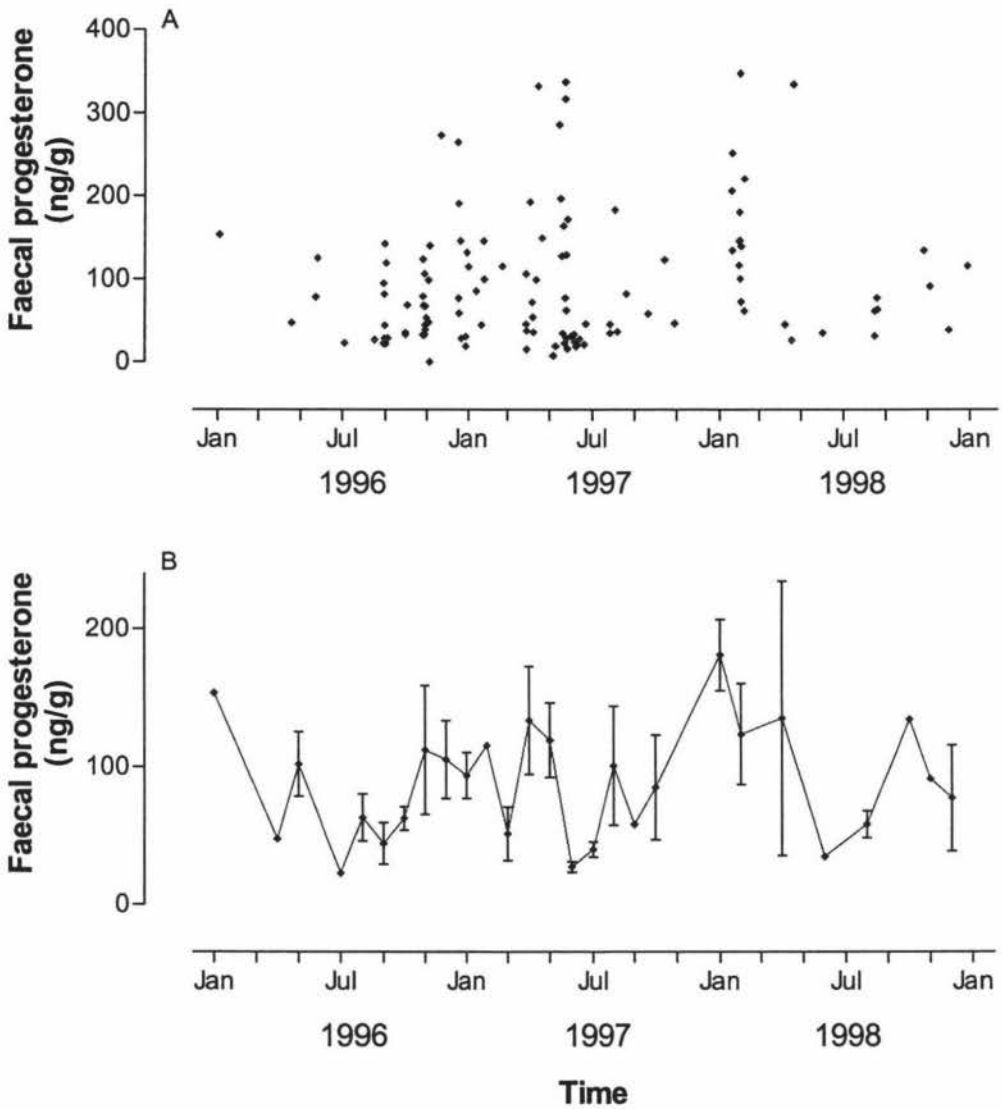

Figure 4.16. Faecal progesterone concentrations in female kakapo. A) All individual faecal samples collected over sampling period, plotted on day of collection. B) Mean faecal concentration for each month over the sampling period ± SE.

Table 4.8. Parameter estimates of quadratic equation (Equation 4.2) fitted to mean progesterone concentrations by month for two seasons, 1996-97 and 1997-98.

Variable	Parameter estimate	SE	p
Season 1996-97 ($R^2 = 0.62$)			0.021
Intercept			
a	-15.97	28.45	0.590
b	34.11	10.15	0.010
c	-2.24	0.75	0.018
Season 1997-98 ($R^2 = 0.65$)			0.073
Intercept			
a	-10.48	38.34	0.796
b	45.58	15.03	0.029
c	-3.35	1.15	0.033

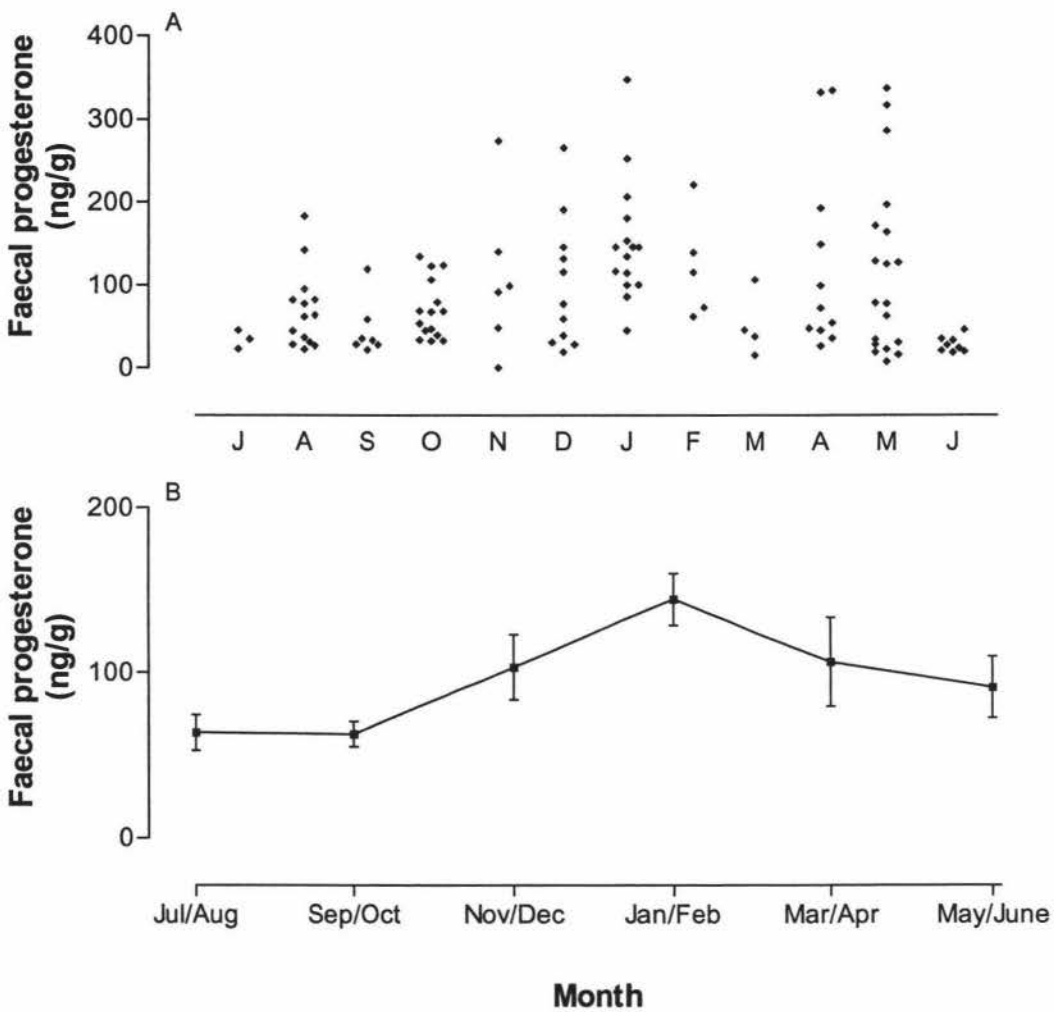

Figure 4.17. Faecal progesterone concentrations in female kakapo. A) All individual faecal samples grouped by month. B) Mean faecal concentration for bi-monthly groupings over all years combined ± SE.

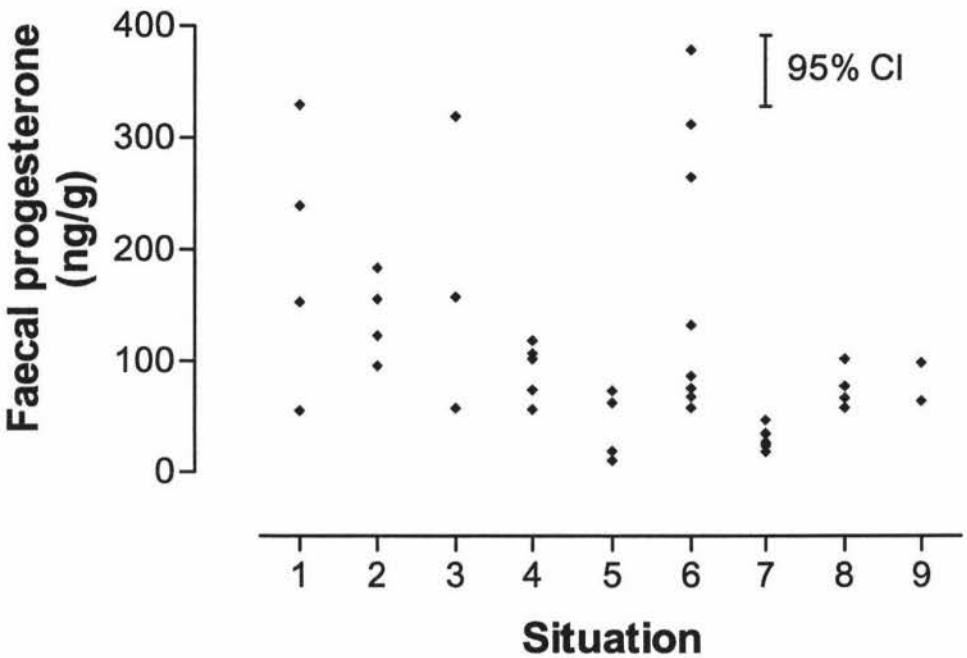

Figure 4.18. Variation in faecal progesterone concentration between fresh droppings from individual birds from the same day or roost site. 95% confidence interval of the mean is indicated.

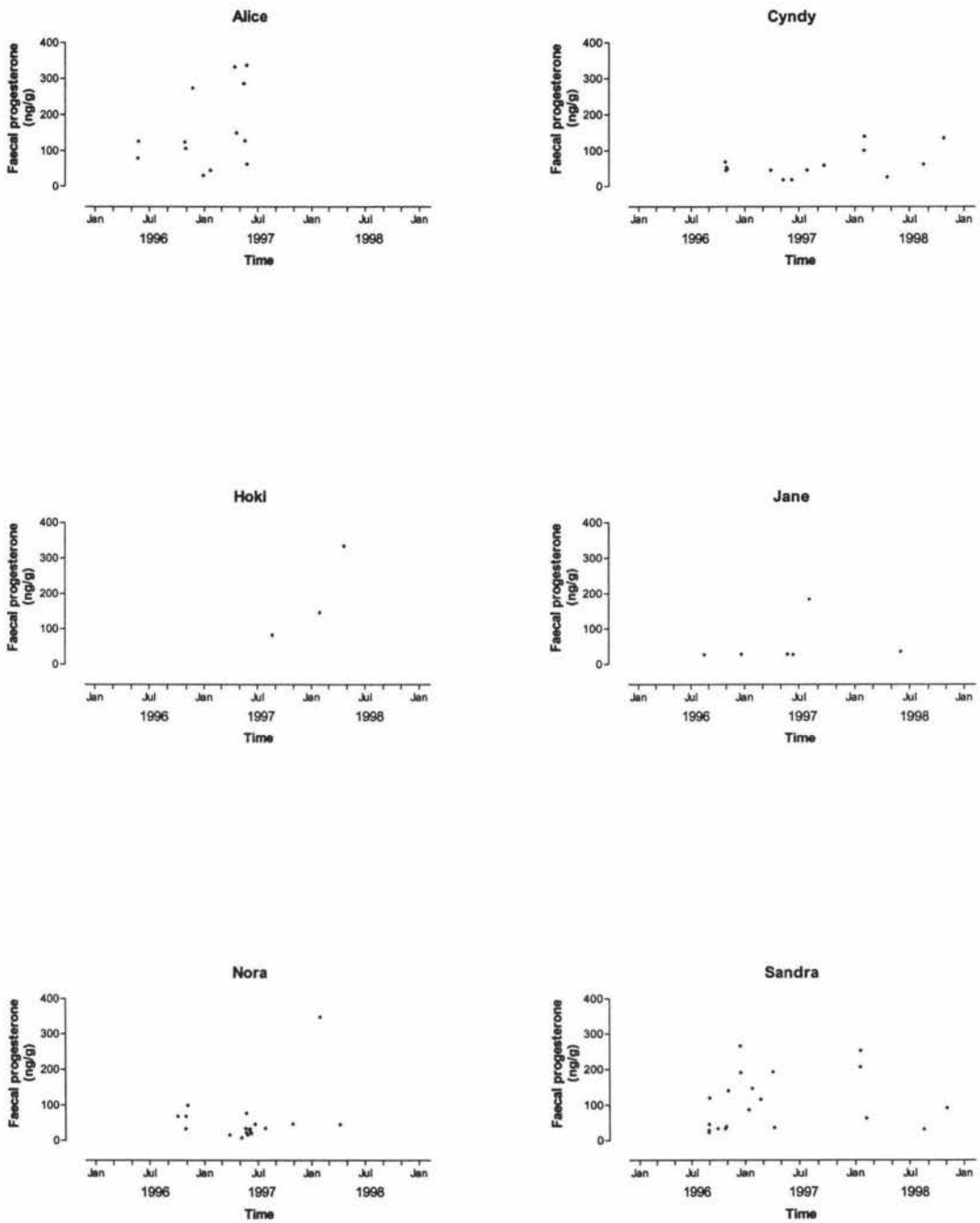

Figure 4.19.A Faecal progesterone profiles for individual female kakapo.

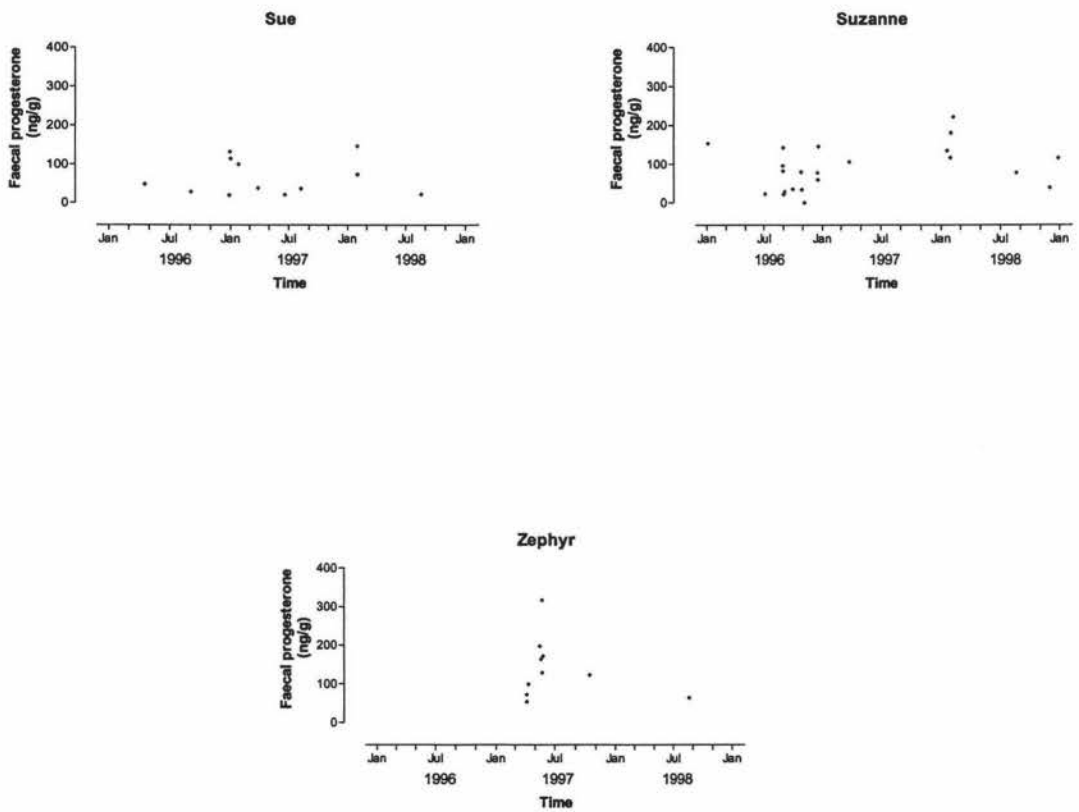

Figure 4.19 B Faecal progesterone profiles for individual female kakapo.

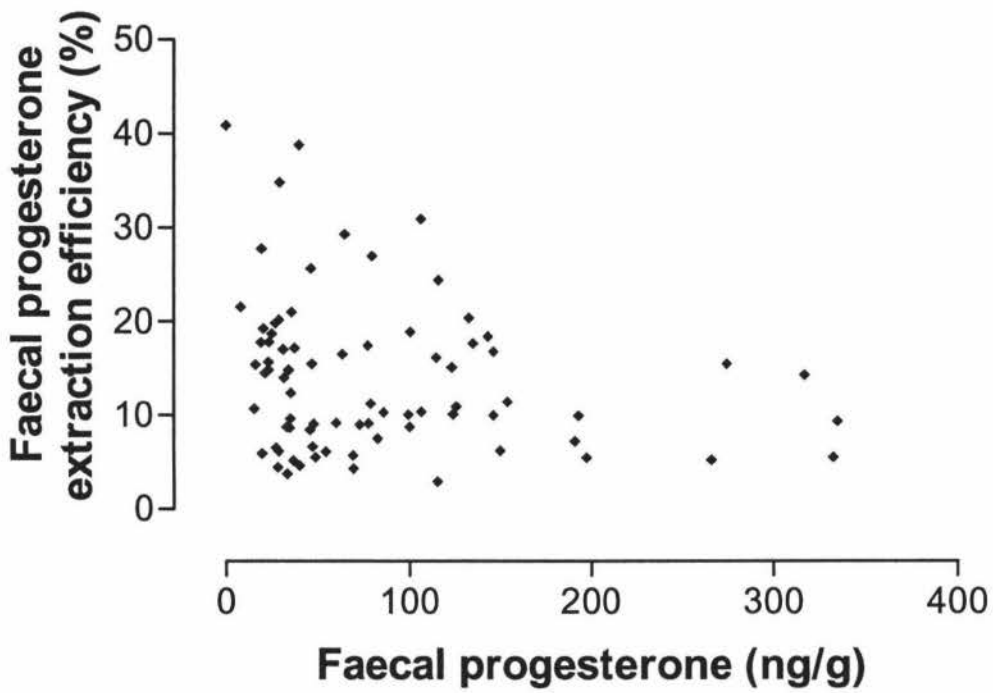

Figure 4.20. Relationship between faecal progesterone concentration and faecal testosterone extraction efficiency. Correlation coefficient $r = 0.22$, $p = 0.060$.

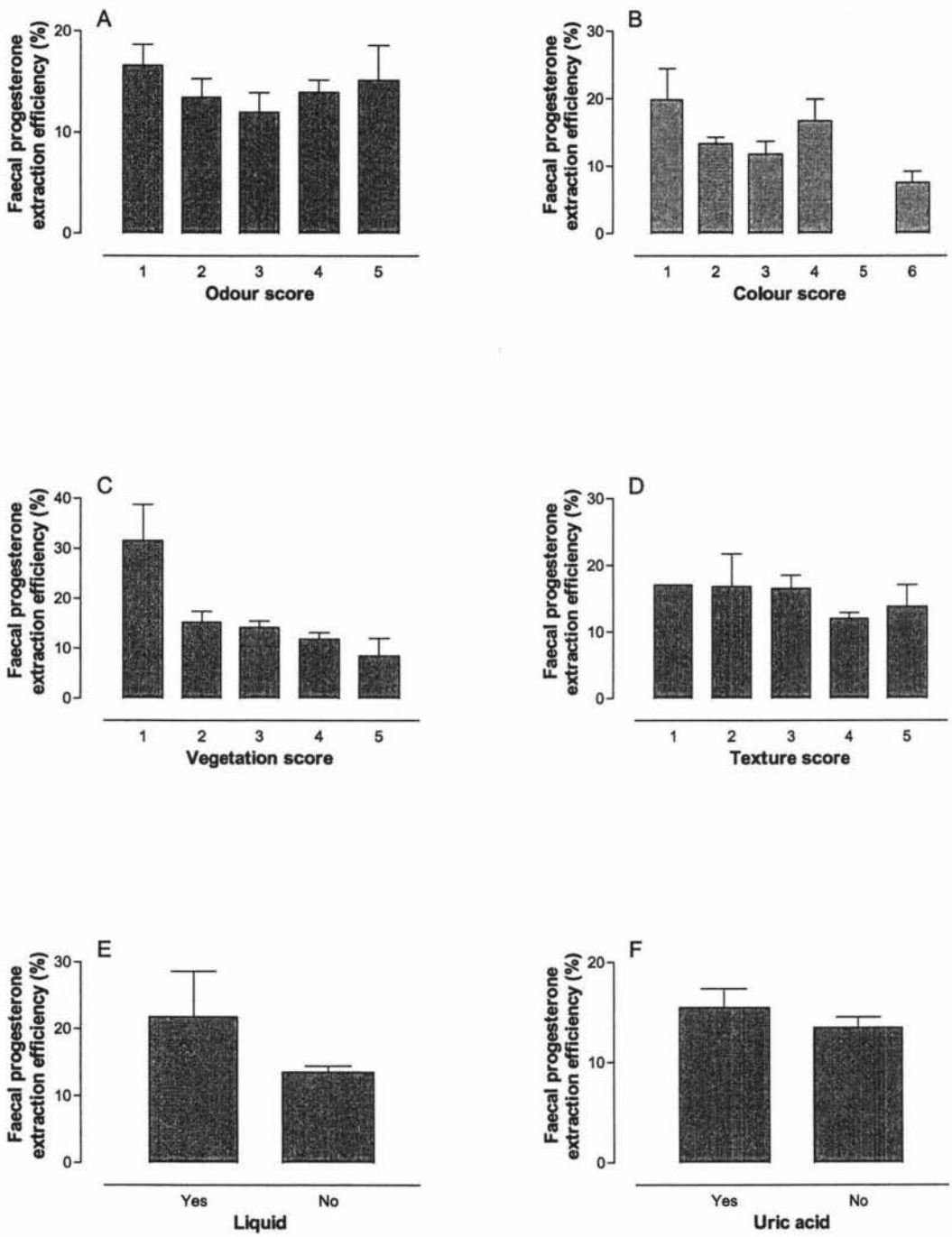

Figure 4.21. Faecal progesterone extraction efficiencies for different scores of physical characteristics of individual droppings (mean % ± SE). A) Odour; B) Colour; C) Size of vegetation; D) Texture; E) Presence of liquid; F) presence of uric acid. Details of the scores for characteristics can be found in Table 4.1.

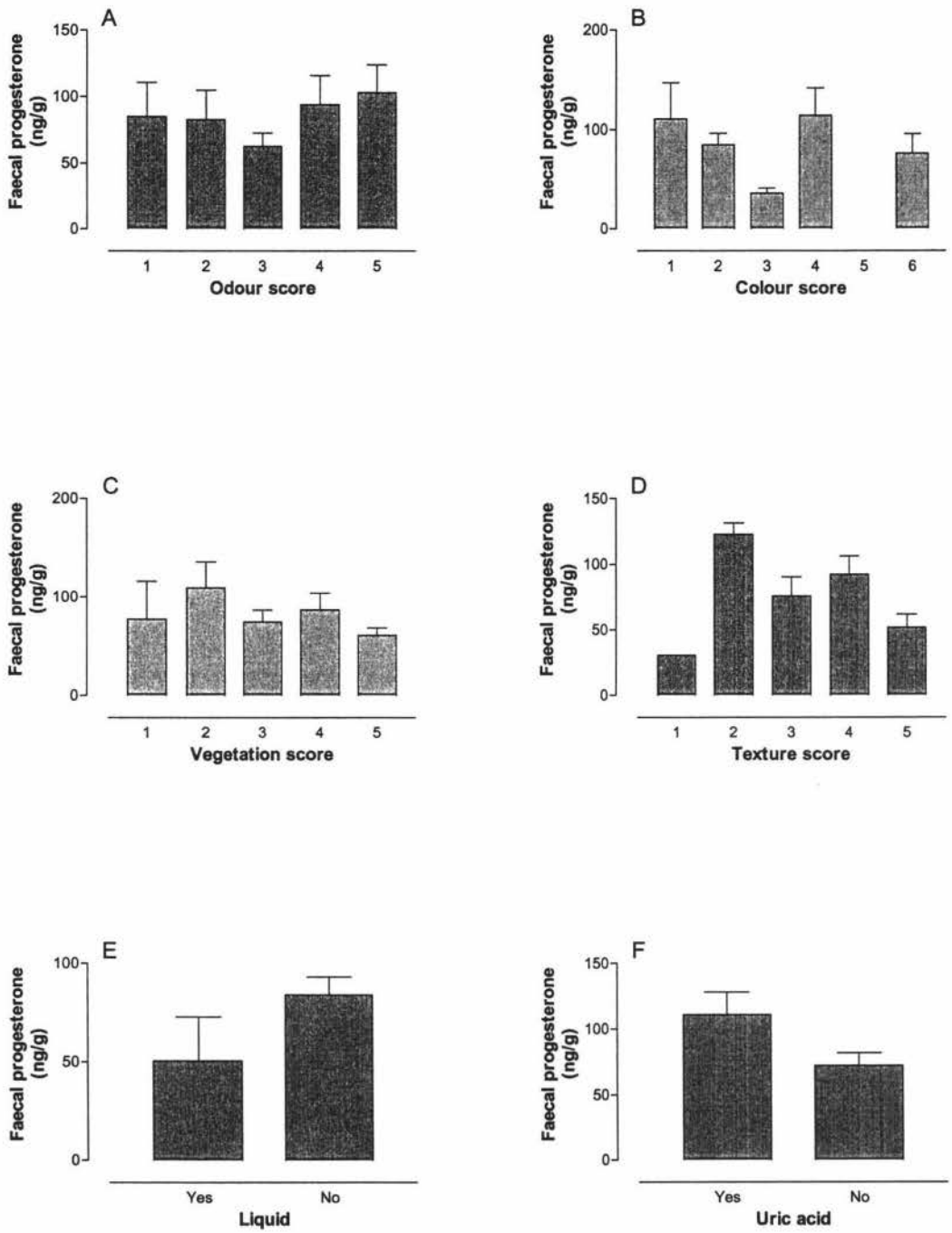

Figure 4.22. Faecal progesterone concentrations for different scores of physical characteristics of individual droppings (mean ± SE). A) Odour; B) Colour; C) Size of vegetation; D) Texture; E) Presence of liquid; F) presence of uric acid. Details of the scores for characteristics can be found in Table 4.1.

Table 4.9. Summary of one-way ANOVA and t-tests of faecal progesterone concentrations and extraction efficiencies across different scores of physical characteristics of individual droppings. Categories with fewer than two cases were excluded from the analysis.

	Progesterone concentration				**Extraction efficiency**			
	F	**t**	**df**	**p**	**F**	**t**	**df**	**p**
Odour	0.658		4, 73	0.623	0.727		4, 73	0.577
Colour	2.240		4, 73	0.073	2.099		4, 73	0.090
Vegetation	0.517		4, 73	0.723	18.513		4, 73	0.001
Texture	1.204		3, 73	0.315	1.570		3, 73	0.217
Liquid		-0.863	76	0.391		1.563	76	0.122
Uric acid		2.052	76	0.044		0.742	76	0.460

4.4 Discussion

The goals of this study were to determine the annual pattern of steroid hormone concentrations in free-living kakapo and relate this to breeding activity in individual birds. Faecal steroid concentrations were measured in samples collected from known male and female kakapo. Annual cycles of reproductive steroids were found that coincided with stages of the breeding season. No differences in hormone cycle between breeding and non-breeding years were found, supporting the idea that birds undergo a degree of gonadal development each year.

4.4.1 Testosterone

There were no differences in faecal testosterone concentration between the two seasons with complete data collections from July - July. The 1996-97 season was a successful breeding season during which the males boomed from December to March and had testosterone concentrations that rose from August to a peak in December before dropping to lower concentrations by March. The 1997-98 season showed a very similar pattern of testosterone, rising from July to a peak in December before dropping to lower concentrations by April, although no booming or nest building occurred. This suggests that there is a degree of testicular development in male kakapo each year. The development of a fully functioning testis is controlled by supplementary information (Wingfield, 1980), and clearly supplementary information that stimulates full gonadal development for successful breeding is present for kakapo in some years but not in others. With more regular and frequent collections during the critical period from July to March, the extent to which testicular development is occurring each year and the role of supplementary information could be more accurately determined.

There was an annual pattern of faecal testosterone concentrations, with lower concentrations in March/April and May/June than other months. Periods of male booming activity that occurred in the 1996-97 and 1997-98 breeding seasons coincided with periods of elevated testosterone. Cockrem and Rounce (1995) identified an annual cycle of faecal testosterone in droppings from unidentified kakapo without information on the exact date of production. Concentrations rose from October-November to a peak

in February, before declining in March to lowest concentrations by April. In the present study, collection of samples from male kakapo was of lower priority than collection from female kakapo, with infrequent collections especially during the period of greatest interest from October – January over the three seasons. The available data were combined into bimonthly units, thereby lowering the resolution of the annual hormone cycle. In both studies, testosterone concentrations were lowest around April. In the Cockrem and Rounce study, concentrations remained low until October. In the present study concentrations rose from May/June and were elevated without a clear peak until January/February. The most obvious difference between annual cycles of the two studies was that Cockrem and Rounce identified a significant peak in February. Despite infrequent sampling in the current study, there was a trend towards an earlier peak in November/December.

Cockrem and Rounce reported values of faecal testosterone ranging from <60 ng/g to 441 ± 163.4 ng/g. These values are ten-fold higher than in the present study. However, the cross reaction of the testosterone antibody with 5α-dihydrotestosterone in the previous study was 34% compared with 3.4% in the present study. Differences in cross-reaction with 5α-dihydrotestosterone and potentially with other steroids may account for the differences in measured testosterone concentrations between the two studies. Faecal testosterone concentrations in male greylag geese (Hirschenhauser *et al.* 1999a, 1999b) and domestic ganders (Kotrschal *et al.* 2000) are similar to those measured in kakapo in this study. However reported concentrations in other species, for example quail (Bishop and Hall, 1991; Hawke, chapter three), and chickens (Cockrem and Rounce 1994) are approximately ten-fold higher in magnitude. These differences may relate to the diets of the study birds, with geese and ducks eating a largely herbivorous diet and the kakapo an entirely herbivorous diet whereas quail and chickens eat more concentrated commercial diets.

Testosterone concentrations varied markedly between samples collected on one day from an individual bird. The mean coefficient of variation for male kakapo was 38.5 ± 4.3%, whereas in quail the equivalent value was 19.9 ± 2.0% (see section 2.3.5). The greater variation in the kakapo samples is to be expected due to variation in extraction

efficiency of testosterone in droppings, diet, time from defecation to sample collection and environmental conditions.

Measurements taken from individual droppings are accurate enough to detect differences in hormone concentration that are greater than 16.8 ng/g. However, collections of multiple pooled droppings from a roost site are capable of detecting a difference of 7.4 ng/g. Therefore, pooled samples are likely to give more accurate information when determining small changes in hormone concentrations, for example using profiles from individual birds for the prediction of reproductive status. In the previous two chapters, the variation associated with the determination of faecal hormone concentrations was similar for individual or pooled faecal samples in quail. Therefore, individual and pooled samples were capable of detecting similar differences in faecal hormone concentration in quail. However, the relationships between faecal hormone concentrations and both plasma hormone concentrations and gonad size became stronger as the number of droppings that were pooled increased. The quail samples were collected from birds eating a constant diet in a controlled, captive environment, whereas the diet of free-living kakapo will vary. Other variables that could affect steroid concentrations in kakapo faeces included the time from defecation to sample collection and weather conditions.

Insufficient droppings were collected to give clear testosterone profiles for individual males and compare these to individual breeding activity. However, a peak in testosterone was indicated for Ken in February 1997 and 1998, for Merv in August 1998, for Nog in December 1996 and August 1998 and for Whiskas in November 1996. No booming activity was recorded for Ken from July 1996 to January 1999. However, booming was recorded for the other males when faecal testosterone concentrations appeared to be elevated. Mating was also recorded for Nog in both years. A lack of data in other months limits the information these data can provide.

Extraction efficiency of faecal testosterone was lower and more variable than for quail faecal samples. This was most probably due to variation in diet between the two species and between individual kakapo. All quail were fed on a quail pellet layer mash, whereas kakapo eat a variety of plant species and supplementary food items such as kumara,

apple, nuts and formulated pellets. High lipid content has been shown to interfere with the extraction process and cause low extraction efficiencies in faecal samples from rock ptarmigan (M. Kikuchi, pers. comm.). During the extraction process, a layer of fat-like film was present on the surface of many of the kakapo extracts after the final spin in the eppendorf tube. This fat-like film was not present in any of the quail extracts. The physical characteristics that might contribute to variation in extraction efficiency or concentration were investigated. Although testosterone concentration did not vary across any of the characteristics, extraction efficiency was lower in samples with a strong pungent odour than in samples with a light or mildly pungent odour. The odour of an individual dropping is very subjective and although only one person evaluated this, the interpretation of this result must be taken with care. The presence of a layer of fat-like film was not recorded for each extract, and this may be of use in future studies to determine the relationship between this film and any of the other characteristics evaluated. Colour was also related to extraction efficiency. Tan samples were extracted at a lower efficiency than black samples and green samples were of lower efficiency than brown, black or red samples. The presence of uric acid reduced faecal testosterone extraction efficiency. With greater numbers of samples, a clearer relationship between odour, colour or uric acid and extraction efficiency may emerge that may be useful as an index of faecal testosterone concentration.

4.4.2 Oestradiol and progesterone

No differences in faecal oestradiol or progesterone were found between the two seasons with complete data collections from July - July. The 1996-97 season was a successful breeding season during which five females produced chicks and mean oestradiol concentrations tended to rise from July to a peak in December before dropping to lower levels by March. Progesterone concentrations also tended to rise from July and although no distinct peak was attained, levels appeared elevated from November to February before dropping to lower levels by June. During the 1997-98 season, no nest building or laying occurred. Data were limited during the crucial months from October to January for the 1997-98 season, although patterns of oestradiol and progesterone similar to those of the previous season were apparent before and after the period of missing data. These results support the suggestion by Cockrem and Rounce (1995) that ovarian growth is occurring to an extent each year. This development would be controlled by initial

predictive information, with the development of a fully functioning ovary and ovulation controlled by supplementary information. The time scale of this process is unclear, although rapid yolk deposition and ovulation may occur over a period as short as two weeks. With more regular and frequent faecal sample collections during the critical period from July to March, the degree to which ovarian development is occurring each year and the role of supplementary information could be more accurately determined.

There were clear annual cycles of faecal oestradiol and progesterone. Oestradiol concentrations rose from September and there was a trend towards a peak in November/December before dropping to lowest concentrations by March. Progesterone followed a similar pattern with concentrations tending to rise from September to highest concentrations later than oestradiol in January/February, before dropping to lowest concentrations by June. The periods of elevated steroid concentrations coincided with the periods of female nesting, laying and chick rearing activity that occurred in the 1996-97 and 1998-99 breeding seasons. Patterns of oestrogen and progesterone in female birds during the breeding cycle are well established (Farner and Wingfield, 1980; Dawson, 1983). In general, both increase during the onset of the breeding season and decrease after egg laying. The roles each play in reproduction were discussed in section 1.4. Faecal oestradiol and progesterone in kakapo were elevated prior to laying and decreased once incubation had begun. Cockrem and Rounce (1995) did not identify a clear annual cycle of oestradiol, as it was likely that most of the faecal samples collected were from unidentified male kakapo. In addition, droppings were collected without information on the exact date of production and no data were available for December or January. However, concentrations were high in November and decreased by March, which is consistent with the annual cycle of oestradiol identified in this study. Cockrem and Rounce reported mean values of faecal oestradiol ranging from <10 ng/g to 23.3 ± 8.7 ng/g, which are similar to those measured in the present study. Faecal progesterone concentrations in female Japanese quail (Bishop and Hall, 1991; Hawke, chapter three) are similar to those measured in kakapo in this study. However, faecal oestrogen concentrations were approximately ten-fold lower in comparison to those measured in chickens (Cockrem and Rounce, 1994) and quail (Bishop and Hall, 1991; Hawke, chapter three). Maximum concentrations of faecal oestradiol measured in the brown dipper (Kofuji *et al*, 1993) were similar to the minimum concentrations detected in kakapo.

There was a possibility that phytoestrogens present in supplementary food or natural food sources would affect oestrogen concentrations detected in faecal samples. Levels of oestrogenic activity in both supplementary food and in a variety of phytochemicals that are likely to occur in the natural diet of kakapo were measured by Fidler *et al.*, (2000). No oestrogenic activity was detected in adult supplementary food and only very weak oestrogenic activity was detected in two phytochemicals, podocarpic acid and its reduced derivative, podocarpinol. It is unlikely that phytoestrogens contributed to the measured faecal oestradiol concentrations in the present study.

There was considerable variation in oestradiol concentration in individual droppings. The mean coefficient of variation for female kakapo was 26.1 ± 2.2%, whereas in quail the equivalent value was 21.6 ± 2.2% (see section 2.3.8). This is in contrast to the increased level of variation found in testosterone concentrations in droppings from kakapo compared with quail, indicating that oestradiol concentrations may be less responsive than testosterone to variation in diet, time from defecation to sample collection and environmental conditions.

The variation in progesterone concentrations between individual droppings was greater than that found in oestradiol and testosterone. The mean coefficient of variation for female kakapo was 47.03 ± 7.7%. This may be due to lower and more variable extraction efficiency for progesterone than the other two hormones. The coefficient of variation in progesterone concentrations in individual samples for female quail was substantially lower, 27.0 ± 4.8% (see section 2.3.11). This is probably due to variation in extraction efficiency of progesterone in droppings, diet, time from defecation to sample collection and environmental conditions.

Measurements taken from individual droppings are accurate enough to detect differences in hormone concentration that are greater than 8.8 ng/g for oestradiol and 272.9 ng/g for progesterone. However, collections of multiple pooled droppings from a roost site are capable of detecting a difference of 2.4 and 127.9 ng/g and for oestradiol and progesterone respectively. Therefore, pooled samples are likely to give more accurate information than individual samples when determining small changes in hormone concentrations.

Insufficient droppings were collected to give clear oestradiol and progesterone profiles for individual females. However, the profiles for Sandra indicate a peak in faecal oestradiol and progesterone over the breeding season of 1996-97, during which Sandra produced two eggs that did not hatch. That patterns were even apparent in individual birds shows the potential of faecal steroid analysis on droppings collected from free-living birds. The profile for Sandra indicates elevated faecal progesterone concentrations in January 1998, during which no breeding occurred. Profiles from Sandra suggest an increase in progesterone over the summer months in two successive years, supporting the idea that there is a degree of ovarian development each year. The profile from Nora shows one elevated progesterone value in January 1998, which may indicate a peak. Nora did not breed at all during the course of the study. A peak in faecal oestradiol was indicated in Suzanne's profile for the 1996-97 season, which was the maximum oestradiol concentration measured in any individual. There was evidence of a possible peak in progesterone the following season, 1997-98. Suzanne did not breed in either season. Profiles from Alice showed a peak in oestradiol in January of 1996-97 and a possible peak in progesterone in April 1997. Alice produced three eggs during that season. No further droppings were collected, so comparisons between breeding and non-breeding years were not possible. Within a season, all birds on an island receive the same initial predictive information and similar supplementary information. Therefore, individual variation in the response to this supplementary information may be influencing the successful breeding of one bird compared to another.

An investigation into faecal steroid excretion in the common marmoset (*Callithrix jacchus*) identified a clear diurnal variation in both cortisol and progesterone excretion (Sousa and Ziegler, 1998). It was demonstrated that to avoid false results when investigating ovarian cyclicity, faecal samples had to be collected at a set time each day. Diurnal variations in plasma steroid hormones have been identified in Japanese quail (Doi *et al.*, 1980; Gulati *et al.*, 1981). Similar diurnal variations may also occur in kakapo, which may affect faecal steroid concentrations in individual droppings. Collecting individual droppings at a set time each day from kakapo in the wild is impractical. Therefore, the collection of multiple pooled droppings from a roost site would reduce the possible effect of diurnal fluctuations in steroid concentrations. In the previous chapter, all droppings for 24 hours were collected from quail, so in addition to steroid concentrations, total faecal hormone production over 24 hours could be

calculated, thereby avoiding problems that diurnal fluctuations can create. Collecting all droppings over 24 hours is impossible from free-living kakapo, so pooled droppings from a roost site are the best alternative. Ratios of steroid to creatinine concentrations are commonly used with urinary steroid analysis in mammalian species to correct for variations in dilution and sample size, as creatinine is excreted at a relatively constant rate over 24 hours (Erb *et al.*, 1970; Metcalf, 1976, Peter *et al.*, 1996). However, birds excrete very low quantities of endogenous creatinine so this method is unsuitable.

As with testosterone, extraction efficiencies of faecal oestradiol and progesterone were lower and more variable in kakapo than in quail faecal samples. The physical characteristics that might contribute to variation in extraction efficiency or concentration were investigated. Oestradiol or progesterone concentration did not vary across any of the characteristics, although progesterone extraction efficiency was higher in samples without traces of vegetation than in samples with vegetation. Vegetation also affected oestradiol extraction efficiency. With a greater number of samples, a clearer relationship between extraction efficiency and physical characteristics might emerge that could be useful as an index of faecal hormone concentration.

In the previous chapter a canonical discriminant analysis was performed on data from quail to measure the prediction accuracy of reproductive status from faecal steroid hormone concentrations. With more frequent and regular faecal collections from kakapo, a similar model could be applied to data from both male and female birds at specific times for predicting reproductive status. Currently, the location of each female is determined on a daily basis, and nesting is assumed once a female has been in the same location for three to four days. Therefore, reproductive status in females is not generally determined until a few days before laying. There is the potential for reproductive status to be evaluated from steroid concentrations substantially earlier than from field observations alone.

4.5 Conclusion

The main aims of this study in the kakapo were to:

- Determine the annual pattern of steroid hormone concentrations.
- Verify the annual cycle of testosterone reported by Cockrem and Rounce (1995).
- Determine the feasibility of collecting droppings identified to individual birds.
- Compare reproductive hormone cycles between successive years.
- Compare reproductive hormone profiles between reproductively active and inactive birds.
- Determine the reliability of individual faecal samples for estimating steroid concentrations.
- Evaluate the potential of faecal steroid analysis as a diagnostic tool for kakapo.

Annual cycles of faecal testosterone, oestradiol and progesterone were found which coincided with stages of the breeding season and breeding behaviour. No differences in the pattern of hormone secretion were found for oestradiol, progesterone or testosterone between a breeding and a non-breeding year. This supports the suggestion by Cockrem and Rounce (1995) that gonadal growth is occurring to an extent each year. Although there was insufficient sampling to give clear hormone profiles for individual birds, profiles from several of the birds suggest an increase in steroid concentrations over the summer months, supporting the idea that there is a degree of gonadal development each year. Differences within birds between seasons highlight the probable importance that

supplementary information has on the onset of breeding activity. Differences between birds within a season highlight the importance individual variation in the response to the supplementary information may have on the onset of breeding activity.

For all three steroids individual droppings appeared to be accurate enough to detect large differences in hormone concentrations, such as those in the height of the breeding season compared to those during non-breeding months. However, collections of multiple pooled droppings were capable of detecting smaller differences and are recommended, especially when determining profiles for individual birds for the prediction of reproductive status.

Extraction efficiency of all three faecal steroids was low and varied in comparison to extraction efficiencies using the same extraction method with quail faecal samples in the previous study. Several physical characteristics such as odour, colour, the amount of vegetation and the presence of uric acid appeared to effect extraction efficiency. A more detailed analysis on a greater number of samples needs to be completed before these effects can be evaluated in detail.

This is the second study in which seasonal changes in faecal reproductive steroid concentrations have been reported for a free-living nocturnal bird species. The first study was that by Cockrem and Rounce (1995), also in kakapo. Several other studies have reported seasonal faecal hormone changes for the purpose of estimating the breeding cycle or changes in gonadal activity in a variety of avian species (bald eagles, Bercovitz *et al.*, 1982; brown dipper, Kofuji et al., 1993; greylag geese, Hirschenhauser et al., 1999a; rock ptarmigan, Kikuchi et al., 1996). However, only the latter three have been conducted on free-living species and individual hormone profiles were only determined in brown dippers.

With more frequent data collection during the onset of the breeding season, especially from November to February, a better understanding of the extent of gonadal development in individual kakapo may be gained. Additional sampling may also provide the data required for the prediction of reproductive status in individual birds. Given the difficulties with collecting faecal samples from a wild nocturnal bird in a

dense bush habitat, that hormone cycles were able to be identified and inferences made about gonadal activity shows the power and value of faecal steroid analysis as a diagnostic tool.

Ros Cole with Sirocco, photo: E.J. Hawke.

5 General discussion

The kakapo is an endangered parrot endemic to New Zealand. Although kakapo reproductive behaviour has been documented, little is known of its reproductive physiology. Breeding attempts are infrequent and usually occur at intervals of three to four years, although breeding in successive years occurred on Little Barrier Island in 1990 and 1991. There is evidence to suggest that kakapo undergo a degree of gonadal development each year, although the extent of this development is unclear (Cockrem and Rounce, 1995). In addition, the role that environmental supplementary information plays in stimulating the final phases of gonadal growth is unclear. Steroidogenesis occurs in the ovaries and testes of reproductively active birds and clear relationships between reproductive steroids and gonadal status have been demonstrated in several species, including Japanese quail (Follett and Robinson, 1980; Wingfield *et al.*, 1987). Therefore, the measurement of reproductive steroids may give valuable information on the reproductive status of individual kakapo.

Conventionally endocrine data has been obtained through analysis of blood samples. However, due to handling and capture stress, non-invasive methods by which samples are collected have been developed. Steroids are easily measured in waste products as they are deactivated in the liver and excreted as metabolites conjugated to sulphates or glucuronides, or as free steroids. However, the use of faecal steroid analysis assumes a positive relationship between plasma and faecal steroid concentrations. This relationship has only been demonstrated successfully in two avian species (Japanese quail, Bishop and Hall, 1991; Ishii *et al.*, 1994; domestic fowl, Cockrem and Rounce, 1994). Relationships between faecal steroid concentrations and gonad size in birds have not yet been described.

The main aims of this research were to develop and validate a faecal extraction method for measuring oestradiol, progesterone and testosterone using Japanese quail. The relationships between faecal and plasma steroid concentrations and gonadal development, including the value of individual and pooled sample collections were

examined. This extraction method was then applied to faecal samples from free-living kakapo and annual hormone cycles in relation to breeding status were described.

5.1 Conclusions

The faecal extraction method used in the laboratory at the outset of this study was modified for faecal samples from Japanese quail. Both the amount of time needed in the laboratory and extraction efficiency were taken into account when deciding on the appropriate extraction method. The following method was chosen: quail faecal samples were freeze-dried and ground to an homogenous texture in a centrifugal grinder. 0.05 g of sample was shaken with 2.5 ml of ethanol, followed by a 1.25 ml rinse, and reconstituted in 800 µl of PBSG with a 200 µl rinse the following day. Kakapo samples were prepared in a similar manner, except the volume of sample and initial two aliquots of ethanol were doubled.

No relationship was found between plasma and faecal steroid hormone concentrations in quail kept on different photoperiods in the first experiment. However, results suggested that the strain of Japanese quail used in these experiments require manipulation of both photoperiod and temperature to ensure birds with regressed gonads and low reproductive hormone concentrations are produced. Individual faecal samples appeared to be as reliable as pooled 3-hour samples for determining steroid concentration.

The results of the first experiment were used to plan a more detailed sampling protocol for quail held under conditions of controlled temperature and photoperiod. 24-hour faecal samples were collected and faecal and plasma steroid concentrations were compared. Clear relationships were found between plasma and faecal steroid hormone concentrations. However, relationships were not apparent over small ranges in plasma and faecal values. Clear relationships were also found between hormone concentrations and gonadal size, in particular for faecal concentrations. Although positive relationships were found between plasma and faecal steroid concentrations and between steroid concentrations and gonad size for 24-hour samples, stronger relationships were found when multiple samples were pooled from individual birds. Variation within a bird was

greater for plasma than faecal steroid concentrations, but variation between birds was greater for faecal steroid concentrations. Gonad size in quail was predicted with greater accuracy from faecal steroid concentrations than from plasma steroid concentrations.

This is the first study to show clear relationships between plasma and faecal testosterone, oestradiol and progesterone together in one species and to show relationships between steroid concentrations and gonad size. It is also the first study to quantify the value of collecting multiple faecal samples. Results from individual droppings and 24-hour samples show that both are as reliable as the respective pooled samples when reflecting hormone concentrations. However, in chapter three, when demonstrating relationships between hormone concentration and other variables, for example gonad size or faecal – plasma relationships, pooled 24-hour samples gave better results than a single 24-hour sample.

The collection of 24-hour samples was impossible for kakapo and even collecting multiple individual droppings from individual birds on a given day was difficult. In the majority of cases, only single droppings were collected. Annual cycles of testosterone, oestradiol and progesterone were identified in faecal samples from male and female kakapo. These cycles corresponded with events of the breeding season. Oestradiol and progesterone concentrations rose from September and tended to reach peaks in November/December and January/February respectively. These periods corresponded with the onset of the breeding season, egg laying, incubation and chick rearing. Testosterone concentrations were elevated during periods of booming and mating in male kakapo.

The comparison of hormone cycles between a breeding and a non-breeding year revealed no differences, supporting the idea that birds undergo a degree of gonadal development each year. There were too few samples to provide detailed annual profiles for individual birds, but profiles from several birds support the idea of annual gonadal development. Large differences in hormone concentration could be determined from individual droppings. Where possible, the collection of multiple pooled droppings would provide a more accurate measure of faecal hormone concentration.

Seasonal hormone cycles have been estimated by faecal steroid analysis in only three other free-living avian species (brown dipper, Kofuji *et al.*, 1993; greylag geese, Hirschenhauser *et al.*, 1999a; rock ptarmigan, Kikuchi *et al.*, 1996). Hormone concentrations were determined in individual droppings in all cases, including the previous study on kakapo by Cockrem and Rounce (1995). However, the current study is the first to address the value of collecting individual in comparison to multiple droppings.

In order to gain a clearer understanding of the extent of gonadal development that occurs each year and to be able to predict reproductive status in individual birds, more frequent sampling is needed during the onset of the breeding season.

5.2 Directions for future research

The kakapo population was 62 birds before the 2002 breeding season. In late April 2001, 11 birds were transferred from Maud Island to Whenua Hou in response to an expected abundance of rimu fruit in the forthcoming spring. There was a very successful breeding season, with 20 from 21 females of breeding age laying 26 fertile eggs. 24 eggs hatched and fledged, raising the population to 86. Breeding seasons with this level of success may only occur once every 20 years, so conservation efforts are still important for this highly endangered bird.

A more precise understanding of the level of gonadal development that occurs in an individual bird each year would be beneficial in several areas of the research objectives and goals outlined in the current kakapo recovery plan. Firstly, it would help identify factors that stimulate breeding. The timing and extent of gonadal activity in kakapo varies between seasons, so the role of supplementary information is extremely important. Details of which factors are important at specific times of the breeding cycle are central to the understanding of why kakapo breed infrequently and how their breeding frequency can be increased. Secondly, faecal steroid concentrations could be used to evaluate sexual maturity and could be used alone or combined with other variables to create a model that could predict reproductive status in individual birds earlier in the season than current methods. Accurate diagnosis of reproductive status would also

optimise the benefits of translocations of individual birds between islands for mating. Concurrent research at Massey University into the hormonal stimulation of ovulation in Japanese quail has shown promising results (Bennett, 2002), which may be applicable in the future to endangered species such as the kakapo. It may be possible that in the absence of appropriate initial predictive or supplementary information, the application of an exogenous hormonal stimulus could be used to stimulate full ovarian growth and ovulation in individual females. A detailed knowledge of the ovarian status of individual birds would be essential to ensure precise timing and dosage requirements.

Although the level of gonadal development can be assessed by faecal hormone concentrations, determination of exact relationships between faecal steroid concentrations and gonad size, as has been achieved in Japanese quail, is very difficult without visualisation of the ovary or testes. In order to further define this relationship in kakapo, it may be necessary to use ultrasound measurements on several birds to determine gonad size and relate this to faecal steroid concentrations. Although this may be difficult with current capture and handling protocols, especially during the breeding season, as long as participating birds were strategically selected, the benefit to the whole population would be immense.

Variation in hormone concentration between individual kakapo droppings from a roost site and between individual and pooled droppings may have resulted from variation in diet, weather conditions and time from defaecation to sample collection. In the field situation, although the first two cannot be controlled, the time from defaecation to sample collection can, to an extent. Trials involving droppings identified at the time of defaecation from birds during routine handling procedures that have been left out in the field and sub-sampling at intervals up to 48 hours (the maximum time in this study between defaecation and collection was 30 hours, however 48 hours may be of interest for convenience) may quantify the effect that increased time to collection has on variation in faecal steroid concentrations. A more precise estimation of the accuracy with which individual and pooled kakapo faecal samples can detect differences in hormone concentrations could be carried out with greater numbers of samples.

The current extraction method takes approximately five days to determine the steroid concentration from a raw faecal sample. This does not include transport time from the site of collection to a suitable laboratory for analysis. In a diagnostic situation, for example, determining the timing of ovulation in a bird to aid management practices, timely knowledge of an event is often crucial. Therefore the development of a quicker extraction method or a field extraction method may be useful.

In order to optimise the value of faecal sample collection in kakapo, samples should be collected at least once or twice a week, especially during the period from November to February. Where possible, multiple samples should be collected from roost sites and pooled for analysis. This would provide sufficient data to create annual profiles with greater resolution, evaluate the extent of gonadal development occurring in individual birds, allow comparisons between breeding and non-breeding years and help to understand the factors which control the onset of breeding in the kakapo.

This study has shown the value and potential of faecal steroid analysis as a diagnostic tool, which can be used to monitor reproductive status in a non-invasive manner and contribute to the survival of endangered species, such as the kakapo.

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