Plasma and Faecal Corticosterone in Chickens (*Gallus domesticus*)

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ABSTRACT

The overall aim of this thesis was to investigate stress, and plasma and faecal corticosterone secretion in chickens. It is known that social rank may be correlated with glucocorticoid levels and glucocorticoid responses to a stressor in both birds and mammals. The relationship between social rank and glucocorticoid secretion may only be evident during periods of increased environmental stress, such as that caused by social disruption. In the first experiment of the present study, the relationship between social rank index and corticosterone responses to a handling stressor was investigated before and after mixing together two unfamiliar groups of hens. Handling elicited corticosterone responses in most hens and the levels of corticosterone during a response and the magnitude of the response varied between individuals. Middle ranking hens tended to have elevated corticosterone responses to handling relative to low or high ranking birds, but overall, the results did not demonstrate a significant relationship between social rank and corticosterone levels during a corticosterone response or between social rank and the magnitude of the response. Mixing two groups of hens did not produce a sustained period of social stress so there was no concurrent increase in the number of aggressive interactions, corticosterone levels or corticosterone responses to handling after mixing. It was thought that this was because corticosterone responses and behavioural interactions were measured over days rather than hours, and the sampling protocol may have missed any changes in these variables that occurred within a few hours of mixing.

The second experiment investigated the non-invasive measurement of corticosterone in cockerel droppings and the relationship between plasma and faecal corticosterone responses to an ACTH injection. An intramuscular dose of ACTH produced a peak in faecal corticosterone levels whereas two smaller intravenous doses of ACTH, which increased plasma corticosterone levels, did not significantly alter faecal corticosterone. This showed that faecal corticosterone levels may give a non-invasive measure of plasma corticosterone in chickens although the increase in plasma corticosterone levels might need to be large and sustained to be detected in the droppings. The magnitudes of the plasma and faecal corticosterone responses to ACTH were not correlated. Problems with the method used to extract corticosterone from droppings in this study may have affected faecal corticosterone measurements and the radioimmunoassay was not validated. As a result, a different extraction method is now used in our laboratory.

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1 GENERAL INTRODUCTION

An individual faces many challenges from the environment in daily life. These challenges may be coped with through activation of specific homeostatic mechanisms, such as sweating in response to increased environmental temperature. A sufficiently threatening challenge may be coped with additionally through a generalised non-specific response. The state of the individual leading to such a response has been historically referred to as 'stress' (Selye, 1950). Hormone mediators of this generalised response can be measured in order to infer the level of stress an animal is experiencing. Corticosterone is the principal glucocorticoid hormone released by birds during such a response. Corticosterone is secreted from the cortices of the adrenal glands into the blood and can be measured in plasma, urine and faeces. The pattern of corticosterone secretion in response to a threat is often dependent on the stimulus eliciting its secretion and tends to vary between species and individuals.

The first aim of the present study was to examine the variation in corticosterone secretion between individual chickens. In particular, the relationship between the social status of each individual and the pattern of corticosterone secretion elicited by handling was investigated. The second aim was to ascertain whether corticosterone could be measured in chicken droppings. It was intended that this method of corticosterone measurement could be used to non-invasively assess responses to stress and in particular to define individual differences in corticosterone secretion.

1.1 What is stress?

The use of the term 'stress' in the literature is often confusing as it is not applied systematically to describe the same phenomenon. Definitions of stress tend to fall into one of three categories as follows:

1. Stress as an environmental stimulus, internal or external, causing a specified response, for example "an environmental effect on an individual which overtaxes its control systems and reduces its fitness or appears likely to do so" (Broom and Johnson, 1993). Examples might

include "psychological stress", "social stress" or more specifically "restraint and isolation stress" (Minton, 1994).

2. Stress as the psychological and/or physiological state of the individual producing a specified response. For example, "a state of non-specific tension in living organisms" (Selye, 1952). Alternatively the term 'distress' can be used to refer to the psychological state of the individual (Mellor and Reid, 1993) and 'stress' to refer to the physiological result.

3. Stress as the physiological or behavioural responses to environmental stimuli. For example, "the physiological resultant of demands that exceed an organism's regulatory capacities" (Bradshaw, 1996) and "the homeostatic, physiological, and behavioral responses detectable in the animal resulting from its interactions with environmental stressors" (Stephens, 1980).

In reality, there is no rigorous definition of stress and indeed there is still further discussion on what degree of perturbation is necessary for the individual to be classified as 'stressed'. For example, does exercise cause stress or does there need to be a change in the individual's likelihood of survival before it experiences stress? Moreover, there is debate about whether the word 'stress' should be maintained in scientific language at all (eg. Rushen, 1986), as the stress response is often stimulus dependent (Wingfield, 1994) and it has proved difficult to define non-specific features of the response. Attempts have been made to isolate the non-specific features of the stress response underlying the stimulus specific responses (Kopin, 1995). It seems however that too many stimuli that can still be classified as 'stressful', in that they involve significant perturbation of normal body processes, induce strictly specific responses, rather than the generalised response to stress. Conversely, some classic indicators of the generalised response to stress, such as increased glucocorticoid secretion, may be involved in non-stress-associated specific responses to certain stimuli (Rushen, 1986; Broom and Johnson, 1993; Bradshaw, 1996).

Despite this disagreement, it is most widely accepted that stress is the state of the individual occurring as a result of a potential or realised disturbance of homeostasis that probably exceeds the body's normal coping strategies and is sufficient to elicit recognised psychological, physiological and behavioural responses. This is the definition of stress used in the present study. The physiological responses may include both stimulus-specific components and non-specific components common to many stress responses. The function of these responses is to allow the organism to maintain or rapidly return to a normal internal environment. Within this

definition, environmental stress, as described in definition one above, may be used with a descriptor, as in "social stress in hens".

1.2 Stressors

The stimulus eliciting a stress response is a 'stressor'. Potential stressors include extremes in temperature (Beuving and Vonder, 1978; Levine, 1985), exercise, injury (Levine, 1985), immobilisation, crating, dehydration, starvation (Beuving and Vonder, 1978), handling and blood sampling (Lagadic *et al.*, 1990), social stress (Henry, 1993), disease, high altitude, electric shock, loud noise (Harvey *et al.*, 1984), exposure to predators and biting flies (Kavaliers and Colwell, 1996) and pain (McMeekan *et al.*, 1998).

1.3 Stress responses

When animals are exposed to a potential threat to homeostasis, the sympathetic-adrenomedullary-system (SAS) and/or the hypothalamic-pituitary-adrenal (HPA) axis are activated. These initiate a wide variety of responses in the body to counteract potentially dangerous effects of the threat. The activation of these axes and the physiological consequences constitute the 'stress response'. Traditionally, the stress response was thought of as a series of non-specific events occurring in response to a stressor that eventually lead to adaptive or pathological changes in the individual, termed the General Adaptation Syndrome (GAS) by Selye (1950). According to the GAS, an individual experiences three stages after exposure to the stressor. The first stage, the 'alarm reaction' (first classified by Cannon, 1935), is a reflex type reaction involving mainly the SAS. The individual is able to adjust to the stressor using short-term reversible changes in physiology and fight or flight behaviour. On longer exposure, the individual has committed to a fight or flight response and must adapt to the stimulus (Siegel, 1971). It does this primarily by activation of the HPA axis which allows passive submission if necessary. This is the 'stage of resistance'. If the individual is still exposed to the stressor and has not adapted, it exhibits features of the 'stage of exhaustion', usually states of disease such as gastric ulceration, or death.

It is now known that the response to stress can be more complicated than is proposed in the GAS. However the fundamental principles, in terms of activation of the SAS and HPA axis, still hold true. The HPA axis and SAS are anatomically and functionally associated and interact at a

number of levels during a stress response. However, the two systems are discrete and may react differently and independently in different situations (Henry, 1993). In general, the SAS is thought to be responsible for inducing changes to cope with serious but controllable threats, such as sudden attack by a predator, when a fight or flight response is necessary (Henry, 1993). Longer exposure to uncontrollable stressors such as a serious threat, or significant injury is more likely to involve HPA axis activation (Henry, 1993). This induces changes to deal with healing requirements and larger challenges to homeostasis and enables psychological coping responses, and "conservation-withdrawal" type behaviour (Henry, 1993) and learning (Rushen, 1986). The effects of HPA axis activation take significantly longer to manifest themselves than the effects of SAS activation. This is due in part to transport time of ACTH through the circulation and in part to the time taken for glucocorticoids to interact with cell nuclei to alter protein synthesis (O'Malley and Schrader, 1979).

The first step in HPA axis and SAS activation is the stimulation of higher centres of the brain. Stimulation occurs both reflexly to maintain basal activity, and in reaction to a stressor to elicit the stress response. It is believed that the perception of a stressor as being potentially threatening to normal functioning or survival is important in HPA axis activation (Chadwick *et al.*, 1993). By contrast, SAS activation occurs as a kind of reflex reaction to certain stressors such as exercise (Chadwick *et al.*, 1993). The perception of a stressor as 'stressful' depends largely on the individual and is due to factors such as control over the stressor (Levine, 1985; Henry, 1993), coping mechanisms, social status (Sapolsky, 1982), prior experience (Dallman, 1993) and genetics (Davison *et al.*, 1980; Manteca and Deag, 1994; Muir, 1997). Furthermore, the response to a stressor is likely to change over time as a result of habituation and facilitation of both HPA axis activity and noradrenergic responsiveness by prior stress (Dallman, 1993; Kavaliers and Colwell, 1996). This probably reduces the response to non-lethal stressors which has the adaptive function of allowing conservation of body reserves.

Sympathetic-adrenomedullary-system activation usually begins with the mass discharge of the catecholamines, dopamine, adrenaline and nor-adrenaline from neurons in most of the sympathetic nervous system. The catecholamines are released directly onto tissues supplied by the sympathetic nervous system. Chromaffin cells in the adrenal medulla are thereby stimulated to release adrenaline and nor-adrenaline into the systemic circulation. The effects of catecholamine release are similar regardless of the route taken and mainly relate to their function by allowing sudden, vigorous muscle activity. These include increased mental alertness, piloerection, increased heart rate and contractility, vaso- and venoconstriction in the periphery

and gut, vasodilatation in skeletal muscle, increased glycogenolysis in liver and muscle and increased blood glucose, contraction of the spleen, copious sweating, pupil dilation and bronchial dilation (Guyton, 1991; Minton, 1994; Kopin, 1995). Activation of the SAS may also be responsible for some of the immunosuppression previously attributed to glucocorticoid activity (Minton, 1994).

Activation of the hypothalamic-pituitary-adrenal system begins with the secretion of a releasing hormone from neurons in the hypothalamus into the primary capillary plexus of the hypophyseal portal system in the median eminence. The releasing hormone then passes into the anterior pituitary where it stimulates both the synthesis of mRNA for pro-opiomelanocortin, the precursor of adrenocorticotropic hormone (ACTH), and release of ACTH from the corticotrope cells into the systemic circulation. The release of catecholamines prior to activation of the HPA axis may potentiate the effects of CRH to enhance ACTH secretion (Harvey *et al.*, 1984).

The primary ACTH releasing hormone in most species is corticotropin-releasing-hormone (CRH). CRH is a neuropeptide that is similar in structure and function in birds and mammals (Harvey *et al.*, 1984). It is predominantly secreted by neurons in the parvocellular division of the paraventricular nucleus of the hypothalamus (Chadwick *et al.*, 1993). This region receives a varied afferent nerve supply potentially allowing, for example, visceral, somatic and special sensory input to impinge upon CRH secretion. Many different substances are thought to affect CRH gene expression including serotonin and acetylcholine, and the cytokines, interleukins 1 and 6 (Chadwick *et al.*, 1993). In turn, CRH fibres synapse with other neuropeptide-releasing neurons within the hypothalamus. CRH performs functions outside of its role as a central ACTH secretagogue. The effects depend on the area in which it acts and include the stimulation of ACTH production by leucocytes (Hendricks *et al.*, 1995), depressed food and water intake, reduced sexual receptivity and increased cardiac output and gut motility (Muller, 1987). Other peptides, including arginine-vasopressin (Minton, 1994), vasoactive-intestinal-peptide and cholecystokinin may act as ACTH secretagogues either separately or in concert with CRH and their expression may be modified by CRH (Chadwick *et al.*, 1993).

ACTH moves in the systemic circulation to the adrenal glands where it initiates the production of corticosteroids, largely glucocorticoids, from the adrenal cortices in mammals or from interrenal tissue in the avian adrenal gland (Harvey *et al.*, 1984). The main glucocorticoid secreted by mammals is cortisol and the main avian glucocorticoid is corticosterone (Siegel, 1971; Harvey *et*

al., 1984). The secretion of corticosterone (or glucocorticoids) in response to stress is termed a corticosterone response (or glucocorticoid response).

Glucocorticoids are largely transported in the blood bound to carrier proteins including albumin and transcortin (Harvey *et al.*, 1986). Fluctuations in the level of carrier protein directly affect both the level and effects of glucocorticoids in the blood, since bound hormone is not as biologically active as free hormone and is cleared less rapidly from the blood (Harvey *et al.*, 1986).

Basal levels of glucocorticoids are secreted in a pulsatile manner. They exhibit a circadian rhythm with a peak in secretion normally occurring just before waking to prepare the individual for action on rising (Cockrem, 1990; Dallman, 1993). During a stress response, glucocorticoids have three main actions, namely modification of immune function, metabolic alteration and behavioural adjustment (Wingfield, 1994; Siegel, 1995). These function primarily to augment some homeostatic mechanisms and prevent overactivity of others in order to alleviate stress and prevent further damage (Harvey *et al.*, 1984; Munck, 1984; Wingfield *et al.*, 1997). Immune function is regulated by changes such as inhibition and suppression of inflammation (McMeekan *et al.*, 1998) and an increase in the ratio of heterophils to lymphocytes (Gross and Siegel, 1983). Major metabolic changes involve carbohydrate, fat and protein metabolism. The main result is an elevation in the level of substrates in the blood, particularly glucose, needed for reaction against threats and tissue repair, through the stimulation of gluconeogenesis and through glucose sparing, fatty acid liberation and increased protein catabolism (Guyton, 1991; Wingfield *et al.*, 1997). Glucocorticoids modify behaviour to reduce immediately unnecessary activities, such as reproduction, and enhance those needed for survival, such as foraging (Wingfield, 1994).

Glucocorticoid secretion is regulated by negative feedback, operating by the inhibition of CRH release or the inhibition of ACTH release (Chadwick *et al.*, 1993). Negative feedback may be specific to certain stimuli. In this case, important physical stressors may be relatively unresponsive to glucocorticoid feedback whereas psychological stressors which involve purely cognitive stimuli and to which individuals may adapt, are susceptible to glucocorticoid feedback inhibition (Chadwick *et al.*, 1993). Glucocorticoids also cause downregulation in the number of corticosterone receptors in the brain, act on the adrenal cortex itself to reduce sensitivity to ACTH, and act on the liver to reduce the synthesis of transcortin (Harvey *et al.*, 1984). The net effect is a reduction in available circulating glucocorticoids. However, this may be stressor and species specific, since Minton (1994) reported differences between cortisol and ACTH secretory responses (indicating adrenal and pituitary habituation), depending on species, type of stressor

and frequency and duration of exposure. Finally, endogenous opioids may also help regulate HPA axis activity (Pierzchala-Koziec *et al.*, 1996).

If baseline glucocorticoid secretion or the glucocorticoid response is suppressed, the animal ultimately dies. For example, Astheimer *et al.* (1994) found a high mortality rate in birds subjected to capture and restraint when the glucocorticoid responses were suppressed by dexamethasone. Conversely, oversecretion of glucocorticoids can lead to pathological changes described by Selye (1950) as the stage of exhaustion. The three main changes are gastric ulceration, enlargement of the adrenal cortices and shrinkage of the lymphatic tissues (Siegel, 1971). Oversecretion of glucocorticoids occurs if there is a decline in feedback sensitivity, an excessive production of ACTH, or if exposure to a stressor continues for hours or days, or for short periods over a long time (as in repeated immobilisation; Klemcke, 1994).

The stress response often involves other systems in the body as an aid to cope with long term challenges, such as starvation and dehydration during migration in birds (Levine, 1985; Wingfield, 1994). A typical outcome of exposure to stress in many animals is the suppression of reproduction (Rzasa *et al.*, 1983; Harvey *et al.*, 1984; Guillette *et al.*, 1995; Wingfield *et al.*, 1997), indicating the inhibition of the hypothalamic-pituitary-gonadal axis at the level of the hypothalamus, the pituitary or the gonads (Rivier and Rivest, 1991). Other systems that may be involved in chronic stress include the oxytocinergic (Henry, 1993) and endogenous opioid systems (Pierzchala-Koziec *et al.*, 1996).

1.4 Measurement of stress

The measurement of stress, like the definition of stress, is the subject of debate. There are two approaches to the measurement of stress. The physiological or behavioural responses to a stressor can be measured or observed (for example, to see a rise in CRH or locomotor behaviour), or the actual effect of the stressor on the physiology of the individual can be measured. It is argued that this is the more real measure of stress since it is an objective measure of the degree of perturbation of the internal environment of the body (the '*milieu interieur*'), that is of interest to the observer. Conversely, when we measure an animal's response to stress, we must account for possible individual variation. Furthermore, we are not measuring the effect of the stressor *per se*, but rather the individual's reaction to and (probably)

perception of the stressor, and we are not measuring the intensity of stress but rather its mere presence (Bradshaw, 1996, Rushen, 1986).

Measures of stress can be made in animals that are stimulated by environmental stressors (where results are subject to multiple variation such as stressor specific or individual variation) or stimulated by exogenous stress hormones. These results are also subject to individual variation unless a pharmacological dose is given to override the normal variation. Either basal values, or single values over a long period of time, can be taken. Alternatively, the individual's response over time to a stressor may be followed through serial sampling. Basal levels can be misleading due to confounding factors such as circadian rhythms and stress induced by the sampling procedure. Furthermore, basal levels do not always parallel changes in response to a stressor (Beuving and Vonder, 1986; Wingfield *et al.*, 1992).

Measuring the stress response is potentially a far more informative indicator of stress since it allows assessment of basal levels, maximum levels, rate of increase and decrease, duration of elevation, and relative percentage increase in the variable of interest (Wingfield, 1994; Wingfield *et al.*, 1997). It also allows estimation of the total hormone secreted over time, by integrating the area under the stress response curve (Klemcke, 1994; McMeekan *et al.*, 1998). Deliberate exposure to environmental stressors and exogenous hormone administration are very useful tools in this method. A high dose of ACTH, for example, can allow assessment of an individual's capacity to respond to a stressor, as opposed to its individual response to an environmental stressor (Breitenbach, 1962; Davison *et al.*, 1980; Zenoble *et al.*, 1985a,b). By contrast, an individual showing a higher than normal adrenocortical response to a low dose of ACTH is considered to be in a state of stress. This method of assessment (the 'ACTH challenge') is a common tool for assessing individual responses to different husbandry conditions in welfare research (Broom and Johnson, 1993).

There are a number of physiological and behavioural variables that can be used to define or infer the amount of stress that an animal is experiencing. Many responses are species or stressor specific and as a result, there can be no single measure of stress for every situation.

1.4.1 Physiological indicators of stress

As most mediators of the stress response are carried in the blood, plasma samples yield physiological measurements that directly indicate the degree of activation of the HPA axis and the

SAS. For example, CRH and other releasing factors, ACTH and glucocorticoid levels increase during HPA axis activation and tyrosine hydroxylase (Broom and Johnson, 1993) and phenylethanolamine-N-methyl-transferase (Harvey *et al.*, 1984) are depleted during nor-adrenaline synthesis. Neurotransmitters which modulate the stress response or its effects, including glutamate, nitric oxide, serotonin and melatonin (Cook and Hodgetts, 1996) and nor-adrenaline and dopamine (Broom and Johnson, 1993), can also be measured. Many components of the effects of HPA axis and SAS activation, such as glucose, are also blood borne. While these may not provide a measure of the magnitude stress response *per se*, they may be correlated with glucocorticoid secretion and when taken together, may indicate whether or not the animal is perceiving stress, or whether normal functions have been modified in response to stress.

A potential problem with blood sampling is that it is invasive and the handling and bleeding procedure itself can cause stress (Beuving and Vonder, 1978; Lagadic *et al.*, 1990). This can be avoided by taking blood samples rapidly (generally within three minutes) or by cannulating veins (Le Maho *et al.*, 1992) or brain sinuses (Irvine and Alexander, 1987) to allow remote and repeated sampling.

The collection of urinary and faecal samples provides a useful non-invasive method for the assessment of stress. Adrenal steroids are degraded mainly in the liver, and both degraded and unaltered steroid may be further conjugated to form glucoronides and sulphates. Both unaltered and degraded steroid can then be measured in urine and faeces using a tailored extraction process followed by normal radioimmunoassay. As a result of increased deamination in the liver, the excretion of urea also increases. The pattern of excretion in urine or faeces should closely correlate with levels found in plasma and in most cases it does (Bojeson and Egense, 1960; Carlstead *et al.*, 1992; Lasley and Kirkpatrick, 1994). Unaltered cortisol has also been measured directly in milk (Verkerk *et al.*, 1996) and saliva (Fell *et al.*, 1986).

Several other physiological variables, again pertaining to the activation of the HPA axis and SAS, and the consequences of this activation, may indicate the level of stress that an animal is experiencing. Less obvious indicators include enlargement of the anterior pituitary gland in response to increased ACTH production (Stephens, 1980), enlargement of the adrenal gland by up to two or three times normal mass (Harvey *et al.*, 1984), an increase in the expression of mRNA for CRH (Chadwick *et al.*, 1993) and hormone concentration, receptor numbers and

degree of hormone-receptor binding in brain (Henry, 1993; Romero *et al.*, 1995) and adrenal gland tissue (Harvey *et al.*, 1984).

1.4.2 Behavioural indicators of stress

Changes in the type and occurrence of certain behaviours can indicate both the presence and degree of stress in an animal, to an experienced observer. However, the behaviour of an animal can be misleading in that different stressors may elicit the same response regardless of the threat posed to the animal (Cockrem and Silverin, 1998), and the behavioural response may be absent if animals have habituated to a stressor. Nevertheless, certain behaviours including vocalisations, freezing and sudden escape behaviour may indicate SAS activation, and other behaviours have been correlated with glucocorticoid secretion (Wingfield, 1996). For example, tonic immobility, an indicator of fearfulness, may correlate with corticosterone secretion (Jones *et al.*, 1988). Some behaviours may indicate stress without necessarily being indicative of SAS or HPA axis activation. For example, stereotypies such as head flicking and pacing in caged hens may indicate endogenous opioid release paralleling HPA axis activation (Broom and Johnson, 1993).

1.5 Outline of thesis

This thesis consists of four chapters; a general introduction, two experimental studies and a general discussion. The overall aim of the thesis was to investigate stress and corticosterone secretion in chickens. In particular, the first experiment (Chapter 2) considered the effect of social disruption on corticosterone secretion in laying hens, and the variation in corticosterone secretion between individual laying hens. The second and third experiments (Chapter 3) considered the measurement of corticosterone in cockerel droppings and the relationship between corticosterone levels in the droppings and in the plasma after ACTH administration. The final chapter discusses the practical implications of these findings and provides some direction for future research.

2 SOCIAL STRESS AND INDIVIDUAL VARIATION IN CORTICOSTERONE SECRETION IN CHICKENS

2.1 Abstract

Glucocorticoid levels generally differ between individual animals in the same situation. One factor that could contribute to these differences is the animal's position in a social hierarchy. Relationships have been found between social status and glucocorticoid secretion in mammals and birds, yet no studies have investigated the relationship between the corticosterone response to stress and position in the hierarchy in birds. In this experiment, two floor penned groups of laying hens were mixed together to form one group. The social hierarchy of the groups and social rank index of each hen were determined before and after mixing by group behavioural observations. Hens were subjected to a handling stressor six days before mixing and one, seven and 21 days after mixing. They were blood sampled at 0 minutes before handling, and at 15 and 40 minutes after handling, to determine their corticosterone response. All hens showed clear corticosterone responses to handling. These differed between and within individuals, but the variation could not be explained by social rank. It is likely that a relationship could have been observed during the period of social stress within the first few hours after mixing, but this period was not observed in this experiment due to potentially confounding variables. There was no relationship between body weight and social rank index or any corticosterone variable. This was probably due to the similarity in individual body weights. There was no relationship between the number of behaviours received by hens and their corticosterone response, indicating that the level of aggression was not sufficient to affect corticosterone secretion.

2.2 Introduction

Both basal and stress-induced elevations in glucocorticoid levels are subject to within-individual and between-individual variation. This variation has not been widely studied. If glucocorticoid basal levels and glucocorticoid responses to stress are to be used as reliable indicators of the state or responsiveness of the hypothalamic-pituitary-adrenal axis, it is important to know whether a single level or response is repeatable over time. It is known that basal and stress-induced corticosterone responses are easily affected by a number of factors including social conditions (Siegel and Siegel, 1971; Harvey *et al.*, 1984; Wingfield *et al.*, 1997). This study was designed to investigate whether relationships exist between position in the social hierarchy and both basal levels of corticosterone (the principal glucocorticoid secreted by birds) and the corticosterone response to stress.

For many social species, dominance hierarchies are the principal method of social organisation. An animal's position in the hierarchy determines its priority of access to limited resources such as food, water and reproduction opportunities and its roles in the society (Syme, 1974; Rohwer and Wingfield, 1981; Mench, 1991; Sapolsky, 1993; Hansen, 1994;). For example, Gibson *et al.* (1988) found that subordinate hens tended to have reduced ranges in loose-housed flocks kept in a strawyard. These hierarchies can be short or long term, and as such, the effects of position in the hierarchy will alter depending on the chronic nature of the stressor.

Many factors influence or are influenced by social position. These include the resource under competition (Syme, 1974; Sapolsky, 1982), sex (Schwabl *et al.*, 1988), age (Williams *et al.*, 1977; Schwabl *et al.*, 1988), body weight and condition (Ramenofsky, 1984; cf. Cristol, 1995), prior experience (Sapolsky, 1993), genetics (Freeman and Flack, 1980) and distance from home range. For example, Romero *et al.*, (1995) reported that rats remaining in their home cage were dominant over intruders in 69 $\pm$ 6% of interactions. Moreover, sex, age, body weight and condition, prior experience and genetics are often correlated with glucocorticoid secretion independently of their relationship with social status (see reviews by Beuving and Vonder, 1978; Harvey *et al.*, 1984; Mench, 1991; Wingfield, 1994).

Animals will be subject to different stressors and hence will have different glucocorticoid secretory profiles depending on their position in the hierarchy. Stressors may include increased activity, such

as agonistic interactions, vigilance or foraging (Ely and Henry, 1978; Lee *et al.*, 1982; Hansen, 1994), level of aggression (Fernandez *et al.*, 1994), aggressive encounters (Sapolsky, 1993), disruption of the social group (Siegel and Siegel, 1961; Williams *et al.*, 1977; Gross and Siegel, 1983; Satterlee *et al.*, 1983; Harvey *et al.*, 1984; Rogers *et al.*, 1993; Sapolsky, 1993; Romero *et al.*, 1995), injury, pain (McMeekan *et al.*, 1998), population density (Flickinger, 1961; Gibson *et al.*, 1988; Koelkebeck and Cain, 1984), decreased social contact and therefore increased parasite load (Sapolsky, 1993) and reduced access to food and water (Beuving, 1980), a physiological and psychological stressor (Mench, 1991). For example, where dominance is maintained primarily by non-physical behaviours such as displacements or threats (as in chickens; Williams *et al.*, 1977; Mench and Ottinger, 1991), subordinate animals will not be subject to high aggression and hence may not have elevated glucocorticoid levels. By contrast, middle ranking animals may interact with dominants in attempts to increase their rank (Sapolsky, 1983). They will be subject to increased aggression and hence may have elevated glucocorticoid secretion.

The relationship between glucocorticoid secretion and social position is further complicated by other factors capable of influencing or modulating glucocorticoid secretion independently of social status. These include inherent rhythmicity (circadian, circannual, seasonal and diurnal rhythms; Assenmacher and Jallegeas, 1980; Beuving, 1980; Cockrem, 1990), reproductive state (Beuving and Vonder, 1978; Guillette *et al.*, 1995), prior exposure to stress (Dallman, 1993), genetic makeup (Davison *et al.*, 1980; Muir, 1997), novelty (Levine, 1985; Romero *et al.*, 1995) and environmental conditions (Wingfield, 1994). For example, in situations where food is a limited resource and access is accorded by rank, distribution of food may increase or decrease rank related stress, perhaps based on increased or decreased aggression, or changes in the perception of food availability or controllability. Schwabl *et al.* (1988) reported that middle ranked white-throated sparrows, *Zonotrichia albicollis*, had increased corticosterone levels when food was dispersed throughout their environment. Furthermore, the negative correlation between rank and corticosterone in wintering Harris' sparrows, *Zonotrichia querula*, changed to positive when food became scarce (Rohwer and Wingfield, 1981).

Any relationship between social rank and glucocorticoid secretion may only be exhibited, or may change, when individuals are exposed to stressors or social competition situations (Mench, 1991; Mench and Ottinger, 1991). For example, Sapolsky (1983) suggests that a general correlation

between low basal cortisol, increased responsiveness to stressors and high rank, at least in primates, is shown when a society is stable. The converse is true in situations of social instability. This was supported by findings that dominant male Olive baboons, *Papio anubis*, showed a change from low basal cortisol levels and high responsiveness, to high basal cortisol levels and reduced responsiveness to stressors during a period of social instability (Sapolsky, 1983). Moreover, Ely and Henry (1978) reported that adrenocortical responsiveness was similar in dominant and subordinate mice during hierarchy formation, but that subordinate mice showed higher corticosterone levels in response to a stressor once the hierarchy was established. By contrast, Mench (1991) suggested that a lack of any relationship between social rank and glucocorticoid secretion may be due to individuals adapting to their position in a stable hierarchy. Alternatively, the relationship between glucocorticoid secretion and social position might not be linear (Gross and Siegel, 1983).

The pattern of corticosterone secretion seen in relation to rank in birds may also depend on the method of assessment. In staged dyadic encounters, subordinate birds may not be able to escape aggressive attacks and hence may have increased corticosterone levels. For example, subordinate male House finches, *Carpodacus mexicanus*, had increased corticosterone levels during dyadic encounters yet there was no relationship between corticosterone and rank outside of the staged competitions (Belthoff *et al.*, 1994). Ramenofsky (1984) reported that aggressive or subordinate behaviour in male Japanese quail, *Coturnix coturnix japonica*, that had not previously encountered each other, was not correlated with corticosterone levels during staged dyadic competitions.

Finally, the method of measurement or assessment of corticosteroid secretion may affect the relationship between social rank and corticosterone. Handling is known to elicit an increase in circulating glucocorticoid levels within a few minutes (Beuving and Vonder, 1978; Wingfield *et al.*, 1982; le Maho *et al.*, 1992), hence, levels may be very high if blood sampling takes longer than this. Furthermore, baseline levels do not indicate the responsiveness of the hypothalamic-pituitary-adrenal axis. The response to a handling stressor is commonly used to examine the responsiveness of this axis in wild animals as it allows assessment of basal levels, maximal levels, the rate of increase and decline, the relative percentage increase in glucocorticoid levels and duration of the elevation (Sapolsky, 1982; Wingfield, 1994).

In mammals, relationships have been found between cortisol secretion and low, middle and high social status. Studies on primates found low basal levels of cortisol and increased adrenocortical

responsiveness in dominant squirrel monkeys, *Saimiri scuireus* (Manogue *et al.*, 1975), and rhesus monkeys, *Macaca mulatta* (Golub *et al.*, 1979), as compared to subordinates. Sapolsky (1982, 1983) reported that dominant male Olive baboons had lower basal cortisol levels yet a more rapid response to stressors with cortisol increasing to similar levels seen in subordinates by 60 minutes after immobilisation. Conversely, Coe *et al.* (1979) reported that high-ranking squirrel monkeys had reduced responsiveness to stressors and higher basal cortisol levels than subordinate animals. Dominant mice have also shown lower basal corticosterone levels, decreased adrenocortical responsiveness to stressors and relatively smaller adrenal weights than subordinates (Davis and Christian, 1957; Louch and Higginbotham, 1967; Ely and Henry, 1978). Fernandez *et al.* (1994) showed that subordinate pigs tended to have higher basal cortisol and greater levels in response to a stressor while dominant pigs tended to have a more rapid response to a lower peak than did subordinates. Romero *et al.* (1995) reported that rats remaining in their home cages were dominant over intruders and that the intruders had higher circulating corticosterone levels than home rats.

In birds, plasma corticosterone has also been correlated with low, middle and high social status. The relationship between adrenocortical responsiveness and social rank has not been well elucidated. Low social status has been correlated with increased adrenal gland weight (indicating increased corticosterone secretion) in wood pigeons, *Columba palumba* (Murton *et al.*, 1971) and chickens (Flickinger, 1961). Conversely, Siegel and Siegel (1961) found no correlation between adrenal weight and social status in male chickens. Increased corticosterone levels in subordinate birds have been found in white-throated sparrows (Schwabl *et al.*, 1988) and wintering Harris' sparrows (Rohwer and Wingfield, 1981). In both sparrows, the relationship was dependent on certain conditions, with Harris' sparrows showing a negative correlation when food was readily available and the opposite when food was scarce. Similarly, middle ranked white-throated sparrows had elevated corticosterone levels when food was dispersed throughout their environment. Schwabl *et al.* (1988) also reported a more rapid increase in corticosterone levels in response to capture and handling in juvenile and low ranking female white-throated sparrows.

Marsteller *et al.* (1980) reported a slight negative relationship between basal corticosterone levels and social status in juvenile laying hens. Others have reported no relationship in male chickens (Williams *et al.*, 1977; Cunningham *et al.*, 1987), white laying hens (Mench and Ottinger, 1991) and dark-eyed juncos, *Junco hyemalis* (Cristol, 1995). Ramenofsky (1984) found no relationship between

aggressive or submissive behaviour and corticosterone levels in male Japanese quail, despite the finding that the direction of dominance within each dyad did not change once established in the initial encounter, indicating that a stable hierarchy had been established. However, the quail in this experiment were not housed together before the experiment, and this may have prevented any permanent dominance relationships from becoming established within the group context, or at least prevented the relationship from influencing chronic corticosterone secretion.

The primary aim of this experiment was to investigate within-individual and between-individual variation in basal and stress-induced rises in corticosterone levels in laying hens. In particular, the experiment was designed to address the following questions with these predictions:

1. Are basal corticosterone levels or corticosterone responses to handling related to social status? It was predicted that both basal corticosterone levels and the magnitude of the corticosterone response to handling would be greater in middle ranking birds.

2. Do the corticosterone response to handling and basal corticosterone levels change during the establishment of a new social hierarchy? It was expected that basal corticosterone levels would increase when the two groups formed a single new hierarchy and that the corticosterone response to handling might also increase in magnitude.

3. Does the number of aggressive interactions per minute change during the establishment of a new social hierarchy? It was predicted that the number of aggressive interactions would increase as a new hierarchy is formed.

4. Does body weight change during the establishment of a new social hierarchy and is body weight related to the corticosterone response to handling stress or social rank? It was predicted that body weight would remain stable and would not be correlated with social rank. It was also predicted that there might be a positive relationship between body weight and both basal corticosterone levels and the magnitude of the corticosterone response.

This experiment was also used to gain experience with radioimmunoassays and blood sampling techniques that would be needed in later experiments. In addition, it was used to assess whether the handling protocol would reliably elicit a corticosterone response in laying hens so that it could be used as a stressor in future experiments.

Chickens were chosen for this experiment as it is well established that groups of chickens organise themselves according to dominance hierarchies or 'peck orders' (Guhl, 1958; Chase, 1982). Furthermore, they are easily obtained and housed and so provide a useful model for other species. A brown laying strain was used, as brown strains are generally known to show more inter-bird aggression and less fearfulness of humans than white strains (D. Thomas, pers. comm.) Hens were used as it was thought group housing of brown males would induce excessive aggression and cause undue distress to the animals.

2.3 Materials and methods

Two groups of laying hens were mixed together so as to examine the effect of a social stressor on the corticosterone response. The corticosterone response to handling was measured before mixing and three times after mixing. All experiments were undertaken following approval from the Massey University Animal Ethics Committee.

2.3.1 Animals and housing

Ten medium hybrid laying hens (Brown Hyline), *Gallus domesticus*, were obtained from Massey University Poultry Research Centre at 51 weeks of age. The birds had previously been housed in pairs in battery cages. They were removed from the cages, legbanded with one coloured plastic band on each leg, weighed and randomly assigned to one of two groups of five hens.

The two groups (groups one and two) were maintained in pens under identical conditions in two separate rooms. Each room contained a round pen of 110cm diameter with one bell drinker and one tube feeder, giving 1429cm^2/hen (the recommended commercial stocking density for loose housed hens) with free access to food and water. The birds were fed standard layer mash (Massey University Feed Supply Unit) and kept under a lighting regime of 18:6 hours L:D. They remained in lay throughout the experiment.

After eight weeks from April 3 1995 to May 30 1995 (days -57 to 0; weeks -8 to -1) the two groups were mixed together (day 0). The ten birds were placed into plastic transport crates while a larger pen allowing the same area per bird (1429cm^2/hen) with two feeders and two drinkers was

constructed in one of the rooms. All birds were then placed together in the new pen and maintained under the same conditions for a further three weeks from May 30 1995 to June 21 1995 (days 0 to 21; weeks 0 to 2).

2.3.2 Behavioural observations

Behaviour of the hens in separate and mixed groups was recorded each week throughout the study (except the third before mixing (week –3) when the observer was ill). The hens were watched from the edge of their pen by the same observer for all recordings to avoid inter-observer effects. A five minute settling period was allowed after the entrance of the observer. The hens were continuously observed for 20 minutes on at least two days each week, except on week –4 when hens were only observed once. The data from this observation were combined with those from week –5 for all analyses. Observations were always performed between 0900 and 1200 hours to minimise the effects of diurnal rhythms in behaviour. The observation days were varied each week and the order of observation was alternated at each period so that neither group was observed at the same time over two consecutive periods. The mixed group was watched for an additional six 30 minute periods beyond normal observation hours over five days, beginning immediately after the two groups of five were mixed together in the new pen.

During each observation period all pecks, threats, chases, displacements and exploratory pecks (see below) between birds were recorded. For each interaction, the bird initiating the interaction and the bird receiving were recorded. The number of pecks, threats, chases and displacements between each pairwise combination of birds was calculated for each observation period. From this, the sum of occurrences of each behaviour and of all pecks, threats, chases and displacements ('total behaviours') between each pairwise combination of birds was calculated for each week.

The sum of exploratory pecks occurring in all observation periods between each pairwise combination of hens was calculated for each group before mixing and for the mixed group to determine which pairwise combinations performed exploratory pecking and the direction of these interactions.

The behaviours were classified as follows:

Peck: forceful physical contact occurred between the initiator's beak and the receiver's body surface. Aggressive pecking was usually done with a closed beak and directed at areas around the head, neck and cloaca. The receiver usually attempted to avoid or escape the peck.

Threat: the initiator held its head above that of the receiver and showed a peck intention movement toward the receiver but no physical contact occurred. The receiver usually attempted to avoid or escape the initiator in response to a threat.

Chase: the initiator advanced toward the receiver and both birds moved more than two steps with the initiator in pursuit of the receiver.

Displacement: the initiator made no intention movement but the receiver moved away from its previous position, whether sitting or lying, on arrival of the initiator.

Exploratory Peck: the initiator pecked at any part of the receiver's body and often exhibited consummative behaviour (moving the beak as if swallowing some particle it had found). There was never contact with the skin under the feathers (in areas other than the head) and the receiver did not show any avoidance. Exploratory pecking did not elicit avoidance or retaliation from the receiver.

2.3.3 Determination of the dominance hierarchy

The total number of behaviours occurring between each pairwise combination of hens in groups one and two before mixing and in the mixed group after mixing were used to determine the proportion of interactions in which each bird dominated the other. One bird was considered dominant when it initiated more than 75% of all interactions with another. The social rank index (X) was then calculated as follows (Lee et al., 1982): $X = ½ (D - S + N + 1)$

where D = the number of birds dominated by one individual, S = the number of birds dominating that individual and N = the total number of birds in each group. The social rank index was only calculated

once before and once after mixing, since the direction of dominance did not change during either period before or after mixing. Therefore, the calculated social rank index was taken as representative of the birds overall position in each hierarchy.

2.3.4 Blood sampling

Blood samples (0.5-3.0 ml) were taken from the right and left ulnar vein of each hen using a heparinised needle (23g x 3/4") and a heparinised 3 ml syringe. Samples were collected within three minutes of the bird being picked up in order to minimise the effects of handling on corticosterone levels, and between 1300 and 1600 hours each day to avoid the effects of ovulation and oviposition, and minimise the effects of circadian rhythm on corticosterone levels. Blood was expelled into a heparinised 5 ml plastic test tube. All samples were centrifuged within twenty minutes of collection and the plasma drawn off with a Hamilton glass syringe. The plasma was frozen immediately at −20°C in 1 ml plastic titre tubes and was later transferred without thawing to −70°C for long-term storage.

The corticosterone response to handling was determined in all birds on the sixth day before mixing (day -6) and on days 1, 7 and 21 after mixing. On each of these days, the birds were caught individually and bled immediately. After the first sample was collected, the bird was placed by itself in a 29 x 39 x 29 cm (w x l x h) opaque white plastic box with a lid. The birds were handled every two minutes for fifteen minutes by removing them from the box, being turned upside down and then replaced in the box. A second blood sample was collected 15 minutes after the first sample. The birds were then left in the box with no disturbance for a further 25 minutes at which time another blood sample was taken. They were then returned to their pen.

All hens were weighed at the end of each blood sampling day before being returned to their home pens.

2.3.5 Radioimmunoassay of corticosterone

Corticosterone levels were measured in extracted chicken plasma by radioimmunoassay. The method used was a modification of that described by Wingfield *et al.* (1992). 300 µl plasma samples were extracted into 2 ml of dichloromethane. The dichloromethane and plasma were then shaken together for 1h in a Chiltern Scientific SS70 orbital shaker and centrifuged at 3000 rpm for 10 minutes in an Heraeus Christ 5000S refrigerated centrifuge. A 1.5 ml aliquot of dichloromethane was removed from each extracted sample and dried at 37°C under a stream of air. Dried extracts were

reconstituted in 2 ml of phosphate-buffered saline with gelatine (PBSG; 0.1M, pH 7.0), shaken for 1 h at room temperature and then left overnight at 4°C. 100 µl aliquots were removed into plastic test tubes and assayed immediately or frozen at -20°C. The extraction efficiency, measured using a spike of tritiated corticosterone, was 71.98 ± 2.1% (n=10) for hen plasma.

All samples were assayed in triplicate and were randomly distributed throughout 3 assays. Reconstituted extracts were incubated with 100 µl of antibody (Dr. R.J. Etches, University of Guelph, Ontario, Canada; 1:18 000 final dilution) and 100 µl of tritiated corticosterone (approximately 5000 cpm; Amersham, UK) at 4°C overnight. Steroid bound to the antibody was separated from unbound steroid by addition of 500 µl of dextran-coated charcoal (2.5 g/l charcoal, 0.25 g/l dextran in PBSG) for 15 minutes at 4°C. Tubes were then centrifuged at 3000 rpm for 15 minutes at 4°C and the supernatant poured off. Separation by addition of dextran-coated charcoal was performed for each triplicate in succession. 3 ml of scintillant (5 g/l PPO, 0.3 g/l dimethyl POPOP) in toluene) were added, the samples were shaken for 1h, left for 1h at room temperature, and then counted for five minutes in a Wallac 1409-411 liquid scintillation counter.

Serial dilutions of extracted chicken plasma in assay buffer provided results which were parallel to the corticosterone standard curve (n=4). Recovery of corticosterone added to hen plasma was 96.12 ± 2.17% (n=4). The limit of sensitivity, defined as the smallest amount of steroid on the standard curve distinguishable from the method blank and expressed as corticosterone concentration in plasma, for hen plasma was 0.13 ng/ml. The intra-assay and inter-assay coefficients of variation were 3.87% (n=15) and 15.42% (n=17) respectively.

Cross-reactions of the antibody with other steroids were previously determined by Etches (1976). These are deoxycorticosterone (27.9%), cortisol (6.9%), progesterone (37.6%), 11β-hydroxyprogesterone (21.3%) and <5% for oestradiol, testosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, aldosterone and pregnenalone. Cross-reactivity with 11-dehydrocorticosterone was 2.1% (Lewis *et al.*, in press). Avian plasma has only very low levels of cortisol and deoxycorticosterone has not been reported (Etches, 1976). Progesterone is poorly extracted in dichloromethane and 11β-hydroxyprogesterone is an unstable intermediate in avian adrenal steroid biosynthesis (Wingfield *et al.*, 1992), thus corticosterone is the main steroid measured in this assay.

2.3.6 Calculation of area under the corticosterone response curve

Corticosterone levels at each sampling time and the area under the corticosterone response curve were used to quantify individual variation in corticosterone secretion. The area under the corticosterone response curve was determined by numerical integration using MathCad (MathSoft Inc., Massachusetts). Both the total area (total integrated response) and the total area minus the area attributable to basal corticosterone levels (corrected area or corrected integrated response) were calculated. Figure 2.1 shows the areas calculated by MathCad. The total integrated response is calculated from areas ***a*** plus ***b***. The corrected integrated response is equivalent to area ***a***.

2.3.7 Statistical analyses

Statistical analyses and graphing were performed using GraphPad Prism version 2.01 (1996; GraphPad Software Inc.) and Systat version 5.0 (Systat Inc., Illinois). Relationships between non-normal data were investigated using Spearman rank correlations. Relationships between normal or tied data were analysed using Pearson correlations. Sample sizes were too small to allow comparison of behavioural observations using one-way ANOVA so Kruskal-Wallis non-parametric analyses were used to investigate the change of behaviours over time. Differences in corticosterone secretion over time and between birds were investigated using a two-way repeated measures ANOVA.

2.4 Results

Some results are graphed in more one manner in order to draw attention to pertinent points.

2.4.1 Behavioural interactions

The hens were taken from pairs in cages and placed in pens on the floor in groups of five. They were observed for 20 minutes and did not move during this period. Eight hours later all hens were moving around, feeding and drinking, but were not interacting with other hens. Interactions between hens were first observed on the second (group one) or third (group two) day after group establishment. The direction of the interaction between each pair of hens in their first observed interaction was maintained thereafter, and a well-defined hierarchy was rapidly established within each group. When the two groups of hens were mixed together they began interacting within 30 minutes and formed a single clear hierarchy that day.

2.4.1.1 Aggressive interactions

There was wide variation in the number of behaviours between observations occurring each week. The number of pecks, threats, chases, displacements and total behaviours each week did not change significantly during the experiment (Figure 2.2; Table 2.1). The hens began interacting within the 30 minute observation period immediately after the two groups were placed together in a floor pen on day 0 (Figure 2.3). During the second observation six hours after mixing, the frequency of pecks and chases increased markedly. The hens were next observed 22 hours after mixing at which time the frequency of all interactions had declined and was similar to frequencies observed in the previous weeks.

2.4.1.2 Exploratory pecking

16 of the 20 possible pairwise combinations in group one (80%), 14 (70%) in group two, and 21 of 90 (23.33%) in the mixed group were observed performing exploratory pecking. Of these some pairs,

such as WB – GW, were observed exploratory pecking more often than were others. Nine of these pairwise relationships were between a bird of higher rank toward a bird of lower rank; one was between hens of the same rank and six were from a low ranking hen toward a high ranking hen. In group two, eight were from a higher ranked hen toward one of lower rank and six were from a lower ranked hen toward a higher ranked hen (there were no hens of the same rank). Of 21 observed in the mixed group, 12 were from a higher hen toward a lower hen, one between hens of the same rank and eight from a lower ranked hen toward a higher ranked hen.

2.4.2 Hierarchies

The sum of all behaviours initiated and received by each hen was used to organise the hens into dominance matrices for groups one (Table 2.2) and two (Table 2.3) before mixing and for the combined group after mixing (Table 2.4). Ten dyadic combinations of hens were possible for groups one and two. Nine pair combinations (90%) of hens in group one were observed interacting at least once over the seven weeks prior to mixing (Table 2.2) and eight pairs (80%) in group two (Table 2.3). Thirty seven (82.22%) of the 45 possible pairwise combinations were observed interacting at least once in the mixed group over the three weeks after mixing (Table 2.4).

For each observed dyad in all groups, one hen won at least 75% of the interactions and was considered to be dominant whilst the other hen was deemed subordinate. The number of hens dominated by and dominating each other hen and hence the social rank index of each hen, were calculated from these data (Table 2.5; Figure 2.4). The direction of dominance in each pairwise combination observed was established soon after group formation at both times. As the direction did not change after it was established, the social rank index was only calculated once before and once after mixing so that the maximum number of observations could be used in each calculation.

On no occasion before mixing did a subordinate hen initiate or win an interaction over a dominant hen (a dominance reversal). In the mixed group however, there were reversals on three occasions where a lower ranking hen pecked or displaced a higher ranking hen (Table 2.4). The first reversal (WW pecked GG two times) occurred during the initial encounters between hens at the time the two groups were mixed. The other two reversals occurred when hen RR was showing stereotypic pacing

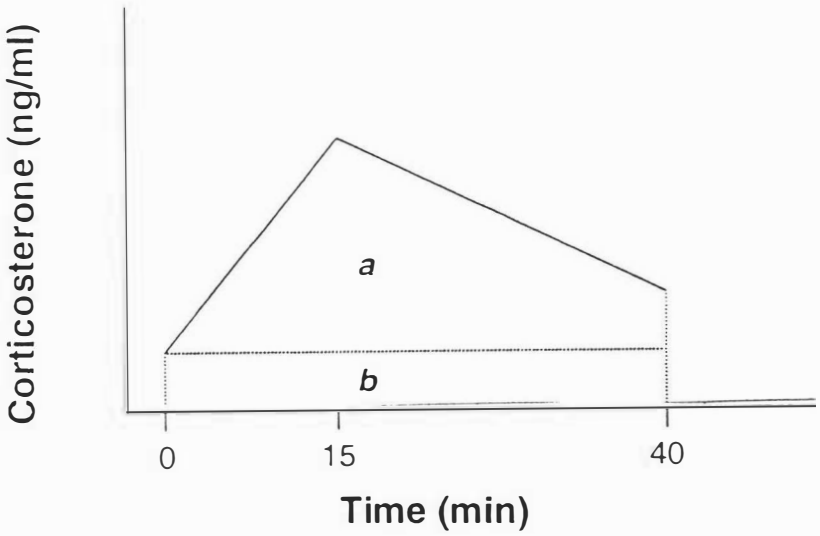
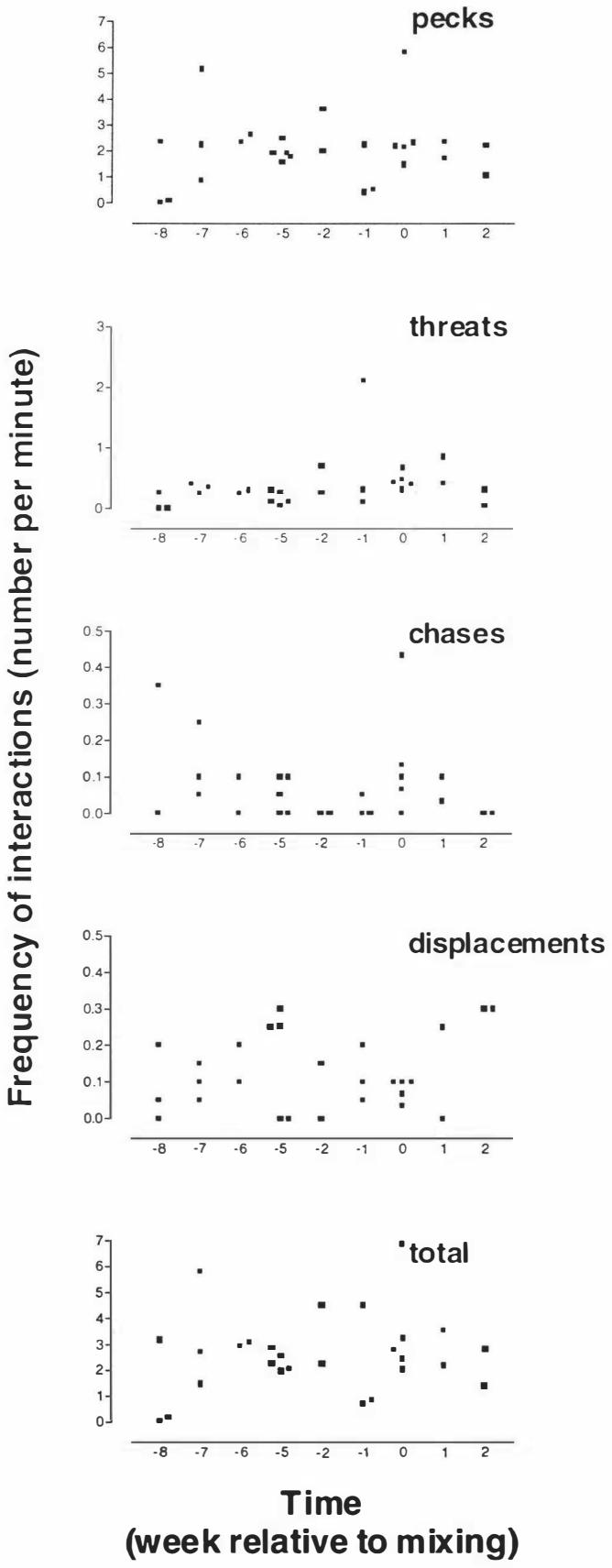


Figure 2.1: Diagram of the total and corrected areas under the corticosterone versus time curve. Total area under the curve = *a* + *b*; corrected area under the curve = *a*.

Figure 2.2: Weekly behavioural interactions between hens before and after mixing two groups of hens at week 0. Each point represents data collected on one observation day. (Note different scales on y axis.)

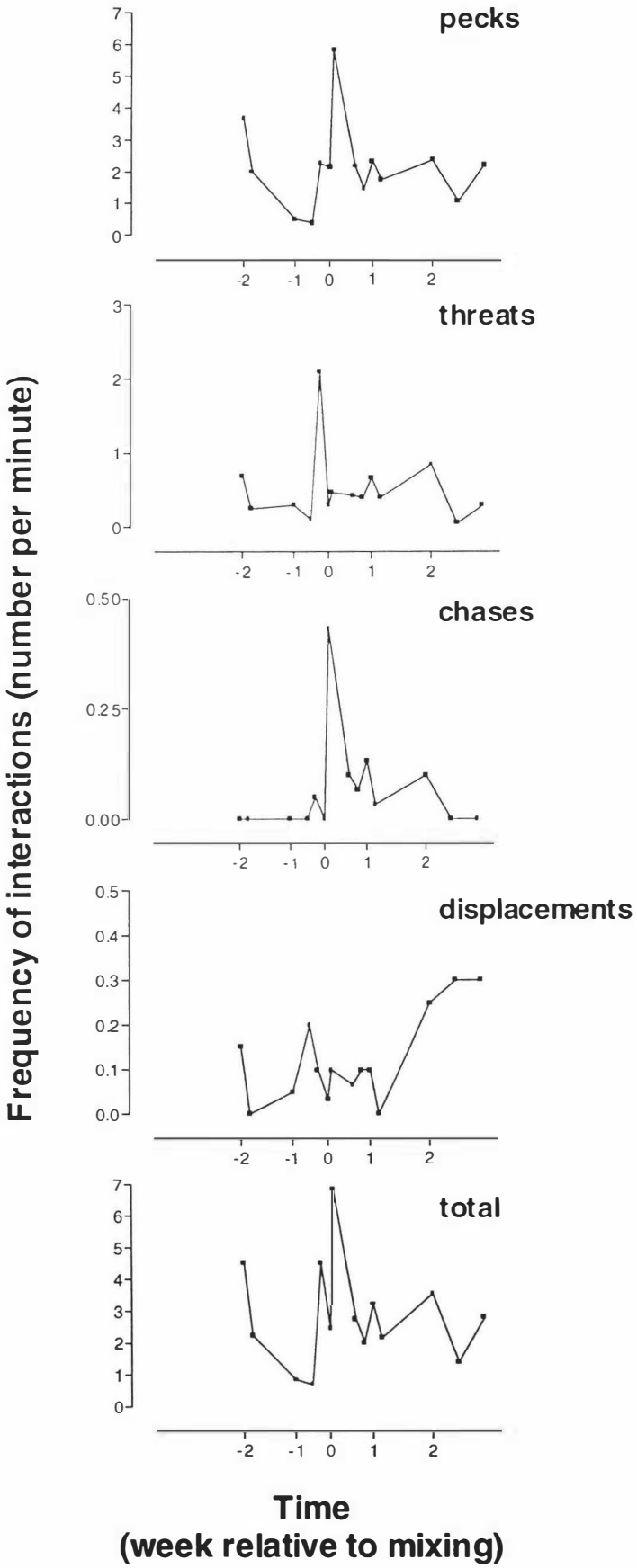

pecks
threats
chases
displacements
total
Frequency of interactions (number per minute)
-8 -7 -6 -5 -2 -1 0 1 2
Time
(week relative to mixing)

Behaviour	K^*	P
pecks	7.548	0.479
threats	15.240	0.055
chases	9.710	0.286
displacements	6.913	0.546
total	5.548	0.700

*Kruskal-Wallis one-way ANOVA comparisons of weekly total of each behaviour per minute and total behaviours per minute between all weeks.

Table 2.1: Results of Kruskal-Wallis one-way ANOVAs of the difference between weeks in the number of behavioural interactions observed between all hens (sample size varies, see Figure 2.3).

Figure 2.3: Behavioural interactions each day in the week before and the week after mixing two groups of laying hens on day 0 (the first point on week 0). Each point represents data collected in one observation session. (Note different scales on y axis).


pecks
threats
chases
displacements
total
Frequency of interactions (number per minute)
Time
(week relative to mixing)

Subordinate hen

Dominant hen	RG*	RR	BB	WW	BR
RG	-	28	150	165	87
RR	0	-	36	6	1
BB	0	0	-	0	11
WW	0	0	0	-	19
BR	0	0	0	0	-

Table 2.2: Dominance matrix for group one laying hens before mixing. Each cell represents the total number of interactions between a pair of hens. *Letters refer to coloured legbands used for individual identification. R=red, B=blue, W=white, G=green.

Subordinate hen

Dominant hen	BG*	GG	GW	WB	WR
BG	-	50	117	49	47
GG	0	-	19	4	44
GW	0	0	-	0	2
WB	0	0	0	-	0
WR	0	0	0	0	-

Table 2.3: Dominance matrix for group two laying hens before mixing. Each cell represents the total number of interactions between a pair of hens. *Letters refer to coloured legbands used for individual identification. R=red, B=blue, W=white, G=green.

Subordinate hen

Dominant hen	RG*	BG	GG	WW	RR	BB	BR	WB	GW	WR
RG	-	8	15	16	23	10	5	48	92	86
BG	0	-	3	20	28	32	29	9	14	9
GG	0	0	-	34	9	35	21	7	6	4
WW	0	0	2	-	3	2	1	10	17	50
RR	3	0	0	1	-	5	2	0	4	6
BB	0	0	0	0	0	-	0	11	10	13
BR	0	0	0	0	0	0	-	0	0	0
WB	0	0	0	0	0	0	0	-	0	0
GW	0	0	0	0	0	0	0	0	-	0
WR	0	0	0	0	0	0	0	0	0	-

Table 2.4: Dominance matrix for mixed group of laying hens. Each cell represents the total number of interactions between a pair of hens. *Letters refer to coloured legbands used for individual identification. R=red, B=blue, W=white, G=green.

and displaced others in its path as it walked through the group. Hens maintained roughly the same relative position in the hierarchy in the mixed group as in groups one and two with the main exception being in the change to a higher position of hen WW (Figure 2.5).

The total number of all behaviours received was calculated for each hen once before mixing and once after mixing. Hens with lower social rank indexes tended to receive more aggression than higher ranked hens in all groups, but this could not be tested due to the inter-relatedness of the two variables (Figure 2.6).

2.4.3 Corticosterone

There was no relationship between the time taken to collect each sample and the corticosterone level in the first blood sample (Figure 2.7; Pearson correlation; r^2= 0.067, P= 0.106) or between order of bleeding and corticosterone level in the first sample (Spearman rank correlation; r^s= -0.267, P=0.096). The handling procedure elicited a corticosterone response in most hens on all four sampling days (Figure 2.8). Plasma corticosterone levels were low when birds were first picked up (range: 0.42 – 6.04 ng/ml), rose to peak at 15 minutes later (1.12 – 19.64 ng/ml) and then fell toward basal levels at 40 minutes (0.94 – 9.03 ng/ml; Figure 2.9). There was a highly significant effect of time on plasma corticosterone levels (two-way ANOVA for repeated measures; F=95.25, P<0.001, df=2,72) but no effect of day of sampling (F=1.683, P=0.188, df=3,36). There was no interaction between these two variables (F=0.991, P=0.438, df=6,72). Corticosterone levels rose significantly between 0 and 15 minutes (F=35.548, P<0.001, df=4,36) and fell significantly from 15 to 40 minutes (F=28.354, P<0.001, df=4,36). Corticosterone levels were higher at 40 minutes than 0 minutes (F=7.507, P<0.001, df=4,36), indicating that corticosterone had not returned to basal levels 40 minutes after the birds were first picked up. Corticosterone levels at 0 minutes but not at 15 or 40 minutes were significantly higher on day –6 than on the other days (Figure 2.10; post hoc linear contrast; F=6.738, P=0.014, df=1,36).

Both total and corrected integrated corticosterone responses tended to be higher on day –6 than on other days when responses were similar (Figure 2.11). There were significant differences between days in the total integrated corticosterone response (repeated measures ANOVA, F= 3.479, P=0.030) but not corrected integrated corticosterone response (F= 2.756; P= 0.073).

Hen	Number of hens dominated	Number of hens dominating	Social rank index
group one			
RG*	4	0	5
RR	3	1	4
WW	1	2	2.5
BB	1	2	2.5
BR	0	4	1
group two			
BG	4	0	5
GG	3	1	4
GW	1	2	2.5
WB	0	2	2
WR	0	3	1.5
mixed group			
RG	9	0	10
BG	8	1	9
GG	7	2	8
WW	6	3	7
RR	4	4	5.5
BB	3	5	4.5
BR	0	5	3
WB	0	5	3
GW	0	6	2.5
WR	0	6	2.5

Table 2.5: Number of laying hens dominated by and dominating each hen and social rank index of each hen. *Letters refer to coloured legbands used for individual identification. R=red, B=blue, W=white, G=green.

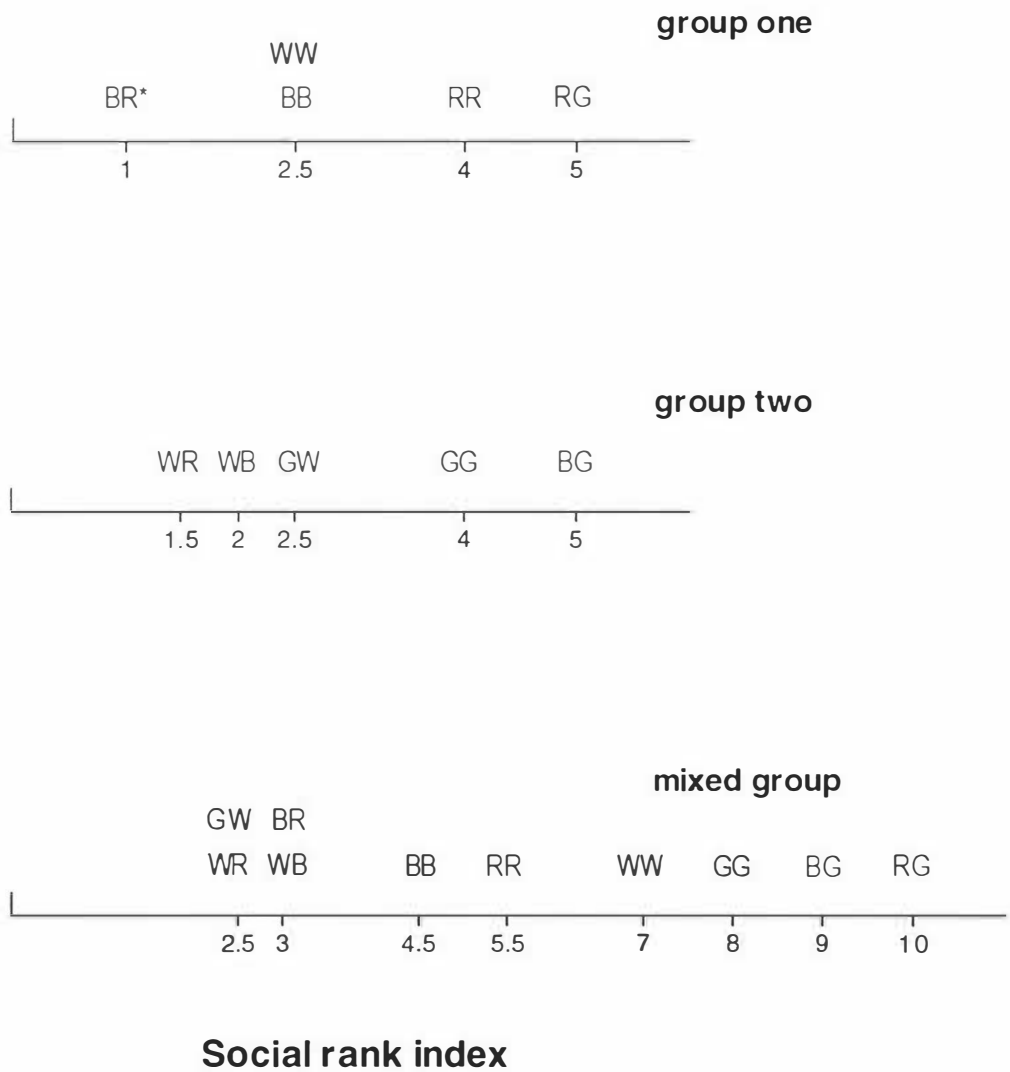


Figure 2.4: Social rank indexes of laying hens penned in two groups of five and mixed together to form one group of ten. *Letters refer to coloured legbands used for individual identification. R=red, B=blue, W=white, G=green.

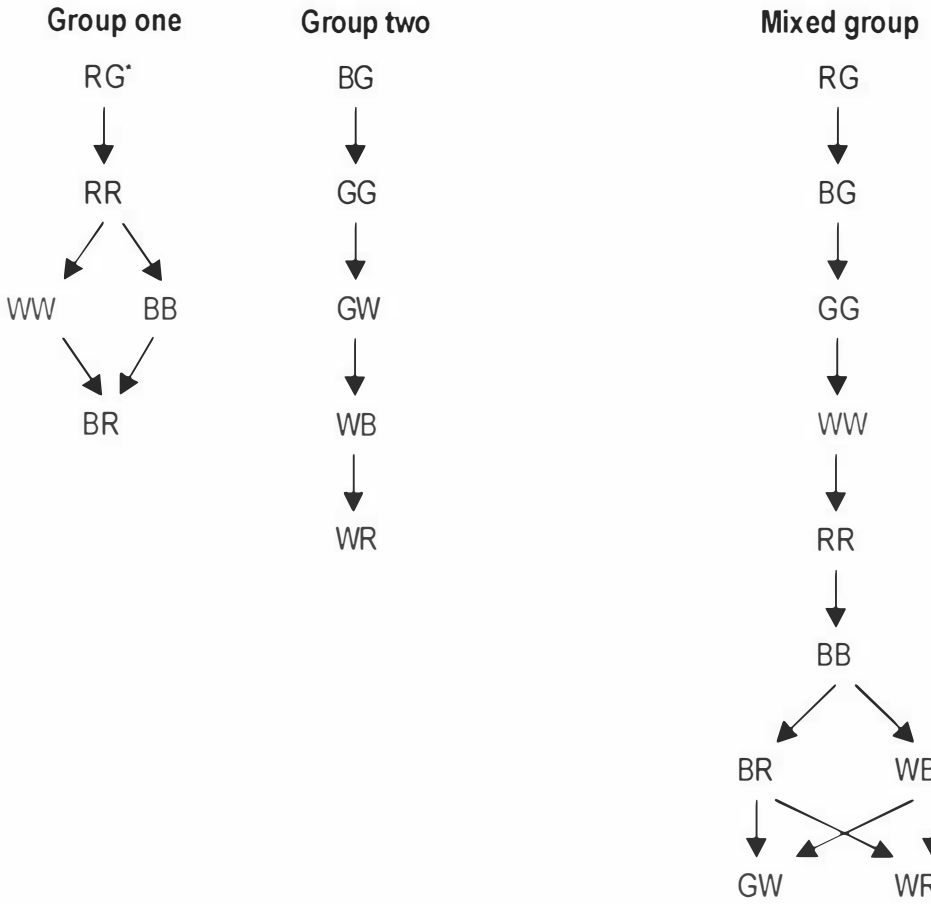


Figure 2.5: Social hierarchies derived from social rank indexes of two groups of five laying hens before mixing and one group of ten laying hens after mixing the two groups together. *Letters refer to coloured legbands used for individual identification. R=red, B=blue, W=white, G=green.

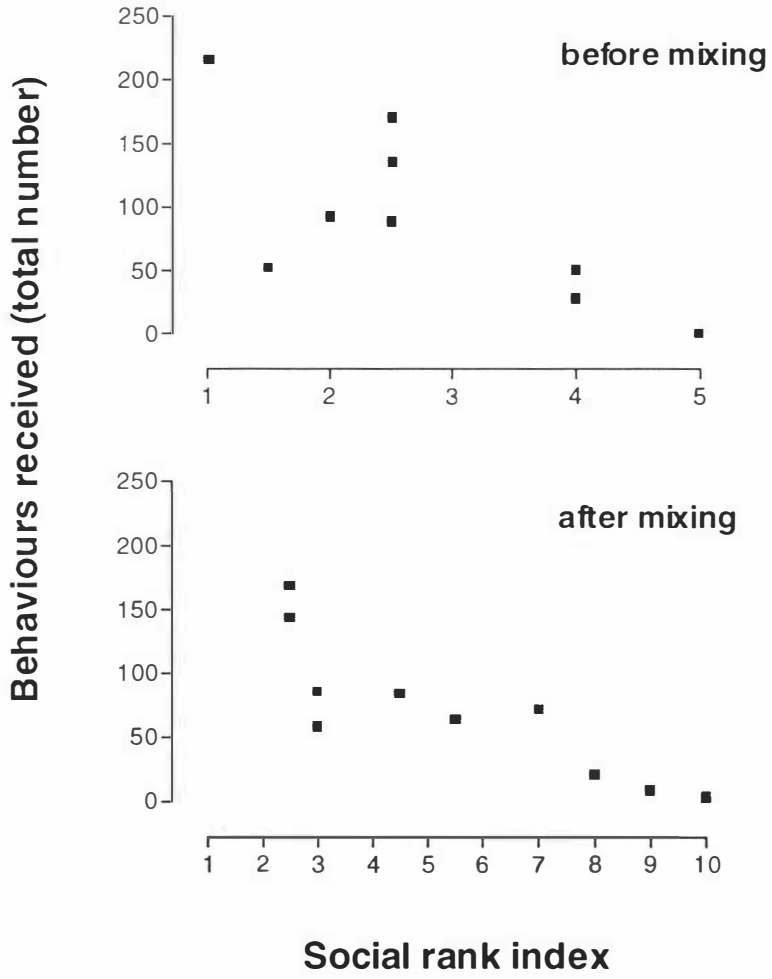


Figure 2.6: Relationship between social rank index and total number of behaviours received by each individual before and after mixing two groups of laying hens. (Note different scales on x axis).

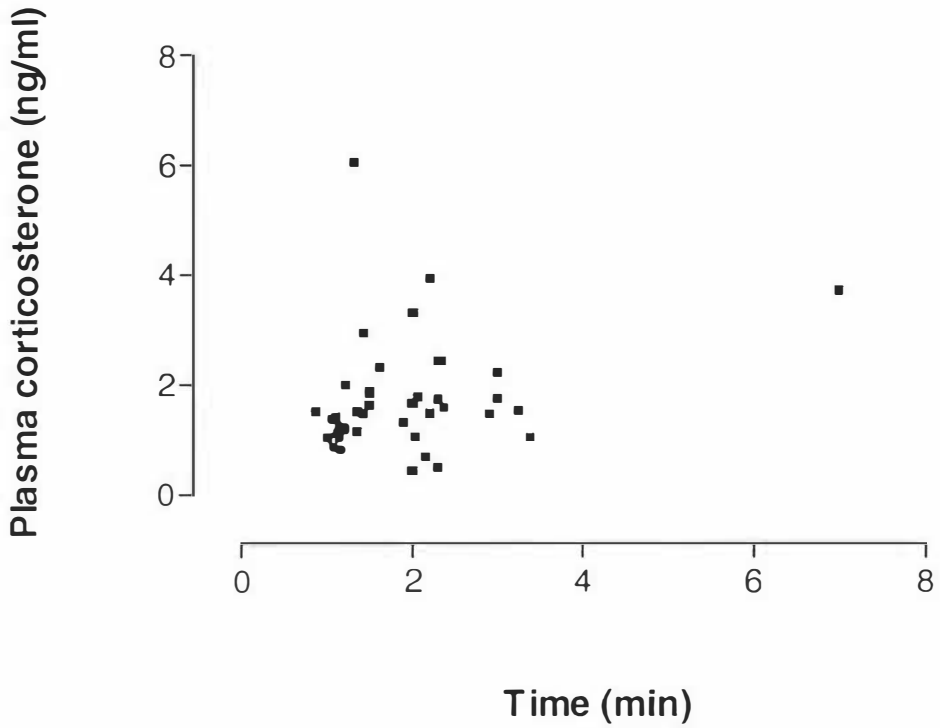


Figure 2.7: Relationship between basal plasma corticosterone levels and time elapsed from first disturbance to completion of blood sample for all hens on all days.

Figure 2.8: Corticosterone responses to handling of each hen on all days. *Letters refer to coloured legbands used for individual identification.

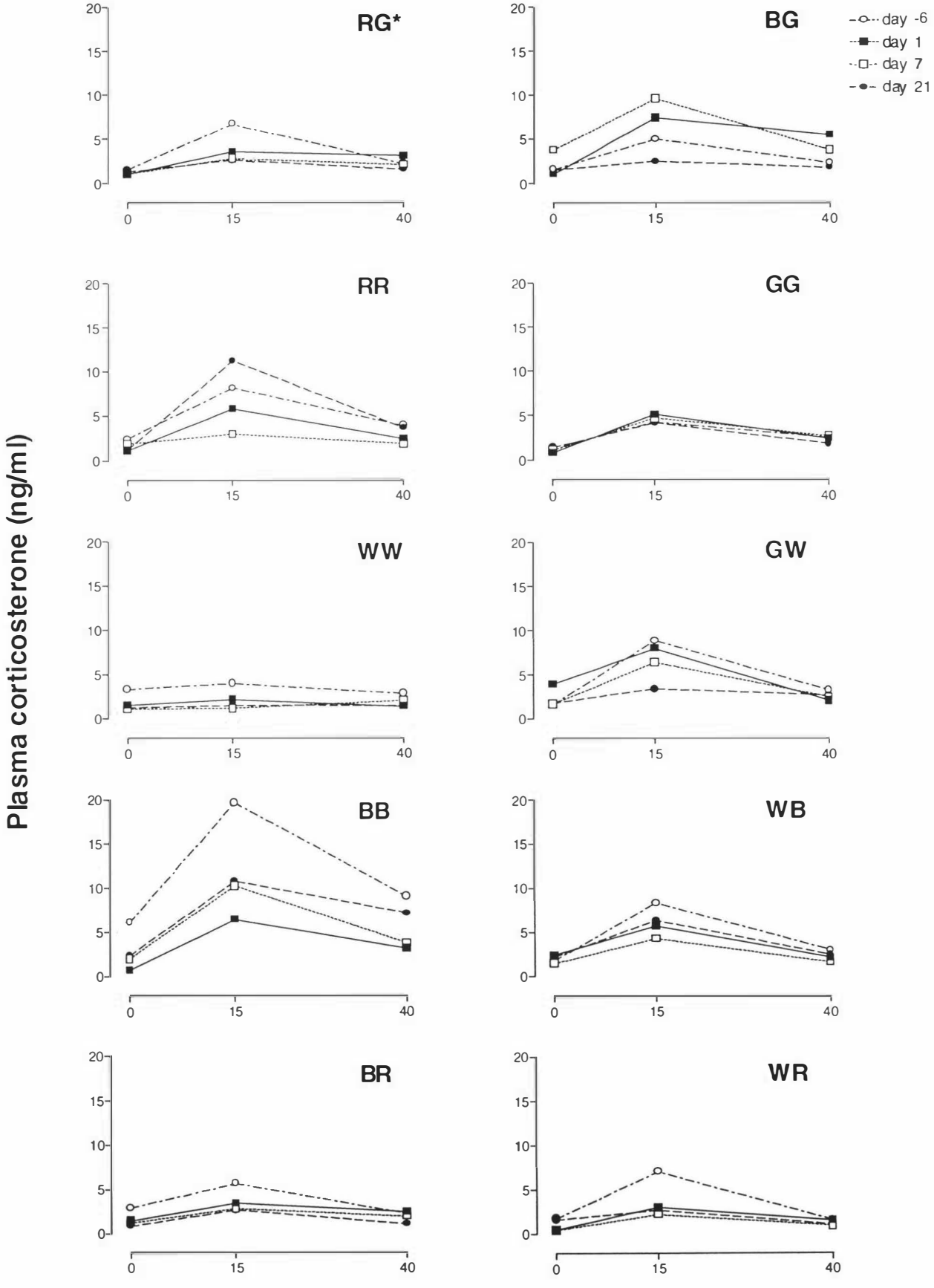

RG*
BG
RR
GG
WW
GW
BB
WB
BR
WR
day -6
day 1
day 7
day 21
Plasma corticosterone (ng/ml)
Time (min)
0
5
10
15
20
40

Table 2.6: Variation in corticosterone levels at 0, 15 and 40 minutes after handling and total and corrected integrated corticosterone responses to handling of all hens on all days. *Letters refer to legbands used for individual identification. R=red, B=blue, W=white, G=green.

Bird	0 min corticosterone				15 min corticosterone				40 min corticosterone				Total integrated response				Corrected integrated response			
	mean	SE	CV	n	mean	SE	CV	n	mean	SE	CV	n	mean	SE	CV	n	mean	SE	CV	n
RG*	1.22	0.11	18.10	4	3.86	0.96	49.55	4	2.13	0.32	30.07	4	112.97	20.63	36.53	4	64.11	18.04	56.28	4
BG	1.94	0.60	61.96	4	6.06	1.54	50.66	4	3.22	0.83	51.47	4	176.35	41.48	47.04	4	98.72	33.72	68.32	4
GG	1.17	0.13	22.61	4	3.80	0.83	43.81	4	2.03	0.28	27.36	4	127.46	5.10	8.00	4	80.49	10.13	25.17	4
WW	1.77	0.52	58.79	4	2.14	0.63	59.03	4	1.85	0.33	35.69	4	79.18	19.84	50.12	4	9.66	2.28	47.21	2
RR	1.65	0.31	36.98	4	6.99	1.76	50.29	4	2.88	0.47	32.89	4	188.36	40.11	42.59	4	122.37	42.91	70.14	4
BB	2.77	1.15	83.08	4	11.77	2.80	47.60	4	5.76	1.40	48.54	4	328.05	80.47	49.06	4	217.46	35.05	32.24	4
BR	1.62	0.46	56.98	4	3.62	0.67	37.15	4	1.88	0.31	32.59	4	108.03	19.35	35.83	4	43.37	3.42	15.77	3
WB	1.97	0.22	22.24	4	6.14	0.84	27.39	4	2.27	0.29	25.42	4	165.91	20.78	25.04	4	95.71	22.57	47.15	3
GW	2.23	0.57	51.19	4	6.62	1.20	36.29	4	2.55	0.25	19.53	4	181.01	25.94	28.67	4	103.81	30.55	58.87	3
WR	1.07	0.36	67.16	4	3.72	1.11	59.91	4	1.26	0.15	24.15	4	97.12	26.03	53.60	4	52.40	7.47	28.50	2
All birds																				
mean	1.74				5.47				2.58				156.44				96.53			
SE	0.17				0.56				0.25				14.72				12.37			
CV	60.55				65.28				61.11				59.52				73.61			
n	40				40				40				40				33			
Average variation between birds (mean, SE and CV of individual bird means)																				
mean	1.74				5.47				2.58				156.44				88.81			
SE	0.17				0.86				0.39				22.64				17.76			
CV	30.03				49.87				48.34				45.76				63.23			
n	10				10				10				10				10			
Average variation within birds (mean of individual bird CVs)																				
mean	47.91				46.17				32.77				37.65				44.96			
SE	6.93				3.24				3.24				4.44				5.95			
CV	45.75				22.16				31.30				37.31				41.85			
n	10				10				10				10				10			

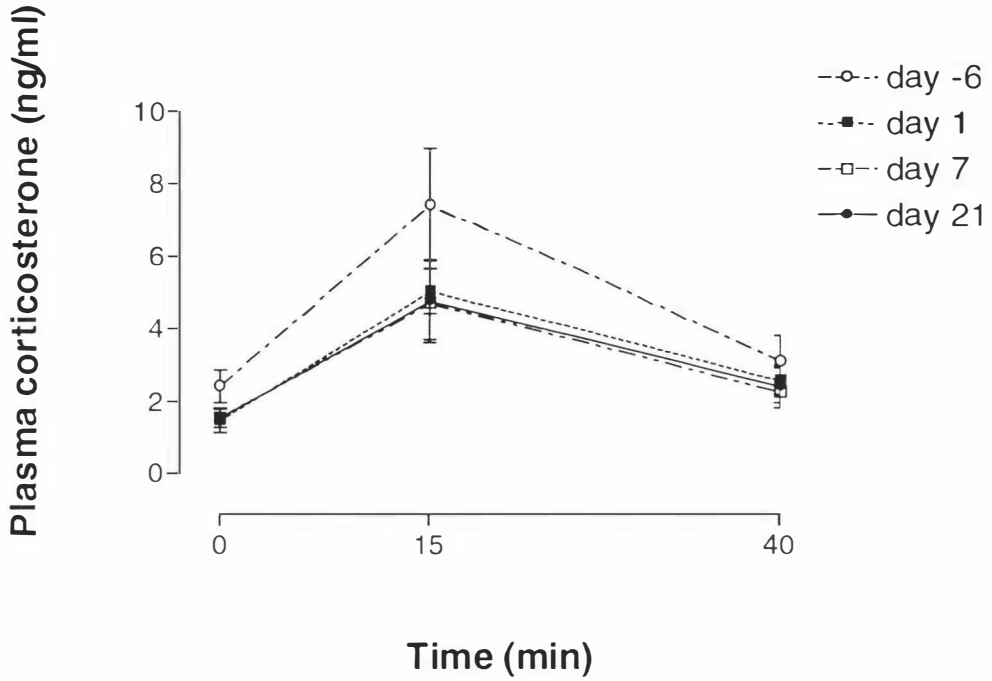


Figure 2.9: Corticosterone response to handling curves of all hens each day (mean ± SE; n=10 each day).

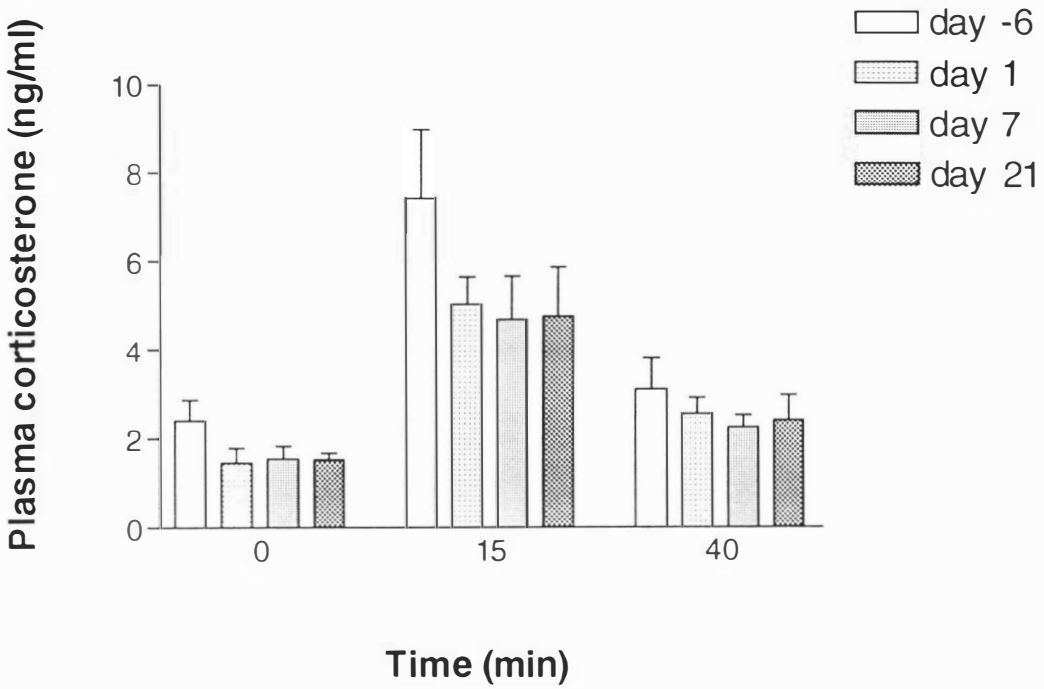


Figure 2.10: Corticosterone levels at 0, 15 and 40 minutes after handling on each day (mean ± SE; n=10).

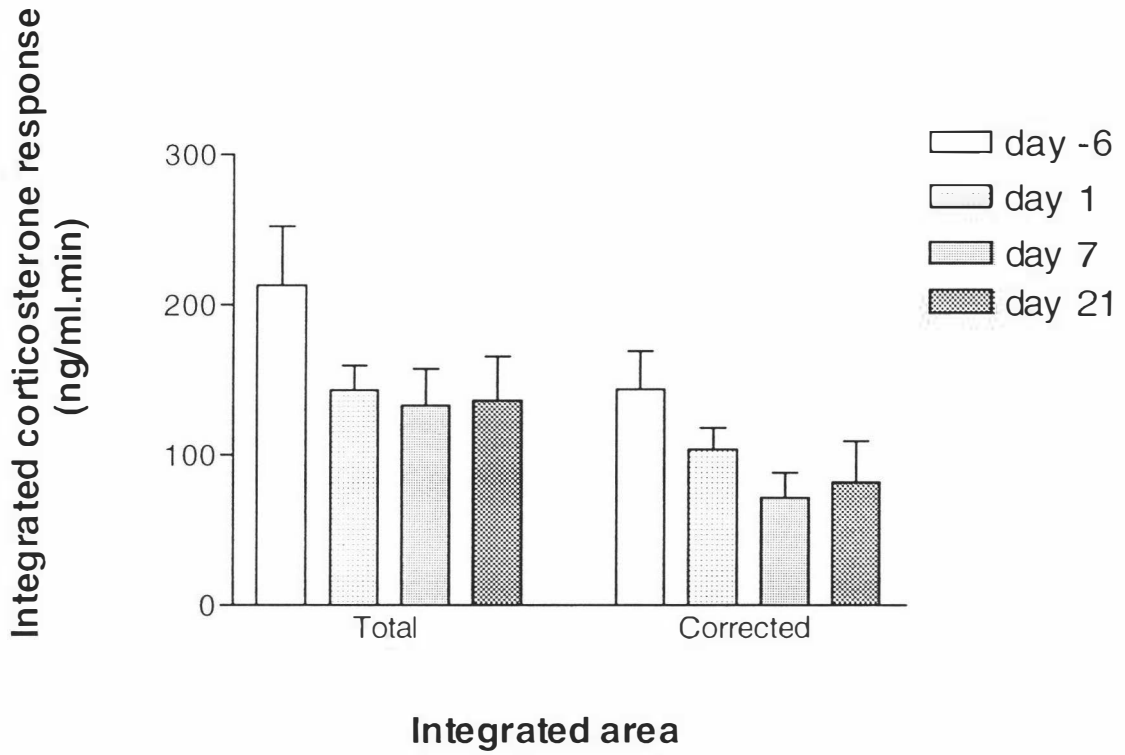
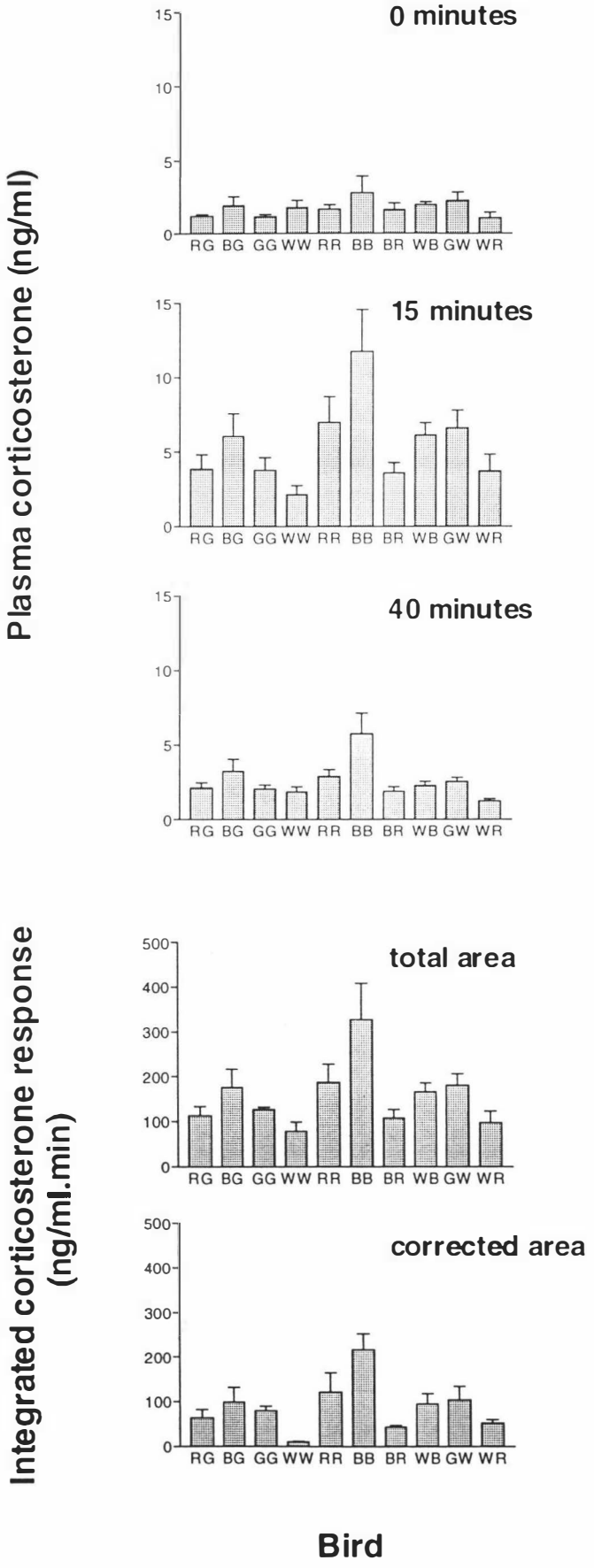


Figure 2.11: Total and corrected integrated corticosterone responses on each day (mean ± SE; sample size varies - total area: all days, n=10; corrected area: day –6, n=8; day 1, n=7; day 7, n=10; day 21, n=9).

Figure 2.12: Corticosterone levels at 0, 15 and 40 minutes after handling, and total and corrected integrated corticosterone responses for individual birds on all days combined (mean ± SE; n=4). *Letters refer to coloured legbands used for individual identification. R=red, B=blue, W=white, G=green.


Plasma corticosterone (ng/ml)
0 minutes
15 minutes
40 minutes
15
10
5
0
RG BG GG WW RR BB BR WB GW WR
Integrated corticosterone response (ng/ml.min)
total area
corrected area
500
400
300
200
100
0
Bird

Corticosterone variable	F*	P
total area	24.60	0.003
corrected area	14.62	0.102
corticosterone at 0 min	11.07	0.271
corticosterone at 15 min	23.78	0.005
corticosterone at 40 min	18.38	0.031

*Friedman's one-way repeated measures ANOVA of difference between hens in total and corrected integrated corticosterone responses and corticosterone at 0, 15 and 40 minutes after handling on all days combined.

Table 2.7: Results of Friedman's one-way repeated measures ANOVAs of differences between hens in total and corrected integrated corticosterone response and corticosterone levels at 0, 15 and 40 minutes after handling on all days combined.

There was marked variation within and between hens in their corticosterone responses (Figure 2.8; Table 2.6). The corticosterone responses of some hens were almost identical on each of the four sampling days (for example GG) whereas corticosterone responses of others varied considerably between days (for example BB, BG, RR). The shape of the corticosterone response also varied from nearly no response (WW) to a marked rise and fall in corticosterone levels (BB).

The magnitude of the variation was quantified by the calculation of coefficients of variation (Table 2.6). The average variation between birds was less than the average variation within birds at 0 minutes, but greater at 15 and 40 minutes. The total and corrected integrated corticosterone responses also varied more between than within birds. Variation between individuals in corticosterone levels at 15 and 40 minutes was similar and both were greater than variation at 0 minutes (Figure 2.12; Table 2.6). This between-bird difference was significant at both 15 and 40 minutes (Table 2.7). Variation between individual integrated responses was greatest for corrected integrated corticosterone responses but the difference between individuals was only significant for total integrated responses (Table 2.7). The values from each sampling day were treated as replicates for each bird in order to assess the repeatability of corticosterone measurement. Variation within birds was similar in basal and 15 minute corticosterone levels and these varied more than levels at 40 minutes. Corrected integrated responses varied more than total integrated responses (Table 2.6).

2.4.3.1 Corticosterone and social rank index

There was no significant relationship between corticosterone levels at each time and social rank index on any of the sampling days or on all days combined (Figure 2.13; Table 2.8). Similarly, there was no relationship between either total or corrected integrated corticosterone response and social rank index on each day or on all sampling days combined (Figure 2.14; Table 2.9).

2.4.3.2 Corticosterone and social status

The relationships between social rank and corticosterone variables were further clarified by dividing hens into either high, middle or low social status based on their social rank indexes as follows: before mixing: high, social rank indexes 4-5; middle, 2.5-3.5; low, 1-2; after mixing: high, 7.5-10; middle, 4-7; low, 1-3.5. There did not appear to be any relationship between social status and either

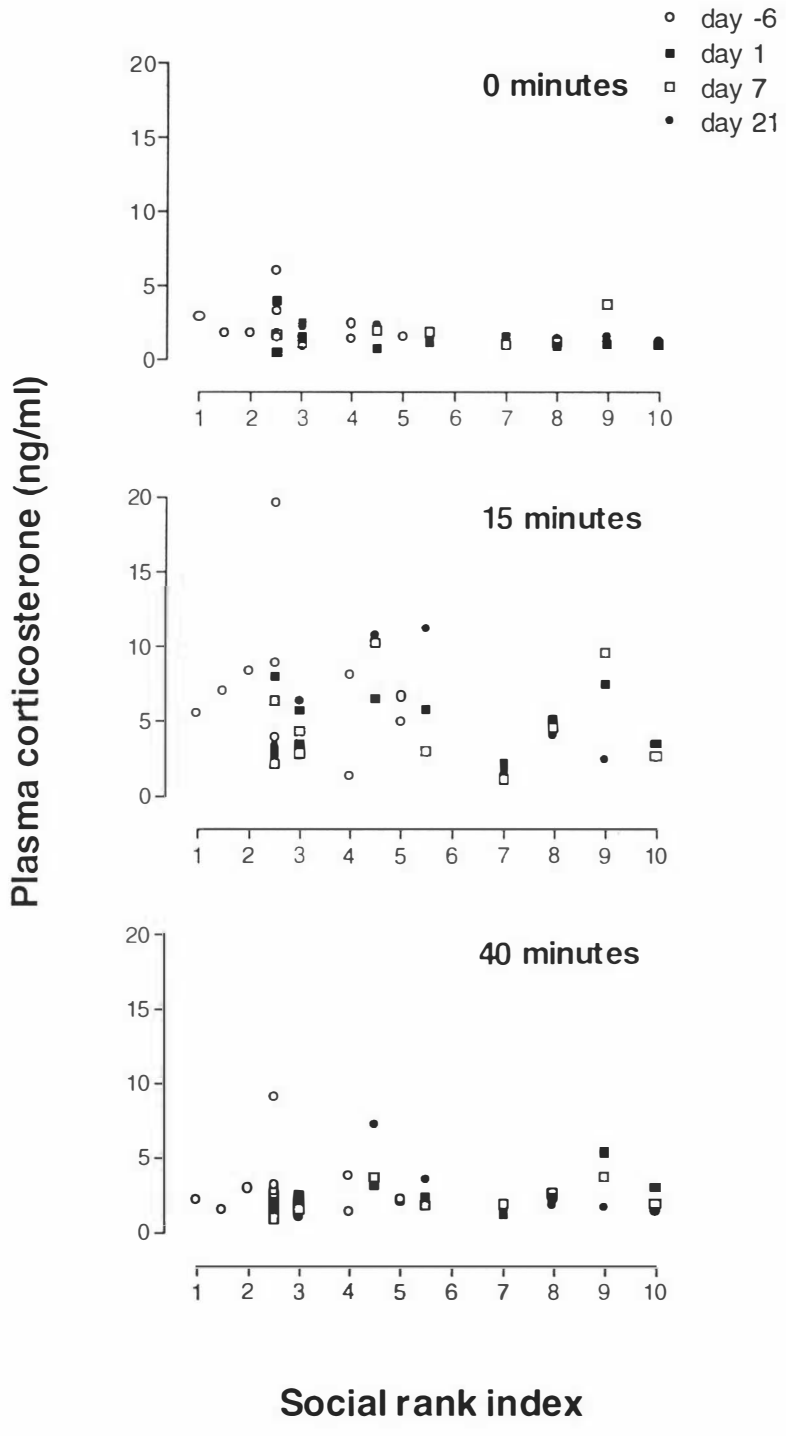


Figure 2.13: Relationship between social rank index and corticosterone levels at 0, 15 or 40 minutes after handling of all hens on all days.

	0 min		15 min		40 min	
	r^2*	*P*	r^2	*P*	r^2	*P*
-6	0.105	0.362	0.048	0.543	0.015	0.734
1	0.177	0.226	0.009	0.798	0.272	0.123
7	0.100	0.373	0.005	0.850	0.147	0.275
21	0.099	0.376	0.044	0.560	0.027	0.653
all	0.076	0.085	0.047	0.181	0.000	0.988

*Pearson correlations between social rank index and plasma corticosterone at 0, 15 or 40 minutes after handling of all hens on each sampling day and on all days combined.

Table 2.8: Results of Pearson correlations between social rank index and corticosterone levels at 0, 15 or 40 minutes after handling of all hens for each day and on all days combined.

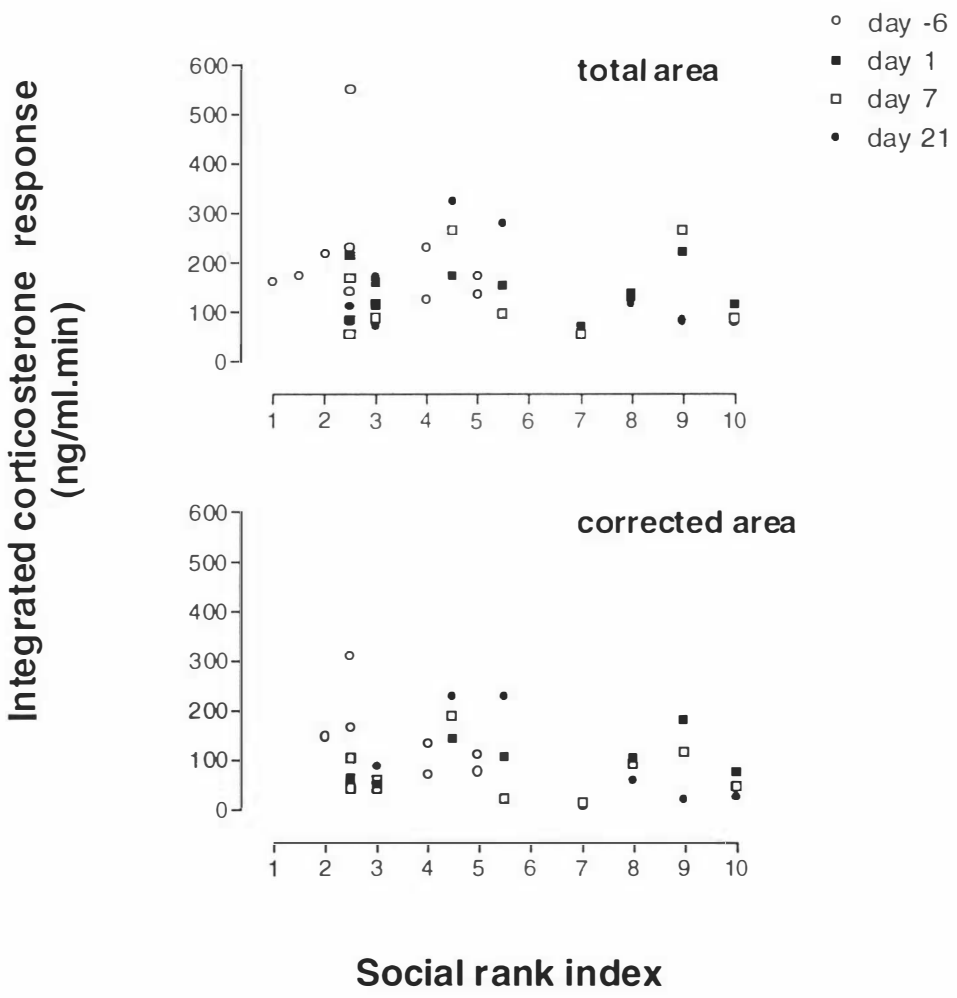


Figure 2.14: Relationship between social rank index and total and corrected integrated corticosterone responses to handling of all hens on all days.

Day	Total area under curve		Corrected area under curve	
	r^{2*}	P	r^2	P
-6	0.033	0.615	0.409	0.122
1	0.000	0.976	0.179	0.345
7	0.021	0.692	0.000	0.979
21	0.045	0.555	0.083	0.452
all	0.038	0.229	0.044	0.244

*Pearson correlations between social rank index and total integrated response or corrected integrated response for all hens combined on each sampling day and on all days combined.

Table 2.9: Results of Pearson correlations between social rank index and total and corrected integrated corticosterone responses to handling of all hens on each day and on all days combined.

corticosterone levels at 0, 15 or 40 minutes (Figure 2.15), or total or corrected integrated corticosterone response (Figure 2.16).

2.4.3.3 Corticosterone and behaviours received

There was no significant relationship between the total number of behaviours received by each hen before mixing and corticosterone levels at each time on day –6. Neither was there any relationship between the total number of behaviours received after mixing and corticosterone levels at each time on days 1, 7 or 21 (Figure 2.17; Table 2.10). Similarly, there was no relationship between total or corrected integrated responses on each day and behaviours received before or after mixing on any of the sampling days or on all days combined (Figure 2.18; Table 2.11).

2.4.3.4 Corticosterone and body weight

Body weights changed significantly in both groups during the experiment (Figure 2.19; repeated measures ANOVA; group 1: F= 3.428, P= 0.033; group 2: F= 5.068, P= 0.008). In both groups, body weights declined from day –24 to day 1 and remained relatively constant thereafter (Tukey's multiple comparison post-hoc test; group one, day –24 vs. day 1: q= 4.554, P<0.05; group two, day –24 vs. day –6: q= 5.356, P<0.05; day –24 vs. day 1: q= 4.783, P<0.05).

There was no relationship between social rank index and body weight (Figure 2.20; Table 2.12). There was no relationship between corticosterone levels at 0, 15 or 40 minutes and body weight (Figure 2.21; Table 2.13), or between total or corrected integrated corticosterone responses and body weight (Figure 2.22; Table 2.14).

2.5 Discussion

Handling elicited corticosterone responses in most hens (Figure 2.8). Hens showed individual differences in these corticosterone responses and in corticosterone levels (Figure 2.12; Table 2.6). However, the results do not support the prediction that social rank influences these differences (Figures 2.13; 2.14). Mixing two groups of hens did not produce a prolonged period of social stress;

correspondingly, the number of aggressive interactions (Figures 2.2, 2.3) and corticosterone secretion (Figures 2.10, 2.11) were not chronically altered in response to mixing. Any increase in aggression and corticosterone secretion probably persisted for a period of hours rather than days.

2.5.1 Behavioural interactions

There was no significant effect of mixing two groups of hens on the occurrence of the behaviours observed. Nevertheless, the highest numbers of pecks (5.8/min), chases (0.4/min) and total behaviours (6.8/min) were recorded on the day of mixing (Figures 2.2 and 2.3). Moreover, all aggressive interactions increased immediately after mixing and declined within 22 hours (Figure 2.3), indicating that a hierarchy was established within this period (Guhl, 1958). This follows the generally accepted pattern of a rapid increase in agonistic interactions during hierarchy establishment followed by a decrease over a few hours (Chase, 1982; Guhl, 1958) or days (Williams *et al.*, 1977). In this experiment, behavioural and physiological variables were measured over days and weeks rather than hours. The sampling protocol therefore would miss any temporary changes in these variables that may have occurred within a few hours after mixing. Repeating this experiment with more observations per group and more groups, could determine conclusively whether mixing two unfamiliar groups of hens leads to an increase in behavioural interactions. Observations could be made more frequently around the time of mixing. For example, scan sampling could be used, with groups being scanned for interactions once every ten minutes for the 24 hour periods immediately preceding and following mixing. Scan samples could be taken at less frequent intervals in the weeks preceding and following mixing in order to establish baseline interaction levels.

It has been suggested that there is a decline in more aggressive behaviours and a concurrent increase in avoidance or non-contact behaviours after the initial period of increased activity immediately following mixing of unfamiliar chickens. Williams *et al.* (1977) reported a peak after mixing of 0.25-0.45 pecks per minute between six cockerels, and a decline to 0.05-0.15 pecks per minute within the first week after mixing. Threats subsequently increased as pecks declined beyond the first week of mixing. In the present experiment, the number of threats was not noticeably reduced on the day of mixing and did not obviously increase thereafter (Figures 2.2, 2.3).

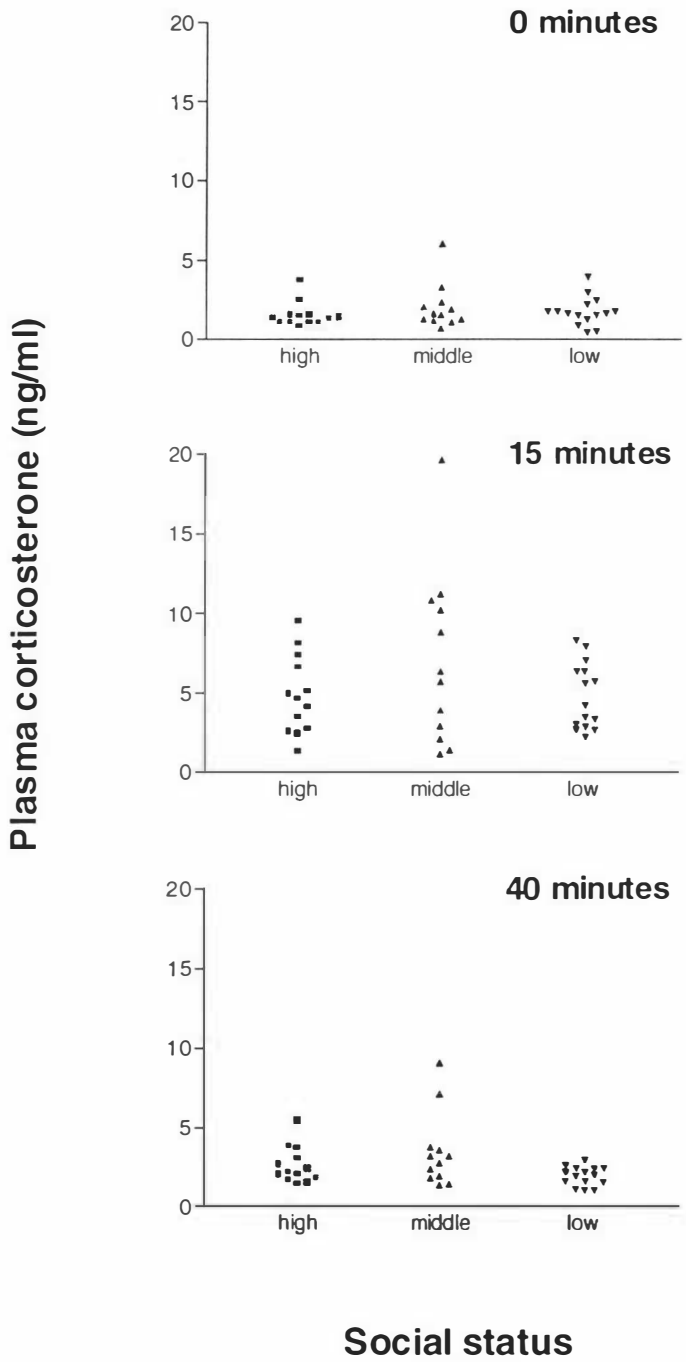


Figure 2.15: Corticosterone levels at 0, 15 and 40 minutes after handling on each day for hens of low, middle and high social status.

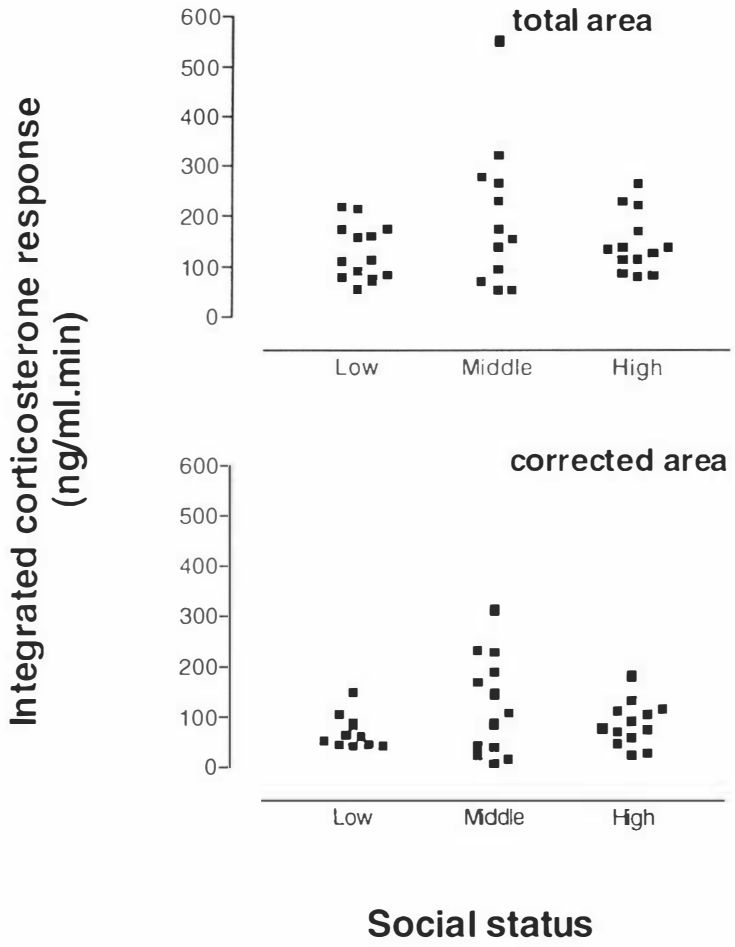


Figure 2.16: Relationship between social status and total and corrected areas under the corticosterone response to handling curve.

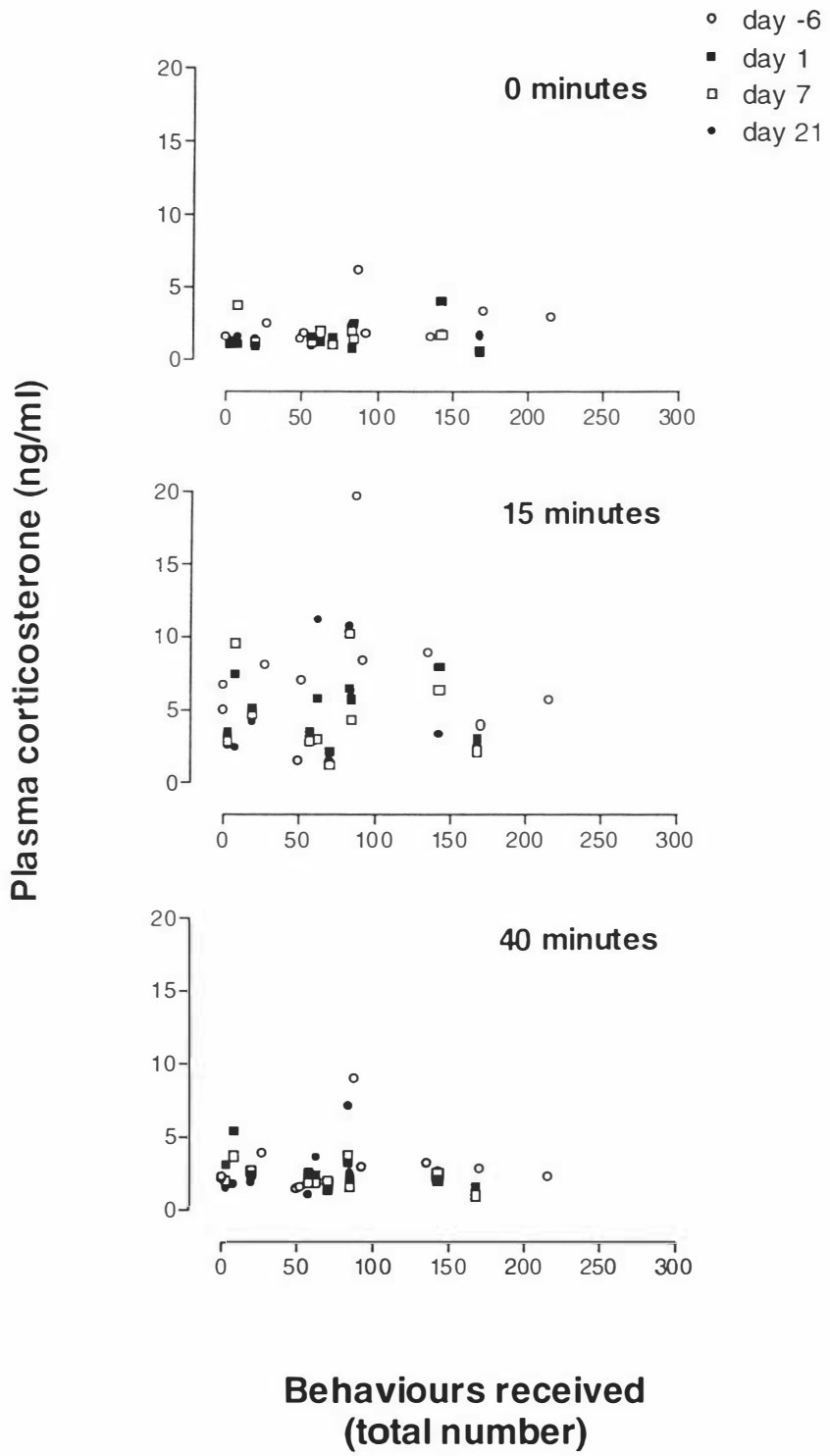


Figure 2.17: Relationship between the number of behaviours received by each hen and corticosterone levels at 0, 15 or 40 minutes after handling on all days.

Day	0 min		15 min		40 min	
	r^2*	*P*	r^2	*P*	r^2	*P*
-6	0.117	0.335	0.000	0.975	0.006	0.834
1	0.125	0.316	0.000	0.985	0.365	0.065
7	0.163	0.247	0.016	0.731	0.220	0.181
21	0.128	0.311	0.004	0.867	0.011	0.772
all	0.044	0.195	0.001	0.867	0.005	0.667

*Pearson correlations of number of behaviours received by each hen versus plasma corticosterone at 0, 15 and 40 minutes after handling for all hens on each sampling day and on all days combined.

Table 2.10: Results of Pearson correlations between behaviours received and corticosterone levels at 0, 15 or 40 minutes after handling of all hens on each day and on all days combined.

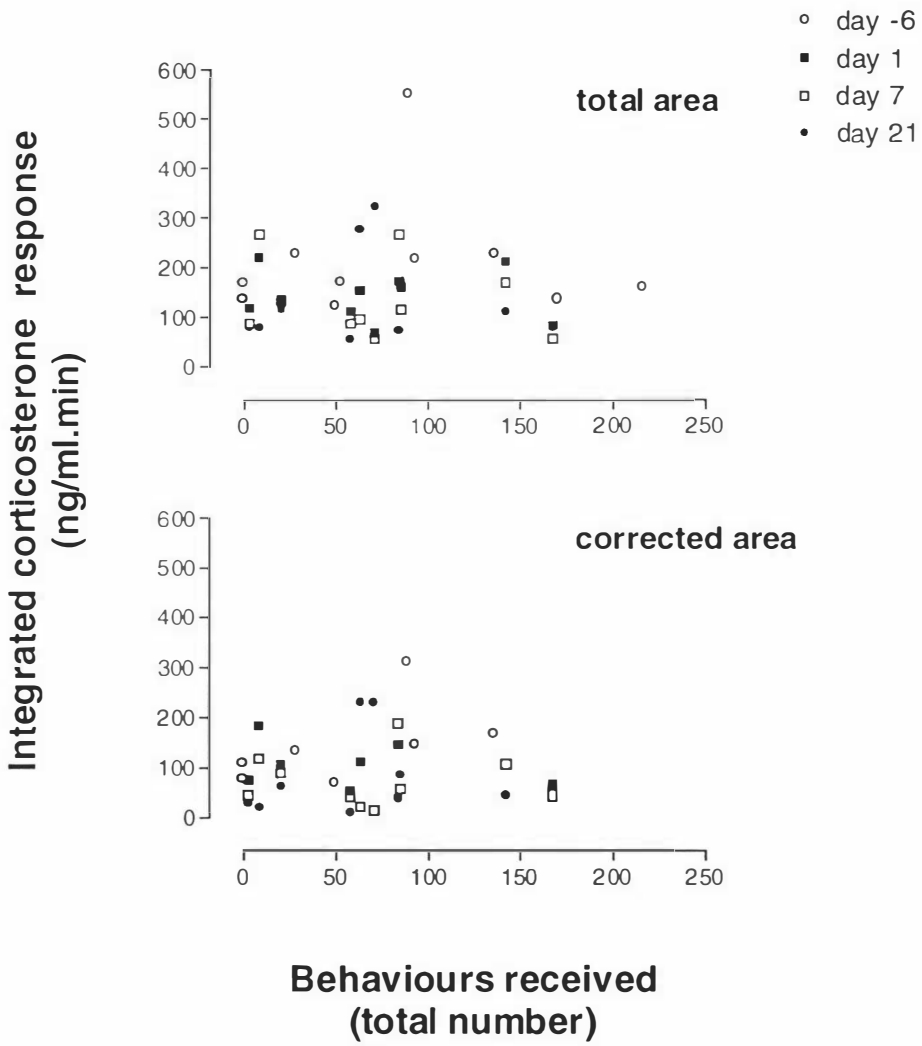


Figure 2.18: Relationship between number of behaviours received and total or corrected integrated corticosterone responses to handling of all hens on all days.

Day	Total area under curve		Corrected area under curve	
	r^{2*}	P	r^2	P
-6	0.001	0.942	0.294	0.209
1	0.012	0.761	0.123	0.440
7	0.043	0.568	0.000	0.962
21	0.007	0.813	0.056	0.540
all	0.000	0.932	0.009	0.593

*Pearson correlations between number of behaviours received by each hen and total or corrected integrated corticosterone responses to handling of all hens on each day and on all days combined.

Table 2.11: Results of Pearson correlations between behaviours received and total and corrected integrated responses to handling for all hens on each day and on all days combined.

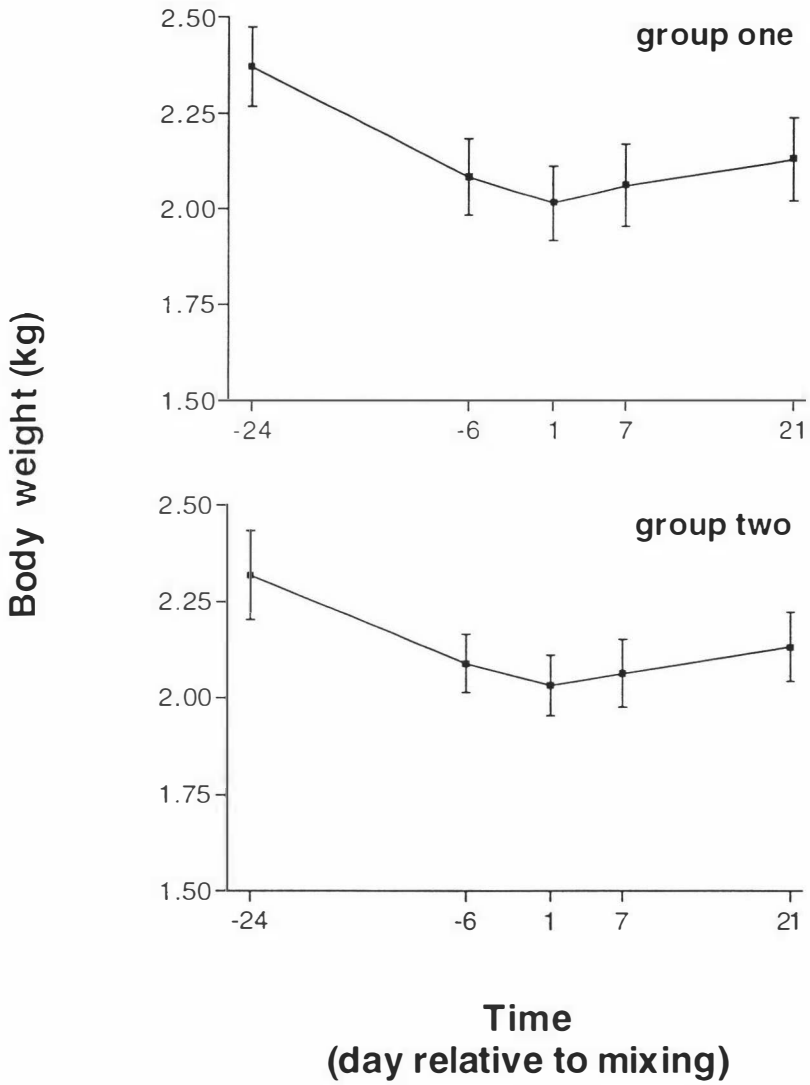
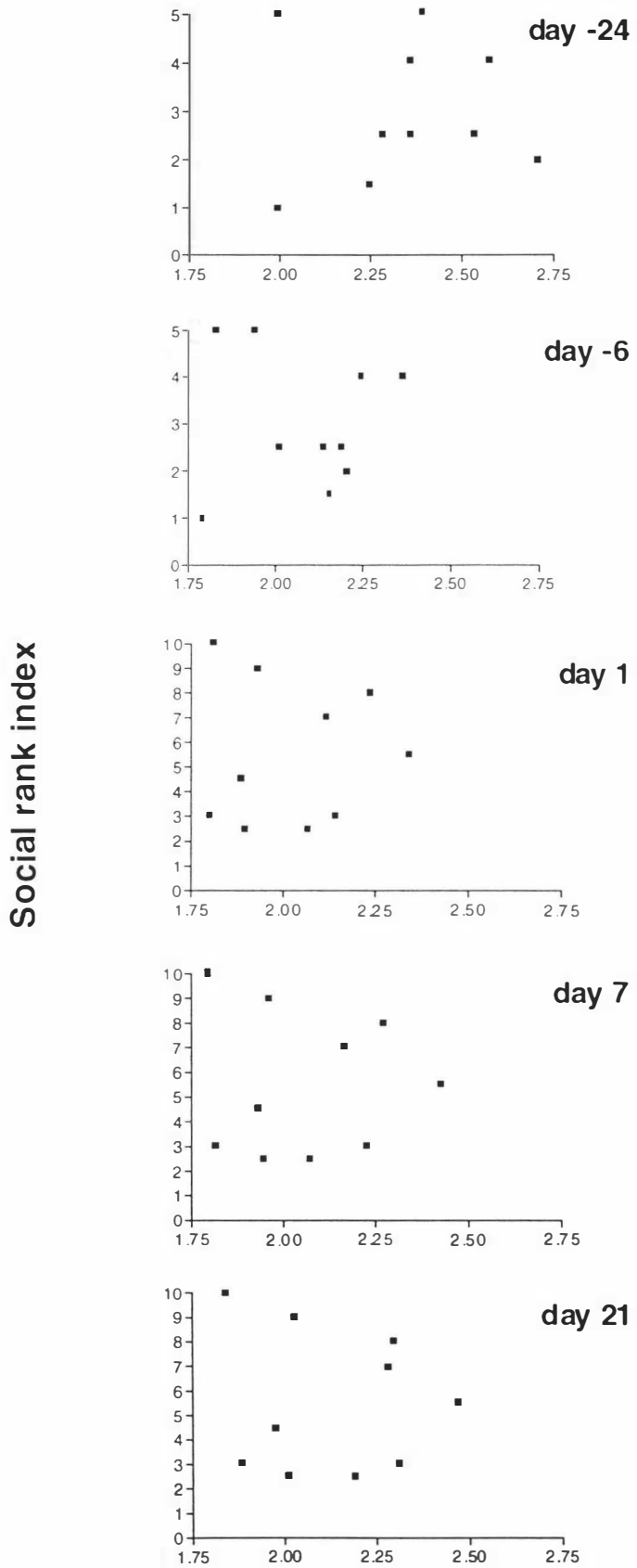


Figure 2.19: Body weights of hens before and after mixing two groups of hens together (mean ± SE; n=5).

Figure 2.20: Relationships between body weight and social rank index of laying hens weighed on five days before and after mixing. (Note different scales on y axis).


day -24
day -6
day 1
day 7
day 21
Social rank index
Body weight (kg)
1.75
2.00
2.25
2.50
2.75

Day	r_s^*	P
-24	0.111	0.759
-6	0.056	0.892
1	0.061	0.865
7	-0.018	0.973
21	-0.073	0.838

*Spearman rank correlations of body weight of all hens on each day and social rank index.

Table 2.12: Results of Spearman rank correlations between body weight and social rank index of all hens on each day.

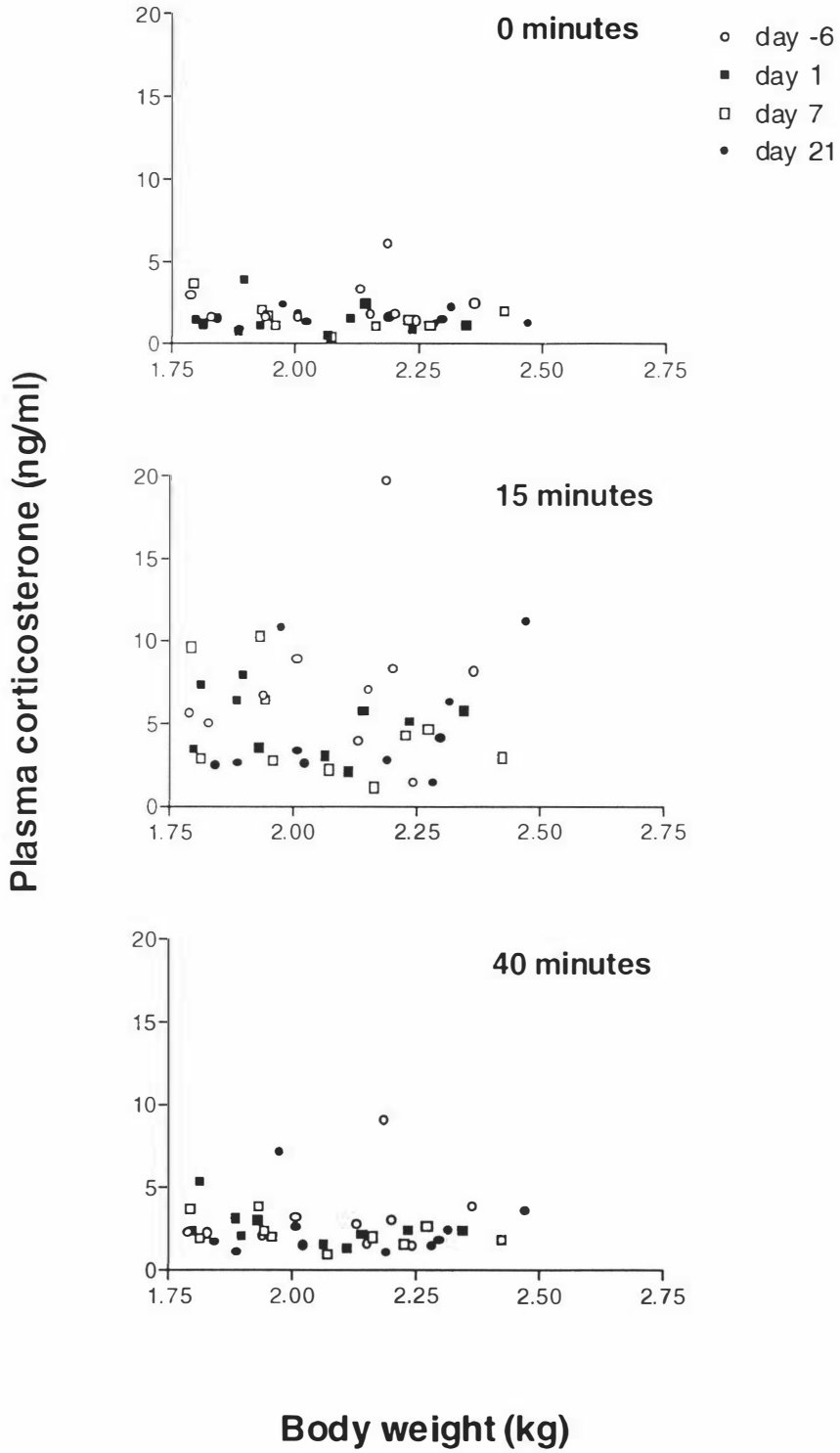


Figure 2.21: Relationships between body weight and corticosterone levels at 0, 15 or 40 minutes after handling of all hens on all days.

Day	r_s^*	P
-6	0.056	0.892
1	0.061	0.865
7	-0.018	0.973
21	-0.073	0.838

*Pearson correlations of body weight and corticosterone at 0, 15 or 40 minutes after handling on each sampling day.

Table 2.13: Results of Pearson correlations between body weight and corticosterone levels at 0, 15 or 40 minutes after handling of all hens on all days.

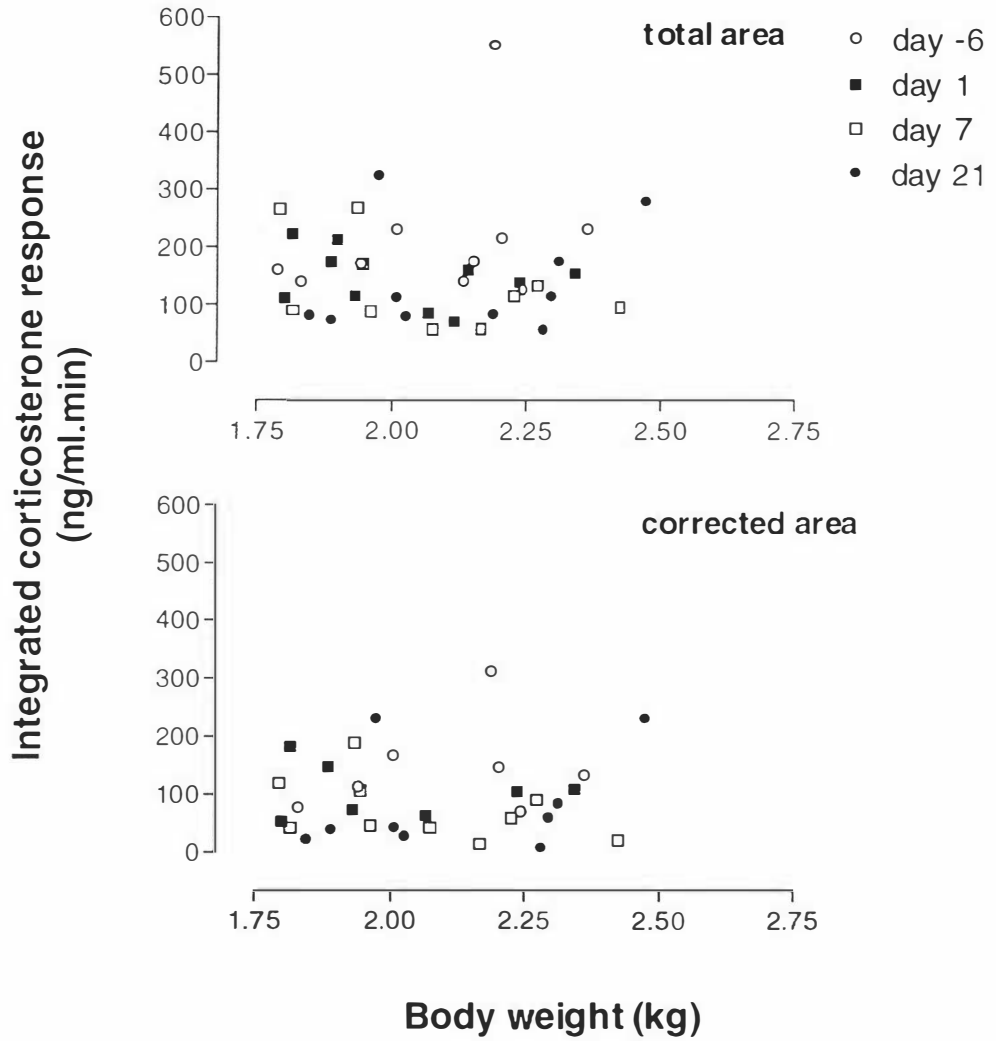


Figure 2.22: Relationships between body weight and total and corrected areas under the corticosterone response curve in laying hens weighed and blood sampled on four days.

Day	Total area under curve		Corrected area under curve	
	r^*	P	r	P
-6	0.280	0.433	0.244	0.597
1	-0.296	0.407	-0.132	0.778
7	-0.457	0.184	-0.439	0.204
21	0.268	0.454	0.336	0.377

*Pearson correlations of body weight and total area, and body weight and corrected area for all hens on each sampling day.

Table 2.14: Pearson correlations between body weight and total and corrected areas under the corticosterone response curve of all hens on each day.

However, the number of displacements, while not being substantially lower on the day of mixing relative other days, did show a non-significant increase in the second week after mixing to levels similar to those seen five weeks prior to mixing. This tendency may be an indication that avoidance or non-aggressive behaviours are more important than aggressive behaviours in maintaining an established hierarchy, as suggested by Mench and Ottinger (1991).

Numbers of interactions were generally low in the present experiment, with pecks, the most common behaviour, reaching a peak of around 6 pecks per minute on the day of mixing, rapidly falling to a plateau of around two pecks per minute the next day (Figure 2.3). Others have reported similar low numbers of interactions between chickens (Williams *et al.*, 1977; Mench and Ottinger, 1991). It has been suggested that hens watch known hens interacting with unknown hens, apparently in order to determine the unknown hen's relative social position (Chase, 1982). This may indicate that hens do not need to interact with all unknown hens in order to form a hierarchy. This could contribute to the low number of interactions and may explain why some pairwise combinations were never seen interacting. Alternatively some interactions may not have been recorded or may have occurred outside of observation periods.

In general, there was substantial variation between observation sessions within each week in the number of behaviours recorded (Figure 2.2) and this, together with the predominantly low level of interaction, may have contributed to the absence of any significant effect of mixing on observed behaviours. The irregularity of both the level and type of activity performed by the hens during each session seemed mainly responsible for the variation. The irregularity may have been partially due to differences in the time of day each group was observed before mixing, despite all observations being taken between 0900 and 1100 and the order of observation being altered each time. Such problems could be avoided in the future by observing both groups simultaneously, for example by keeping both groups in the same room or by having one observer watch each group.

Features of the present experimental design may have contributed to the wide variation. As the numbers of all behaviours were low, a change by only a few interactions could have influenced the overall result. As the hens were confined, the behaviour of certain hens could directly influence the behaviour of others. Both the type of activity and the identity of the hen performing it appeared to be important. For example, if hens were resting during the observation period, they did not interact aggressively but tended to show more exploratory pecking. A high number of dominant hens feeding

tended to reduce the number of subordinate hens feeding and nesting hens frequently attracted the attention of pen-mates, leading to a high density of hens in the immediate area and hence an increase in the number of interactions. If a greater number of dominant hens were active at any one time, the number of interactions observed tended to increase as they moved around the pen and either interacted with other hens, or caused subordinate hens to move away and in turn receive more aggression from other dominant hens. Conversely, if mainly subordinate hens were active, they tended to avoid contact with others, thereby reducing the number of interactions observed. If they did not move away from dominant hens, or avoid a threat, they were often chased or pecked aggressively, again influencing the number of interactions recorded. One hen (WR; one of the two lowest ranking hens; Figure 2.4), was often seen hiding under the feeder or drinker. It did not seem to move freely about the pen but rather moved hurriedly from one hiding place to another. When it moved across the pen, it was often pecked more frequently and with more force than were the other hens.

Occasionally hens showed idiosyncratic behaviours during one or two observation sessions only. This affected the number of interactions occurring during that session. For example, hens RG and WW repeatedly pecked and chased one or two particular subordinate hens in one session but not in subsequent session. Hen RR periodically showed stereotyped pacing and this tended to displace others in its path and cause the other hens to clump in other areas of the pen. This again increased the density in the immediate area, which may have inflated the number of interactions.

There was also a marked increase in feeding behaviour in response to an increase in the amount of feed visible in the tube feeder. Occasionally hens knocked the feeder or removed a substantial amount of feed at once, causing an influx of feed from the tube into the feeder pan. Again an increase in the density in the immediate area could affect the number of interactions. In a future experiment, incidents of increased feeding activity could be closely monitored to exclude this possibility.

Another incidental but obvious factor which reduced the number of interactions for a short period on three occasions was external noise, such as that from tractors, aeroplanes and neighbouring cockerels. However, the overall level of interaction during observations when hens experienced external noise fell within the range of the other observation periods.

Finally, there may have been a change in observer accuracy over time ('observer drift'). Inaccuracies could arise if behaviours were incorrectly categorised or if some interactions were not seen and recorded. It was unlikely that behaviours were incorrectly categorised as hens were always watched from the same position outside of the pen and strict, simple descriptions of the behaviours observed were adhered to throughout the experiment. Observer drift could be assessed in a future experiment by cross-checking written observations against video recordings of at least one observation session each week in order to ensure the behaviours were consistently categorised. If all observations were recorded on video, it would be possible to cross-check the number of interactions that occurred in each observation session. It is not easy to identify individual hens from video recordings however, and the use of identification markers such as paint could incite pecking.

It is possible that some interactions were not recorded during the observation sessions as it was necessary to record the occurrence, direction and type of each interaction. Accordingly, it was difficult to record more than two or three interactions occurring concurrently. Immediately following mixing it was particularly difficult to simultaneously observe and record as the frequency of interactions increased substantially. This could be avoided in the future if two observers were used with one watching each group. The agreement between observations made by both observers ('inter-observer reliability') would need to be checked would need to be checked in this case.

Moreover, if it were possible to determine the hierarchy in another manner, it would not be necessary to record the individual identification of each hen involved in the interactions. Rather, only the occurrence and type of each interaction would be noted, in which case a counter or tally device could be used to reduce observer error. However, in the early stages of the present experiment, we attempted to incite dyadic interactions between hens in an arena in order to define the hierarchy. When placed in the arena, a ring of metal sheeting approximately 50 cm high and 100 cm in diameter, the hens generally remained inactive and did not interact. If a different strain of chicken was used, or they were male rather than female, we may have provoked competition. For example, Mench and Ottinger (1991) determined hierarchies in young white male chickens using arena tests. Alternatively, observer presence may have affected the outcome in our arena tests. In their home pens, the hens appeared to ignore the observer after a five minute acclimation period, yet in the arenas, hens seemed more interested in the observer than in each other. If the observer were able to hide from view, such as behind a one-way mirror, the hens may have interacted as they normally

did in the pens. However, it was decided to observe the direction of interactions within the pens rather than persevere with arena tests.

With regard to exploratory pecking, subordinate hens were observed initiating interactions with dominants, although the reverse was more common. The higher incidence of dominant-subordinate directed exploratory pecks suggests that aggressive pecking could have been mistakenly recorded as exploratory pecking. However, the two behaviours are quite distinct to an experienced observer, so it is unlikely that this occurred. Given that subordinates were seen initiating exploratory pecking with dominants, there is some indication that dominance hierarchies do not control this form of pecking. It has been suggested that exploratory pecking (often termed 'feather pecking') is a displacement behaviour for foraging for caged hens or that it is redirected ground pecking (Appleby *et al.*, 1993). To my knowledge, it has not been suggested that exploratory pecking has a component of aggression, which supports the findings in the present experiment.

2.5.2 Hierarchies

The direction of dominance relationships in the present experiment was established during the first few observed interactions as seen by Chase (1982), who reported that chickens established a linear hierarchy after no more than three interactions with any one hen.

The relative position of each hen within the hierarchy did not change markedly after mixing (Figure 2.5), indicating that the social status of an individual hen is fairly stable. Social status may be derived from some inherent individual features, including psychological variables such as the ability to cope with stressors (Sapolsky, 1992), or physical features such as comb appearance (Williams *et al.*, 1977). In this experiment there was no relationship between body weight and social rank (Figure 2.20) indicating that body weight is not an important feature in determining social rank in hens. Chase (1982) suggested that physical features are not important in determination of hen social rank, but rather that winning hens go on to win whereas losers tend to keep losing. Certainly, hens do not appear to use body weight to their advantage in social competition in the way some other species do. Furthermore, commercial laying hens are selected for similar body weight and the differences in body weight may be not substantial enough to influence social rank.

All hierarchies were basically linear (Figure 2.5), that is one hen was dominant in each pair, relationships were transitive and there were no dominance reversals. There were three occasions when a subordinate hen pecked or displaced a dominant hen, but these were isolated incidents and were not repeated when the pairs next interacted. It has been suggested that missing interactions and small group numbers, as were present in this experiment, increase the likelihood of the appearance of linearity in a randomly organised social group (Appleby, 1983). However, dominance relationships were observed repeatedly once established, supporting the notion of linearity. Furthermore, it is commonly accepted that chickens normally form linear hierarchies (Guhl, 1958; Chase, 1982; Lee *et al.*, 1982).

2.5.3 Corticosterone

Handling elicited corticosterone responses in most hens (Figure 2.8). Corticosterone responses to handling have been detailed previously for many bird species (Wingfield, 1994; Wingfield *et al.*, 1997) including chickens (Beuving and Vonder, 1978, 1986; Beuving, 1980; Freeman and Flack, 1980; Webb and Mashaly, 1984). The average basal corticosterone level in this experiment was 1.74 ± 0.17 ng/ml (range: 0.42 to 6.04 ng/ml). Corticosterone peaked at 5.47±3.57 ng/ml (1.12 to 19.64 ng/ml) and had fallen to 2.58±1.58 ng/ml (0.94 – 9.03 ng/ml), not reaching basal levels, by 40 minutes. These values are similar to previously reported basal corticosterone levels and those seen in response to handling for chickens (eg. Beuving, 1980). Beuving and Vonder (1986) observed basal corticosterone levels of around 0.7 ng/ml in white leghorn hens and a peak of around 3 ng/ml by 6 minutes after handling. The level approached basal at 18 minutes. Webb and Mashaly (1984) reported basal corticosterone levels of 2.48±0.20 ng/ml in white leghorn hens and corticosterone was still elevated by around 1 ng/ml one hour after handling.

Chickens generally have lower basal corticosterone levels, lower peaks in response to stressors and levels return to basal more rapidly than wild birds (Astheimer *et al.*, 1994; Wingfield *et al.*, 1997). For example, grey ducks, *Anas superciliosa*, sampled in the same way as the hens in this experiment had higher basal corticosterone levels (6.26±0.57 ng/ml), peaked at higher levels (21.68±1.25 ng/ml at 15 min) and took longer to return to undisturbed levels (16.78±1.80 ng/ml at 40 min; R. Osborne, unpublished data). Domestication or habituation to human contact is probably responsible for this

difference and it has been shown that wild birds have lower basal corticosterone levels after several months in captivity compared to leveis at initial capture (Forman, 1994).

Basal corticosterone levels and total integrated corticosterone response were highest on day –6 and were similar on subsequent days (Figure 2.9), indicating that mixing two groups of hens had no effect on corticosterone secretion in the hens. This result is consistent with the lack of significant increase in aggressive interactions after mixing. Moreover, it appears that the first exposure to handling and blood sampling was a more significant stressor than group mixing. Venipuncture (Webb and Mashaly, 1984), handling (Beuving, 1980) and exposure to a novel stimulus have been shown to elicit glucocorticoid responses (Levine, 1985), and thus could have contributed to the relatively larger corticosterone responses to handling on the first day of sampling. On subsequent days, the hens may have at least started to habituate to handling (Webb and Mashaly, 1984; cf. Beuving and Vonder, 1978, and Freeman and Manning, 1979). Cockrem and Silverin (1998) and M. Welch (pers. comm.) also observed relatively greater corticosterone responses to handling and bleeding on the first day of sampling relative to subsequent days in great tits, *Parus major*, and blackbirds, *Turdus merula*, respectively. This effect could be avoided in future experiments by habituating chickens to handling prior to the start of the experiment. The hens could also be subjected to blood sampling before the experiment started, provided the sample could be used to provide useful information, rather than functioning simply to acclimatise hens to bleeding *per se*.

It is well established that indicators of stress increase in response to social disruption in birds (Siegel and Siegel, 1961; Williams *et al.*, 1977; Gross and Siegel, 1983; Satterlee *et al.*, 1983; Harvey *et al.*, 1984; Romero *et al.*, 1995). It is likely that basal corticosterone levels and the corticosterone response may have been elevated during the few hours immediately following mixing and then returned to previous levels by the time samples were collected the next day. Corticosterone responses were not measured on the day of mixing in this experiment so as to avoid confounding the possible stressors of environmental change, social disruption, transportation and handling (Beuving and Vonder, 1978; Mench, 1991). If each group were kept in one half of a large pen and the central barrier removed in order to mix the two groups, this would avoid stress caused by transport and a change in environment. Consequently, it would be possible to measure corticosterone levels on the day of mixing.

There was marked within-individual and between-individual variation in corticosterone responses and some birds varied more in their responses than others (Figure 2.8). A similar pattern has been reported for Japanese quail and great tits in our laboratory (Cockrem, unpublished data). Variation in the current experiment was generally greater between birds than within birds (Table 2.6), suggesting that corticosterone responses are more repeatable in individuals. Similarly, Beuving and Vonder (1986) reported that white leghorn hens subjected to immobilisation by hand or given a submaximal intra-arterial dose of ACTH on consecutive days had greater variation in corticosterone responses between hens than within hens.

Corticosterone levels at 15 and 40 minutes varied more between hens than did basal corticosterone levels. This supports previous suggestions that baseline levels do not provide adequate information about individual differences in responses to stressors (Wingfield, 1994; Wingfield *et al.*, 1997).

There was no relationship between basal corticosterone levels and either the time taken to collect each blood sample (Figure 2.7) or the order in which hens were bled. It has been shown that a gradual reduction of group size is a stressor to chicks (Jones and Harvey, 1987). The stressor effect is abolished if birds are bled within around three minutes and replaced in the group after blood sampling (Jones and Harvey, 1987; Lagadic *et al.*, 1990).

2.5.3.1 Corticosterone, social rank and social status

The position of hens in the social hierarchy was not significantly correlated with their corticosterone responses (Figures 2.13, 2.14; Tables 2.8, 2.9), suggesting that social rank did not contribute to the variation between individuals in corticosterone levels. However, the three greatest total and corrected integrated areas were shown by middle ranking hens (Figure 2.16). Hens in the middle rank would be expected to have higher corticosterone responses if their position in the hierarchy were more labile relative to either high or low ranked hens. It is possible that middle ranking hens are the most active, as they are interacting with lower and higher ranked birds and may be attempting to elevate their social status. This has been reported in olive baboons (Sapolsky, 1983) and grey ducks (R. Osborne, unpublished data). Middle ranking hens may also receive the most aggression as a consequence of their attempts to improve their ranking. In the present experiment, two of the three middle ranking hens received more behaviours than the remaining low or high ranked hens before

mixing but there was no pattern after mixing and overall, low ranked hens tended to receive the most aggression (Figure 2.6). It is possible that middle ranking hens only assert themselves when they have already established a position in the hierarchy. Observations in the current experiment, from which the 'behaviours received' data were calculated, were taken during the period of social disruption when the hens may not have been attempting to elevate their position. It would only be possible to confirm this tendency by repeating observations and perhaps observing hens for an extended period after mixing.

Some studies have reported a relationship between increased indices of stress and low social status in birds (Flickinger, 1961; Siegel and Siegel, 1961). For example, Schwabl *et al.* (1988) reported a more rapid increase in corticosterone levels in response to capture and handling in juvenile and low ranking female white-throated sparrows. However, other studies found no relationship between social status and indices of stress (Siegel and Siegel, 1961; Williams *et al.*, 1977; Marsteller *et al.*, 1980; Cunningham *et al.*, 1987, 1988; Mench and Ottinger, 1991). As a result, it is difficult to predict whether a relationship should have been observed in the present experiment. The discrepancy between these findings is likely due to extrinsic modification of basal corticosterone secretion, or due to differences in stability of the hierarchy. It has been suggested that relationships between social rank and glucocorticoid secretion are often only seen during periods of social stress (Williams *et al.*, 1977). Again, it may be that such a relationship would have been apparent in the chickens during the few hours following mixing.

An absence of correlation between social position and corticosterone levels or responses may be expected if chronic stressors are not associated with social rank. This is likely to be relevant in a linear hierarchy (Mench, 1991) and in situations where there is no intense competition for resources. This was probably the case in the small social groups in this experiment. It has also been shown that the relationship may not be linear (Gross and Siegel, 1983). However, in this experiment, there was no apparent relationship at all between any corticosterone variable and social position.

The variation in corticosterone secretion between individual hens in this experiment was not related to social rank, but it may have been influenced by other factors such as sex, genetics, previous background, and individual differences in perception of the stressor. The hens in the present study were all from the same genetic stock, of the same age and had the same background, hence it is likely that differences in corticosterone secretion reflected inherent differences between the birds.

Individual variation in glucocorticoid secretion can be mediated by the higher centres in the brain, the hypothalamus, for example by downregulation in the number of glucocorticoid receptors (Sapolsky *et al.*, 1984), the pituitary gland, for example by a change in sensitivity to CRH, or the adrenal glands (Sapolsky, 1992; Astheimer *et al.*, 1994). Beuving and Vonder (1986) demonstrated that individual hens had different corticosterone responses to an ACTH injection. As ACTH stimulates the adrenal glands, the individual variation must have arisen due to differences in adrenal gland function, for example by an alteration in adrenal gland sensitivity to ACTH, or in the secretory capacity of the adrenal glands.

2.5.3.2 Corticosterone and behaviours received

Lower ranked hens tended to receive the most aggression overall but did not have the highest or lowest corticosterone levels or responses (Figure 2.6). It appears that aggression in hens is not an important component in the modulation of corticosterone secretion, as previously suggested by Ramenofsky (1985) and Mench and Ottinger (1991). Marsteller *et al.* (1980) also reported that aggression received was negatively correlated with social rank index in cockerels, yet there was no correlation between corticosterone levels and aggression received or social rank index.

2.5.3.3 Corticosterone and body weight

There was no relationship between body weight and corticosterone secretion in hens in the current experiment (Figures 2.21, 2.22; Tables 2.13, 2.14). Corticosterone is an important modulator of protein, carbohydrate and fat metabolism and birds may modulate secretion in response to changes in body fat or condition depending on seasonal changes in corticosterone secretion and environment condition (Wingfield *et al.*, 1997). Negative relationships between corticosterone responses or basal corticosterone levels and body condition or body weight are often reported for wild birds (Smith *et al.*, 1994; Heath and Dufty, 1996), although others have found no correlation (Dawson and Howe 1983; Rehder *et al.*, 1986). The discrepancy between these findings is likely due to changing environmental influences impinging on the different species observed (Wingfield, 1994). The hens in the current experiment were housed indoors in constant conditions and with continuous access to food. Relationships between corticosterone levels or responses and body weight have not been

reported for birds in these conditions. Moreover, it is probable that the differences in body weight between hens were not large enough to affect corticosterone secretion.

2.5.4 Conclusions

In conclusion, the findings of this experiment did not generally support the predictions, although this was likely due to features of the experimental design. Handling elicited corticosterone responses in most hens that were similar to those reported in the literature. There was substantial variation between hens in the level of corticosterone measured during corticosterone responses to handling and in the magnitude of these responses. However, there was no relationship between these corticosterone variables and the social rank of each hen, suggesting that social rank did not contribute to the observed variation. The number of aggressive interactions did not change when two unfamiliar groups of hens were mixed together, and there was no change in corticosterone levels or the magnitude of corticosterone responses during this time. Body weight declined over time in all hens before mixing and remained relatively stable after mixing. There was no relationship between body weight and social rank, or between body weight and either corticosterone levels during the corticosterone response or the magnitude of the corticosterone response.

3 THE NON-INVASIVE MEASUREMENT OF CORTICOSTERONE IN COCKEREL DROPPINGS

3.1 Abstract

Blood sampling is known to elevate plasma levels of corticosterone. It is possible to non-invasively measure many steroid hormones in urine, faeces and droppings, in order to avoid the effects of sampling. Until recently, corticosterone had not been measured in avian droppings. This study was intended to ascertain whether an established method of extracting sex steroids from faecal samples followed by radioimmunoassay could be applied to the measurement of corticosterone in chicken droppings. It was also designed to determine whether there was a relationship between the pattern of plasma and faecal corticosterone secretion, and between the total amount of corticosterone secreted in the plasma in response to a stressor, and the total amount of corticosterone excreted in the droppings. In two experiments, cockerels were given intramuscular or intravenous ACTH injections and droppings were collected throughout each experiment. In the first experiment, blood samples were collected at 0 hours before intravenous ACTH or saline (control) injection and at 1, 2, 5 and 24 hours after injection. Cockerels showed an obvious and dose-related increase in plasma corticosterone levels in response to ACTH followed by a decline to basal levels by two hours post-injection. There was no marked parallel increase in faecal corticosterone levels, and there was no relationship between the total amount of corticosterone secreted in the plasma and the total amount measured in the droppings. In the second experiment, birds were not bled. One group of cockerels were not handled, and the remaining groups received an intramuscular injection of either ACTH or saline (control). Cockerels given ACTH had higher peak levels of faecal corticosterone than unhandled birds or those given saline. However, the total amount of faecal corticosterone excreted was similar for all groups. The consistency of droppings changed markedly after the ACTH injections and this may have affected the outcome. The method used to extract corticosterone from droppings before radioimmunoassay also influenced the results.

3.2 Introduction

In order to determine the effects of a stressor, it is often necessary to measure hormone levels in blood or tissue samples from living or dead animals. It is recognised that hormone levels fluctuate over time, often negating findings based on single level data (Wingfield *et al.*, 1997) and that handling and sampling themselves affect endocrine measures (Holmes *et al.*, 1990; Lagadic *et al.*, 1990; Le Maho *et al.*, 1992; Tuli *et al.*, 1995). Furthermore, increasing awareness of animal welfare has encouraged a move by some to adopt the 'three R's': a reduction in the number of animals used in experiments, the refinement of protocols in order to minimise suffering and the replacement of animals where possible (see Monamy, 1996). The result, particularly in response to refinement, is increasing interest in the development of non-invasive methods of endocrine assessment (Lasley and Kirkpatrick, 1994; Peter *et al.*, 1996).

Hormone levels can be measured in many substances other than blood or plasma, including egg yolk (Lipar *et al.* 1996), saliva (Fell *et al.*, 1986), milk (Verkerk *et al.*, 1996), faeces or droppings and urine (Peter *et al.*, 1996). There are potential problems associated with non-invasive steroid assessment, and in particular with the use of urine and faecal samples. For example, the amount of fibre (Wasser *et al.*, 1993) and lipid (M. Kikuchi, pers. comm.) in the animal's diet and the method used to store samples (R. Osborne, unpublished data) can affect steroid measurement. The rate of removal of the steroid from the blood ('metabolic clearance rate') can fluctuate due to a number of factors such as clearance organ capability, and only a small proportion of steroid is excreted in an unaltered form (Helton and Holmes, 1973; Wasser *et al.*, 1994; Brown *et al.*, 1994). Moreover, methods of extracting the steroid from the raw sample can be time consuming and often need to be tailored to species (M. Kikuchi, pers. comm.) Finally, the timing of faecal collection is controlled by the animal rather than by the experimenter and collection of samples from free-living animals can be difficult.

Steroid hormones readily lend themselves to measurement in faeces, droppings or urine of birds and mammals as they are excreted as identifiable metabolites or conjugates and they are generally stable in urine or faeces, at least in mammals (Lasley and Kirkpatrick, 1994). Peptide hormones are less easy to measure in this way as they are excreted primarily as uric acid or urea (Guyton, 1991), but there are exceptions. For example, levels of the glycoprotein human chorionic gonadotropin (hCG) increase during human pregnancy and a laboratory test is used to confirm pregnancy based on urine analysis.

Non-invasive steroid measurement has an important practical application in the field of conservation. The survival of endangered species relies on adequate monitoring of the reproductive status of both captive and free-living animals (Brown *et al.*, 1994; Ishii *et al.*, 1994; Cockrem and Rounce, 1995) but the effects of invasive sampling are potentially lethal or debilitating, as in capture myopathy. The measurement of sex steroids and metabolites in urine and faeces has been used successfully to monitor reproduction in a number of species (Lasley and Kirkpatrick, 1994). Non-invasive measurement is also being used to investigate the effects of stress on the reproduction and welfare of captive and endangered animals (Carlstead *et al.*, 1992; Lasley and Kirkpatrick, 1994; Wasser *et al.*, 1997).

3.2.1 Steroid hormone synthesis

The pathways of steroid hormone synthesis are generally similar in function in both birds and mammals. Steroid hormones are derived from cholesterol through a series of conversions resulting first in pregnenolone then progesterone. Progesterone is converted into the mineralocorticoids, glucocorticoids or androgens, and androgens are converted into oestrogens (McKerns, 1969). Cholesterol may be synthesised in hepatic, intestinal or adrenocortical cells then taken up from lipoproteins in the blood and stored as cholesterol esters in lipid droplets in the cytoplasm of steroid secreting cells (McKerns, 1969).

Cholesterol is converted to steroid hormones on the endoplasmic reticulum and in the mitochondria of cells in the adrenal glands, testes, ovaries and placenta. The product released depends on the cell type and hence on the enzymes that are available. Some steroids can be carried in the blood and further modified in other tissues. For example, androgens produced in the adrenals can be converted to oestrogens by ovarian follicles. The sex steroids are largely synthesised in the sex organs whereas corticosteroids, comprising mineralocorticoids (such as aldosterone) and glucocorticoids (such as cortisol and corticosterone), are synthesised respectively in the glomerular and fascicular cells of the adrenal cortex of mammals (Guyton, 1991), or the subcapsular and inner zones of the cortex-like interrenal tissue in bird adrenals (Harvey *et al.*, 1986). It is generally accepted that there is no substantial storage of corticosteroids in the adrenals, and thus the rate of release is a fair indication of the rate of synthesis (Harvey *et al.*, 1986).

In mammals, the principal glucocorticoid is cortisol. This is synthesised via the conversion of cholesterol to pregnenolone, progesterone, 17α-hydroxyprogesterone and 11-deoxycortisol (McKerns, 1969). In birds, the main glucocorticoid is corticosterone. This is synthesised through pregnenolone, progesterone and then 11-deoxycorticosterone or 11β-hydroxyprogesterone (Harvey *et al.*, 1986). The latter pathway involving 11β-hydroxyprogesterone is not as important as the former (Nakamura *et al.*, 1978). Young birds synthesise cortisol and cortisone until around 14 days after hatching, but the reduction in activity of an essential enzyme prohibits further synthesis after this time (Nakamura *et al.*, 1978). Aldosterone is synthesised from corticosterone (Harvey *et al.*, 1986).

3.2.2 Steroid hormone transport and action

All steroid producing glands secrete their products directly into the blood. Steroids are insoluble in blood and so are transported bound to specific binding globulin proteins or non-specific proteins such as albumin. Corticosterone is transported by transcortin (also called corticosteroid-binding globulin, CBG) which is synthesised in the liver (Harvey *et al.*, 1986). The binding capacity of this protein in birds is maximal between 25-56 days of age in fowl and tends to be greater in males (Freeman, 1984). Bound hormone is less biologically active than free hormone and is cleared less rapidly (Harvey *et al.*, 1986). As a consequence, it is important to establish whether bound, free or total steroid is being measured when determining steroid levels using a radioimmunoassay, in order to assess the biological relevance of the measurement.

As a result of their lipid solubility, steroids can permeate any cell membrane, but they are only retained in target organ cells where the appropriate receptor proteins are present (O'Malley and Schrader, 1979). At the target cell, the steroid is bound to a receptor protein in the cytoplasm, forming a steroid-receptor complex and any steroid not immediately bound remains in the blood to be transported to the liver and kidneys for deactivation and excretion (O'Malley and Schrader, 1979). The steroid-receptor complex moves into the nucleus to directly affect gene transcription (O'Malley and Schrader, 1979). Following activation or alteration of gene transcription, the complex may be sequestered in the cytoplasm or nucleus of the target cell indefinitely (O'Malley and Schrader, 1979). This may explain why less steroid is excreted than that injected in radiolabelled steroid metabolism experiments. For example, Helton and Holmes (1973) found that mallard ducks, *Anas platyrhynchos*, excreted around 70% of an injection of radiolabelled corticosterone and Holmes and Slikker (1976) reported a yield of around 54%.

3.2.3 Steroid hormone catabolism

Steroids are deactivated and catabolised by oxidation, reduction, degradation, methylation or conjugation (McKerns, 1969). The main site of steroid deactivation and degradation in birds is the liver (Bojeson and Egense, 1960; Harvey *et al.*, 1986) with the spleen and intestine also being important in some animals (Bojeson and Egense, 1960). Typically, most steroid is conjugated in the liver to glucoronides or sulphates, before excretion (Guyton, 1991). Excretion occurs via the kidneys in urine, or in bile in faeces. The principal route taken, and the resultant products, including altered and unaltered forms of the steroid, are species and steroid dependent. Typically, conjugates are excreted in urine whereas unconjugated forms are excreted in faeces (Lasley and Kirkpatrick, 1994). In general, steroids are excreted largely in urine by mammals (Brown *et al.*, 1994) and birds (Gorsline *et al.*, 1989). For example, Holmes and Slikker (1976) showed that mallard ducks excreted more than 85% of an injection of tritiated corticosterone via the kidneys as conjugated unaltered corticosterone and conjugated and unconjugated metabolites.

3.2.4 Excretion of steroid hormones in faeces

The deactivated steroid or metabolite moves into the blood, and is incorporated into bile in the liver or in the intestine (Hill, 1984). Bile salts in the bile attach to the deactivated steroid making it soluble in water (Stryer, 1988). The steroid then passes with bile into the duodenum or is stored in the gall bladder (Guyton, 1991). Bile is secreted directly into the duodenum or out of the gall bladder in response to food (Guyton, 1991). Bile, incorporating the deactivated steroid, then moves along the intestine, where the solubilising action of bile makes the steroid available to lipolytic enzymes (Stryer, 1988). The bile may be reabsorbed along the small intestine into the enterohepatic circulation and recycled back to the liver, and the steroid may be lipolysed or otherwise modified in the mucosal cells (Helton and Holmes, 1973; Hill, 1984). In chickens, a considerable amount of bile is reabsorbed through the intestine at the jejunum and ileum into the enterohepatic circulation, hence only low levels are generally found in the large intestine and faeces (Hill, 1984).

Steroids and metabolites may be further modified by microbial action as they pass down the intestine; for example conjugated corticosteroids can be hydrolysed through microbial action. As a result, the intestinal microflora may be important in determining both the timing of excretion and

relative amounts of different substances excreted (King and McLelland, 1975). Bile and steroids may also be subject to microbial modification in the caecae, where present. Digesta, and consequently metabolised steroids, reach the caecae through peristalsis (Hill, 1984). If bacterial modification significantly affects steroid excretion profiles, it follows that the timing of peristaltic contractions will affect clearance time thereby affecting the correlation between plasma secretion and faecal excretion.

In birds, digesta passes from the rectum into the most anterior part of the cloaca, the coprodaeum (King and McLelland, 1975). Further modification of the digesta occurs according to the section of intestine involved. For example, droppings from the coprodaeum are a green colour, probably due to a breakdown product of bile, biliverdin, whereas fresh cloacal droppings are usually light brown but rapidly turn dark brown on exposure to the air (Hill, 1984). Moreover, the rectum and the cloaca may be able to absorb water in chickens, thereby modifying dropping consistency (King and McLelland, 1975).

3.2.5 Excretion of steroid hormones in urine

Steroids are excreted in urine largely as soluble conjugated sulphate or glucoronide compounds (Lasley and Kirkpatrick, 1994). The principal product of nitrogen catabolism excreted in urine of mammals is urea, which is soluble in urine. Conversely, birds largely excrete uric acid in a relatively solid form. Birds pass urine through the ureter into the most posterior portion of the cloaca, the urodaeum. In the urodaeum, urine combines with faeces to form droppings (King and McLelland, 1975) hence avian droppings contain both faecal and urinary steroids.

3.2.6 Factors affecting steroid hormone turnover rates

The concentration of a hormone in plasma is a function of the rate of secretion from the endocrine organ and the metabolic clearance rate (Guyton, 1991). The biological half-life of a hormone is the time taken for the plasma concentration of the hormone to decrease by one half. Hence, the half-life is a function of the metabolic clearance rate. The half-life of corticosterone in avian plasma is around 15 minutes (Harvey *et al.*, 1986). As the metabolic clearance rate is itself a function of the excretion of the hormone by the liver or kidneys into bile or urine respectively, catabolism by the tissues and binding to the tissues, it follows that the half-life is affected by these variables. Factors that impinge upon these variables, and hence influence the half-life of steroids

in plasma, include the average proportion of steroid bound to binding proteins and the amount of binding protein available, blood flow rate through clearance organs, sex (Freeman, 1984; for example, the half life of corticosterone in plasma of female rats is approximately 50% less than that of males; Glenister and Yates, 1961) and clearance organ capability. The rate of steroid excretion is normally proportional to plasma concentration (Bojeson and Egense, 1960) and the pattern of excretion generally follows the pattern of secretion (Bojeson and Egense, 1960; Carlstead *et al.*, 1992; Lasley and Kirkpatrick, 1994; Wasser *et al.*, 1997).

3.2.7 The measurement of urinary steroids

Urine is collected from mammals by means of trays or plastic litter, by training animals to urinate into collection vessels or after catheterisation of the ureter or bladder. Collecting urine samples from unanaesthetised or unsedated wild animals is often not possible, so the use of urinary analysis is limited in wild situations. Using these collection methods, sex steroid excretion profiles have been assessed in a number of mammals (Munro *et al.*, 1991; Lasley and Kirkpatrick, 1994; Wasser *et al.*, 1994; Peter *et al.*, 1996). Urinary glucocorticoid levels and responses to stressors have been measured in humans and non-human animals including captive rhesus monkeys (Mason *et al.*, 1968), domestic pigs (Jones-Witters *et al.*, 1975) and domestic and wild cats (Carlstead *et al.*, 1992).

In birds, urine combines with faeces to form droppings in the cloaca, hence it is not possible to collect pure urine samples from birds without surgical alteration such as catheterisation of the ureter in order to collect urine directly (Gorsline *et al.*, 1989). As a result, droppings, rather than uncontaminated urine, are usually collected from free-living birds. The steroids and steroid metabolites found in droppings are commonly referred to as faecal steroids, despite this being strictly incorrect. Urinary excretion of aldosterone (Gorsline *et al.*, 1989) and corticosterone (Daniel and Assenmacher, 1971; Holmes and Slikker, 1976) have been characterised in surgically altered birds. Corticosterone is excreted as conjugated unaltered corticosterone and conjugated and unconjugated metabolites (Holmes and Slikker, 1976). Low levels of metabolites, including 11-dehydrocorticosterone and tetrahydrocorticosterone in ducks, are conjugated as steroid ester sulphate and some may be conjugated as glucoronides (Daniel and Assenmacher, 1971).

3.2.8 The measurement of faecal steroids

Faeces and droppings are relatively easier to collect than urine and as a result have been used extensively in wild animal research. They are collected indirectly from trays or the ground, or directly from the rectum. Two main concerns with sample collection are the length of time between excretion and collection and the extraction method used before assay.

By far the majority of work on faecal steroids has involved sex steroid assessment, primarily as an aid to reproductive monitoring (Lasley and Kirkpatrick, 1994; Peter *et al.*, 1996). Faecal excretion profiles of progesterone and oestrogen have been assessed in many mammalian species (Lasley and Kirkpatrick, 1994; Peter *et al.*, 1996) including yellow baboons (Wasser *et al.*, 1994), domestic cats (Shille *et al.*, 1990; Brown *et al.*, 1994), pigs (Hulten *et al.*, 1995), elk (White *et al.*, 1995) and shiba goats (Hirata and Mori, 1995). Faecal analysis of sex steroids in birds was originally developed to assess the sex of sexually monomorphic birds (Bishop and Hall, 1991). There has been a lot of work on the excretory profiles of sex steroids in a number of species including kakapo (Cockrem and Rounce, 1995), white-crowned sparrows (Lee *et al.*, 1994), domestic fowl (Cockrem and Rounce, 1994), Japanese quail (Bishop and Hall, 1991; Ishii *et al.*, 1994), Japanese ibis' (Ishii *et al.*, 1994), bald eagles (Bercovitz *et al.*, 1982) and brown dippers (Kofuji *et al.*, 1993).

Glucocorticoids have not been extensively measured mammalian faeces (Jurke *et al.*, 1997). Similarly, the measurement of glucocorticoids in bird droppings is a relatively new field in avian endocrinology. Kikuchi *et al.* (1996) reported seasonal changes in faecal corticosterone levels in rock ptarmigan, *Lagopus mutus*, and Wasser *et al*. (1997) demonstrated that free-living northern spotted owls, *Strix occidentalis caurina*, had elevated faecal corticosterone levels in areas of frequent human disturbance. It was also shown that the pattern of corticosterone excretion in the droppings of a captive owl closely resembled the rise in plasma corticosterone in response to a change in enclosure.

3.2.9 Validating non-invasive measures of steroid secretion

In order to validate a non-invasive procedure for steroid assessment, it is important to ensure that the assay is measuring the steroid of interest, and to determine the relationship between both the level of steroid and the pattern of secretion in the plasma and excretion in faeces or urine (Lasley and Kirkpatrick, 1994). To do this, plasma and faecal samples are collected simultaneously

during a natural or artificial elevation of the steroid of interest. It is desirable to chronically alter secretion as this ensures faecal excretion, which lags behind plasma elevation, can be adequately monitored. Steroids are naturally elevated during significant physiological events such as social disruption or pregnancy and this can be measured in faeces, urine and plasma (Munro *et al.*, 1991; Carlstead *et al.*, 1992; Brown *et al.*, 1994; Lasley and Kirkpatrick, 1994; Jurke *et al.*, 1997; Pickard *et al.*, 1998). Bishop and Hall (1991) showed that plasma and faecal testosterone in Japanese quail, *Corturnix corturnix japonica*, both rose in response to exposure to long daylength and were significantly correlated. Wasser *et al.* (1997) showed that faecal and plasma corticosterone levels were elevated after moving a captive Northern spotted owl to a new enclosure.

Artificial elevation is achieved with chronically acting implants or injections of steroid, pituitary polypeptides or hypothalamic releasing hormones. It is well established that corticosterone secretion can be stimulated by exogenous ACTH administration to many birds including parrots (Zenoble *et al.*, 1985a), eagles and condors (Zenoble *et al.*, 1985b), black ducks (Spelman *et al.*, 1995) and chickens (Breitenbach, 1962; Zachariasen and Newcomer, 1974; Davison *et al.*, 1980; Kalliecharan, 1981). For example, Wasser *et al.* (1997) gave 100µl of ACTH intravenously to a northern spotted owl and observed a rise in faecal corticosterone within two hours to 140 ng/g and a peak at 12 hours of 720 ng/g.

The primary aim of the present study was to assess whether there was a similarity in the patterns of corticosterone in plasma and droppings of cockerels treated with ACTH. It was intended that the non-invasive assessment of corticosterone secretion could be used to investigate individual differences in corticosterone secretion and excretion. In particular, the study was designed to address these questions with the following predictions:

1. Do cockerels respond to an intravenous injection of 2 IU/kg ACTH or 5 IU/kg ACTH with an increase in plasma and faecal corticosterone? It was predicted that the levels of corticosterone in plasma and droppings would rise in response to both concentrations of ACTH. The rise in response to 2 IU/kg ACTH was expected to be smaller than the response to 5 IU/kg ACTH.

2. What is the relationship over time between plasma and faecal corticosterone levels? It was thought that the level of corticosterone in plasma would rise immediately in response to ACTH, and the level in droppings would increase after a lag of around one to two hours.

It was predicted that the total amount of corticosterone excreted would be correlated with the magnitude of the plasma corticosterone response to ACTH.

3. Do handling and bleeding after an intramuscular injection of 30 IU/kg ACTH cause droppings to become more liquid? Does the consistency of the dropping affect the measurement of corticosterone in a dropping sample?

3.3 Materials and Methods

Cockerels were given intravenous or intramuscular doses of adrenocorticotropic hormone (ACTH) in two separate experiments. Blood samples were taken at regular intervals in the first experiment and droppings were collected throughout both experiments. The change in dropping consistency was determined and corticosterone levels in the plasma and droppings were compared in order to assess the reliability and practicality of faecal sampling as a non-invasive measure of corticosterone secretion in chickens. All experiments were undertaken with prior approval from the Massey University Animal Ethics Committee.

3.3.1 Animals and Housing

24 layer-breeder light sussex strain cockerels were housed indoors in numbered cages under 24 hour light and ambient temperatures before and during two experiments. They were given free access to layer mash (Massey University Feed Supply Unit) and water. Cockerels were kept in every second cage in two tiers to prevent neighbour-neighbour contact and to facilitate dropping collection.

Four days before the start of each experiment, cockerels were weighed and randomly allocated to one of three groups. The same birds were used for both experiments, but were allocated to different groups each time.

3.3.2 ACTH administration

Individual doses were made up to a total volume of 2 ml and prepared no more than one hour prior to use. Saline was prepared within 24 hours prior to each experiment. Doses comprised sterile isotonic saline alone (0.154 M) or synthetic ACTH (Synacthen, Ciba-Geigy, Switzerland)

diluted in sterile isotonic saline. The groups receiving injections were dosed simultaneously in each experiment, with the first bird of each group being dosed at 0900 and the following birds being dosed at four-minute intervals. The groups were dosed and bled by separate operators.

3.3.3 Blood sampling

Blood samples (0.5-3.0 ml) were taken from the right and left ulnar veins of each cockerel using a heparinised needle (23g x 3/4") and a heparinised 3 ml syringe. All samples, except for three initial samples which were difficult to collect, were taken within no more than three minutes of the bird being picked up in order to minimise the effects of handling on corticosterone levels. Blood was expelled into a heparinised 10 ml plastic test tube. All samples were centrifuged within twenty minutes of collection at 10 000 rpm for five minutes and the plasma drawn off with a Hamilton glass syringe. The plasma was frozen immediately at -20°C in 1 ml plastic titre tubes. The frozen plasma was later transferred without thawing to -70°C for long-term storage.

3.3.3.1 Experiment 1 treatment groups

Three groups of eight cockerels received an intravenous injection into the brachial vein. The first group ('i.v. saline') acted as a control and received 2 ml of isotonic saline only. The second group ('2 IU/kg i.v. ACTH') received 2 IU/kg of ACTH and the third group ('5 IU/kg i.v. ACTH') was given 5 IU/kg of ACTH. All birds were blood sampled immediately after removal from their cages before administration of ACTH (0 hours) and again at 1, 2, 5 and 24 hours after dosing. As the time between the 0 h sample and ACTH dosing was considered to be insignificant in relation to the duration of the response, the time at which the 0 h sample was taken also served as the starting time for dropping collection.

3.3.3.2 Experiment 2 treatment groups

There were three groups in this experiment. The first group ('unhandled') of eight cockerels was not handled for the duration of the experiment. The second group ('i.m. saline'), containing seven cockerels was given 2 ml of isotonic saline alone, injected intramuscularly into the pectoral muscle. The third group of eight cockerels ('30 IU/kg i.m. ACTH') received an intramuscular injection into the pectoral muscle of 30 IU/kg ACTH. Cockerels were not handled after the

injection and were not blood sampled. It was initially intended that the i.m. saline group have eight cockerels. However, one cockerel died before the present experiment commenced, reducing the number of animals available.

3.3.4 Dropping collection

A clean, galvanised aluminium tray was placed under each cage for dropping collection. Individual droppings were collected manually with plastic or metal scrapers into labelled plastic specimen containers. All faecal and liquid material associated with each dropping was collected every time so that the tray was cleaned between droppings. Droppings were frozen within 60 minutes of collection at -20°C and were later transferred without thawing to -70°C for long-term storage.

3.3.4.1 Experiment 1

Droppings were collected from all groups once before saline or ACTH administration and continuously for 24 hours after saline or ACTH administration. The first dropping excreted after 0900 on the day before dosing was collected from each bird and this was used to represent the 0 h pre-treatment sample. On the day of ACTH administration, all droppings were collected individually from each tray as soon as possible after excretion from the time of ACTH injection until 24 hours later (24h). The time of collection of each sample, relative to the 0 h blood sample, was recorded next to the individual identification number of each dropping. As collection was continuous, the time between excretion and collection was considered to be insignificant and the time of collection was used as an estimate of the time of excretion. As it was impractical and inefficient to assay every dropping, they were later grouped according to collection time as shown. All droppings excreted by one bird during one time interval were pooled and treated as a single sample. The time intervals were chosen based on the estimated period of peak corticosterone secretion. There were 16 pools in total as follows:

Pool number	Time (h.min)	Pool number	Time (h.min)
1	0.00-0.20	9	2.41-3.00
2	0.21-0.40	10	3.01-3.30
3	0.41-1.00	11	3.31-4.00
4	1.01-1.20	12	4.01-4.30
5	1.21-1.40	13	4.31-5.00
6	1.41-2.00	14	5.01-6.00
7	2.01-2.20	15	6.01-21.00
8	2.21-2.40	16	21.00-24.00

3.3.4.2 Experiment 2

Collection started at the time the i.m. saline and 30 IU/kg i.m. ACTH groups were dosed (0 h) and finished 12 hours later (12h). All excreted material on each tray was collected at the end of 11 time periods as follows.

Pool number	Time h.min	Pool number	Time h.min
1	0.00-0.30	7	4.01-5.00
2	0.31-1.00	8	5.01-6.00
3	1.01-1.30	9	6.01-7.00
4	1.31-2.00	10	7.01-8.00
5	2.01-3.00	11	8.01-12.00
6	3.01-4.00		

The consistency of each dropping was graded and recorded at the time of collection according to the scale described below.

3.3.5 Extraction of corticosterone from droppings

The extraction procedure was based on that of Shideler *et al.* (1993). The extraction of droppings from each experiment was performed within the same week. Droppings from one to five birds each time were first thawed overnight at 4°C and were held at 4°C when not being processed. Droppings from experiment 1 were then combined together in pools. Pooled droppings were thoroughly stirred with a spatula to create a homogenous mixture. The consistency of each pooled sample was determined according to the following scale:

1. solid
2. soft
3. loose
4. liquid incorporating some solid particles (liquid + solids)
5. liquid

All pooled droppings were then weighed and a subsample of approximately 1 g taken and weighed to ±0.0001g. Two extra subsamples of approximately 1 g were taken from 12 randomly selected droppings in order to crosscheck sample homogeneity. There was close agreement between corticosterone levels in these subsamples to within 10% (coefficient of variation). 5 ml/g of 'faecal buffer' were added. Faecal buffer comprised 800 ml phosphate-buffered saline with gelatine (PBSG; 0.1 M, pH 7.0), 200 ml methanol, 1.25 ml Tween 80 and 175 µl tritiated testosterone corrected to pH 7.0. Methanol was used to increase the percentage of corticosterone recovered from each dropping ('extraction efficiency'). Tween is a surfactant that helps emulsify the dropping. Tritiated testosterone was used to allow estimation of the extraction efficiency, with the concentration of tritiated testosterone in the assay buffer being approximately 700 cpm in 500 µl of buffer. The proportion of 700 cpm recovered gives an estimation of the percentage of corticosterone recovered (the 'extraction efficiency') in each sample. The buffer-dropping mixture was vortexed thoroughly and shaken overnight at room temperature on a Chiltern Scientific SS70 orbital shaker.

The next day, tubes were centrifuged for 30 minutes at 3000rpm in an Heraeus Christ 5000S refrigerated centrifuge. The colour of the supernatant was graded according to the following scale:

1. pale green

2. green
3. dark green
4. pale brown
5. brown
6. dark brown
7. pale yellow
8. yellow
9. colourless.

Dark colours were opaque when held up to a light source. Pale colours were clear and pale relative to normal colours. It was possible to easily distinguish all colours described according to this classification.

A 500 µl aliquot of each extract was removed for measurement of extraction efficiency. The average extraction efficiency was 36.6±0.01%. Two additional 500 µl aliquots were removed from six extracts to determine the agreement between aliquots. There was close agreement between aliquots (± 5% coefficient of variation). Three 100 µl aliquots were removed from each extract for radioimmunoassay of corticosterone. All aliquots and extracts were stored for no longer than three weeks at -70°C until assayed.

3.3.6 Extraction of corticosterone from plasma

Corticosterone levels were measured in extracted chicken plasma by radioimmunoassay. The extraction method used was a modification of that described by Wingfield *et al.* (1992). 300 µl plasma samples were extracted into 2 ml of dichloromethane. The plasma in dichloromethane was shaken for 1 h in a Chiltern Scientific SS70 orbital shaker and centrifuged at 3000 rpm for 10 minutes in an Heraeus Christ 5000S refrigerated centrifuge. A 1.5 ml aliquot of dichloromethane was removed from each and dried at 37°C under a stream of air. Dried extracts were reconstituted in 2 ml of phosphate-buffered saline with gelatine (PBSG; 0.1M, pH 7.0), shaken for 1 h at room temperature and then left overnight at 4°C. 100 µl aliquots were removed into plastic test tubes and assayed immediately or frozen at -20°C. The extraction efficiency, measured using a spike of tritiated corticosterone, was 98.69 ± 1.57% (n=10) for cockerel plasma.

3.3.7 Radioimmunoassay of corticosterone

All extracts were assayed in triplicate. All dropping extracts from one bird were assayed within one assay and all droppings from each bird were randomly distributed throughout six assays. Plasma extracts were randomly distributed throughout three assays.

Reconstituted extracts were incubated with 100 µl of antibody (Dr. R.J. Etches, University of Guelph, Ontario, Canada; 1:18 000 final dilution) and 100 µl of tritiated corticosterone (approximately 5000 cpm; Amersham, UK) at 4°C overnight. Steroid bound to the antibody was separated from unbound steroid by addition of 500 µl of dextran-coated charcoal (2.5 g/l charcoal, 0.25 g/l dextran in PBSG) for 15 minutes at 4°C. Tubes were then centrifuged at 3000 rpm for 15 minutes at 4°C and the supernatant poured off. Separation by addition of dextran-coated charcoal was performed for each triplicate in succession. 3 ml of scintillant (5 g/l PPO, 0.3 g/l dimethyl POPOP in toluene) were added, the samples were shaken for 1 h, left for 1 h at room temperature, and then counted for five minutes in a Wallac 1409-411 liquid scintillation counter.

Serial dilutions of extracted cockerel plasma in assay buffer provided results which were parallel to the corticosterone standard curve (n=4). Recovery of corticosterone added to cockerel plasma was 96.12 ± 3.7% (n=4). The limit of sensitivity (smallest amount of steroid on the standard curve distinguishable from the method blank expressed as corticosterone concentration in plasma) for cockerel plasma was 9.5 pg/ml (0.095 ng/ml). The intra-assay and inter-assay coefficients of variation were 3.87% (n=15) and 15.42% (n=17) respectively.

Serial dilutions of extracted chicken droppings in assay buffer provided results which were not parallel to the corticosterone standard curve (Figure 3.1; n=4) until the extract was diluted at least eight fold. Recovery of corticosterone added to extracted droppings was 30.38 ± 7.2% (n=4), which is unacceptable for the validation of a novel radioimmunoassay. The limit of sensitivity for cockerel droppings was 10.5 pg/ 100µl.

Cross-reactions of the antibody with other steroids were previously determined by Etches (1976). These are deoxycorticosterone (27.9%), cortisol (6.9%), progesterone (37.6%), 11β-hydroxyprogesterone (21.3%) and <5% for oestradiol, testosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, aldosterone and pregnenolone. Cross-reactivity with 11-dehydrocorticosterone was 2.1% (Lewis *et al.*, in press). Avian plasma has only very low levels of cortisol and deoxycorticosterone has not been reported (Etches, 1976). Progesterone is poorly extracted in dichloromethane and 11β-hydroxyprogesterone is an unstable intermediate in avian adrenal

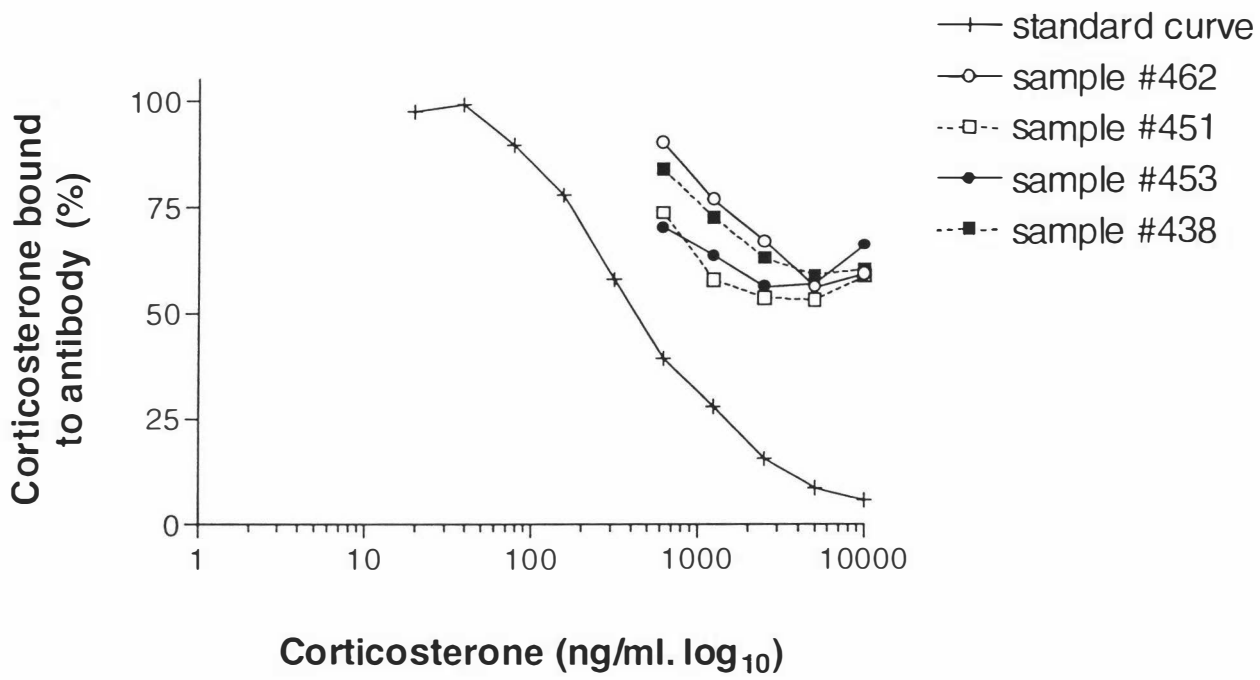


Figure 3.1: Plot of four extracted cockerel dropping samples serially diluted with normal PBSG buffer calculated against a normal PBSG buffer standard curve to validate radioimmunoassay using parallelism.

steroid biosynthesis (Wingfield *et al.*, 1992), thus corticosterone is the main steroid measured in this assay.

3.3.8 Calculation of area under the corticosterone response curve

Corticosterone levels at each sampling time and the area under the corticosterone response curve were used to quantify individual variation in corticosterone secretion. The area under the corticosterone response curve was determined by numerical integration using MathCad (MathSoft Inc., Massachusetts). Both the total area (total integrated response) and the total area minus the area attributable to basal corticosterone levels (corrected area or corrected integrated response) were calculated (Figure 3.2). The total integrated response is calculated from areas ***a*** plus ***b***. The corrected integrated response is equivalent to area ***a***. Cockerels with incomplete data sets were omitted from these calculations.

3.3.9 Statistical analyses

Statistical analyses and graphing were performed using GraphPad Prism version 2.01 (1996; GraphPad Software Inc.) Relationships between non-normal data were investigated using Spearman rank correlations. Relationships between normal or tied data were analysed using Pearson correlations. Comparisons between group means were performed using a non-parametric test, the Kruskal-Wallis one-way ANOVA, as data did not appear to be normally distributed and some sample sizes were small and variable. Mann-Whitney U tests were used as a *post hoc* test to compare groups with the highest and lowest medians after Kruskal-Wallis analyses. Differences between mean extraction efficiencies and proportions of corticosterone bound to antibody in relation to different temperatures and durations of shaking during extraction were analysed using a two-way ANOVA. Chi-square tests were used to investigate the association between treatment and faecal consistency. Data were normalised for the analysis of the effects of faecal consistency and extract colour on the efficiency of corticosterone extraction using a log transformation of data ($x = \log_{10}x$). Differences between group means were then analysed using one-way ANOVAs.

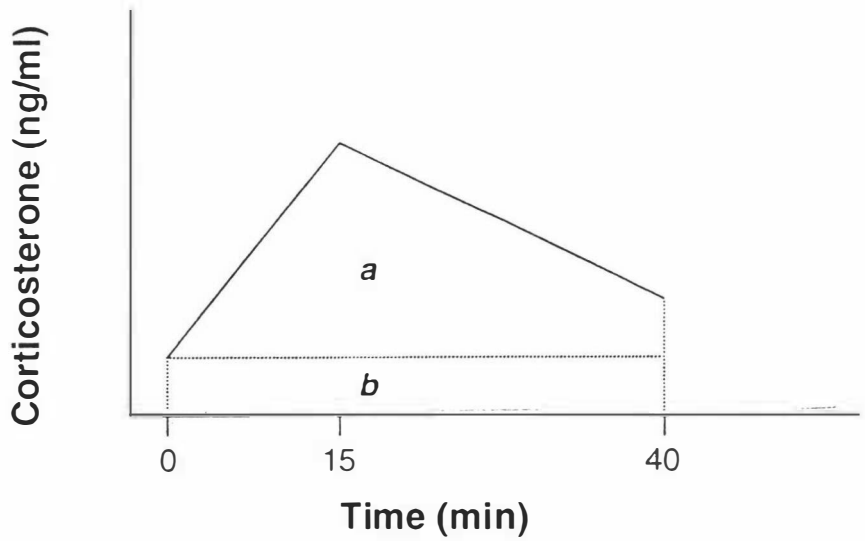


Figure 3.2: Diagram of the total and corrected areas under the corticosterone versus time curve. Total area under the curve = *a* + *b*; corrected area under the curve = *a*.

3.4 Results

3.4.1 Experiment 1: the relationship between plasma and faecal corticosterone secretion

3.4.1.1 Plasma corticosterone

There was no significant relationship between the time taken to collect each sample and the corticosterone level in the first blood sample (Figure 3.3; Pearson correlation; r^2 =0.019, P=0.543) or between the order of bleeding and the corticosterone level in the first sample (Figure 3.4; Spearman rank correlation; r^s=-0.315, P=0.154). Only three samples from the experiment took longer than three minutes to collect. All were time 0 samples from birds given ACTH. These were not discarded as there was no effect of time on basal corticosterone levels and the increase in corticosterone secretion due to ACTH would have been greater than the increase due to the time taken to bleed the birds.

All birds had low basal plasma corticosterone levels initially (ranges, i.v. saline: 0.66-2.69 ng/ml; 2 IU/kg i.v. ACTH: 1.10-1.50 ng/ml; 5 IU/kg i.v. ACTH: 0.76-2.70 ng/ml) and there was no significant difference in basal plasma corticosterone levels between groups (Figure 3.5; Kruskal-Wallis one-way ANOVA; K=0.540, P=0.763). Three of the seven birds in the i.v. saline group showed a slight rise in plasma corticosterone levels to 3.01, 3.03 and 7.54 ng/ml but the remainder had uniformly low levels of 1.57–2.15 ng/ml (Figure 3.6). Cockerels injected with ACTH responded with a rise in plasma corticosterone levels within 1 hour to peaks of 4.54 – 8.25 ng/ml for group 2 IU/kg i.v. ACTH (Figure 3.7) and 7.14-16.02 ng/ml for group 5 IU/kg i.v. ACTH (Figure 3.8). The peak level of corticosterone differed significantly between groups (Kruskal-Wallis one-way ANOVA; K=15.12, P<0.001) with 5 IU/kg i.v. ACTH birds having significantly higher median peak corticosterone levels than i.v. saline birds (Mann-Whitney U test; U=1.0, P<0.001).

All levels had returned to basal by two hours and a paired t-test showed no difference between mean plasma corticosterone for all three groups combined at 0 h or 24 h (t= 0.552, P=0.588, df=18). There was also a significant effect of treatment on the total and corrected integrated responses (Figure 3.9; Kruskal-Wallis one-way ANOVA; total integrated response, K=7.088, P=0.017; corrected integrated response, K=6.280, P=0.021) with 5 IU/kg i.v. ACTH birds having

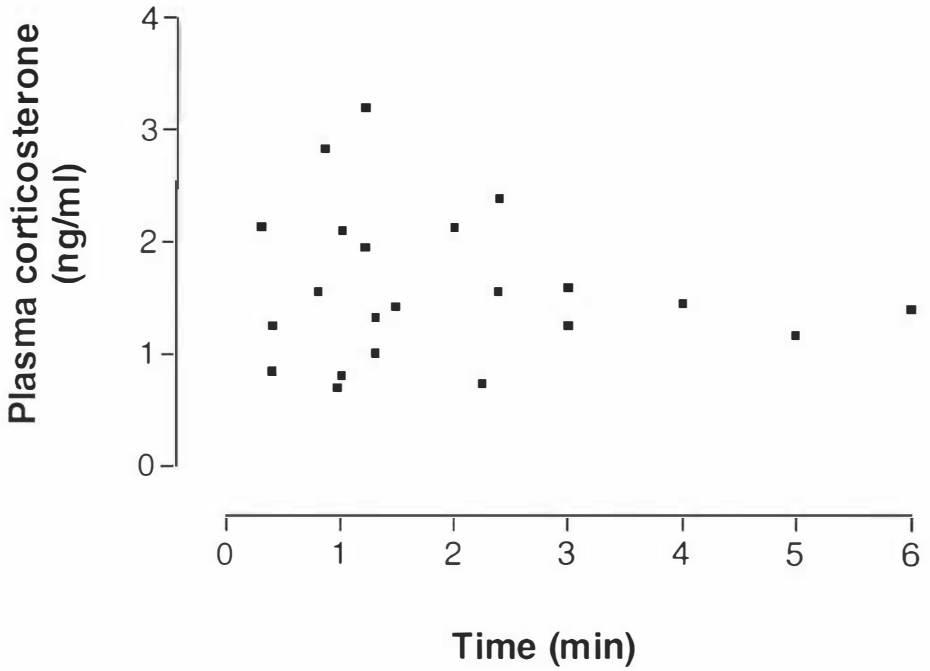


Figure 3.3: Relationship between basal plasma corticosterone levels and time from first disturbance of cockerels to completion of blood sample.

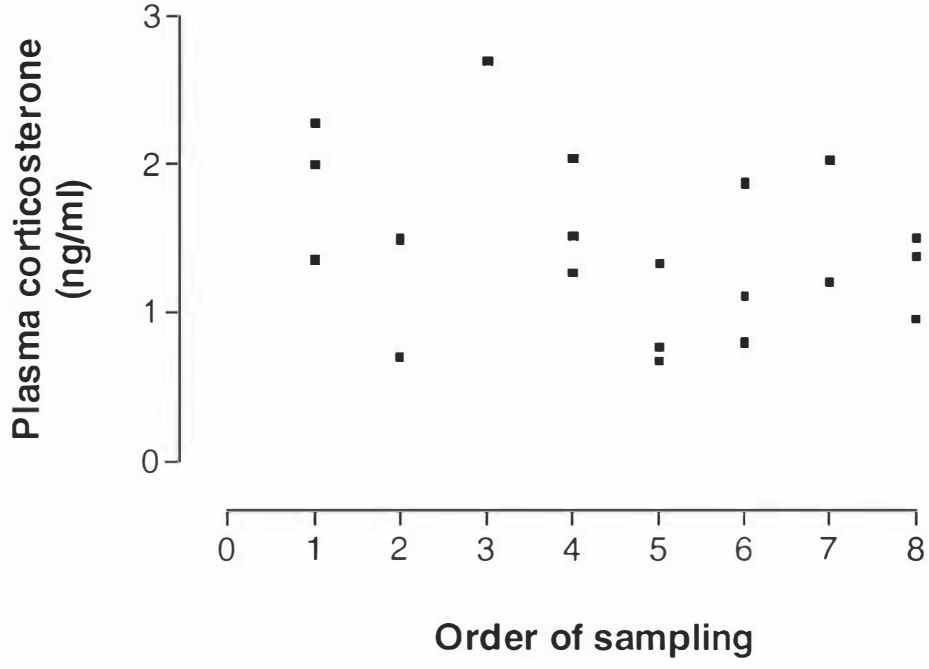


Figure 3.4: Relationship between basal plasma corticosterone levels and the order in which cockerels were blood sampled.

significantly higher median integrated responses than i.v. saline birds (Mann-Whitney U test; total integrated response, U=4.0, P=0.019; corrected integrated response, U=0.0, P=0.017).

3.4.1.2 Faecal corticosterone

Faecal corticosterone was initially low in all groups (range of 0 h corticosterone levels; saline: 1.22 – 1.63 ng/g; 2 IU/kg ACTH: 0.84 – 1.67; 5 IU/kg ACTH: 0.13 – 3.96) and there was no difference between groups in corticosterone levels on the day before injection (Kruskal-Wallis one-way ANOVA; K=2.362, P=0.321). Three birds given 5 IU/kg i.v. ACTH responded with a discrete peak in secretion (Figure 3.8) so that the highest mean level reached was slightly greater than the maximum of either i.v. saline or 2 IU/kg i.v. ACTH cockerels (Figure 3.5). However, there was no significant effect of treatment on the peak level of corticosterone of any group (Kruskal-Wallis one-way ANOVA; K=4.620, P=0.099). Moreover, total and corrected integrated faecal corticosterone responses did not differ between groups (Figure 3.9; Kruskal-Wallis one-way ANOVA; total integrated response, K=2.450, P=0.294; corrected integrated response, K=0.503, P=0.793).

The total amount of excreted corticosterone was calculated by multiplying the level of corticosterone in each sample in ng/g by the total wet weight of the dropping and then summing individual values for each bird and for each pooled time period. There was no significant difference between groups in total corticosterone excreted (Figure 3.9; Kruskal-Wallis one-way ANOVA; K=2.540; P=0.281) despite birds given 5 IU/kg ACTH having slightly higher levels (10.08-148.10 ng) than either saline (20.33-101.80 ng) or 2 IU/kg ACTH (11.91-126.30 ng) treated birds.

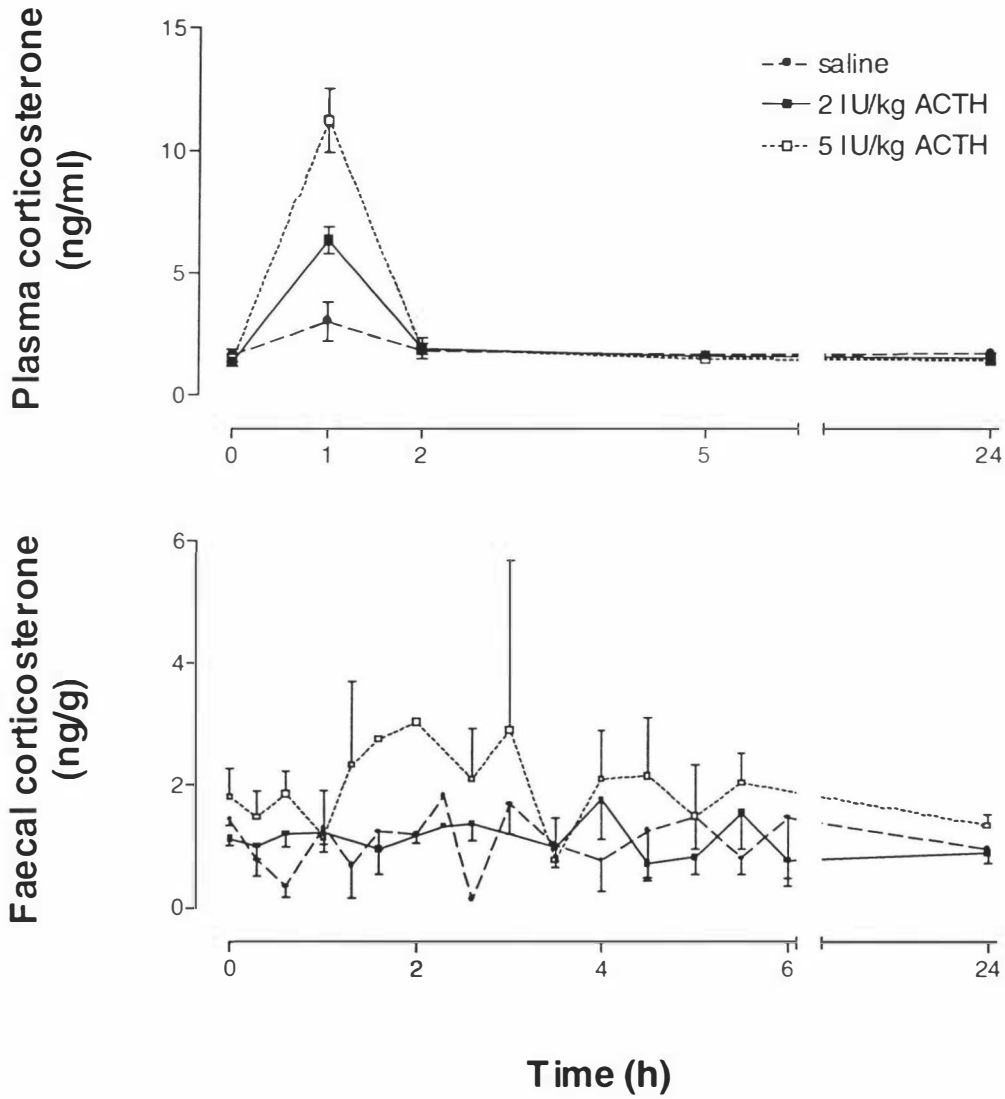


Figure 3.5: Plasma and faecal corticosterone responses of cockerels to intravenous administration of isotonic saline, 2 IU/kg ACTH or 5 IU/kg ACTH at 0 h (mean ± SEM).

Figure 3.6: Individual plasma and faecal corticosterone responses of cockerels to intravenous administration of isotonic saline at 0 h. *Numerals represent the individual identification number of each cockerel.

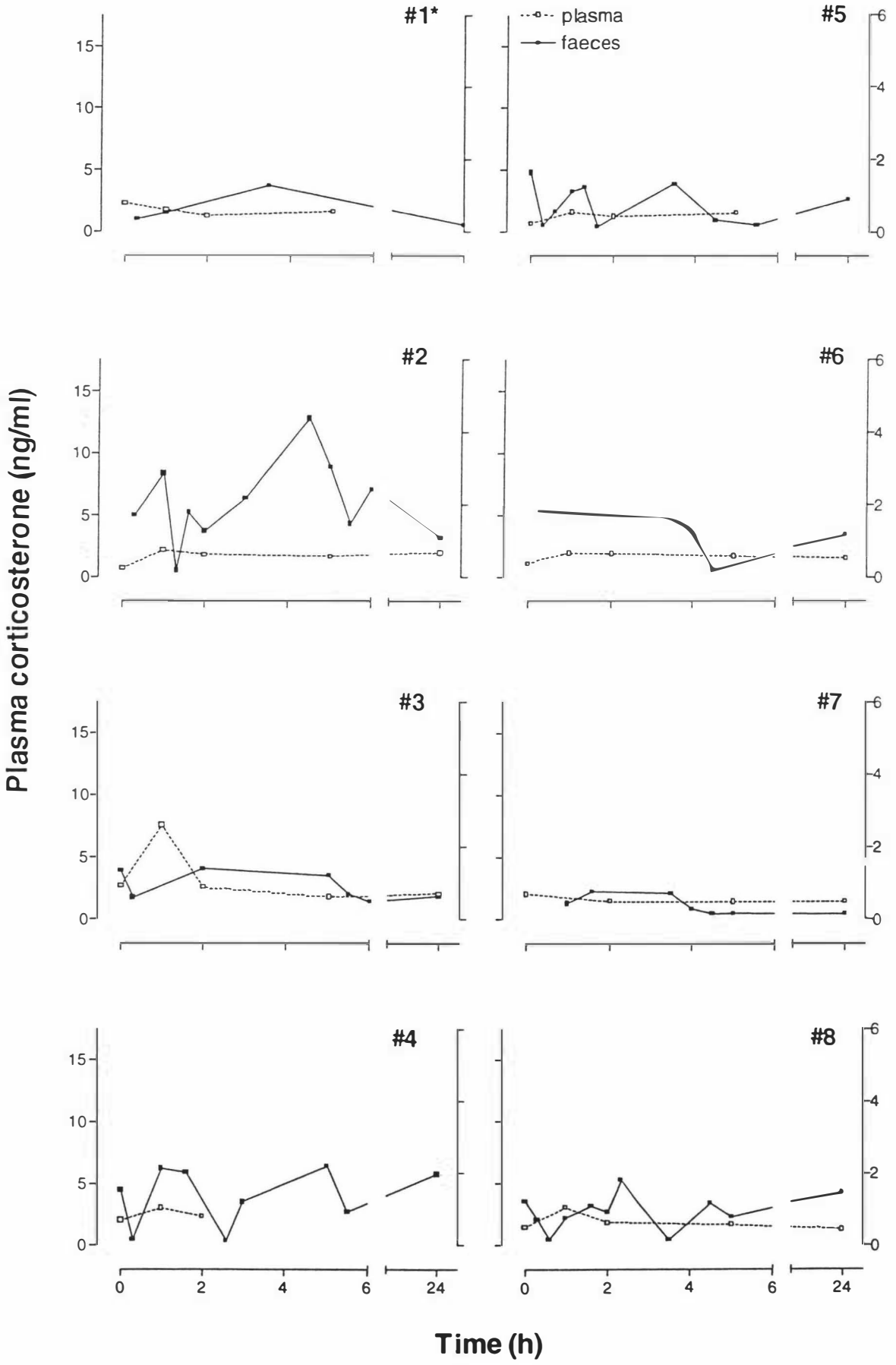

#1*
plasma
faeces
#5
#2
#6
#3
#7
#4
#8
Plasma corticosterone (ng/ml)
Faecal corticosterone (ng/g)
Time (h)
0
2
4
6
24
0
5
10
15
0
2
4
6

Figure 3.7: Individual plasma and faecal corticosterone responses of cockerels to intravenous administration of 2 IU/kg ACTH at 0 h. *Numerals represent the individual identification number of each cockerel.

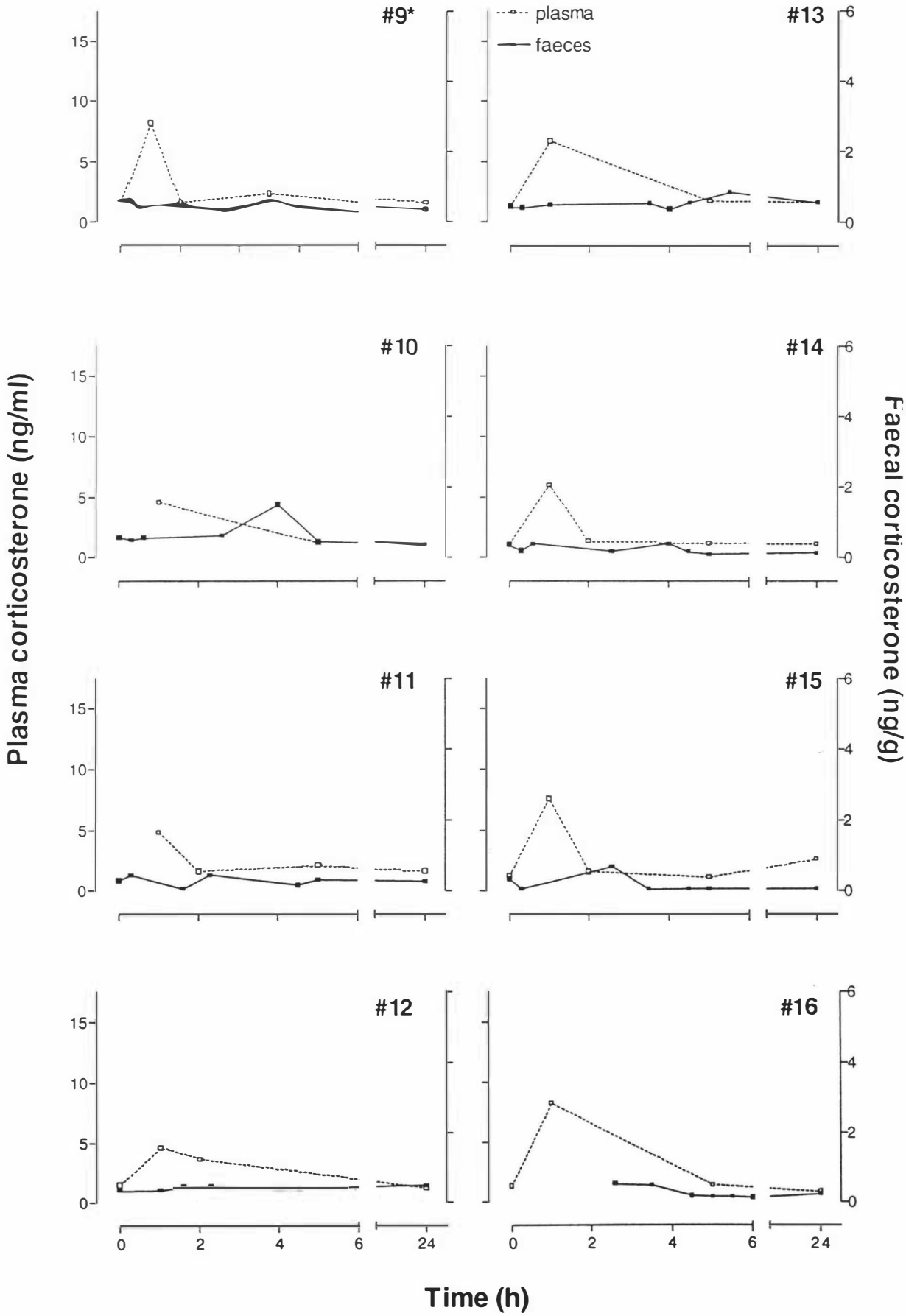
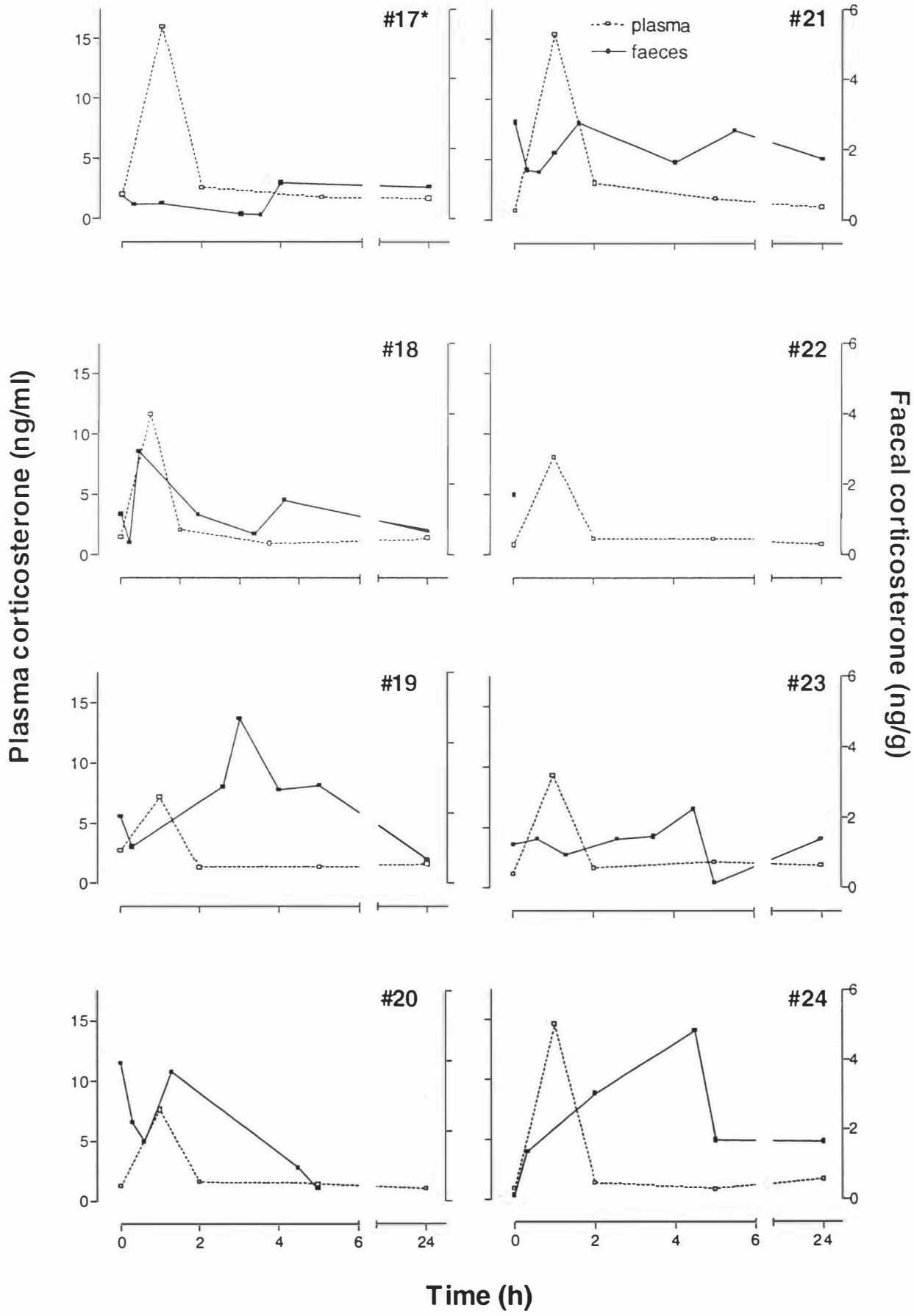

#9*
plasma
faeces
#13
#10
#14
#11
#15
#12
#16
Plasma corticosterone (ng/ml)
Faecal corticosterone (ng/g)
Time (h)
0
2
4
6
24
5
10
15

Figure 3.8: Individual plasma and faecal corticosterone responses of cockerels to intravenous administration of 5 IU/kg ACTH at 0 h. *Numerals represent the individual identification number of each cockerel.


#17*
#21
#18
#22
#19
#23
#20
#24
plasma
faeces
Plasma corticosterone (ng/ml)
Faecal corticosterone (ng/g)
Time (h)
0
2
4
6
24
0
5
10
15
0
2
4
6

3.4.1.3 Relationship between plasma and faecal corticosterone

Faecal corticosterone excretion did not follow the same general pattern as plasma corticosterone secretion (Figure 3.5). Furthermore, there was no correlation between either total integrated plasma and total integrated faecal corticosterone responses (Figure 3.10; Spearman rank correlation; r^s=0.216, P=0.322), or between corrected integrated plasma and corrected integrated faecal corticosterone responses (Figure 3.10; Spearman rank correlation; r^s=0.085, P=0.754).

3.4.1.4 Faecal consistency

Droppings were graded for consistency after being mixed into pooled samples according to time period. A higher grade indicated a more liquid dropping. Droppings from all cockerels were more liquid and therefore of a higher grade, after handling and injection (Figure 3.11) than on the day before injection (0 h). In general, 5 IU/kg i.v. ACTH cockerels had more liquid droppings with a greater number of soft and loose droppings than birds in the other groups (Table 3.1). The association between treatment and faecal consistency was significant (Chi-square test; df=12.77,4, P=0.012).

3.4.2 Experiment 2: the effect of blood sampling on faecal consistency

3.4.2.1 Faecal corticosterone

Birds given 30 IU/kg ACTH i.m. responded with a clear increase in faecal corticosterone to higher peak levels (0.933 – 3.275 ng/g) than either unhandled (0.303 – 1.530 ng/g) or i.m. saline cockerels (0.135 – 1.211 ng/g; Kruskal-Wallis one-way ANOVA; K=11.70, P=0.003; Figures 3.12 – 3.15). The high level of faecal corticosterone in the ACTH-treated birds was sustained throughout the experiment and corticosterone levels at 12 h differed significantly between groups (Kruskal-Wallis one-way ANOVA; K=7.133, P=0.028). As a result, both total and corrected integrated responses (Figure 3.16; Kruskal-Wallis one-way ANOVA; total integrated response, K=10.73, P=0.0047; corrected integrated response, K=7.875, P=0.020) and total excreted corticosterone (Figure 3.16) differed significantly between groups (Kruskal-Wallis one-way ANOVA; K=9.583, P=0.008).

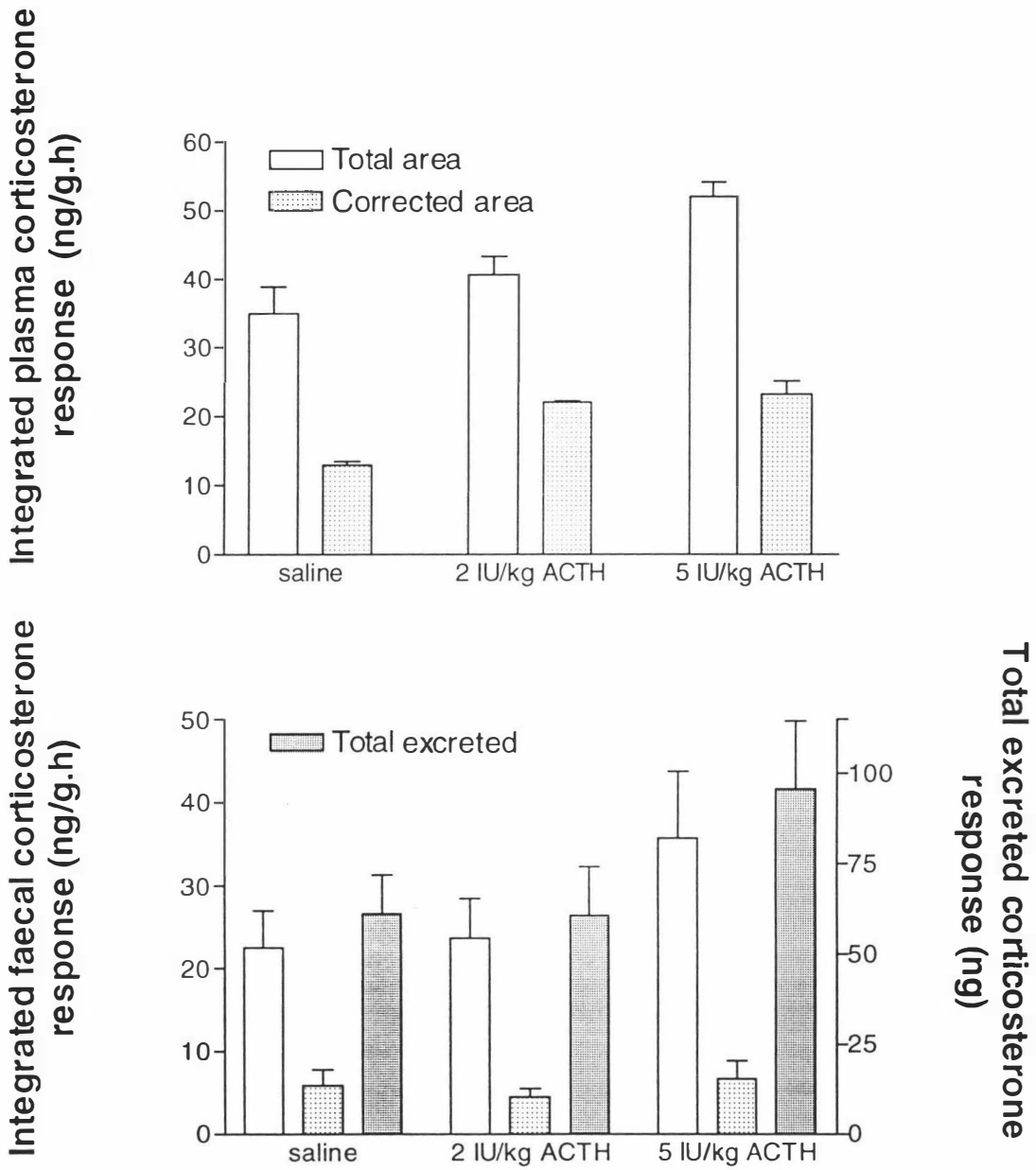


Figure 3.9: Integrated plasma and faecal corticosterone responses and total excreted corticosterone of cockerels given intravenous injections of isotonic saline, 2 IU/kg ACTH or 5 IU/kg ACTH (mean ± SEM).

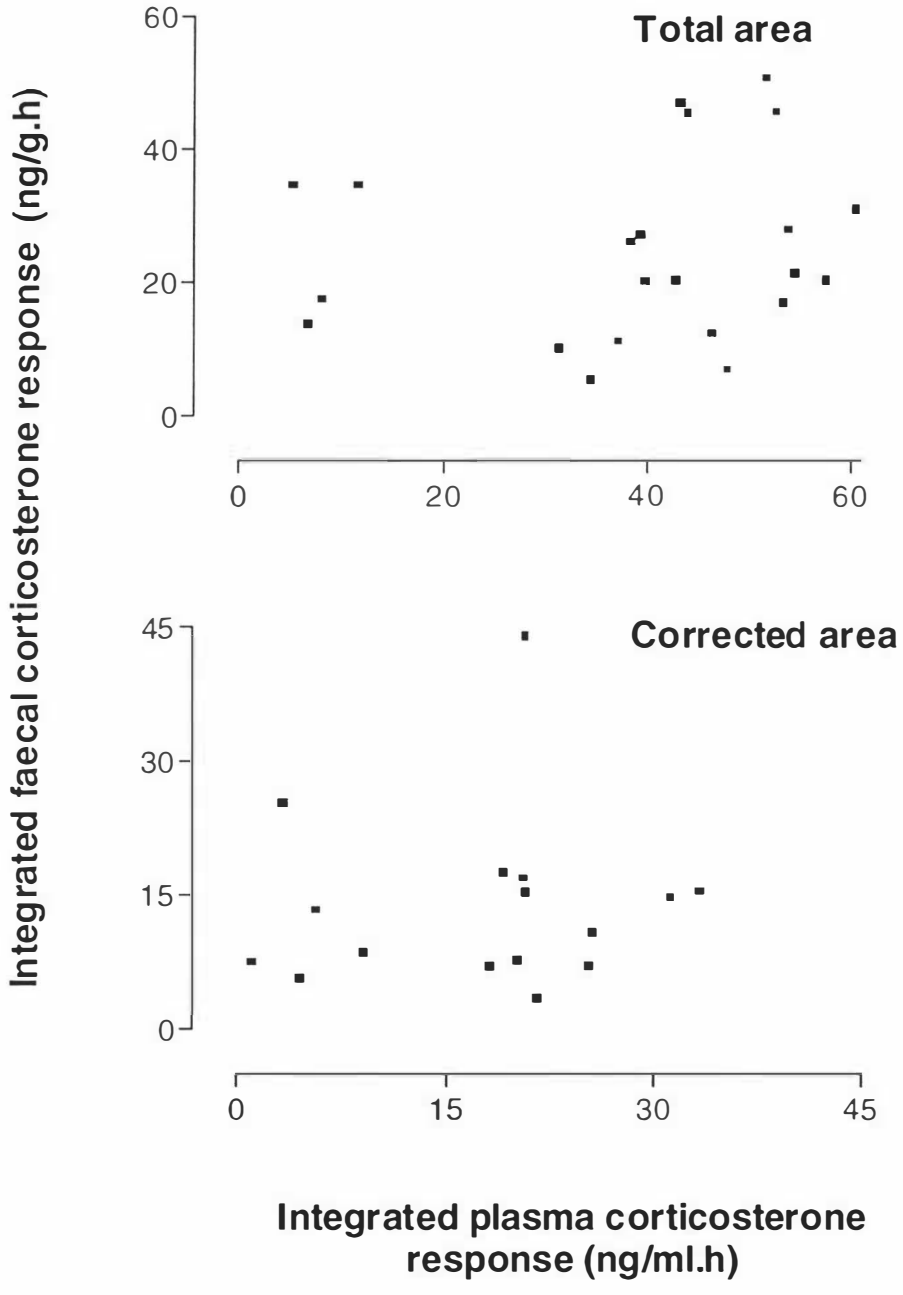


Figure 3.10: Relationship between total integrated plasma and faecal corticosterone responses of cockerels to intravenous administration of isotonic saline, 2 IU/kg ACTH or 5 IU/kg ACTH at 0 h. Note different scales on y-axes.

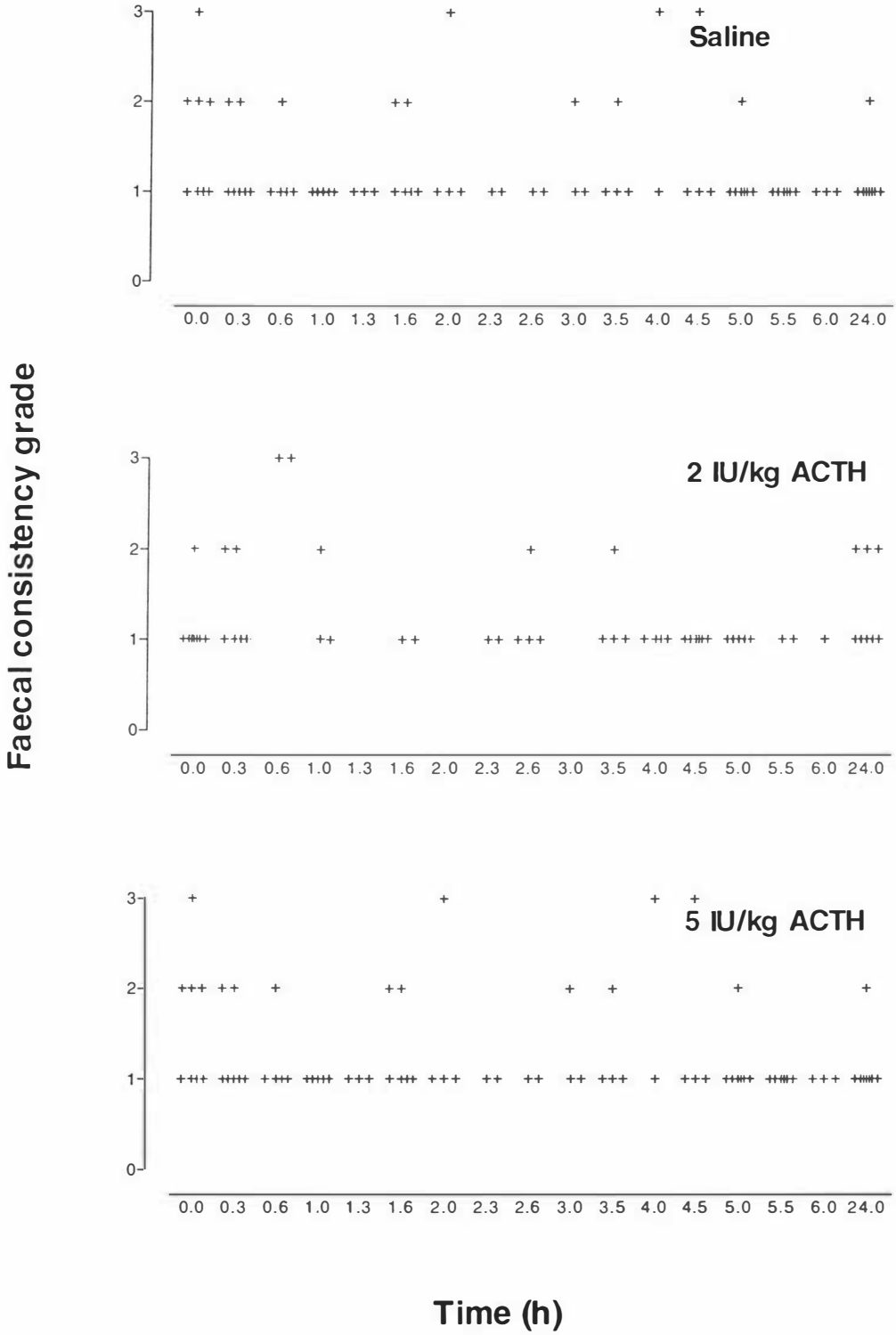


Figure 3.11: Change in faecal consistency over time of cockerels after intramuscular administration of isotonic saline, 2 IU/kg ACTH or 5 IU/kg ACTH at 0 h. Grade 1: solid; 2: soft; 3: loose.

Group	Consistency grade		
	solid	soft	loose
saline	76.83	18.29	4.88
2 IU/kg ACTH	80.70	15.79	3.51
5 IU/kg ACTH	55.38	30.77	13.85

Table 3.1: Percentage of droppings of each faecal consistency grade for each group in experiment 1.

A Mann-Whitney U test showed that the median corticosterone response values of i.m. saline cockerels differed significantly from those of 30 IU/kg i.m. ACTH cockerels (total integrated faecal corticosterone response, U=3.0, P=0.002; corrected integrated faecal corticosterone response, U=4.0, P=0.007; total excreted corticosterone, U=6.0, P=0.009).

3.4.2.2 Faecal consistency

Droppings were graded for consistency as they were collected in order that a finer grading scheme than that used in experiment 1 could be applied. As in the previous experiment, droppings became more liquid after birds were injected. In general, cockerels given 30 IU/kg i.m. ACTH had more liquid droppings than birds in other groups (Figure 3.17). There were relatively fewer solid droppings and more soft, loose, liquid + solids and liquid droppings collected from this group than other groups (Table 3.2). The association between treatment and faecal consistency was very highly significant (Chi-square test; df = 104.8,8, P<0.0001).

3.4.3 Effect of faecal consistency and extract colour on extraction efficiency

There was a significant difference in extraction efficiency between faecal consistency grades (Figure 3.18; One-way ANOVA on log-transformed data; F=40.85, P<0.0001). However, there was no difference in extraction efficiency between extract colour grades (Figure 3.19; One-way ANOVA on log-transformed data; F=1.386, P=0.227).

3.4.4 Effect of ambient temperature and duration of shaking on extraction efficiency and percentage of radiolabelled corticosterone bound to antibody during radioimmunoassay

The purpose of the extraction procedure is to remove corticosterone from the dropping and bring it into solution with the buffer. The buffer-corticosterone solution ('extract') is then put through a radioimmunoassay. The extract is incubated with a known amount of antibody specific to corticosterone and a known amount of radiolabelled corticosterone. Provided there is too little antibody to react with all of the corticosterone in the incubation, the unlabelled corticosterone in the extract competes with the radiolabelled corticosterone for the antibody. The percentage of each that binds to the antibody is proportional to their concentration.

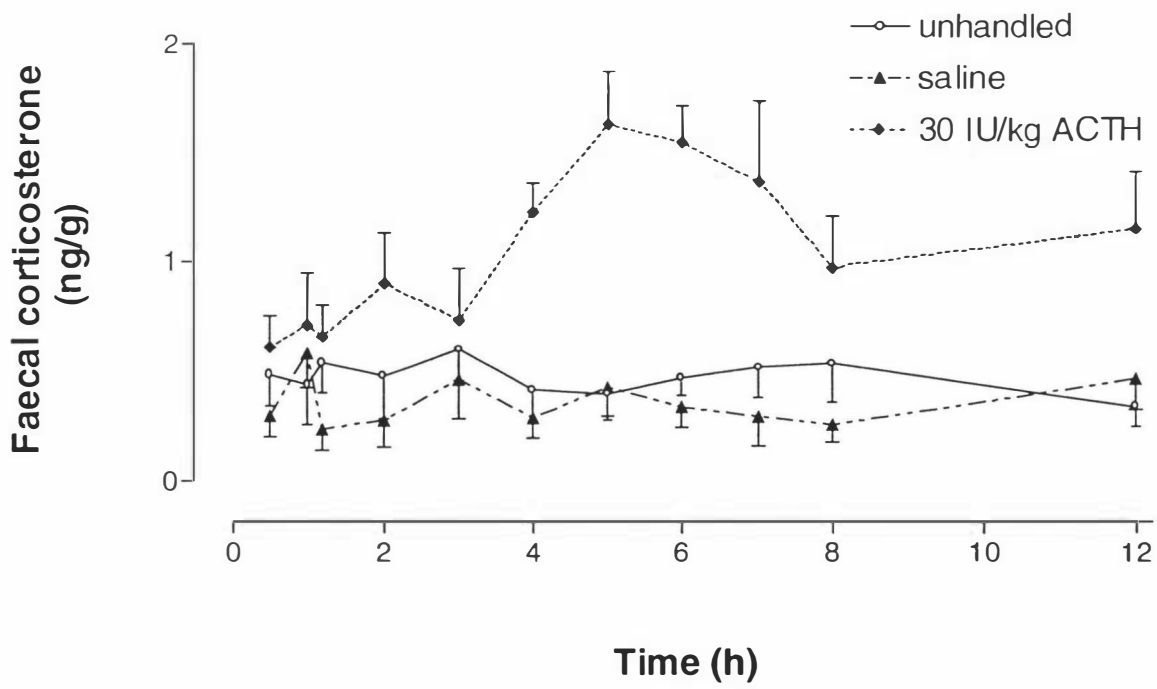


Figure 3.12: Change in faecal corticosterone levels over time of unhandled cockerels or of cockerels given intramuscular injections of isotonic saline or 30 IU/kg ACTH at 0 h (mean ± SEM).

Figure 3.13: Individual changes in faecal corticosterone over time of unhandled cockerels. *Numerals represent the individual identification number of each cockerel.

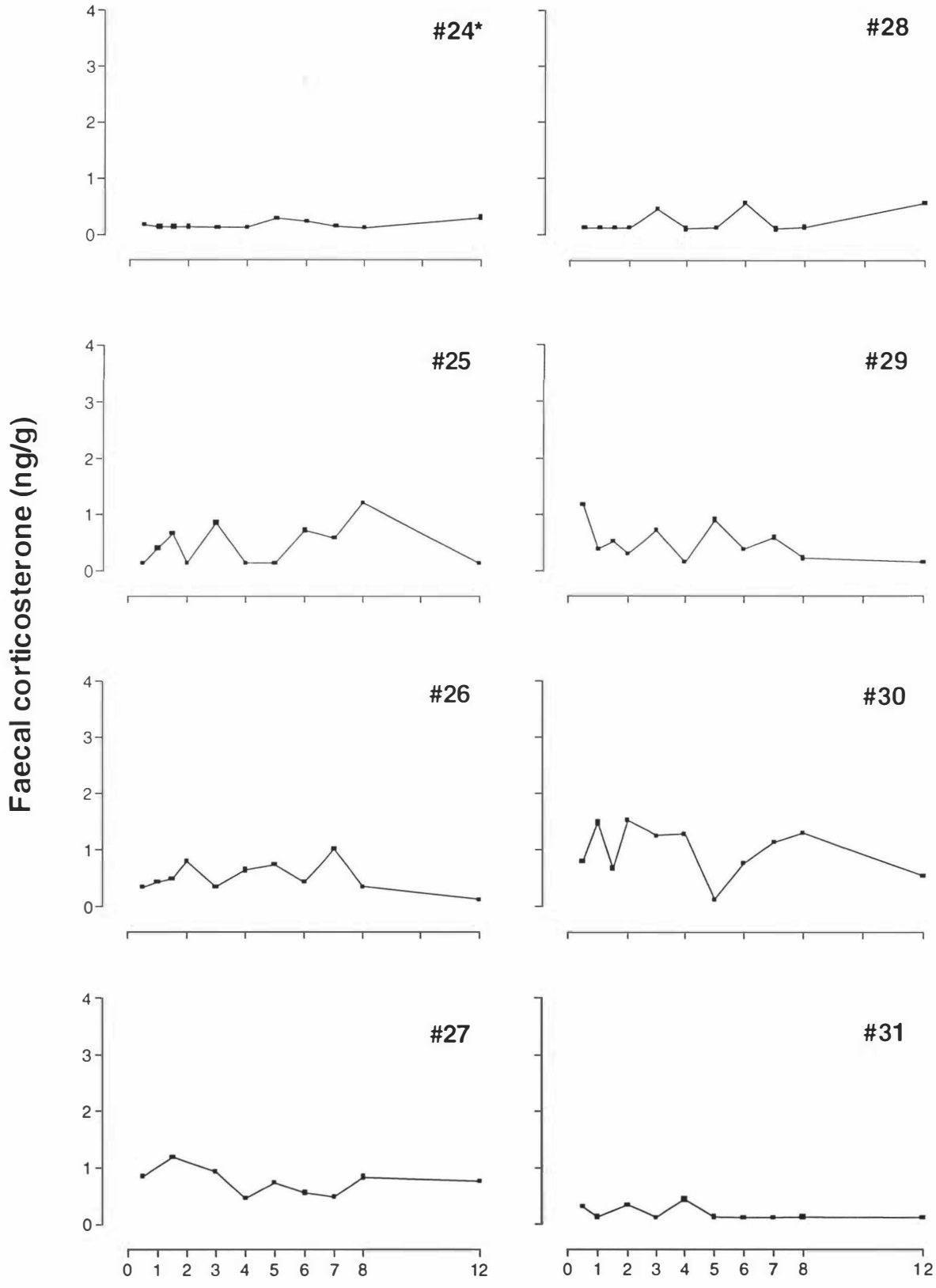

#24*
#28
#25
#29
#26
#30
#27
#31
Faecal corticosterone (ng/g)
Time (h)
0
1
2
3
4
5
6
7
8
12

Figure 3.14: Individual faecal corticosterone responses of cockerels to intramuscular administration of isotonic saline at 0 h. *Numerals represent the individual identification number of each cockerel.

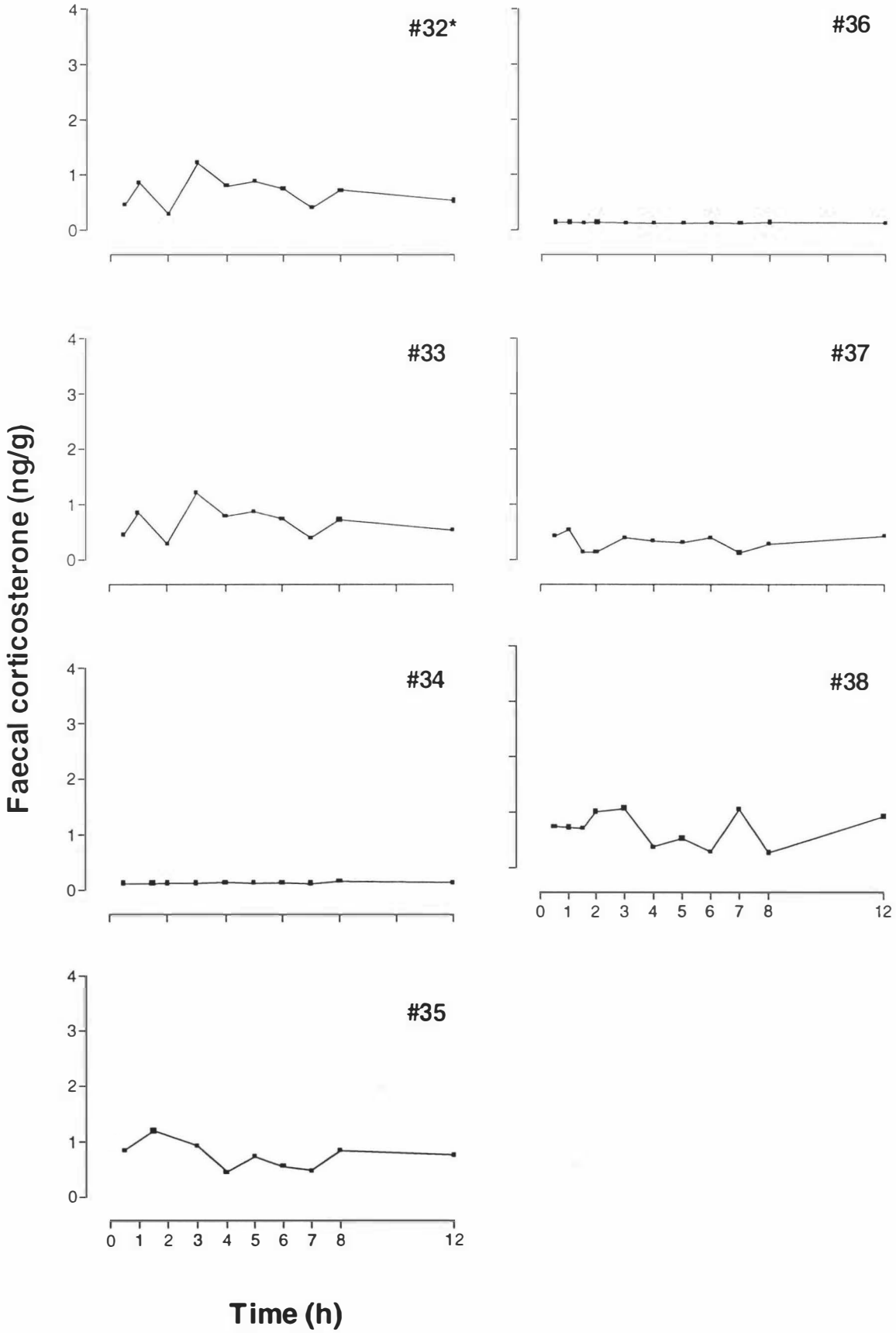
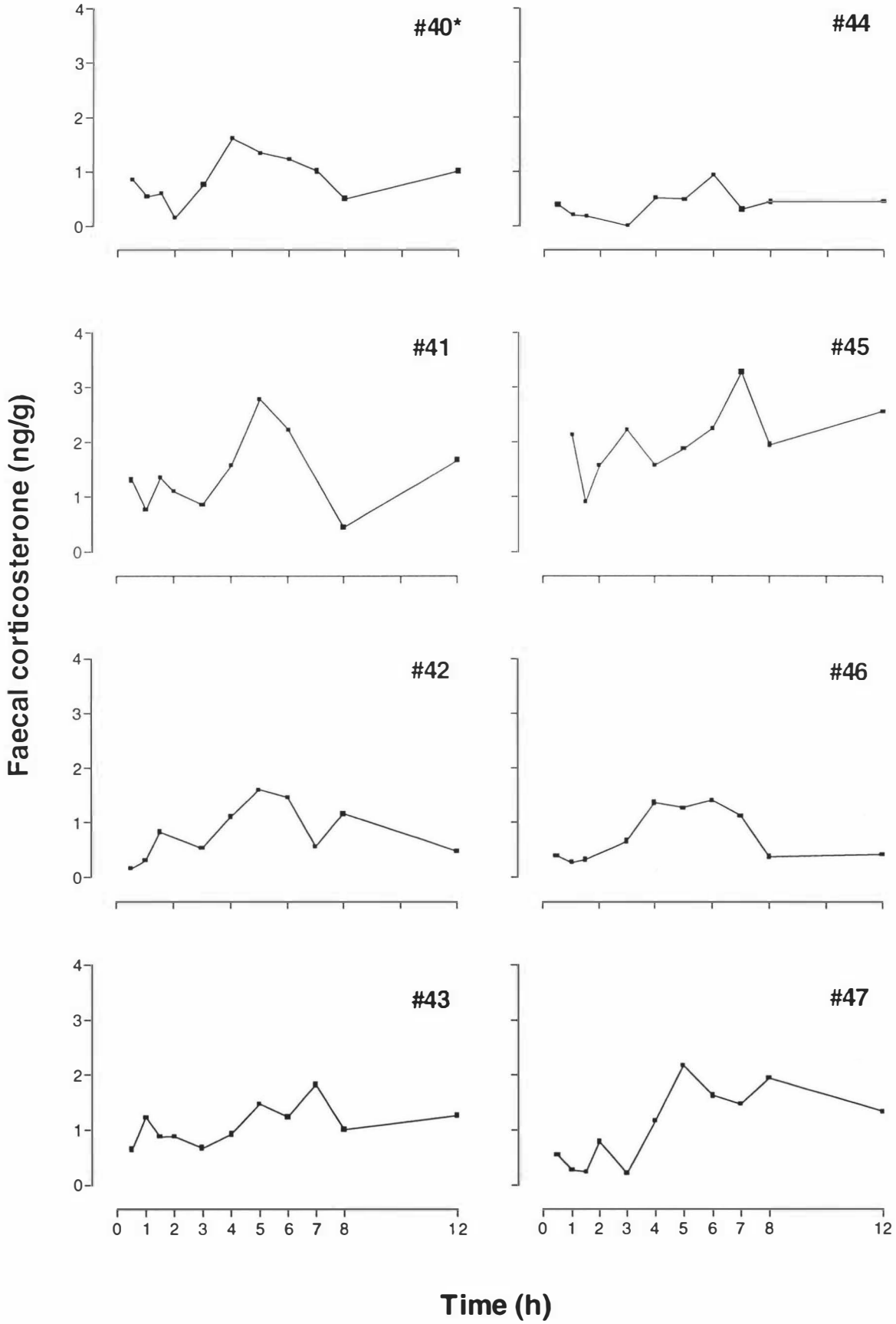

#32*
#36
#33
#37
#34
#38
#35
Faecal corticosterone (ng/g)
Time (h)
0
1
2
3
4
5
6
7
8
12

Figure 3.15: Individual faecal corticosterone responses of cockerels to intramuscular administration of 30 IU/kg ACTH 0 h. *Numerals represent the individual identification number of each cockerel.


#40*
#44
#41
#45
#42
#46
#43
#47
Faecal corticosterone (ng/g)
0
1
2
3
4
0 1 2 3 4 5 6 7 8 12
Time (h)

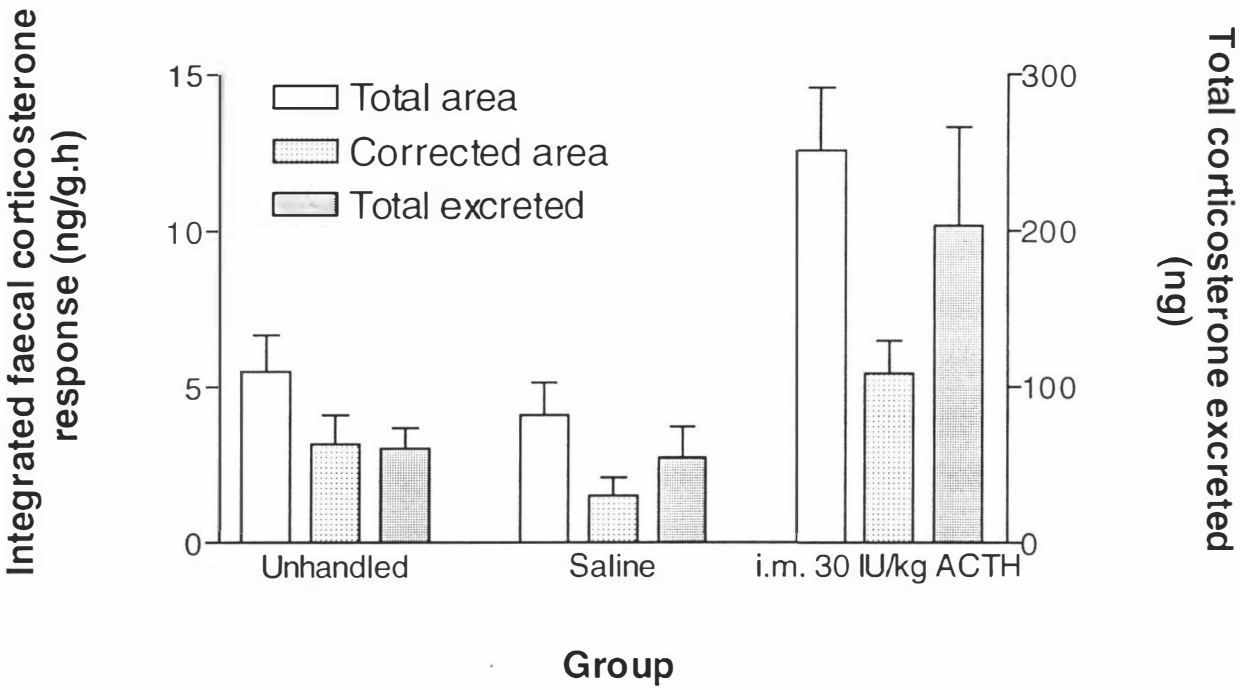


Figure 3.16: Integrated faecal corticosterone responses of unhandled cockerels or of cockerels given intramuscular injections of isotonic saline or 30 IU/kg ACTH at 0 h (mean ± SEM).

The concentration of corticosterone in the extract is therefore determined by measuring the relative amount of radioactivity left after the bound and unbound corticosterone are separated. The smaller the percentage of radiolabelled corticosterone bound to the antibody and hence the smaller the amount of radiation measured in counts per minute (cpm), the larger the percentage of endogenous corticosterone in the buffer extract, and vice versa. A standard curve is produced by incubating known amounts of corticosterone from a serial dilution with antibody and radiolabelled corticosterone. From this curve, the proportion of corticosterone bound to the antibody can be converted into the concentration.

It was thought that corticosterone might be adsorbed onto the plastic tubes used for extracting droppings during the overnight shaking process, or that the temperature during extraction might affect the extraction efficiency. This was tested by measuring extraction efficiency and the proportion of radiolabelled corticosterone bound to antibody after 1, 6 or 15 hours of shaking during the normal extraction procedure at either warm (around 20-25°C) or cool temperatures (around 4°C). Three droppings were each divided into six subsamples and each subsample was submitted to one combination of treatments according to a latin square design. All subsamples were assayed in the same assay. It was intended that the process would be repeated if an effect of treatment was found. Times were chosen on the basis that one hour was a shortest possible period with which to compare the longer periods, six hours would be an average time a sample could be left to shake during the day while still allowing the entire process to be completed in one day and 15 hours was the average duration of overnight shaking during a normal extraction. The proportion of radiolabelled corticosterone bound to antibody was used as a measure of the level of corticosterone. The concentration of corticosterone in each extract was not used because the samples had been thawed, extracted and assayed previously and the corticosterone had degraded to very low levels that could not be read on the standard curve.

There was a highly significant effect of duration of shake during extraction on the proportion of corticosterone bound to antibody (Figure 3.20) such that the percentage bound decreased (and hence corticosterone concentration increased) as the duration of shaking increased (two-way ANOVA; F=7.35, P=0.008, df=2,12). There was no effect of temperature (F=1.11, P=0.314, df=1,12) and no interaction between these two variables (F=2.48, P=0.125, df=2,12). There was also a highly significant effect of duration of shake on the extraction efficiency (Figure 3.20; two-way ANOVA; F=7.57, P=0.008, df=2,12) and an effect of temperature (F=27.07, P<0.001, df=1,12). There was no significant interaction between these two variables (F=0.45, P=0.648, df=2,12).

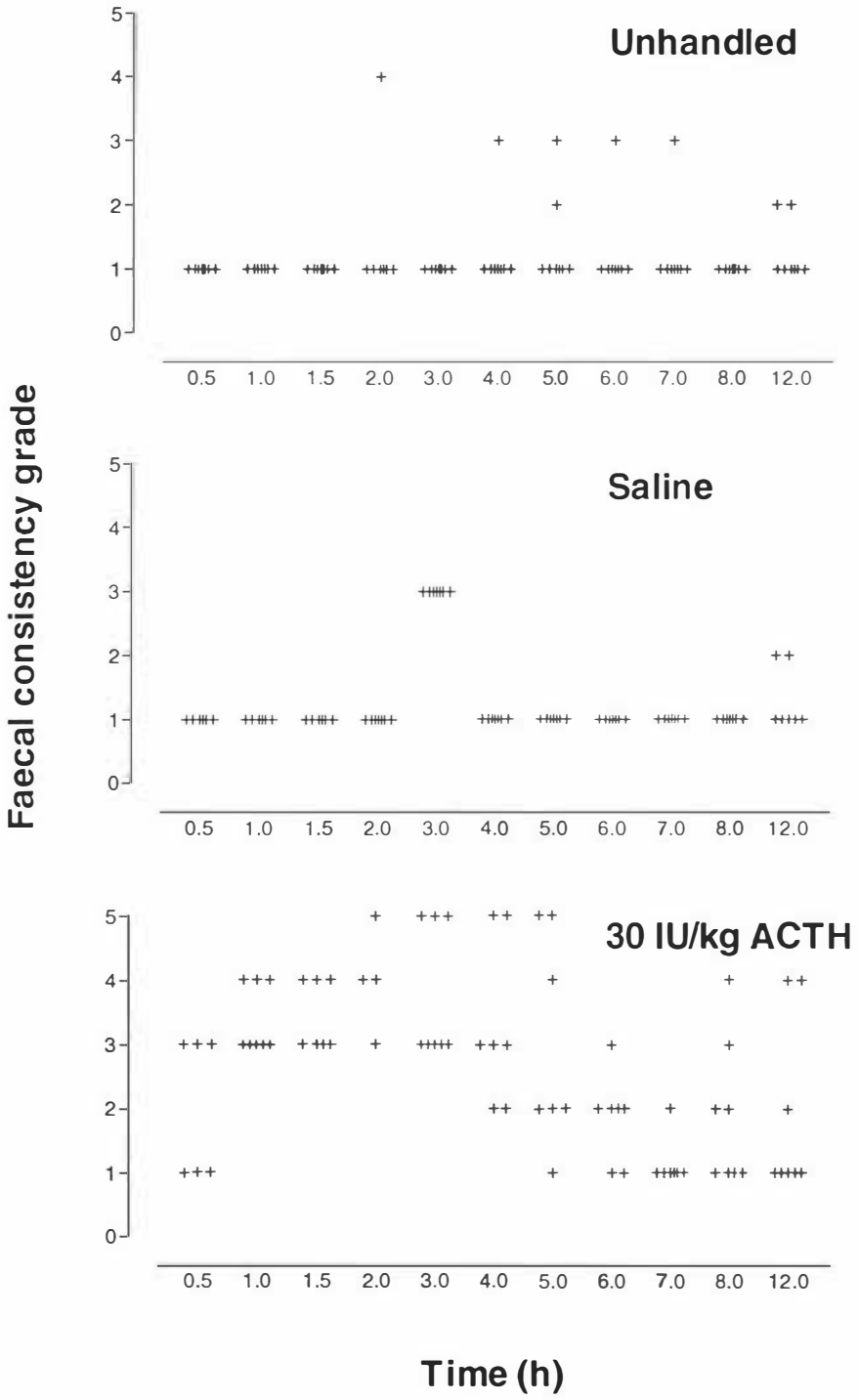


Figure 3.17: Change in faecal consistency over time of unhandled cockerels, or of cockerels after intramuscular administration of isotonic saline or 30 IU/kg ACTH at 0 h. Grade 1: solid; 2: soft; 3: loose; 4: liquid with solid particles; 5: liquid.

Group	Consistency grade				
	solid	soft	loose	liquid+ solids	liquid
unhandled	90.70	3.49	4.65	1.16	0.00
saline	87.84	2.70	9.46	0.00	0.00
30 IU/kg ACTH	25.93	17.28	30.86	16.05	9.88

Table 3.2: Percentage of droppings of each faecal consistency grade for each group in experiment 2.

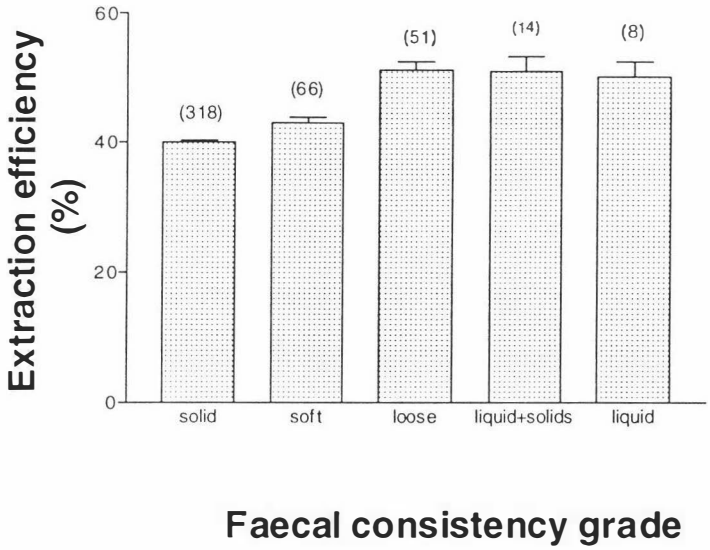


Figure 3.18: Relationship between faecal consistency score and extraction efficiency for extracted droppings from unhandled cockerels and cockerels given ACTH or isotonic saline. Grade 1: solid; 2: soft; 3: loose; 4: liquid with solid particles; 5: liquid. Numbers in brackets indicate sample size.

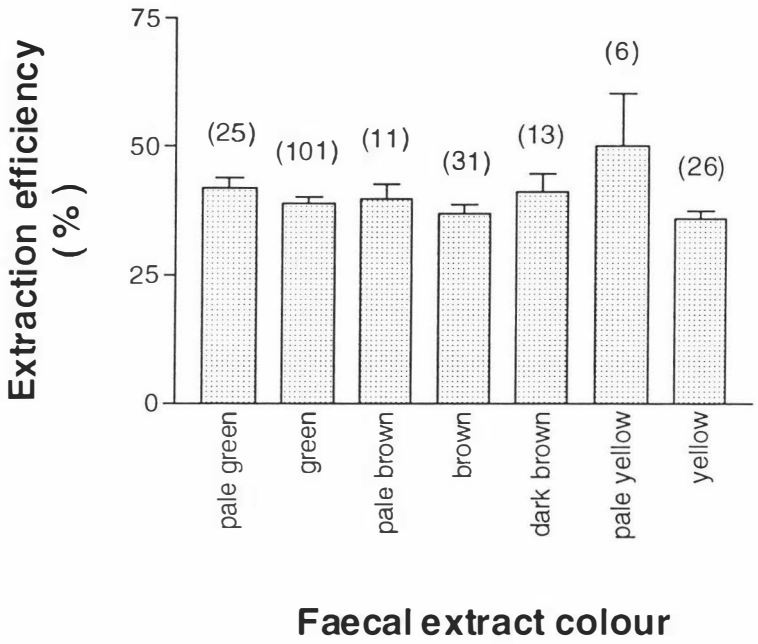


Figure 3.19: Relationship between faecal extract colour and extraction efficiency of droppings from cockerels given ACTH or isotonic saline. Numbers in brackets indicate sample size.

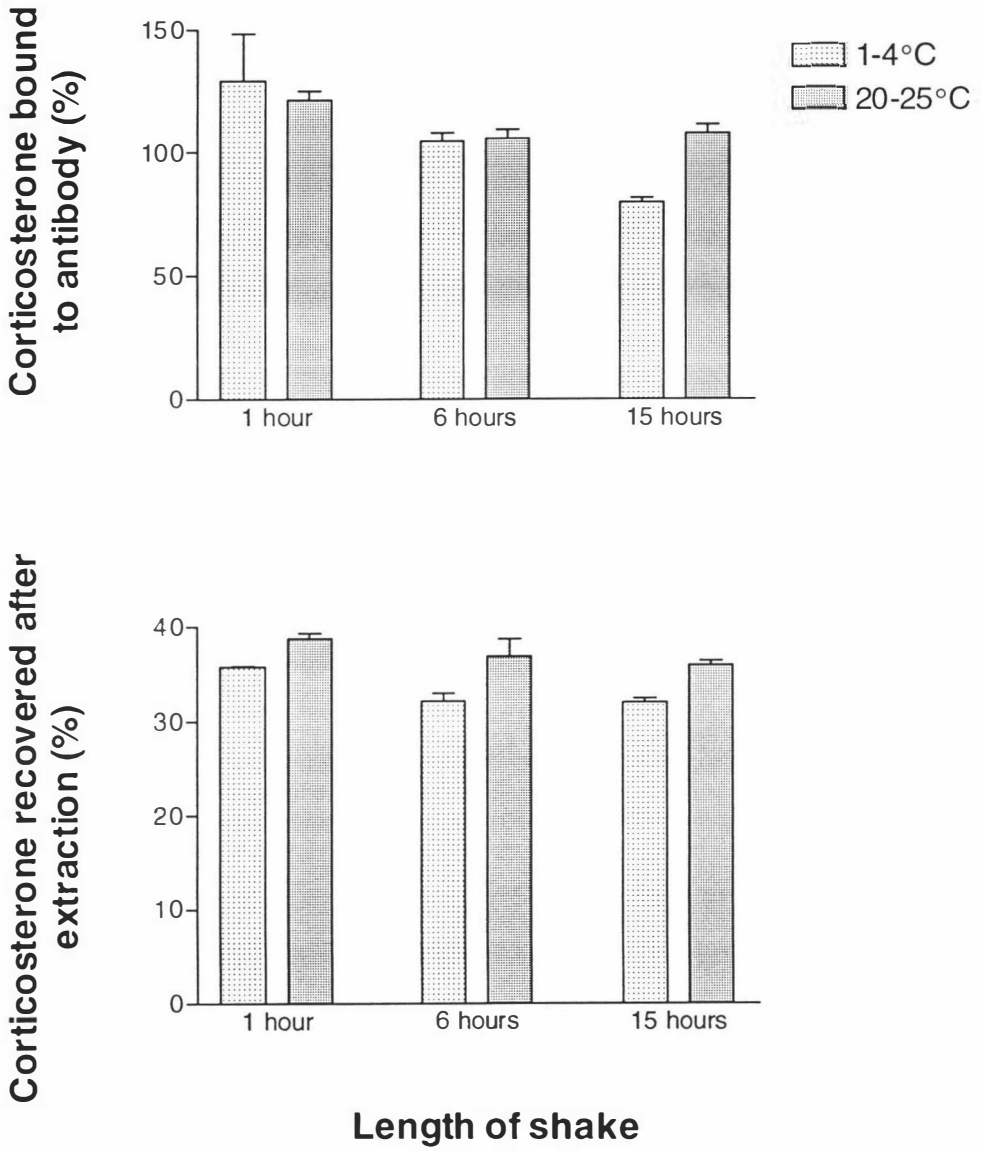


Figure 3.20: Effect of the temperature and duration of shaking on proportion of corticosterone bound to antibody after radioimmunoassay and percentage of corticosterone recovered after extraction (mean±SEM).

3.5 Discussion

The injection of a single intramuscular dose of ACTH into cockerels produced a peak in faecal corticosterone whereas two smaller intravenous doses of ACTH did not significantly alter faecal corticosterone levels. In a previous experiment, 30 IU/kg i.m. ACTH was given to the same cockerels and caused elevated plasma corticosterone levels for at least four hours (R. Osborne, unpub.; Appendix One). Similarly, 2 IU/kg i.v. ACTH and 5 IU/kg i.v. ACTH injections elevated plasma corticosterone levels for at least one hour in the present experiment. This demonstrated that faecal corticosterone levels can provide a non-invasive measure of plasma corticosterone in chickens although it may be that the increase in plasma corticosterone levels needs to be large and sustained to be detected in droppings. There were problems with the method used to extract corticosterone from droppings in this study and these may have affected faecal corticosterone measurements. As a result, a different extraction method is now used in our laboratory.

3.5.1 Validation of the extraction procedure and radioimmunoassay

The method used in the present experiment, to extract corticosterone from droppings followed a previously published and validated protocol (Shideler *et al.*, 1993). However, the method could not be validated for chicken droppings, using serial dilution of extracted droppings against the standard curve ('parallelism'; Figure 3.1), or recovery of a known amount of added corticosterone ('addition'). The method of Shideler *et al.* (1993) was developed to extract sex steroids from mammalian faeces. Methods developed for mammalian faeces have been successfully used for avian droppings in the past (Wasser *et al.*, 1997), and the method of Shideler *et al.* (1993) was applied to the present study because the extraction efficiency was greater than that of the faecal steroid extraction procedure previously used in our laboratory.

Ordinarily, a radioimmunoassay is validated before samples are submitted for measurement. The present study was run concurrently with another MSc student, R. Osborne, and it was intended that the validation procedures be undertaken by myself and R. Osborne before samples were assayed. However, due to time constraints and miscommunication, samples from the present study were assayed before validations were undertaken. The problem with the validation of the extraction method meant that alternative methods had to be evaluated. This led to the adoption of a method developed by Dr. S. Wasser in which droppings are lyophilised and boiled in ethanol

before reconstitution of the extract in buffer. The new method has been validated in our laboratory for a number of steroids in mammalian and avian faeces. However, the application of the new method to samples from the current study was not possible since all samples had already been thawed at least once, processed according to the method of Shideler *et al.* (1993), and refrozen. The samples had remained frozen for some time and would need to be re-thawed and re-extracted. It was felt that the integrity of the droppings could be reduced and moreover time constraints meant the work would not be completed satisfactorily.

In the present experiment, the curve resulting from serial dilution of an extracted dropping sample with normal buffer was not parallel until it had been diluted three times, that is after an eight-fold dilution (Figure 3.1). This suggests that something in the extract was interfering with the antibody-antigen binding reaction. The interfering substance could have arisen from the faecal buffer or from the raw dropping. It is most likely that the substance was present in the faecal buffer as a 'blank' tube, containing only faecal buffer and tritiated corticosterone ('label'), had a substantially higher radioactivity count than a blank containing normal buffer and label and 'zero' tubes containing faecal buffer, antibody and label had lower binding than zero tubes containing normal buffer. This indicates that binding between corticosterone and the antibody was reduced by the presence of faecal buffer.

Tween or methanol are the additives in the faecal buffer that probably caused the interference because the remaining ingredients were also present in normal buffer. It would be possible to test for the effects of Tween or methanol by adding or removing either substance from the faecal buffer and re-running a parallelism test. This was not performed as a new extraction method was by then developed for use in our laboratory. Moreover, Tween and methanol were used to improve extraction efficiency and it would not be expedient to remove them from the faecal buffer without investigating other substances that could perform the same function.

It is also possible that other forms of corticosterone or corticosterone metabolites in the raw dropping may have cross-reacted with the antibody. For example, corticosterone may be catabolised to 11-dehydrocorticosterone in birds (Daniel and Assenmacher, 1971), and cross-reaction of 11-dehydrocorticosterone with the antibody used here is around 2.1% (Lewis *et al.* in press). However, the new method used in our laboratory has been validated for corticosterone in Japanese quail droppings using the same antibody.

3.5.2 Experiment 1

Intravenous ACTH administration successfully stimulated corticosterone secretion and plasma levels in treated birds rose to a peak within one hour and had declined within two hours. Birds treated with 2 IU/kg ACTH (Figures 3.5, 3.7) had peak plasma corticosterone levels of 6.33 ±0.56 ng/ml (mean±SEM). Plasma corticosterone levels peaked at 11.24 ± 1.30 ng/ml one hour after cockerels were injected with 5 IU/kg ACTH (Figures 3.5, 3.8). Similarly, other experiments have shown that the magnitude of the corticosterone response increases with the dose of ACTH given (Beuving and Vonder, 1986). Moreover, the levels reached and the duration of the corticosterone responses in the present experiment are similar to those previously reported for laying hens and cockerels (Culbert and Wells, 1975; Beuving and Vonder, 1986). For example, Beuving and Vonder (1978) injected 1.2 IU/kg ACTH intra-arterially into laying hens and roosters and in all birds plasma corticosterone levels rose to a peak of around 48 ng/ml within 30 to 65 minutes. Corticosterone secretion declined to basal levels in hens by around 100 minutes and corticosterone levels in cockerels were approaching basal at 120 minutes.

Three saline treated birds responded to i.v. saline injections with a slight rise in corticosterone levels (Figure 3.6). This is probably due to the handling and injection procedure acting as a stressor (Beuving and Vonder, 1978; Radke *et al.*, 1985b; Lagadic *et al.*, 1990). Indeed, Zenoble *et al.* (1985a) showed that Andean condors, *Vultur gryphus*, given i.m. saline injections had similar corticosterone levels to condors given 0.125 mg i.m. ACTH.

Overall, there was no apparent elevation in faecal corticosterone in response to either 2 IU/kg i.v. or 5 IU/kg i.v. ACTH administration (Figure 3.5). However, birds #19 and #20 showed a rise in faecal corticosterone in response to 5 IU/kg i.v. ACTH (Figure 3.8) and faecal corticosterone tended to be higher than basal levels between one and three hours after a 5 IU/kg i.v. ACTH injection. By comparison, the peak in plasma corticosterone levels occurred at around one hour after 2 IU/kg i.v. ACTH and 5 IU/kg i.v. ACTH administration. Wasser *et al.* (1997) demonstrated that spotted owls had increased faecal corticosterone levels within two hours of a 100 IU i.v. ACTH injection. Corticosterone levels peaked at around 720 ng/g at 12 hours and had returned to basal levels within 26 hours. There are no other published studies of faecal corticosterone in response to ACTH administration.

There are some explanations for the absence of a clear peak in faecal corticosterone after i.v. ACTH injection. The radioimmunoassay used in the current study measured unaltered

corticosterone, and it is known that very little unaltered corticosterone enters the intestine in birds (Holmes and Slikker, 1976). What little there is will be subject to modification by microbial action, reabsorption through the gut and consequent enzymal modification in the mucosal cells and in the liver (McKerns, 1969; Helton and Holmes, 1973; Hill, 1984). Furthermore, the mean extraction efficiency was low (36.6±0.1%) and the low levels of corticosterone in the droppings were difficult to detect in the radioimmunoassay. It was also shown that the assay was not reading corticosterone levels correctly (Figure 3.1) and that the consistency of the droppings (Figure 3.18) and the temperature and duration of shaking during the extraction procedure may have contributed to this (Figure 3.20).

There was also substantial variation within and between birds in the level of corticosterone in droppings within each time period, which may have hidden an increase in corticosterone. This is supported by the findings of experiment 2 where a higher concentration of ACTH elicited a marked increase in faecal corticosterone levels. Similarly, Jurke *et al.* (1997) reported substantial variation between cortisol levels in faecal samples taken over one day from one animal.

Furthermore, it was possible that corticosterone was degraded by microbial action in the dropping after excretion, although this was limited as much as possible by freezing droppings soon after excretion and keeping them cool during the assay.

Finally, plasma corticosterone levels may not have been sufficient or remained elevated for long enough to raise faecal corticosterone levels above basal. This is supported by the finding that a large dose of i.v. (Wasser et al., 1997) or i.m. ACTH (used in experiment 2) markedly elevated corticosterone levels. A stressor known to elicit a stronger and more prolonged response, such as chronic social stress or repeated handling, could be used to overcome this problem in the future. Alternatively, a different strain of chicken, or a species or individual known to respond more markedly to stressors could be used.

There was no correlation between the total amount of faecal corticosterone excreted and the total amount of corticosterone in the plasma. It is unlikely that there was no relationship between plasma corticosterone and the excretion of unaltered corticosterone measured in droppings, because other studies have demonstrated parallel increases in plasma and faecal or urinary cortisol and corticosterone in response to stressors (Carlstead *et al.*, 1992; Jurke *et al.*, 1997; Wasser *et al.*, 1997). A correlation might be seen if plasma corticosterone was raised to higher levels for a longer duration. In addition, the total integrated and corrected integrated plasma corticosterone responses are only an estimation of the total plasma corticosterone secretion and

this likely contributed to the absence of any significant correlation between plasma and faecal corticosterone responses. It is also possible that the duration of the faecal response would be prolonged relative to the plasma response if corticosterone was cleared more quickly from the plasma than in droppings. In the current experiment faecal corticosterone levels probably did remain elevated for longer than plasma corticosterone levels (Appendix 1; measured in a concurrent experiment; R. Osborne, unpublished data) after 30 IU/kg i.m. ACTH injections. Similarly, faecal corticosterone levels tended to remain elevated relative to plasma corticosterone levels after 5 IU/kg i.v. ACTH.

3.5.3 Experiment 2

Intramuscular ACTH elicited a rise in faecal corticosterone between two and five hours after administration of 30 IU/kg ACTH. The magnitude of the response was significantly greater than the magnitudes of responses of unhandled cockerels or birds given i.m. saline. A dose of 30 IU/kg i.m. ACTH has previously been shown to elicit a rise in plasma corticosterone in chickens to around 100 ng/ml by 30 minutes (Davison *et al.*, 1980). In an experiment run concurrently to the present study, the same cockerels were injected with 30 IU/kg i.m. ACTH or i.m. saline and blood sampled before injection and at 2, 5, 10 and 24 hours after injection. Corticosterone levels peaked by at least 2 hours and remained elevated for at least a further four hours (R. Osborne, unpublished data; Appendix 1), suggesting that there was a lag of around two to five hours between peak plasma corticosterone levels and peak faecal corticosterone levels in the present study. Similarly, Wasser *et al.* (1997) reported that owls given tritiated corticosterone injections had a lag of around two hours between peak plasma and peak faecal corticosterone levels.

The lag between peak plasma and faecal corticosterone levels is partially due to the delay caused by the transport of corticosterone through the systemic circulation to the liver, followed by catabolism of corticosterone and the passage of the metabolites down the intestine. Two features inherent to avian physiology, the metabolic clearance rate of corticosterone and transit time through the digestive tract, affect this delay. Part of the lag is probably caused by the timing of dropping excretion, which may be affected by diet and light regime, for example. The cockerels in the current experiment were allowed free access to food and were given 24 hours light to encourage eating. They appeared to eat in bouts throughout 24 hours although they tended to excrete more droppings through normal daylight hours and after ACTH or saline injections. Some cockerels excreted very few droppings throughout the experiment which may

suggest that corticosterone could be more concentrated in their droppings. There did not appear to be any relationship between the number of droppings and the faecal corticosterone level but this was not statistically tested.

3.5.4 Dropping consistency

The cockerels' droppings became more liquid after they were injected with either saline or ACTH. 5 IU/kg i.v. ACTH cockerels had more droppings of a more liquid consistency than 2 IU/kg i.v. ACTH or i.v. saline cockerels (Table 3.1) and 30 IU/kg i.m. ACTH cockerels had more droppings of more liquid consistency than either i.m. saline or unhandled cockerels (Table 3.2). Birds that were handled and bled throughout experiment 1 had more liquid droppings than birds that remained unhandled or birds that were given i.v. saline and not handled further in experiment 2, suggesting that handling and blood sampling did affect dropping consistency. However, birds given 30 IU/kg i.m. ACTH had the greatest proportion of more liquid droppings over both experiments, despite being handled only once. This suggests that ACTH administration was primarily responsible for the change in faecal consistency. This problem could be avoided in the future by using different methods to elevate corticosterone secretion, such as corticosterone injections or CRF.

The change in dropping consistency was probably mediated by an increase in aldosterone, a corticosteroid mediator of fluid balance, as it is known that aldosterone increases in a dose-related response to handling (Radke *et al.*, 1985a,b) and ACTH injection (Kozma and Pethes, 1976; Radke *et al.*, 1985b). Furthermore, corticosterone increases glomerular filtration rate (Harvey *et al.*, 1986) and this may lead to more liquid droppings. Finally, excessive levels of blood glucose, which can occur in response to elevated glucocorticoids induced by ACTH (Culbert and Wells, 1975), can increase urine output (Guyton, 1991).

The extraction efficiency was higher for more liquid droppings than for solid droppings (Figure 3.18). The concentration of corticosterone (in ng/g) in a dropping sample is calculated as follows:

$$\text{concentration of dropping in pg/tube} \times 5 \div \text{extraction efficiency} \times 10$$

Hence, the corticosterone concentration of liquid droppings could have been artificially lowered due to their higher extraction efficiencies. There were also practical difficulties associated with the liquid droppings; they were difficult to collect and leaked off trays and out of collection pottles.

Moreover, the liquid droppings were passed more frequently than more solid droppings, so corticosterone could have been relatively more diluted as it passed down the intestine. However, the total amount of corticosterone passed over time, measured as the total integrated response or total excreted corticosterone, probably remained the same.

3.5.5 Conclusions

In conclusion, it was possible to measure corticosterone in the droppings of chickens given a high i.m. dose of ACTH. Cockerels responded to 2 IU/kg i.v. ACTH and 5 IU/kg i.v. ACTH with a clear increase in plasma corticosterone and the magnitude of the response was greatest for the highest dose. In general, birds did not respond to a low i.v. dose of ACTH with an increase in faecal corticosterone. However, two birds had elevated faecal corticosterone levels that peaked between one and three hours after ACTH injection in comparison to a peak in plasma corticosterone by one hour. There was no significant correlation between the total amount of plasma corticosterone secreted in response to ACTH and the total level measured in droppings. The radioimmunoassay was not validated and difficulties with the radioimmunoassay probably contributed to the absence of a significant response to i.v. ACTH administration and the absence of a significant relationship between plasma and faecal corticosterone. Finally, it was concluded that bleeding and handling caused droppings to become more liquid, but that an ACTH injection caused cockerels to produce relatively more droppings of a more liquid consistency. The consistency of the dropping probably affected the measurement of corticosterone in the dropping.

4 GENERAL DISCUSSION

Laying hens show individual differences in corticosterone secretion that are not directly related to social status, aggression received or body weight. It may be possible to quantify these differences in corticosterone secretion by measuring changes in the level of corticosterone excreted in the droppings, given a validated extraction procedure and radioimmunoassay. Such findings are important in the field of avian endocrinology as individual differences contribute to variation in experimental results. Furthermore, the measurement of corticosterone in droppings is a relatively new technique that has many important applications in the field, with regard to the effects of stress on the breeding and conservation of endangered species such as the kakapo in New Zealand (Cockrem and Rounce, 1995) and the Japanese ibis or Toki, in Japan (Ishii *et al.*, 1994).

4.1 Major conclusions

The aims of the present study were two-fold. The first experiment examined within- individual and between-individual variation in basal and stress-induced rises in corticosterone levels in laying hens. The second experiment assessed whether there was a similarity in the patterns of corticosterone secretion into the plasma and excretion in the droppings by cockerels during a response to ACTH. The major conclusions are as follows.

1. Chickens showed marked within-individual and between-individual differences in corticosterone secretion. These differences could be quantified by calculating the coefficients of variation for the areas under corticosterone response to handling curves and for corticosterone levels at different times.

2. If mixing two groups of laying hens together induced a change in corticosterone secretion, the change endured for less than 24 hours. There were no changes in corticosterone secretion on the day after mixing, in the week after mixing or in the week following that.

3. There was no relationship between the social rank of each hen and corticosterone responses to handling. Despite the small group numbers, it was evident that middle ranking hens

tended to receive high levels of aggression and tended to have elevated corticosterone responses to handling, whereas there was no particular pattern for lower or higher ranked hens.

4. It was possible to measure changes in corticosterone levels in chicken droppings in response to an intramuscular injection of 30 IU/kg ACTH. There appeared to be a lag of around three to five hours between peak corticosterone levels in plasma and droppings. ACTH injections caused the droppings to become more liquid and this probably affected the measurement of corticosterone in the droppings.

4.2 Considerations for field application

The measurement of stress is useful in at least three situations: experimental studies of the mechanisms of stress, the application of measures of stress to captive breeding programs with regard to the effects of stress on reproduction, and thirdly, the assessment of animal welfare. Glucocorticoid levels can be used in combination with other measures, to indicate both the presence and degree of stress. Basal levels are often not informative as they are subject to modification by external and internal factors and individual differences. The response to a stressor is more informative as it infers the responsiveness of the adrenal glands to stress (Harvey *et al.*, 1986; Wingfield *et al.*, 1997). The area under the stress response curve, basal levels, maximal levels, rate of increase and decrease and relative change in glucocorticoid levels can be calculated from a response curve.

The non-invasive measurement of steroids in the urine or faeces is particularly useful in situations where the elevation of glucocorticoids due to capture, restraint and bleeding is undesirable. For example, non-invasive corticosterone measurement could be useful as one measure for identifying housing conditions that cause stress in laying hens and the measurement of corticosterone in the droppings of wild spotted owls has been used to demonstrate that human interference is a substantial environmental stressor (Wasser *et al.*, 1997). It would be useful to non-invasively identify individual differences in glucocorticoid secretion in particular, in order that factors influencing individual differences could be controlled, either to improve experimental design or to allow better management of animals breeding in captivity (Jurke *et al.*, 1997).

The non-invasive measurement of basal steroid levels is common (Lasley and Kirkpatrick, 1994) and it could be possible to collect all faeces or urine excreted within a certain period after

exposure to a stressor in order to determine a chronic glucocorticoid response (Carlstead *et al.*, 1992). However, it may be difficult to measure short-lived changes in faecal corticosterone levels, such as a corticosterone response to handling, since a small i.v. ACTH injection did not elicit an obvious rise in corticosterone in the present study. Moreover, there was no relationship between the total amount of faecal and plasma corticosterone measured, suggesting that the magnitude of any differences between individuals could be different in faecal corticosterone relative to plasma corticosterone measurements.

There are also a number of practical difficulties associated with the measurement of steroids in urine and faeces. The most obvious difficulty is the collection and storage of samples in a manner that preserves their integrity. It is relatively easy to collect urine and faecal samples from caged animals but the collection of droppings from free-living animals is more difficult and it is unlikely that urine samples can be collected at all. Faecal samples may be distributed throughout the environment in inaccessible areas and contaminated with debris. Moreover, the time of excretion and identity and dietary history of the individual may be unknown and it has been demonstrated that diet, and in particular the level of fibre (Wasser *et al.*, 1992) and lipid (M. Kikuchi, pers. comm.) affects the extraction of steroid from droppings.

In the present experiment, there was substantial variation in faecal corticosterone levels between droppings passed by birds in the same environment. The variation probably contributed to the absence of a significant peak in faecal corticosterone levels during a small and short-lived elevation in plasma corticosterone levels. This suggests that it would not be possible to measure small differences in corticosterone levels between birds. For example, it may not be possible to measure corticosterone responses or differences in faecal corticosterone levels between hens kept in different housing environments as undisturbed hens tend to have low levels of corticosterone and the differences in corticosterone levels between hens in different housing environments also tend to be small, if indeed they exist at all (Appleby *et al.*, 1993). It would be possible to avoid between – individual variation by collecting all of the droppings excreted by one individual within a given time period or by collecting droppings from a large number of birds, for example from all of the broiler chickens in a commercial shed. However, droppings would require frequent collection in order that they did not become contaminated or that corticosterone did not degrade. This would be rather labour intensive and it may be easier from a practical point of view to collect blood samples from a selection of animals.

Studies using faecal measurements are nevertheless useful in the assessment of chronic stressors (Wasser *et al.*, 1997) and have important implications for steroid monitoring in the wild in situations where animals cannot be caught (Lasley and Kirkpatrick, 1994; Wasser *et al.*, 1997).

4.3 Future objectives

The findings of the present study suggest further work could be undertaken both with regard to measuring corticosterone responses in different species in different situations and in the non-invasive measurement of corticosterone responses and corticosterone levels. In particular, it would be worthwhile to validate a method for extracting and measuring corticosterone in bird droppings and to determine the relationship between plasma and faecal corticosterone responses in particular environments.

A new extraction procedure, based on that developed by Dr. Sam Wasser, has already been successfully validated and used to measure corticosterone in Japanese quail droppings in our laboratory (E. Candy, R. Boyd and J. Cockrem, unpublished data). The relationship between faecal and plasma corticosterone is being investigated in chickens by A. Lord and M. Dawkins, together with J. Cockrem. The method used will be similar to that applied in our laboratory and will be based on the experience gained in the present study.

The findings described in this thesis demonstrated that calculating the magnitude of corticosterone responses to handling could be used identify individual differences in corticosterone secretion in different environments. This may be useful in the conservation of birds (Wingfield, 1994), for example to recognise individuals that are unlikely to breed (Forman, 1994) or to identify birds are reacting disadvantageously to captivity. Corticosterone responses to capture and handling of both captive and free-living blackbirds have been determined in our laboratory to determine possible causes of stress during translocation of endangered native birds (M. Welch, unpublished data). Furthermore, the corticosterone responses to handling are being defined in kiwi in order to assess the effects of regular handing and captivity (D. Adams, unpublished data).

In order to investigate the relationship between social rank and individual differences in corticosterone secretion in chickens, it would be useful to repeat the experiment described in chapter 2 according to the suggested modifications. This would indicate whether there other more important features contribute to variation in corticosterone responses to a stressor between

birds. For example, it would be interesting to determine whether individual differences in perception of the stressfulness of a stressor were responsible for variations in corticosterone secretion. This could be investigated by determining the tonic immobility and corticosterone responses to handling of hens kept in a group with a known hierarchy. Tonic immobility provides a measure of the fearfulness of chickens and this may be related to corticosterone secretion in response to a stressor (Jones *et al.*, 1988) and to position in the hierarchy.

In summary, the experiments described in this thesis provided a basis for much further work, despite the fact that predictions were not fulfilled. In particular, they have lead to the development of a validated assay for faecal corticosterone and sex steroid assessment and the use of Japanese quail rather than chickens as a model species in our laboratory.

5 REFERENCES

Appleby, M.C. 1983. The probability of linearity in hierarchies. *Animal Behaviour*, **31**:600-608.

Appleby, M.C., Hughes, B.O. and Elson, H.A. 1992. Poultry production systems, behaviour, management and welfare. CAB International: UK.

Assenmacher, I. and Jallegeas, M. 1980. Circadian and circannual rhythms. In: Avian Endocrinology, pp. 391-413. A. Epples and M.H. Stetson (eds.) Academic Press: New York.

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

Belthoff, J.R., Dufty, A.M. and Gauthreaux Jr., S.A. 1994. Plumage variation, plasma steroids and social dominance in male house finches. *The Condor*, **96**:614-625.

Bercovitz, A.B., Collin, J., Price, P. and Tuttle, D. Noninvasive assessment of seasonal hormone profiles in captive bald eagles (*Haliaeetus leucocephalus*). *Zoo Biology*, **1**:111-117.

Berliner, D.L., Keller, N. and Dougherty, T.F. 1961. Tissue retention of cortisol and metabolites induced by ACTH: an extra-adrenal effect. *Endocrinology*, **68**:621-632.

Beuving, G. 1980. Corticosteroids in laying hens. In: The laying hen and its environment, pp. 65-84. R. Moss (ed.) Martinus Nijhoff: Netherlands.

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

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

Bishop, C.M. and Hall, M.R. 1991. Non-invasive monitoring of avian reproduction by simplified faecal steroid analysis. *Journal of Zoology, London*, **224**:649-668.

Bojeson, E. and Egense, J. 1960. Elimination of endogenous corticosteroids in vivo. *Acta Endocrinologica*, **33**:347-369.

Bradshaw, S.D. 1996. Hormones, stress and their relevance to problems of conservation (abstract). In: Environmental and conservation endocrinology-Proceedings of the 3rd congress of the AOSCE, pp. 1-4. Joss, J. (ed.). AOSCE: Sydney.

Breitenbach, R.P. 1962. The effect of ACTH on adrenocortical secretion and ascorbic acid depletion in normal and testosterone treated cockerels. *Poultry Science*, **41**:1318-1324.

Broom, D.M. and Johnson, K.G. 1993. Stress and animal welfare. First edition. Chapman & Hall: Australia.

Brown, J.L., Wasser, S.K., Wildt, D.E. and Graham, L.H. 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured non-invasively in the feces. *Biology of Reproduction*, **51**:776-786.

Cannon, W.B. 1935. Stresses and strains of homeostasis. *American Journal Medical Science*, **189**:1-14.

Carlstead, K., Brown, J.L., Monfort, Killens, R. and Wildt, D.E. 1992. Urinary monitoring of adrenal responses to psychological stressors in domestic and nondomestic Felids. *Zoo Biology*, **11**:165-176.

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

Chase, I.D. 1982. Dynamics of hierarchy formation: the sequential development of dominance relationships. *Behaviour*, **80**:218-238.

Cockrem,J.F. 1990. Circadian Rhythms in Antarctic Penguins. In: Penguin Biology, pp. 319-344. L.S. Davis & J.T. Darby (eds.) Academic Press: Sydney.

Cockrem, J.F. and Rounce, J.R. 1994. Faecal measurements of oestradiol and testosterone allow the non-invasive estimation of plasma steroid levels in the chicken. *British Poultry Science*, **35**:433-443.

Cockrem, J.F. and Rounce, J.R. 1995. Non-invasive assessment of the annual gonadal cycle in free-living Kakapo (*Strigops habroptilus*) using fecal steroid measurements. *The Auk*, **112**:253-257.

Cockrem, J.F. and Silverin, B. 1998. Corticosterone responses of the great tit (*Parus major*) to handling and to a predator. In: Programme and Abstracts of the New Zealand Society of Endocrinology. Massey University: Palmerston North.

Coe, C., Mendoza, S. and Levine, S. 1979. Social status constrains the stress response in the squirrel monkey. *Physiology and Behavior*, **23**:633-638.

Cook, C.J. and Hodgetts, B.V. 1996. Learning of stress avoidance behaviour: relationship to neurotransmitters (abstract). In: Advances in animal behaviour and welfare in Australasia and Africa. Abstracts of papers presented to the 1st regional meeting of the Australasian African region of the ISAE. AgResearch: Hamilton.

Cristol, D.A. 1995. Costs of switching social groups for dominant and subordinate dark-eyed juncos (*Junco hyemalis*). *Behavioural Ecology and Sociobiology*, 37:97-101.

Culbert, J. and Wells, J.W. 1975. Aspects of adrenal function in the domestic fowl. *Journal of Endocrinology*, **65**:363-376.

Cunningham, D.L., Van Tienhoven, A. and de Goeijen, F. 1987. Dominance rank and cage density effects on performance traits, feeding activity and plasma corticosterone levels of laying hens (*Gallus domesticus*). *Applied Animal Behavior Science*, **17**:139-153.

Dallman, M.F. 1993. Stress update. Adaptation of the hypothalamic-pituitary-adrenal axis to chronic stress. *Trends in Endocrinological Metabolism*, **4**:62-69.

Daniel, J-Y. and Assenmacher, I. 1971. Early appearance of metabolites after single I.V. injection of ^{3}H-corticosterone in rabbit and duck. *Steroids*, **18**:325-340.

Dantzer, R. and Mormède, P. 1983. Stress in farm animals: a need for reevaluation. *Journal of Animal Science*, **57**:6-17.

Davis, D. and Christian, J. 1957. Relation of adrenal weight to social rank of mice. *Proceedings of the Society Experimental Biology and Medicine*, **94**:728-736.

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

Dawson, A. and Howe, P.D. 1983. Plasma corticosterone in wild starlings (*Sturnus vulgaris*) immediately following capture and in relation to body weight during the annual cycle. *General and Comparative Endocrinology*, **51**:303-308.

Ely, D.L. and Henry, J.P. 1978. Neuroendocrine response patterns in dominant and subordinate mice. *Hormones and Behavior*, **10**:156-169.

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

Fell, L.R., Wells, R. and Shutt, D.A. 1986. Stress in calves castrated surgically or by the application of rubber rings. *Australian Veterinary Journal*, **63**:16-18.

Fernandez, X., Meunier-Salaün, M-C. and Mormede, P. 1994. Agonistic behavior, plasma stress hormones, and metabolites in response to dyadic encounters in domestic pigs: interrelationships and effect of dominance status. *Physiology and Behavior*, **56**:841-847.

Flickinger, G.L. 1961. Effect of grouping on the adrenals and gonads of chickens. *General and Comparative Endocrinology*, **1**:332-340.

Forman, M. 1994. Corticosterone responses to captivity and sampling stress in the mallard (*Anas platyrhynchos*) and grey duck (*Anas superciliosa*). MSc thesis, Massey University.

Freeman, B.M. 1984. Adrenal glands. In: Physiology and biochemistry of the domestic fowl, Volume 4, pp. 191-205. B.M. Freeman (ed.) Academic Press: London.

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

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

Gibson, S.W., Dun, P. and Hughes, B.O. 1988. The performance and behaviour of laying fowls in a covered strawyard system. *Research and Development in Agriculture*, **5**:153-163.

Glenister, D.W. and Yates, F.E. 1961. Sex difference in the rate of disappearance of corticosterone-4-C^{14} from plasma of intact rats: further evidence for the influence of hepatic Δ^4-steroid hydrogenase activity on adrenal function. *Endocrinology*, **68**:747-758.

Golub, M., Sassenrath, E. and Goo, G. 1979. Plasma cortisol levels and dominance in peer groups of rhesus monkey weanlings. *Hormones and Behavior*, **12**:50-59.

Gorsline, J., Morris, D.J. and Holmes, W.N. 1989. Metabolism of aldosterone in the colostomised duck (*Anas platyrhynchos*): partial characterization of urinary metabolites. *Comparative Biochemistry and Physiology*, **92B**:773-777.

Gross, W.B. and Siegel, P.B. 1983. Evaluation of the heterophil/ lymphocyte ratio as a measure of stress in chickens. *Avian Diseases*, **27**:972-979.

Guhl, A.M. 1958. The development of social organization in the domestic chick. *Animal Behaviour*, **6**:92-111.

Guillette Jr., L.J., Cree, A. and Rooney, A.A. 1995. Biology of stress: interactions with reproduction, immunology and intermediary metabolism. In: Health and welfare of captive reptiles, pp. 32-81. Warwick, C., Frye, F.L. and Murphy, J.B. (eds.) Chapman and Hall: London.

Guyton, A.C. 1991. Textbook of medical physiology. Eighth edition. W.B. Saunders: Philadelphia.

Hansen, I. 1994. Behavioural expression of laying hens in aviaries and cages: frequencies, time budgets and facility utilisation. *British Poultry Science*, **35**:491-508.

Hanlon, A.J., Rhind, S.M., Reid, H.W., Burrells, C. and Lawrence, A.B. 1995. Effects of repeated changes in group composition on immune response, behaviour, adrenal activity and liveweight gain in farmed red deer yearlings. *Applied Animal Behaviour Science*, **44**:57-64.

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

Harvey, S., Scanes, C.G. and Brown, K.I. 1986. Adrenals. In: Avian physiology, pp. 479-493. P.D. Sturkie (ed.) Springer-Verlag: New York.

Heath, J. and Dufty, A. 1996. Relationship between body condition and adrenal stress response in captive American kestrel juveniles (abstract). In: Abstracts of 6th International Symposium on Avian Endocrinology held in Canada, 1996.

Helton, E.D. and Holmes, W.N. 1973. The distribution and metabolism of labelled corticosteroids in the duck (*Anas platyrhynchos*). *Journal of Endocrinology*, **56**:361-385.

Heinrichs, S.C., Menzaghi, F., Pich, E.M., Britton, K.T. and Koob, G.F. 1995. In: Stress: basic mechanisms and clinical implications. Annals of the New York Academy of Science, Volume 771, pp. 92-104. Chrousos, P., McCarty, R., Cizza, G., Stemberg, E., Gold, P.W. and Kvetnansky, R. (eds.) NYAS: New York.

Hendricks,III,G.L., Mashaly,M.M. and Siegel,H.S. 1995. Effects of corticosterone *in vivo* and *in vitro* on adrenocorticotropic hormone production by corticotropin releasing factor-stimulated leucocytes (43911). *P.S.E.B.M.* **209**: 382-386.

Henry, J.P. 1993. Biological basis of the stress response. *NIPS*, **8**:69-73.

Hill, K.J. 1984. Physiology of the digestive tract. In: Physiology and biochemistry of the domestic fowl, Volume 4, pp. 31-47. B.M. Freeman (ed.)

Hirata, S. and Mori, Y. 1995. Monitoring reproductive status by faecal progesterone analysis in ruminants. *Journal of Veterinary Medical Science*, **57**:845-850.

Holmes, W.N. and Slikker, W. 1976. Some properties of the labelled material excreted by intact and adenohypophysectomized ducks (*Anas platyrhynchos*) given single doses of labeled corticosterone. *General and Comparative Endocrinology*, **29**:128-140.

Holmes, W.N., Broock, R.L. and Devlin, J.M. 1974. Tritiated corticosteroid metabolism in intact and adenohypophysectomized ducks (*Anas platyrhynchos*) given single doses of labeled corticosterone. *General and Comparative Endocrinology*, **22**:41427.

Hulten, F., Zhang, B.R., Forsberg, M. and Dalin, A.M. 1995. Applying a progesterone assay to faecal samples collected from sows during the oestrous cycle. *Reproduction in Domestic Animals*, **30**:101-105.

Irvine, C.H.G. and Alexander, S.L. 1987. A novel technique for measuring hypothalamic and pituitary hormone secretion rates from collection of pituitary venous effluent in the normal horse. *Journal of Endocrinology*, **113**:183-192.

Ishii, S., Wada, M., Wakabayashi, S., Sakai, H., Kubodera, Y., Yamaguchi, N. and Kikuchi, M. 1994. Endocrinological studies for artificial breeding of the Japanese ibis, *Nipponia nippon*, an endangered avian species in Asia. *Journal of Bioscience*, **19**:491-502.

Jones, R.B. and Harvey, S. 1987. Behavioural and adrenocortical responses of domestic chicks to systematic reduction in group size and to sequential disturbance of companions by the experimenter. *Behavioural Processes*, **14**:291-303.

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

Jones-Witters, P., Hurley, M.J., Phillips, R., Brown, B.L. and Erb, R.E. 1975. Characterization of metabolites in domestic sow urine after intravenous administration of radioactive estrogen and corticosteroids. *Journal of Dairy Science*, **58**:41-48.

Jurke, M.H., Czekala, N.M., Lindburg, D.G. and Millard, S.E. 1997. Fecal corticoid metabolite measurement in the cheetah (*Aiconyx jubatus*). *Zoo Biology*, **16**:133-147.

Kalliecharan, R. 1981. The influence of exogenous ACTH on the levels of corticosterone and cortisol in the plasma of young chicks (*Gallus domesticus*). *General and Comparative Endocrinology*, **44**:249-251.

Kavaliers, M. and Colwell, D.D. 1996. Synergism between stress responses induced by biting flies and predator odours. *Ethology*, **102**:89-98.

Kikuchi, M., Sato, F. and Ishii, S. 1996. Seasonal changes in fecal sex steroid and corticosterone levels in relevance to behavioral stages in a wild population of the rock ptarmigan. In: Environmental and conservation endocrinology. Proceedings of the third congress of Asia and Oceania Society for Comparative Endocrinology, p. 196. AOSCE: Sydney.

King, A.S. and McLelland, J. 1975. Outlines of avian anatomy. Cassell and Collier Macmillan Publishers: London.

Klemcke, H.G. 1994. Reponses of the porcine pituitary-adrenal axis to chronic intermittent stressor. *Domestic Animal Endocrinology*, **11**:133-149.

Koelkebeck, K.W. and Cain, J.R. 1984. Performance, behavior, plasma corticosterone, and economic returns of laying hens in several management alternatives. *Poultry Science*, **63**:2123-2131.

Kofuji, H., Kanda, M. and Oishi, T. 1993. Breeding cycles and fecal gonadal steroids in the brown dipper *Cinclus pallasii*. *General and Comparative Endocrinology*, **91**:216-223.

Kopin, I.J. 1995. Definitions of stress and sympathetic neuronal responses. In: Stress: basic mechanisms and clinical implications. Annals of the New York Academy of Science, Volume 771, pp. 56-68. Chrousos, P., McCarty, R., Cizza, G., Stemberg, E., Gold, P.W. and Kvetnansky, R. (eds.) NYAS: New York.

Kozma, M. and Pethes, G. 1976. The effect of ACTH and heparin treatment on the blood plasma aldosterone level in the domestic chicken. *Acta Veterinaria Scientarium Hungaricae*, **26**:415-418.

Kujiyat, S.K., Craig, J.V. and Dayton, A.D. 1983. Duration of tonic immobility affected by housing environment in white leghorn hens. *Poultry Science*, **62**:2280-2282.

Lagadic, H., Faure, J.M., Mills, A.D. and Williams, J.B. 1990. Effects of blood sampling on plasma concentrations of corticosterone and glucose in laying hens caged in groups. *British Poultry Science*, **31**:823-829.

Lasley, B.D. and Kirkpatrick, J.F. 1994. Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids. *Journal of Zoo and Wildlife Medicine*, **22**:23-31.

Le Maho, Y., Karmann, H., Briot, D., Handrich, Y., Robin, J-P., Mioskowski, E., Cherel, Y. and Farni, J. 1992. Stress in birds due to routine handling and a technique to avoid it. *American Journal of Physiology*, **263**:R775-R781.

Lee, Y-P., Craig, J.V. and Dayton, A.D. 1982. The social rank index as a measure of social status and its association with egg production in white leghorn pullets. *Applied Animal Ethology*, **8**:377-390.

Lee, J.V., Whaling, C.S., Lasley, B.L. and Marler, P. 1994. Validation of an enzyme immunoassay for measurement of excreted estrogen and testosterone metabolites in the white-crowned sparrow (*Zonotrichia leucophrys oriantha*). *Zoo Biology*, **14**:97-106.

Levine, S. 1985. A definition of stress? In: Animal Stress. G. Moberg (ed.) American Physiological Society: USA.

Lewis, R.M., Batchelor, D.C., Cockrem, J.F., Bassett, N.S., Johnston, B.M. and Skinner, S.J.M. (in press). Effects of maternal captopril treatment on SHR and WKY foetuses. *Journal of Endocrinology*.

Lipar, J.L., Ketterson, E.D. and Nolan Jr., V. 1996. Steroid hormones in the yolk of red-winged blackbird eggs. In: Environmental and conservation endocrinology. Proceedings of the third congress of Asia and Oceania Society for Comparative Endocrinology. AOSCE: Sydney.

Louch, C. and Higginbotham, M. 1967. The relation between social rank and plasma corticosterone levels in mice. *General and Comparative Endocrinology*, **8**:441-448.

Manogue, K., Candland, D. and Leshner, A. 1975. Dominance status and adrenocortical reactivity to stress in squirrel monkeys (*Saimiri scuireus*). *Primates*, **16**:457-463.

Manteca, X. and Deag, J.M. 1994. Individual variation in response to stressors in farm animals: implications for experimenters. *Animal Welfare*, **3**:213-218.

Marsteller, F.A., Siegel, P.B. and Gross, W.B. 1980. Agonistic behavior, the development of the social hierarchy and stress in genetically diverse flocks of chickens. *Behavioural Processes*, **5**:339-354.

Martin, P.H. and Bateson, P. 1993. Measuring behaviour: an introductory guide. 2nd edition. Cambridge University Press: Cambridge.

Mason, J.W., Brady, J.V. and Tolliver, G.A. 1968. Plasma and urinary 17-hydroxycorticosteroid responses to 72-hour avoidance sessions in the monkey. *Psychonomic Medicine*, **30**:608-630.

McKems, K.W. 1969. Steroid hormones and metabolism. Appleton-Century-Crofts: New York.

McMeekan, C.M., Stafford, K.J., Mellor, D.J., Bruce, R.A., Ward, R.N. and Gregory, N.G. 1998. Effects of regional anaesthesia and/or a non-steroidal anti-inflammatory analgesic on the acute cortisol response to dehorning in calves. *Research in Veterinary Science*, **64**:147-150.

Mellor, D.J. and Reid, C.S.W. 1993. Concepts of animal well-being and predicting the impact of procedures on experimental animals. In: Improving the well-being of animals in the research environment, pp. 3-18. ANZCCART: Glen Osmond.

Mench, J.A. 1991. Stress in birds. In: Acta XX Congressus Internationalis Ornithologici, pp. 1905-1914. New Zealand Ornithological Congress Trust Board: Wellington.

Mench, J.A. and Ottinger, M.A. 1991. Behavioral and hormonal correlates of social dominance in stable and disrupted groups of male domestic fowl. *Hormones and Behavior*, **25**:112-122.

Minton, J.E. 1994. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. *Journal of Animal Science*, **72**:1891-1898.

Monamy, V. 1996. Animal experimentation: a student guide to balancing the issues. ANZCCART: Glen Osmond.

Muir, W.M. 1997. Group selection for adaptation to multiple-hen cages: selection program and direct responses. *Poultry Science*, **75**:447-458.

Muller, O.A. (ed.) 1987. Corticotrophin Releasing Hormone: international workshop, Birkenstein/Fischblau, February 1987. Thième Medical Publishers Inc.: New York.

Munck, A., Guyre, P.M. and Holbrook, N.J. 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrinology Reviews*, **5**:25-44.

Munro, C.J., Stabenfeldt, G.H., Cragun, J.R., Addiego, L.A., Overstreet, J.W. and Lasley, .B.L. 1991. Relationship of serum oestradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. *Clinical Chemistry*, **37**:838-844.

Murton, R.K., Isaacson, A.J. and Westwood, N.J. 1971. The significance of gregarious feeding behavior and adrenal stress in a population of wood pigeons *Columba palumbus*. *Journal of Zoology*, **165**:53-84.

Nakamura, T., Tanabe, Y. and Hirano, H. 1978. Evidence of the *in vitro* formation of cortisol by the adrenal gland of embryonic and young chickens (*Gallus domesticus*). *General and Comparative Endocrinology*, **35**:302-308.

O'Malley, B.W. and Schrader, W.T. 1979. The receptors of steroid hormones. In: Hormones and reproductive behaviour, pp. 51-60. R. Silver and H.H. Feder (eds.) W.H. Freeman and Co.: San Francisco.

Peter, A.T., Critser, J.K. and Kapustin, N. 1996. Analysis of sex steroid metabolites excreted in the feces and urine of nondomesticated animals. *The Compendium for Continuing Education*, **18**:781-792.

Pickard, A.R., Abáigar, T., Holt, W.V. and Cano, M. 1998. Effects of oestrus synchronisation on Mohor gazelle fertility, studied by faecal steroid excretion. *Journal of Reproduction and Fertility, Abstract series* **22**:25.

Pierzchala-Koziec, K., Niezgoda, J., Bobek, S. and Przewlocki, R. 1996. Endogenous opioids may modulate the activity of the hypothalamo-pituitary-adrenocortical axis in domestic fowl. *Journal of Veterinary Medicine*, **43**:139-146.

Radke, W.J., Albasi, C.M. and Harvey, S. 1985a. Haemorrhage and adrenocortical activity in the fowl (*Gallus domesticus*). *General and Comparative Endocrinology*, **60**:204-209.

Radke, W.J., Albasi, C.M. and Harvey, S. 1985b. Stress and ACTH stimulate aldosterone secretion in the fowl (*Gallus domesticus*). *Comparative Biochemistry and Physiology*, **82A**:285-288.

Ramenofsky, M. 1984. Agonistic behaviour and endogenous plasma hormones in male Japanese quail. *Animal Behaviour*, **32**:698-708.

Ramenofsky, M. 1985. Acute changes in plasma steroids and agonistic behaviour in male Japanese quail. *General and Comparative Endocrinology*, **60**:116-128.

Rehder, N.B., Bird, D.M. and Lague, P.C. 1986. Variations in plasma corticosterone, estrone, estradiol-17β, and progesterone concentrations with forced renesting, molt, and body weight of captive female American kestrels. *General and Comparative Endocrinology*, **62**:386-393.

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

Rohwer, S. and Wingfield, J.C. 1981. A field study of social dominance, plasma levels of luteinizing hormone and steroid hormones in wintering Harris' sparrows. *Zeitschrift für Tierpsychologie*, **57**:173-183.

Romero, L.M., Levine, S. and Sapolsky, R.M. 1995. Patterns of adrenocorticotropin secretagog release in response to social interactions and various degrees of novelty. *Psychneuroendocrinology*, **20**:183-191.

Rushen, J. 1986. Some problems with the physiological concept of "stress". *Australian Veterinary Journal*, **63**:359-361.

Rzasa, J., Williams, J. and Etches, R.J. 1983. A study of the ovulation-inhibiting effects of dexamethasone in the domestic hen. *General and Comparative Endocrinology*, **52**:311-314.

Sapolsky, R.M. 1982. The endocrine stress-response and social status in the wild baboon. *Hormones and Behavior*, **16**:279-292.

Sapolsky, R.M. 1983. Individual differences in cortisol secretory patterns in the wild baboon: role of negative feedback sensitivity. *Endocrinology*, **113**:2263-2267.

Sapolsky, R.M. 1992. Individual differences and adrenocortical function: Why do some individuals secrete more glucocorticoids than others? In: Stress, the aging brain and the mechanisms of neuron death. MIT Press: Massachusetts.

Sapolsky, R.M. 1993. Endocrinology alfresco: psychoendocrine studies of wild baboons. *Recent Progress in Hormone Research*, **48**:437-468.

Sapolsky, R.M., Krey, L.C. and McEwen, B.S. 1984. Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology*, **114**:287-292.

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

Schwabl, H., Ramenofsky, M., Schwabl-Benzinger, I., Famer, D.S. and Wingfield, J.C. 1988. Social status, circulating levels of hormones, and competition for food in winter flocks of the white-throated sparrow. *Behaviour*, **107**:107-121.

Selye, H. 1950. The physiology and pathology of exposure to stress. Acta Inc.: Montreal.

Selye, H. 1952. The story of the adaptation syndrome. Acta Inc.: Montreal.

Shideler, S.E., Ortuño, A.M., Morán, F.M., Moorman, E.A. and Lasley, B.L. 1993. Simple extraction and enzymeimmunoassays for estrogen and progesterone metabolites in the feces of *Macaca fascicularis* during non-conceptive and conceptive ovarian cycles. *Biology of Reproduction*, **48**:1290-1298.

Shille, V.M., Haggerty, M.A., Shackleton, C. and Lasley, B.L. 1990. Metabolites of estradiol in serum, bile, intestine and feces of the domestic cat (*Felis cattus*). *Theriogenology*, **34**:779-794.

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

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

Siegel, H.S. and Siegel, P.B. 1961. The relationship of social competition with endocrine weights and activity in chickens. *Animal Behaviour*, **9**:151-158.

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

Spelman, L.H., Fleming, W.J., Davis, G.S. and Stoskopf, M.K. 1995. Effect of exogenous adrenocorticotropic hormone administration on plasma corticosterone concentrations in American black ducks (*Anas rubrides*). *Journal of Wildlife Diseases*, **31**:136-141.

Stephens, D.B. 1980. Stress and its measurement in domestic animals: a review of behavioral and physiological studies under field and laboratory situations. *Advances in Veterinary Science and Comparative Medicine*, **24**:179-210.

Stryer, L. 1988. Biosynthesis of membrane lipids and steroid hormones. In: Biochemistry, 3rd. Edition, pp. 547-574. W.H. Freeman and Company: New York.

Syme, G.J. 1974. Competitive orders as measures of dominance. *Animal Behaviour*, **22**:931-940.

Tuli, J.S., Smith, J.A. and Morton, D.B. 1995. Corticosterone, adrenal and spleen weight in mice after tail bleeding, and its effect on nearby animals. *Laboratory Animals*, **29**:90-95.

Verkerk, G.A., Phipps, A.H. and Matthews, L.R. 1996. Milk and plasma cortisol concentrations following transport or injection of ACTH. In: Advances in animal behaviour and welfare in Australasia and Africa. Abstracts of papers presented to the first regional meeting of the Australasian African region of the International Society of Applied Ethology. AgResearch: Hamilton.

Wasser, S.K., Thomas, R., Nair, P.P., Guidry, C., Southers, J., Lucas, J., Wildt, D.E. and Monfort, S.L. 1993. Effects of dietary fibre on faecal steroid measurements. *Journal of Reproduction and Fertility*, **97**:567-574.

Wasser, S.K., Monfort, S.L., Southers, J. and Wildt, D.E. 1994. Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*) faeces. *Journal of Reproduction and Fertility*, **101**:213-220.

Wasser, S.K., Bevis, K., King, G. and Hanson, E. 1997. Noninvasive physiological measures of disturbance in the Northern Spotted Owl. *Conservation Biology*, **11**:1019-1022.

Webb, M.L. and Mashaly, M.M. 1984. Effect of adaptation to handing on the circulating corticosterone concentration of laying hens. *British Poultry Science*, **25**:425-427.

White, P.J., Garrott, R.A., Kirkpatrick, J.F. and Berkely, E.V. 1995. Diagnosing pregnancy in free-ranging elk using faecal steroid metabolites. *Journal of Wildlife Diseases*, **31**:514-522.

Williams, C.G., Siegel, P.B. and Gross, W.B. 1977. Social strife in cockerel flocks during the formation of peck rights. *Applied Animal Ethology*, **3**:35-45.

Wingfield, J.C. 1994. Modulation of the adrenocortical response to stress in birds. In: Perspectives in comparative endocrinology, pp. 520-528. K.G. Davey, R.E. Peter and S.S. Tobe (eds.) National Research Council of Canada: Ottawa.

Wingfield, J.C. 1996. Corticosterone and behavioural responses to unpredictable events (abstract). In: Abstracts of 6th International Symposium on Avian Endocrinology held in Canada, 1996.

Wingfield, J.C., Vleck, C.M. and Moore, M.C. 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. *Journal of Experimental Biology*, 264:419-428.

Wingfield, J.C., O'Reilly, K.M. and Astheimer, L.B. 1995. Modulation of the adrenocortical responses to acute stress in arctic birds. *American Zoologist*, **35**:285-294.

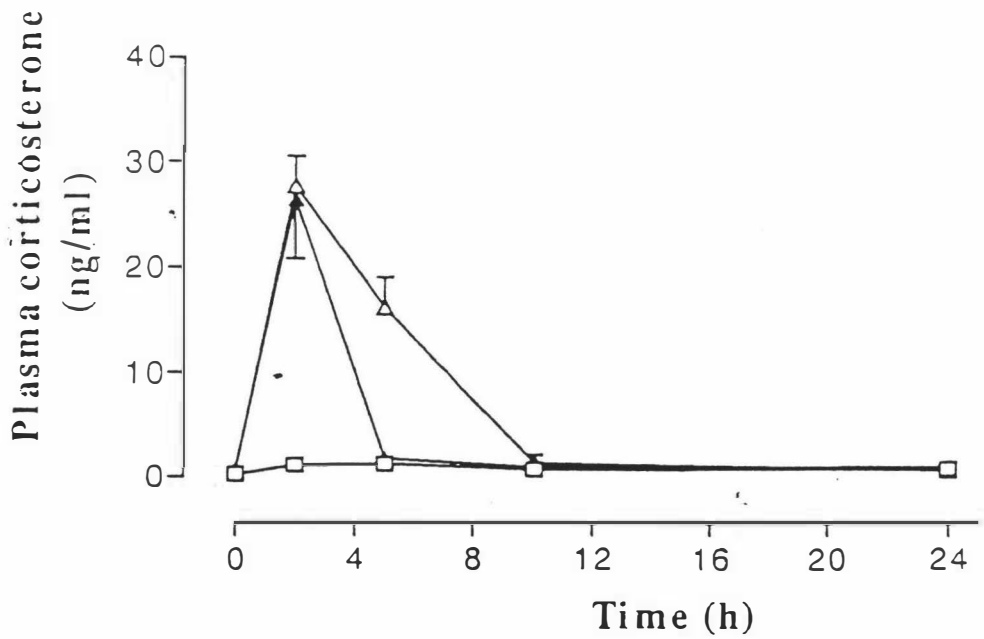
Wingfield, J.C., Hunt, K., Breuner, C., Dunlap, K., Fowler, G.S., Freed, L. and Lepsom, J. 1997. Environmental stress, field endocrinology and conservation biology. In: Behavioral Approaches to Conservation in the Wild, pp. 95-131. J.R. Clemmons and R. Bucholz (eds.) Cambridge University Press: Cambridge.

Zachariasen, R.D. and Newcomer, W.S. 1974. Phenylethanolamine-N-methyl transferase activity in the avian adrenal following immobilization of adrenocorticotropin. *General and Comparative Endocrinology*, **23**:193-195.

Zenoble, R.D., Kemppainnen, R.J., Young, D.W. and Clubb, S.L. 1985a. Endocrine responses of healthy parrots to ACTH and thyroid stimulating hormone. *Journal of the American Veterinary Medical Association*, **187**:1116-1118.

Zenoble, R.D., Kemppainnen, R.J., Young, D.W. and Carpenter, J.W. 1985b. Effect of ACTH on plasma corticosterone and cortisol of eagles and condors. *Journal of the American Veterinary Medical Association*, **187**:1119-1120.

APPENDIX 1



Plasma corticosterone responses of cockerels to intramuscular administration of 30 IU/kg ACTH at 0 h (mean ± SEM) (□ = isotonic saline; △= 30 IU/kg i.m. ACTH; ▲ = 10 IU/kg i.m. ACTH). These results are from an experiment conducted by R.M. Osborne in 1995.