Corticosterone responses, fear behaviour and sociality in laying hens

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Abstract

The overall aim of this thesis was to compare behavioural measures of fear and sociality and corticosterone responses to a stressor in white Leghorn and brown Hyline hens and to examine the relationship between corticosterone and these behaviours. The first set of experiments involved taking behavioural measures of fear and sociality. Tonic immobility and open field tests were conducted to measure fear whist a runway test was used to measure sociality. There was a distinct difference in underlying fear levels with white hens being more fearful than brown, principal components analysis further distinguished this difference. There was no difference in the levels of sociality between the two strains of hen. The second set of experiments investigated the hen's corticosterone response to a 15 min handling stressor and 15 min and 60 min restraint stressors. Corticosterone responses to these stressors were measured by the collection of blood samples at 0. 15, 30 and 60 min after the stressor had begun. Both strains of hen responded to the handling procedure with a greater corticosterone response than to either restraint procedure, with no difference between the strains of hen. There was no difference in corticosterone response to both the restraint procedures but the white hens had a greater corticosterone response than brown hens. The third part of this thesis investigated the repeatability of the tonic immobility test and examined the relationship between corticosterone and fear and sociality. No difference was found in the mean behavioural measures of the first and second tonic immobility test for either strain of hen, but correlations and statistical repeatability calculations indicated that the tonic immobility test was more repeatable for brown than white hens. Correlations were found between corticosterone and behavioural measures of fear and sociality in white hens only.

Principal components analysis supported these findings and indicated that there was a positive relationship between corticosterone and fear and a negative relationship between corticosterone and sociality. The findings of the present study have provided information about the behavioural and physiological responses of white Leghorn and brown Hyline hens and show that the use of derived measures such as principal components analysis can provide useful information about relationships between variables in laying hens.

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Chapter 1: General introduction

When birds encounter a stressor they react with in an increase of corticosterone, if the stimulus elicits an emotional response then the individual may experience the basic emotion of fear. These responses can cause both physiological and behavioural changes in the individual. Different strains of the same species can differ in the magnitude of their response to a stimulus (Korte et al., 1997; Fraisse and Cockrem, 2006), thus it is likely that some individuals will be more successful with interacting with some environments than others.

Differences in fear, sociality and corticosterone responses to stressor were investigated in white Leghorn and brown Hyline hens and the relationships between these variables were examined. This literature review provides an overview of corticosterone and fear on birds with an emphasis on chickens.

1.1 Stress, stressor and stress response

Stress is a commonly used term and many attempts have been made to define it, with different emphases placed on physiological or psychological aspects of stress. It is important therefore to define the terms stress, stressor and stress response for the current study.

An animal's environment is constantly changing and some of these varying conditions may expose the animal to a stimulus or stimuli (stressors) that result in the activation of

the hypothalamo-pituitary-adrenal (HPA) axis. This activation is the basis of a stress response (Axelrod and Reisine, 1984; Siegel, 1995; Gabry et al., 2002).

A stressor is any stimulus that activates the HPA axis and causes an increase in glucocorticoid secretion. Stressors originate from a wide variety of sources and can be environmental (e.g. extremes of temperature), nutritional (e.g. food or water deprivation), physiological (e.g. disease, injury) or psychological (e.g. threat from a predator; Harvey et al., 1984; Mench, 1991). Novelty and uncertainty play a large role in whether an individual will experience a stress response to a potential stressor. The more familiar or predictable an object or the occurrence of an event the smaller the likely stress response. A stimulus that is perceived as a stressor by one individual may not be perceived as a stressor by another individual (Levine, 1985).

A stress response is an animal's response to a stressor. When exposed to a stressor, both the sympathetic nervous system (SNS) and the HPA axis are activated. Activation of the SNS leads to a short-term neural response, which involves the release of catecholamines from the adrenal medulla, whereas the HPA axis response involves the release of glucocorticoids from the adrenal cortex and takes longer to occur. The functions of the catecholamines and glucocorticoids are to restore the homeostatic balance of the animal by either helping the animal to adapt to the new situation or by promoting behaviours that will remove the animal from the stressor to increase the chance of survival (Siegel, 1995; Gabry et al., 2002).

An animal can be said to be experiencing stress when a stress response has been elicited in response to one or more stressors.

1.2 Hypothalamo-pituitary-adrenal (HPA) axis

1.2.1 Components

The components of the HPA axis are the hypothalamus, pituitary gland and adrenal glands. The HPA axis is generally similar throughout all vertebrate species. The hypothalamus is located in a small region in the brain below the thalamus. It is the major integrating link between the nervous and endocrine systems, receiving information from many different areas of the brain. It is involved in the production and regulation of many hormones (Hadley, 1996). The pituitary gland is connected to the hypothalamus by a stalk called the infundibulum. The hormones released from the hypothalamus enter the hypophyseal portal blood system, which provides a passage from the median eminence to the anterior pituitary gland. There are two distinct embryonic origins of the tissues of the pituitary gland with the regions of different origin known as the adenohypophysis and neurohypophysis. They go on to form two anatomically and functionally distinct regions; these are the anterior and posterior pituitary. It is important to note that in birds there is no pars intermedia which in mammals forms part of the anterior pituitary gland (Scanes, 2000).

The adrenal glands are paired structures, which are located anterior and medial to the cephalic lobe of the kidney (Carsia and Harvey, 2000). The adrenal glands are well supplied with blood vessels, and in contrast to mammalian adrenal glands with discrete

regions, the cortical and medullary tissue are intermingled in birds (Freeman, 1971; Mench, 1991; Carsia and Harvey, 2000). Glucocorticoids are secreted from the adrenal cortex (Carsia and Harvey, 2000).

When a stressor activates the HPA axis the hypothalamus secretes corticotrophin releasing hormone (CRH) and, in birds, arginine vasotocin (AVT). CRH and AVT then cause the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH is carried in the blood to the adrenal glands and causes the adrenal cortex to secrete glucocorticoids (Mench, 1991).

1.2.2 CRH and AVT

CRH is an amino acid peptide hormone produced in the hypothalamus (Freeman, 1971; Harvey and Hall, 1990; Mench, 1991). Mammalian CRH consists of 41 amino acid residues and is highly conserved throughout all vertebrates (Hadley, 1996). CRH stimulates the release of ACTH from the anterior pituitary gland (Freeman, 1971; Harvey and Hall, 1990; Mench, 1991). CRH release is inhibited by negative feedback from glucocorticoids (Harvey et al., 1984).

AVT is involved in maintaining water balance in birds. It is secreted by the hypothalamus and has similar actions to CRH in stimulating the secretion of ACTH from the anterior pituitary gland (Harvey and Hall, 1990; Scanes, 2000; Tachibana et al., 2004). The mammalian equivalent is arginine vasopressin (AVP) which differs from the avian structure by one amino acid residue (Scanes, 2000). Although CRH is the primary

regulator of ACTH secretion from the anterior pituitary, AVT also stimulates the secretion of ACTH (Harvey and Hall, 1990; Scanes, 2000). CRH and AVT act synergistically to increase the secretion of ACTH (Harvey and Hall, 1990). There is evidence that the relative proportions of each secretagogue vary with physiological status (Scanes, 2000).

1.2.3 ACTH

Avian ACTH, like mammalian ACTH, is a peptide hormone composed of 39 amino acids synthesised as part of a much larger protein called proopiomelanocortin. The corticotrophic cells of the anterior pituitary produce ACTH (Scanes, 2000). The secretion of ACTH is under hypothalamic control. CRF is the major stimulus but other secretagogues also cause its release. These enter the median eminence and travel to the anterior pituitary gland via the hypophyseal portal blood system (Harvey et al., 1984; Scanes, 2000). The catecholamines (in mammals; Axelrod and Reisine, 1984) and AVT also stimulate ACTH release, adding to the effect of CRF (Scanes, 2000). ACTH stimulates the release of the glucocorticoid corticosterone from the avian adrenal gland (Freeman, 1971; Harvey et al., 1984; Harvey and Hall, 1990; Mench, 1991; Siegel, 1995). The effect of ACTH varies with physiological state (Scanes, 2000). The secretion of ACTH is inhibited by glucocorticoids (Harvey et al., 1984; Carsia and Harvey, 2000; Scanes, 2000) and somatostatin (in mammals; (Axelrod and Reisine, 1984).

1.2.4 Glucocorticoids

Glucocorticoids are secreted by the adrenal cortex. The predominant glucocorticoid secreted is dependent on the species; in birds it is corticosterone (Freeman, 1971; Carsia and Harvey, 2000). Corticosterone has many actions that will be described in the next section.

There are two types of glucocorticoid receptors located in the brain, type I or mineralocorticoid receptor (MR) and type II or glucocorticoid receptor (GR). Activation of these receptors is part of the negative feedback pathway for the control of CRF secretion, so changes in receptor numbers will result in a change of sensitivity to the glucocorticoids. The MR receptors have a discrete distribution and are mainly localized in the hippocampal formation while the GR receptors have a broad distribution throughout the brain and pituitary (Dallman et al., 2002).

1.3 Corticosterone in birds

1.3.1 Synthesis and secretion

Corticosterone is the predominant glucocorticoid synthesised and secreted from the avian adrenal gland (Siegel, 1995; Carsia and Harvey, 2000). Corticosterone is synthesised from cholesterol (Freeman, 1971; Harvey et al., 1984; Carsia and Harvey, 2000) in a complex set of modifications to the cholesterol carbon skeleton. The enzymes involved in the corticosterone synthesis pathway vary in primary structure between species, but there are regions of the enzyme that are homologous between species and are thought to

be responsible for the enzymes' activity. Cholesterol is first converted to pregnenolone by mitochondrial cytochrome P-450_{SCC}. A microsomal dehydrogenase-isomerase enzyme catalyses the conversion of pregnenolone to progesterone. The microsomal enzyme responsible for the conversion of pregnenolone to progesterone can also be found in adrenocortical cell mitochondria. Progesterone is converted to 11-deoxycorticosterone (11-DOC) by microsomal P-450_{C21}. The conversion of 11-DOC to corticosterone is catalysed by the mitochondrial enzyme P-$450_{11\beta}$. 11-DOC is a major secretory product of the avian adrenal gland, but is also the final precursor in the formation of corticosterone, which is secreted at higher levels (Carsia and Harvey, 2000).

Aldosterone is formed from corticosterone in the adrenal glands (Freeman, 1985; Carsia and Harvey, 2000). There are two modes for this conversion in mammals; the porcine-bovine mode (modified P-$450_{11\beta}$ activity) and the rodent-human mode (distinctive enzyme, P-450_{C18} or P-450_{ALDO}). The enzymes that catalyse this conversion in birds may be similar to either one of these modes, but have not been isolated (Carsia and Harvey, 2000).

In most vertebrates, including birds, circulating glucocorticoids bind specifically to corticosteroid binding globulins (CBG) in the blood. It is the free corticosterone that is able to pass through cell membranes and bind to receptors and exert its effects. The effects of corticosterone may therefore depend on the specificity, quantity and binding capacity of CBG (Siegel, 1995; Breuner and Orchinik, 2002).

1.3.2 Corticosterone responses

There may be considerable individual variation in the corticosterone response to the same stressor between individuals of the same species (Littin and Cockrem, 2001; Cockrem and Silverin, 2002). The magnitude of the corticosterone response to a stressor depends on the kind of stressor (Cockrem and Silverin, 2002; Canoine et al., 2002). The corticosterone response of birds to a stressor may depend upon the level of intra-pair aggressiveness (Canoine and Gwinner, 2005), physiological state (before or after a moult; Astheimer et al., 1995; Romero et al., 1998), body condition (Breuner and Hahn, 2003), social role (Pravosudov et al., 2003; Nephew and Romero, 2003; Poisbleau et al., 2005), location, age (Romero et al., 1998) and sex (Pravosudov et al., 2001; Pravosudov et al., 2004). There can also be seasonal differences in the stress response (Breuner and Orchinik, 2001; Canoine and Gwinner, 2005). Glucocorticoid receptor numbers vary between seasons (Breuner and Orchinik, 2001). Changes in receptor numbers will result in changes in the sensitivity to glucocorticoids, thus allowing for an appropriate response depending on physiological needs.

1.3.3 Actions of corticosterone

1.3.3.1 Physiology

Metabolic processes may be significantly altered by the corticosterone. These changes may include an increase in plasma glucose, glycogenolysis, gluconeogenesis, muscle protein catabolism, uric acid secretion, sodium retention, fat deposition, synthesis of fatty acids, changes in saturated to unsaturated fat ratio and calcium transport from the skeleton (adult birds). In young birds there can be a decrease in skeletal calcification.

These changes in metabolism may result in depressed growth and skeletal development in young birds (Siegel, 1995) and weight loss in adult birds (Siegel, 1995; Astheimer et al., 2000).

It has been suggested that corticosterone has a generally inhibitory effect on reproduction in birds (Siegel, 1995). However there a numerous studies that show that this is not necessarily the case with corticosterone being significantly elevated in many species during breeding (Romero, 2002), individuals at different breeding stages (e.g. laying, incubating or chick rearing) having significantly different basal plasma corticosterone concentration (Love et al., 2004), and corticosterone treatment having no effect on reproductive hormone concentration (Astheimer et al., 2000).

Corticosterone can affect the immune system, although the effects appear to be dependent on the type of stressor as well as the genetics of the individual. Effects can include suppression of the both antibody and cell-mediated immunity responses. Corticosterone can reduce inflammation by decreasing macrophage migration, inhibiting phagocytosis and reducing monocyte accumulation at the inflammatory site. These effects appear to be a result of inhibition of the enzymes necessary for those processes (Siegel, 1995).

1.3.3.2 Behaviour

Important effects of corticosterone on behaviour are increases in locomotor activity (Breuner et al., 1998; Kitaysky et al., 2001; Lohmus et al., 2003; Breuner and Hahn, 2003) and foraging behaviour (Pravosudov, 2003). These changes are rapid, in contrast

to some of the other effects of corticosterone (Breuner et al., 1998). The concentration of corticosterone may be important in determining the change in behaviour as it has been found that at intermediate concentrations, corticosterone increases locomotive activity but when an individual had high concentrations of corticosterone then changes in behaviour did not occur (Breuner et al., 1998; Breuner and Wingfield, 2000) In addition, the effect of corticosterone on locomotor activity appears to be less during short days (winter) than long days (summer; Breuner and Wingfield, 2000). Chicks, like adults can also show an increase in activity in response to corticosterone. Chicks with higher levels of corticosterone exhibited more aggressive behaviour (Kitaysky et al., 2003) and higher begging behaviour (Kitaysky et al., 2001; Kitaysky et al., 2003). The increase in begging behaviour resulted in an increase of parents' chick feeding rates (Kitaysky et al., 2001). These chicks also consumed significantly more food but this did not increase their growth rates in comparison to those not treated with corticosterone (Kitaysky et al., 2003).

Food deprivation or a reduction in food availability may result in increased basal corticosterone concentrations in both chicks (Kitaysky et al., 2001) and adults (Kitaysky et al., 1999; Pravosudov et al., 2001; Lynn et al., 2003). This effect is consistent with the increased foraging behaviour seen in adults (Pravosudov, 2003) and increased begging behaviour in chicks (Kitaysky et al., 2001; Kitaysky et al., 2003) which have high corticosterone concentrations, as these actions will lead to an increase the amount of food consumed. However it is important to note that food deprivation does not always lead to increased corticosterone concentration in all chicks. Those chicks that have parents that are insensitive to their demands for food seem to have suppressed HPA axis activity

when they are food deprived (Kitaysky et al., 2005). This means that the chicks will not further deplete their energy reserves by increasing their activity nor will the corticosterone induced catabolic effect on their metabolism be as great. Food caching behaviour also appears to be affected by corticosterone. Food caching birds with higher concentrations of corticosterone consumed significantly more food and cached more than those with lower concentrations. Food caching birds with high concentrations of corticosterone were also able to locate their caches with fewer wrong inspections (showing better spatial memory) than their counterparts with lower concentrations of corticosterone (Pravosudov, 2003).

It appears that corticosterone may be important in regulating migratory behaviour. Individuals with higher corticosterone concentrations showed increased activity, more appropriate seasonal migration orientation (Lohmus et al., 2003), were in a more suitable physical condition and were less responsive to capture and handling stressors (Holberton et al., 1996; Holberton, 1999). Corticosterone concentrations may decrease after migratory birds stop to 'refuel'. Corticosterone may rise in these birds before they depart, possibly in preparation for the next leg of the migration (Landys-Ciannelli et al., 2002).

1.3.3.3 Other actions

Chicks with higher concentrations of corticosterone appear to have decreased cognitive abilities when compared to those not treated with corticosterone. This effect persisted later on in life even when the birds were no longer being treated (Kitaysky et al., 2003).

1.4 Corticosterone in chickens

1.4.1 Stimuli for release

There are a great variety of stressors that may elicit the release of corticosterone in chickens. These potential stressors include immobilisation, prolonged immobilisations, deprivation of water (Beuving and Vonder, 1978; Korte et al., 1997) or food, temperature extremes, shaking, disease (Freeman, 1971) and handling (Freeman, 1971; Beuving and Vonder, 1978).

1.4.2 Corticosterone responses

Not all corticosterone responses to the same stressor will be similar; some individuals will secrete significantly more or less corticosterone at different time periods of the response than other individuals (Littin and Cockrem, 2001). This secretion is also dependent on the type of stressor (Beuving and Vonder, 1978) and strain (Korte et al., 1997).

1.4.3 Actions of corticosterone

1.4.3.1 Physiology

Corticosterone treatment can cause significant changes in the metabolism, immune system function and reproductive function of the chicken. Metabolic effects can include a decrease in plasma growth hormone concentrations (Davison et al., 1980), food efficiency, breast (Gross et al., 1980) and leg muscle weight (Siegel and Van Kampen, 1984), and increases in liver weight (Siegel and Van Kampen, 1984; Williams et al.,

1985; Donker and Beuving, 1989) abdominal fat (Davison et al., 1983; Siegel and Van Kampen, 1984; Williams et al., 1985) excretion of uric acid and water (Siegel and Van Kampen, 1984), concentration of plasma uric acid, protein (Davison et al., 1983), thyroid hormones (John et al., 1987), cholesterol and triglycerides (Davison et al., 1983). Corticosterone may cause a significant decrease in weight of some of the organs of the immune system such as the spleen, thymus and the bursa of Fabricius (Gross et al., 1980; Donker and Beuving, 1989). Other effects can include an increase in the heterophil/lymphocyte ratios (Gross et al., 1980; Jones et al., 1988; Donker and Beuving, 1989; El-Lethey et al., 2001; El-Lethey et al., 2003) and a reduced antibody response to an antigen (Gross et al., 1980). The clearest effect of corticosterone on reproductive function in chickens is a pause in oviposition (Williams et al., 1985; Moudgal et al., 1991), often accompanied by a reduced ovulation rate. These effects, however, were dependent on the concentration of corticosterone (Petitte and Etches, 1991; Moudgal et al., 1991). Other effects of corticosterone have included a reduction in the weight of the ovaries (Petitte and Etches, 1991), oviduct (Williams et al., 1985; Petitte and Etches, 1991) and testes (Gross et al., 1980).

1.4.3.2 Behaviour

Corticosterone can affect behaviour in chickens. In particular there can be an increase in feather pecking behaviour (although this may also be dependent on other factors; El-Lethey et al., 2001), food intake (Gross et al., 1980; Siegel and Van Kampen, 1984; Petitte and Etches, 1991; El-Lethey et al., 2001), water intake (Siegel and Van Kampen, 1984) and tonic immobility duration (Jones et al., 1988; El-Lethey et al., 2003).

1.5 Fear

1.5.1 Basic emotions

Basic emotions are special adaptive behaviours that are vital to survival to combat challenges derived from essential life tasks such as eating, drinking, reproduction and avoiding dangerous situations (LeDoux, 1996). Some basic emotions include fear, hunger and thirst (Rolls, 1999). When external stimuli generate an emotional response the stimuli are first processed in the thalamus. The thalamus then sends rough information to the amygdala to enable the individual to react quickly. At the same time the thalamus also sends information about the stimuli to the cerebral cortex for further processing of the stimuli. The outcome of the processing is also sent to the amygdala for further action. It is the amygdala that mediates the generation of emotions. The initial quick response is vital in case the stimuli put the individual in a threatening situation, since it is better to react than wait for fully processed information (LeDoux, 1996).

1.5.2 What is fear?

Fear is a basic emotion and is thought to be a defensive mechanism. When an animal experiences fear it quickly reacts to a stimulus via the amygdala to increase the probability of survival and lower the chance of injury for the individual (Jones, 1996; LeDoux, 1996). Fear occurs in all animals although only conscious animals will feel afraid. Responses of animals to stimuli vary between animals, so a stimulus that induces fear in one animal may not induce fear in another animal (LeDoux, 1996).

1.5.3 Fear in chickens

1.5.3.1 How to measure fear

Fear is experienced by animals and is difficult to quantify. However once an individual perceives a situation as fearful the fear response takes over and inhibits all other behavioural systems. Individuals can be placed in test situations, with their responses providing a basis to infer how much fear they experience. The majority of tests of fear on chickens are based on novelty. The level of fear a chicken perceives can be quantified by introducing it to a novel situation, such as a new environment, object or person and recording then analysing its behaviour (e.g. avoidance, approach, position or locomotive activity; Jones, 1996). The other major method of quantifying the level of fearfulness is to measure tonic immobility (TI). This is an innate response in which the animal adopts an immobilised posture and has reduced responses to external stimuli. Chickens can be made to go into TI by a brief period of restraint. The time taken for the animal to return back to its normal state is taken to be a measure of its level of fearfulness (Jones, 1986; Jones, 1996). Stimuli that evoke a fear response increase the duration of TI whereas those that reduce it decrease the duration (Gallup, 1979; Jones, 1986).

1.5.3.2 Stimuli that induce fear

Throughout the course of their life all chickens will encounter stimuli that they may consider a threat and activates a fear response. However not all individuals will react in the same way to the potential threat (Jones, 1996). Factors that may affect the magnitude of the response include the individual's strain (Gallup et al., 1976; Murphy, 1977; Jones, 1996), social rank (Cunningham et al., 1988), prior experience, the husbandry system it is

reared (Jones, 1996) or housed in (Sefton, 1976; Jones and Faure, 1981; Kujiyat et al., 1983; Hansen et al., 1993; Jones, 1996) and cage tier (Sefton, 1976; Jones, 1985). The majority of stimuli that have the potential to elicit a fear response in chickens are related to a change in either the social or physical environment. The main changes in the social environment that can elicit fear are the removal of familiar companions or the introduction of unfamiliar birds. Fear responses to changes in the physical environment are usually due to exposure to a predator, unexpected appearances of familiar objects, people, or birds (although familiar the sudden appearance is potentially frightening) or novelty. Novelty includes anything the individual has not previously encountered; examples include a new environment, person, object, bird, odour (Jones, 1996) or noise (Jones, 1996; Campo et al., 2005). Treatment with corticosterone (Jones et al., 1988), adrenaline (Braud and Ginsburg, 1973) or exposure to a stressor (Marin et al., 2001) before a test of fear increases the fear response in chickens.

1.6 Outline of thesis

This thesis consists of five chapters; a general introduction, three experimental chapters and a general discussion. The overall aim of the thesis was to compare fear, sociality and corticosterone responses to a stressor between white Leghorn and brown Hyline hens and to investigate relationships between corticosterone and fear and sociality. The second chapter compares behavioural measures of fear and sociality between the strains of hen whilst the third chapter examines corticosterone responses between the hens to different stressors. Chapter Four examines the relationship between the corticosterone response to

handling and behaviour. The final chapter discusses the thesis results and gives future directions for research.

Chapter 2: Measurement of fear behaviour and sociality in white and brown hens

2.1 Abstract

Behavioural measures of fear and sociality in caged hens on a commercial farm were compared between white Leghorn and brown Hyline strains. Tonic immobility and open field tests were used to measure fear, and a runway test was used to measure sociality. The plasma corticosterone response to the open field was also measured by the collection of a blood sample immediately after the test. The duration, latency to first head movement and number of head movements were greater in white than brown hens in the tonic immobility test, whilst the number of inductions required was less in white hens. Differences between strains were less pronounced in the open field test, with the latency to vocalise and number of vocalisations less in white hens and no differences between strains in three other variables in the test. Plasma corticosterone increased after 10 minutes in the open field in white and brown hens, with no difference between strains in their corticosterone responses. The increase in corticosterone is consistent with the use of the open field test as a measure of fear in hens. There were no differences between strains in any of the four variables in the runway test. Principal components analysis was used to examine relationships between variables in the three behaviour tests. Four principal components were identified that accounted for 69.7% of the total variance. Two of these components were related to fear behaviour. Principal component scores differed between strains for two of the components. This study has clearly shown difference in fearfulness between white and brown hens, and indicates that principal

components analysis is a valuable tool for the measurement of fear in groups of laying hens.

2.2 Introduction

There is growing concern over the welfare of caged hens compared to hens in other housing systems. Although welfare is difficult to define and measure (Barnett and Hemsworth, 2003), some variables are generally accepted as relevant to discussions of the welfare of hens. Fear is one such variable. It is a basic emotion and is thought to be a defensive mechanism (LeDoux, 1996; Jones, 1996). Hens that regularly experience fear can be considered to have a lower state of welfare than hens that rarely experience fear. Social motivation is another variable that can affect welfare. For example, increased contact with other individuals might be detrimental for a bird with low social motivation, so it is important that hens have levels of sociality that are appropriate for their housing system.

Fear and sociality can be measured by conducting behavioural tests. Three tests that have been used in hens are the tonic immobility, open field and runway tests. Tonic immobility, commonly used to assess fear, is an innate response which is triggered by physical restraint where an animal adopts an immobilised posture and has reduced responses to external stimuli (Jones, 1986). It is thought the duration of tonic immobility reflects the degree of fear that an individual is experiencing. The open field test involves removing the hen from its home environment and placing it in a barren enclosure, and then recording particular behaviours (Jones, 1996). Open field behaviour can be interpreted in several ways, the most popular being that the behaviour or lack of behaviour shows the degree of fear in an individual (silent and inactive individuals are more fearful than those that are vocal and active; (Jones, 1996) and that it represents a

compromise between predator avoidance and social motivation (Gallup and Suarez, 1980). The runway test consists of the hen being placed in a runway that has hens at the other end. The times taken to reach particular points in the runway are recorded. The runway test is a measure of sociality in which the time spent near other hens and the time to reach the other hens are thought to reflect underlying levels of sociality (Gallup and Suarez, 1980).

Corticosterone is the predominant adrenal glucocorticoid hormone in birds (Carsia and Harvey, 2000) and is secreted when birds respond to a stressor (Cockrem et al., 2004). Hens treated with corticosterone for several days before a tonic immobility test had increased durations of tonic immobility (Jones et al., 1988), indicating that corticosterone can increase underlying fear levels in hens. This positive association between corticosterone and fear suggests that if the open field test does measure fear as proposed by Jones (1996), then the hens will have an increased plasma corticosterone concentration following the test.

Genetic selection can be used to achieve appropriate levels of fear and sociality in commercial hens (Jones et al., 1999; Hocking et al., 2001). Strains of hens differ in their temperaments (Gallup et al., 1976; Murphy, 1977; Jones, 1987a; Keerkeer et al., 1996), so studies of fear and sociality in different strains of hen can provide information relevant to selection programmes. White Leghorn and brown Hyline hens are commonly commercially used stains of hen, their selection for egg production has resulted in differences in their temperament with white Leghorn and brown Hyline being considered

flighty and docile respectively. This difference in temperament means that one strain may be better suited to this particular environment. Therefore the goal of the present study was to compare behavioural measures of fear and sociality between white Leghorn and brown Hyline hens. Tonic immobility, open field and runway tests were conducted to compare strains, and relationships between behaviour variables were examined using correlations and principal components analysis. Corticosterone responses to the open field test have not previously been measured in adult hens so they were also examined in this study.

2.3 Materials and methods

2.3.1 Animals and husbandry

The study was conducted in a local poultry farm in a shed that could house 28 000 hens. The shed had eight rows of four tiered cages, with 152 cages in each tier. Two rows contained white Leghorn hens and the other six rows contained brown Hyline hens. The cages were 2.00 x 0.59 x 0.55 m (width x depth x height). Each cage was divided into three compartments of two different sizes; the first and third compartment were 0.70 x 0.59 x 0.55 m (width x depth x height) in size and contained 7 hens, whilst the middle compartment was 0.60 x 0.59 x 0.55 m (width x depth x height) in size and contained 6 hens. There was 585 cm^2 floor space per hen in all compartments. Food and water were provided automatically, and eggs and faeces were collected on conveyor belts.

2.3.2 Experimental design

Blood samples were collected for corticosterone measurement and behaviour tests were conducted to compare the two strains of hens. The hens selected for use in this study were from two rows adjacent to one another. One row contained white Leghorn hens and the other brown Hyline hens. Both rows of hens had a row of their respective strain directly behind them. Hens were sampled along each row, with the first hens in each row selected from the first compartment of the second cage. One hen from each 7-bird cage was randomly removed and was banded with a coloured plastic leg band. 50 birds of each strain were used.

All hens of each strain underwent three behavioural tests (tonic immobility, open field and runway). The order of these tests and the handler performing them were randomised for each bird, with a period of two weeks between each behavioural test.

2.3.3 Behavioural observations and sample collection

2.3.3.1 Tonic immobility

Hens were removed from their cage and taken to the end of the shed where they were placed on their side on several layers of cloth. Tonic immobility (TI) was induced by lightly restraining the bird with one hand held over its head and the other hand placed on its keel for 15 sec (Fraisse and Cockrem, 2006). The handler then slowly removed their hands and moved back from the bird. If the bird righted itself within 15 sec then the induction process was repeated up to 5 times. The duration of TI, the latency from induction to the first head movement and the number of head movements were recorded.

If TI was not induced after 5 attempts the hen was given a score of 0 for both duration and latency to first head movement. When the bird remained in TI a maximum 20 min test period was used and the bird was given a score of 20 min for the duration of TI.

2.3.3.2 Open field

Hens were removed from their cages and taken to one end of the shed in which they were placed in a square arena (2.00 x 2.00 m). The arena was barren and had unpainted concrete floors and walls of strong, plain cardboard 0.85 m high. After the hen was placed in the arena the observer sat close by and watched the behaviour of the bird. The latency to first step, the number of steps, latency to first vocalisation, number of vocalisations and number of defecations were recorded in a 10 min period. If the bird did not step or vocalise in this time a score of 10 min was given for the latency to first step or latency to first vocalisation. At 10 min the bird was picked up and a single blood sample (0.5 – 3 ml) taken from the ulnar vein using a heparinised 25 g needle and 3 ml syringe. All samples were taken within three min of the hen being picked up. In addition, blood samples had been collected from the hens up to 8 weeks before or after the open field test to allow the comparison of corticosterone concentrations between undisturbed hens and hens that after 10 min in the open field test. Samples were collected from undisturbed hens by removing a hen from its cage and collecting a blood sample within three min by the same method used for sampling hens after the open field test. All blood samples were expelled into heparinised test tubes, stored on ice then transported to the laboratory where they were centrifuged and plasma frozen at -70°C.

2.3.3.3 Runway

The runway apparatus was 2.40 m long x 0.60 m wide and 0.85 m high. A wire partition 0.4 m from one end created an area was designated as the start box. At the opposite end of the runway there was a plastic chicken crate (0.54 x 0.31 x 0.74 m width x depth x height). Two hens from the same cage as the test bird were placed in the chicken crate and this was designated the goal box. Test hens were individually removed from their cage, carried to the end of the shed and placed in the start box of the runway. After a hen had been in the start box for 2 min the partition was raised. The latency for the bird to leave the start box, time to reach 0.5 m from the start box, time to reach within 0.2 m of the goal box and total time spent within 0.2 m of the goal box were recorded during a 10 min test period.

2.3.4 Plasma sample preparation and corticosterone radioimmunoassay

2.3.4.1 Plasma sample preparation

Each plasma sample was thawed, and spun for 10 min at 13 000 rpm. This process separated lipid from the plasma. The clear plasma from the lipid layer was transferred to a Sterilin RT20 tube and diluted in PBSG by a factor of 6 for the assay of corticosterone.

2.3.4.2 Radioimmunoassay of corticosterone

Corticosterone concentrations in plasma extracts were measured by radioimmunoassay following the method of Fraisse and Cockrem (2006). Samples were assayed in duplicate. 10µl of extract in PBSG was incubated with 20µl of iodinated corticosterone and 20µl of antiserum (^{125}I-corticosterone and antiserum ImmuChem^{TM} Double Antibody

Corticosterone ^{125}I RIA kit for rats and mice, MP Biomedicals, USA; 4000 cpm) for 2 hours at room temperature (22°C – 25°C). 50 µl of precipitant solution (MP Biomedicals, USA) was added and each sample was centrifuged at 3 000 rpm for 15 min at 4°C. 20 µl of starch (2.5 g/l starch in PBSG) was then added to increase adhesion of the pellet to the tube. Samples were then centrifuged for a further 15 min at 3 000 rpm at 4°C, and the supernatant aspirated off. The pellets were counted on a LKB Wallac 1261 Multigamma gamma counter for 2 min each.

2.3.4.3 Assay validation and characteristics

Serial dilutions of plasma in PBSG were parallel to the corticosterone standard curve. The quantitative recoveries of corticosterone in plasma were measured by adding different amounts of corticosterone to two plasma samples in PBSG. The recoveries of added corticosterone were 96.7 $\pm$ 2.5% and 96.6 $\pm$ 7.7%.

The sensitivity of the corticosterone assay was the minimum hormone level that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean - 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity expressed as ng steroid/ml plasma was 0.35 ng/ml.

Solutions of corticosterone in PBSG at concentrations that gave approximately 80, 50 and 20% binding on the standard curve were used as low, medium and high quality controls in every assay. The intra-assay coefficients of variation were 8.4 % (n=20), 6.0 % (n=20), and 5.3 % (n=10) and inter-assay coefficients of variation for ten assays 16.0%, 8.3% and 16.9% for low, medium and high solutions respectively.

The cross-reactivities of the corticosterone antibody with other steroids were reported by ICN Biomedicals as deoxycorticosterone (0.34%), cortisol (0.05%), testosterone (0.01%), aldosterone (0.03%), progesterone (0.02%), androstenedione (0.01%), 5α-dihydrotestosterone (0.01%) and cholesterol, 11-deoxycortisol, dehydroepiandrosterone, dehydroepiandrosterone-sulphate, 20α-dihydroprogesterone, oestrone, oestradiol-17α, oestradiol-17β, oestriol, pregnenolone, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone (<0.01%).

2.3.5 Statistical analysis

Levene's tests indicated that variances were not homogeneous for some behaviour variables, so non parametric statistics were used for all behaviours. Kruskal-Wallis one way ANOVAs were used to determine if there were effects of the number of times a bird had been picked up prior to a test or of the handler performing the test. Differences between strains for the measured variables were examined with Mann-Whitney U tests. Relationships between pairs of variables within each behaviour test were determined with Spearman rank correlations.

Principal components analyses were used to further examine relationships between variables. This approach has been used in studies of feather pecking behaviour in hens (Van Hierden et al., 2002), tonic immobility and open field tests in Japanese quail (Jones et al., 1991), and open field and novel object tests in calves (Miller et al., 2005). Principal components were identified for each behaviour test separately, from analyses of tonic immobility and open field variables combined, and from analyses of variables from

all three behaviour tests combined. Principal components are linear combinations of the original variables, and principal components analysis can summarise many variables into a few factors. The loading of each variable for a principal component indicates the importance of each variable for the component. Principal components analyse were performed on the Pearson correlation matrix for each combination of variables, with principal components with eigenvalues greater than or equal to one retained for further analyses. Components were rotated after the initial analysis and component loadings and the percentage of total variance explained by each component were calculated. Component scores were calculated from the original data by multiplying coefficients for each component by the values of each variable for individual birds. Differences between strains for the component scores were then examined with Mann-Whitney U tests.

Plasma corticosterone concentrations were transformed to logarithms and Levene's tests were performed to check for equality of variances before parametric analyses were performed. A repeated measure two way ANOVA was used to compare corticosterone concentrations between times and strains. Statistical analyses were performed using Prism (GraphPad Software Inc.) and Systat (SPSS Inc.). Data are presented as individual points or as mean $\pm$ S.E.

2.4 Results

2.4.1 Order of tests and comparisons between handlers

The number of times a hen had been picked up at the time of a behaviour test did not influence any variables for the three behaviour tests for each strain (Kruskal-Wallis one way ANOVA $p>0.15$ for all behaviour tests; see Appendix Tables 1, 2 and 3 for details).

There were differences between the three handlers in some measures of tonic immobility (Kruskal-Wallis one way ANOVA white birds number of inductions $K_2 = 6.395$, $P=0.041$; latency to first head movement $K_2 =22.479$, $P<0.01$; number of head movements $K_2 = 17.097$; brown birds latency to first head movement $K_2 = 9.162$, $P = 0.010$). There were also differences between handlers in some measures of the open field behaviour (Kruskal-Wallis one way ANOVA brown birds latency to first step $K_1=367.50$, $P=0.022$; latency to first vocalisation $K_1=381.00$, $P=0.013$; number of steps $K_1=152.00$, $P=0.013$). There were no differences between handlers in measures in the runway test (data not shown; see Appendix Tables 4, 5 and 6 for details). All birds were randomly allocated to handlers for each test, so handler effects were not considered in subsequent comparisons of white and brown hens.

2.4.2 Tonic immobility, open field and runway tests

The duration of tonic immobility, latency to first head movement and number of head movements were greater in white than brown birds in the tonic immobility test, whereas the number of inductions was less (Table 2.1). The latency to vocalise in the open field test was longer and there were fewer vocalisations in white compared with brown birds

(Table 2.1). There were no differences between the strains in the latency to first step, number of steps or number of defecations in the open field. There were no differences between strains in any of the variables for the runway test (Table 2.1).

Plasma corticosterone concentrations were markedly increased after 10 min in the open field test (Fig. 2.1), with no differences between strains in corticosterone in undisturbed birds or after the open field test (see Table 2.2 for statistics).

2.4.3 Principal components analyses

The duration of tonic immobility was positively correlated with the latency to first head movement and with the number of head movements for white birds (see Table 2.3 and Appendix Figs. 1 - 3). These correlations were stronger for brown than white birds and when all birds were considered together. Brown birds and all birds combined had a positive correlation between the latency and the number of head movements, and negative correlations between numbers of inductions and the other variables.

Nine of the ten correlations between pairs of variables in the open field test were significant for white birds, five for brown birds and eight for all birds combined (see Table 2.4 and Appendix Figs. 4 - 6). When correlations were significant the latency to the first step was positively correlated with the latency to first vocalisation. Latencies to first step and to first vocalisation were negatively correlated with numbers of steps, vocalisations and defecations. Numbers of steps were positively correlated with numbers of vocalisations and defecations.

Correlations between pairs of behaviour variables in the runway test were positive for relationships between the latency to leave the start box and the times to reach 0.5 m from the start box and to reach 0.2 m from the goal box. The times taken to reach 0.5 m from the start box and 0.2 m from the goal box were also positively related. There were negative correlations with the time spent within 0.2 m of the goal box and the latency to leave the start box and the times to reach 0.5 m from the start box and 0.2 m from the goal box (see Table 2.5 and Appendix Figs. 7 – 9).

Principal components analyses using the four measures of tonic immobility for white birds, brown birds and white and brown birds together produced two principal components that accounted for 66.5% of the total variance for white birds, 71.4% for brown birds and 72.8% for all birds. Analyses using the five measures in the open field test produced one principal component that accounted for 56.5% of total variance for white birds and two components that accounted for 65.4% of total variance for brown birds and 67.9% for all birds combined. Analyses using the four measures in the runway test produced one principal component that accounted for 82.4% of total variance for white birds, 87% for brown birds and 84.4% for all birds. When all 13 variables for the three tests were used in a principal components analysis for all birds combined four components were identified that accounted for 69.7% of the total variance.

The first principal component for tonic immobility can be considered to represent fear as the number of inductions had a high negative loading whilst duration of tonic immobility and the number of head movements had high positive loadings. Birds that took more

attempts to induce tonic immobility therefore tended to have shorter durations of immobility and to show more head movements during immobility. The numbers of inductions and head movements had positive loadings on the second component whereas duration of tonic immobility and the number of head movements had negative loadings. The second component was thus inversely related to fear in the birds. Numbers of steps and of defecations in the open field test had high positive loadings and time to first step a high negative loading on the first component. This is consistent with an inverse relationship to fear for the first component in the open field test. The number of vocalisations and the time to first vocalisations had high opposite loadings on the second component. The latency to leave the start box and the times to reach 0.5 m from the start box and to reach 0.2 m from the goal box had high positive loadings on the single component in the runway test, whilst the time spent within 0.2 m of the goal box had a high negative loading. The component showed a positive relationship with fear in the runway test and a negative relationship with sociality. The first component for all behaviour tests combined had high positive loadings for four variables associated with fear across the three tests (Table 2.6). The fourth component had high positive loadings for three of these variables and for two other variables. These two components were related to fear behaviour.

Principal component scores were calculated for each bird using component coefficients from the analyses of white and brown birds together. Mean component scores differed between white and brown birds for component one ($U = 1753.00$, $P = 0.001$; see Fig. 2.3) from the tonic immobility test, for components one and two from the open field test ($U =$

1532.50, P = 0.018; U = 600.50, P < 0.001; Fig. 2.3), and for components two and three from all three behaviour tests combined (U = 711.00, P = 0.001; U = 729.00, P = 0.001; Fig. 2.4).

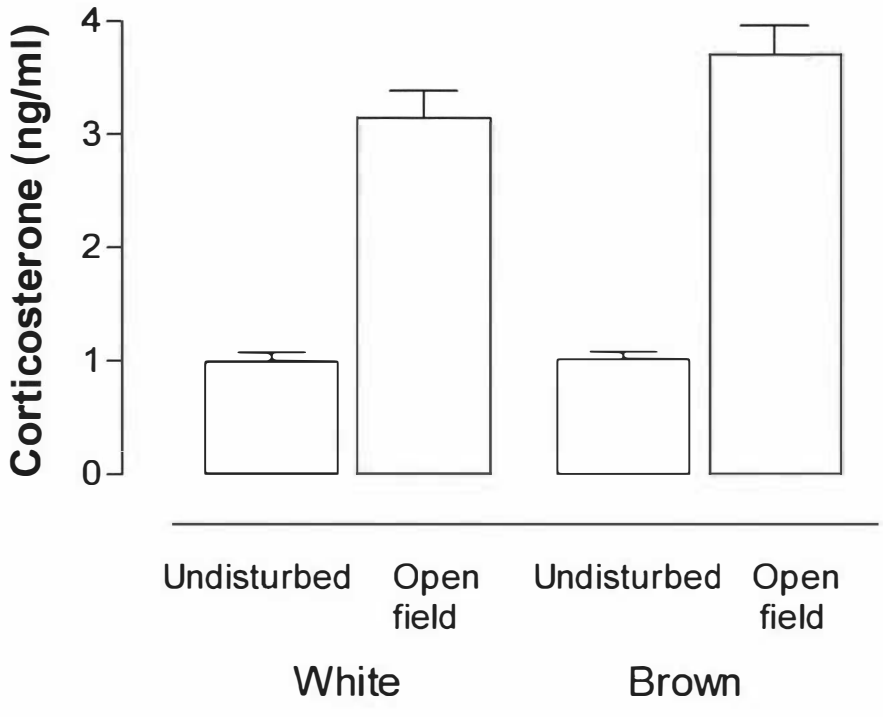
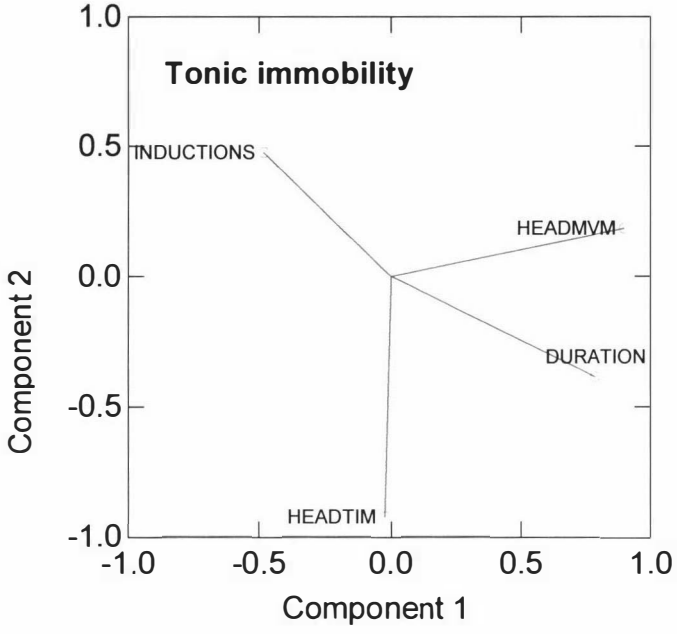


Fig. 2.1. Plasma corticosterone concentrations in undisturbed white Leghorn and brown Hyline hens and in the hens after 10 min in an open field. n = 49 for each strain.

Fig.2.2. Distributions of behavioural variables in relation to the first two principal components of principal components analyses of tonic immobility and open field variables for white and brown birds combined. Labels indicate the mean positions of variables in relation to the first two components. Each variable has a specific loading on the X- and Y-axis. The label abbreviations for tonic immobility are INDUCTIONS = number of inductions required to induce tonic immobility; HEADTIM = Latency to first head movement; HEADMVM = Number of head movements; DURATION = duration of tonic immobility. the abbreviations for open field are FIRSTSTEP = Latency to first step; FIRSTVOCAL = Latency to first vocalisation; NOSTEPS = Number of steps; NOVOCALS = Number of vocalisations; DEFECATIONS = Number of defecations.


Tonic immobility
INDUCTIONS
HEADMVM
DURATION
HEADTIM
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0
Component 2
Component 1

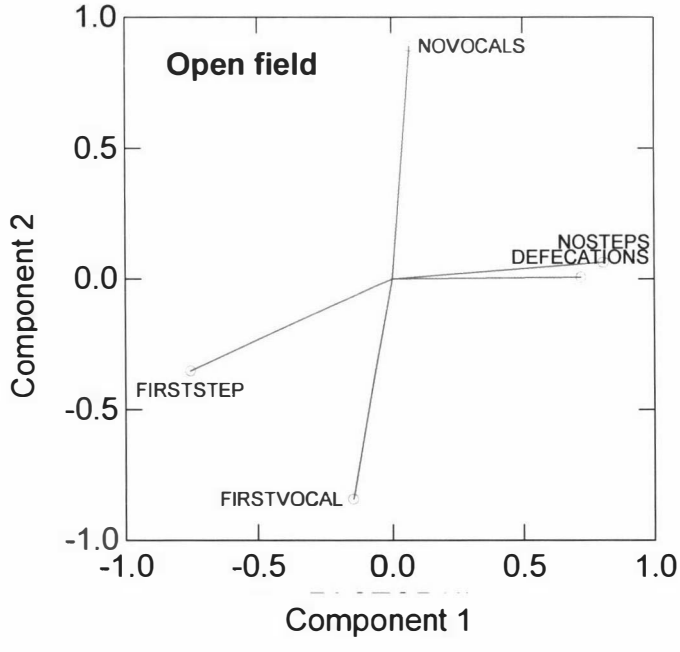
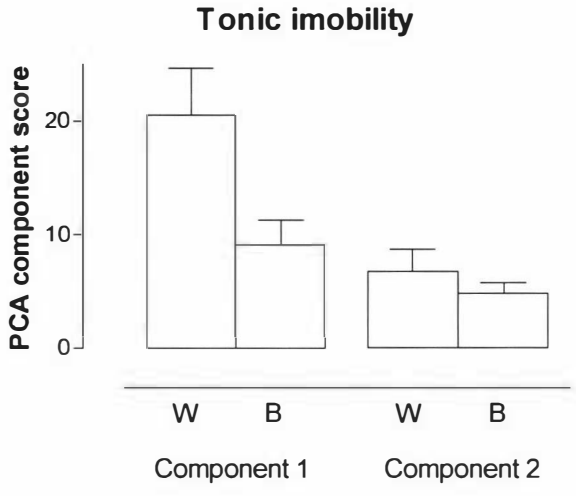
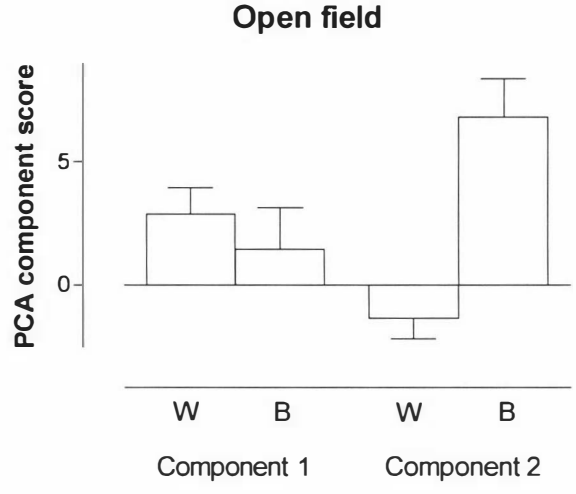
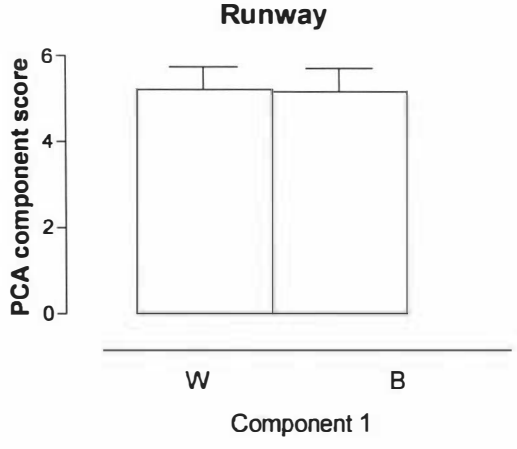

Open field
NOVOCALS
NOSTEPS
DEFECATIONS
FIRSTSTEP
FIRSTVOCAL
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0
Component 2
Component 1

Fig. 2.3. Mean scores for white and brown birds for components identified in principal component analyses of variables in tonic immobility, open field and runway tests. W = white and B = brown hens.


Tonic imobility
PCA component score
20
10
0
W
B
W
B
Component 1
Component 2


Open field
PCA component score
5
0
W
B
W
B
Component 1
Component 2


Runway
PCA component score
6
4
2
0
W
B
Component 1

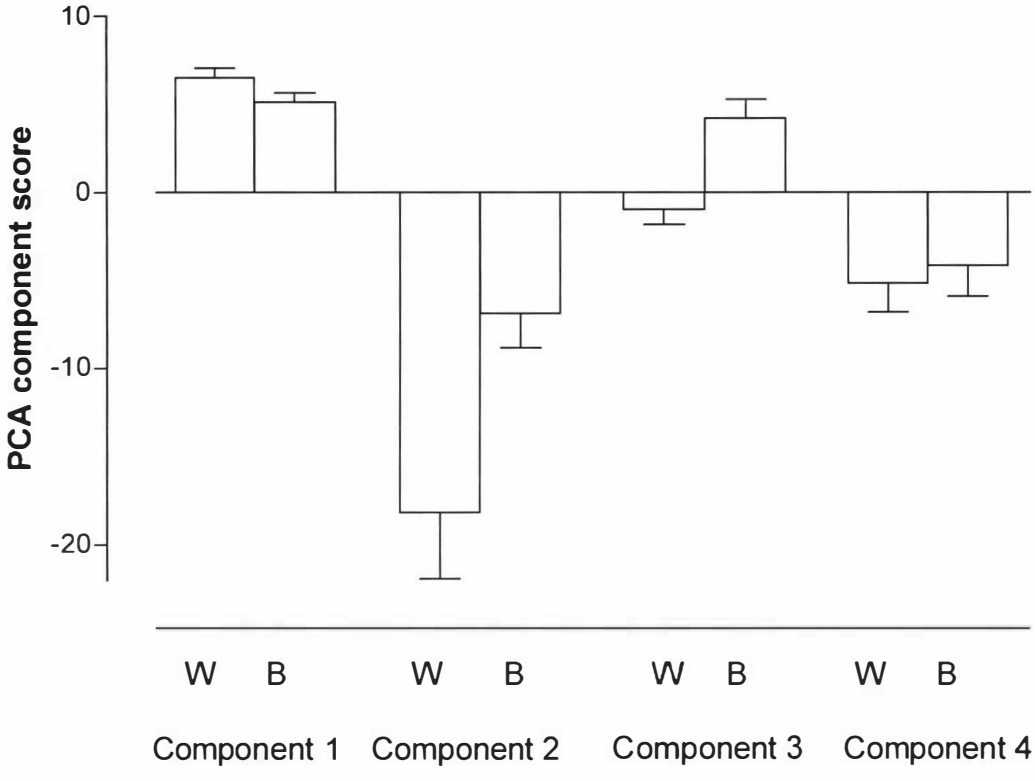


Fig. 2.4.Mean scores for white and brown birds for components identified in principal component analyses of all variables from tonic immobility, open field and runway tests combined. W = white and B = brown hens.

Table 2.1. Mean values and statistics for behavioural measures of tonic immobility, open field and runway tests in white Leghorn and brown Hyline hens. n = 50 for each strain of hen in the tonic immobility and runway tests. n = 49 for each strain of hen in the open field test.

	White		Brown			
	Mean	SE	Mean	SE	U	P
Tonic immobility						
Duration (min)	9.53	1.00	2.85	0.62	2003.50	<0.001
Latency (min)	1.03	0.19	0.27	0.10	1865.50	<0.001
Number of inductions	1.64	0.16	3.32	0.23	548.00	<0.001
Number of head movements	27.92	6.34	14.22	3.27	1660.50	0.004
Open field						
Latency to first step (min)	5.68	0.57	5.66	0.57	1142.50	0.673
Latency to first vocalisation (min)	5.08	0.61	1.78	0.43	1820.00	<0.001
Number of steps	10.24	1.91	10.65	3.20	1341.00	0.307
Number of vocalisations	5.51	1.03	16.45	2.41	631.00	<0.001
Number of defecations	1.12	0.13	1.14	0.15	1213.50	0.922
Runway						
Latency to leave start box (min)	6.47	0.54	5.98	0.58	1300.50	0.720
Latency to reach 0.5 m (min)	6.49	0.55	6.66	0.56	1164.00	0.530
Latency to reach 0.2 m of goal box (min)	7.77	0.50	8.07	0.46	1144.00	0.391
Time spent within 0.2 m of goal box (min)	1.58	0.42	1.79	0.45	1306.00	0.647

Table 2.2. Two way repeat measures ANOVA for plasma corticosterone concentrations in white leghorn and brown Hyline hens in two situations. Hens were sampled when undisturbed and after 10 min in an open field test.

Effect	F	df	P
Strain	2.37	1,96	0.127
Situation	307.82	1,96	<0.001
Interaction of strain and situation	0.85	1,96	0.358
Comparisons between situations for each strain			
White	138.14	1,96	<0.001
Brown	170.53	1,96	<0.001
Comparisons between strains for each situation			
Undisturbed	0.22	1,96	0.639
10 min open field	3.22	1,96	0.076

Table 2.3. Spearman rank correlations[1] between four behaviour variables measured in tonic immobility tests for each strain and for all birds. n = 50 for each strain.

Variable	Latency to first head movement	Number of head movements	Duration of tonic immobility
White			
Number of inductions	-0.084	-0.223	-0.025
Latency to first head movement		-0.020	0.288*
Number of head movements			0.662***
Brown			
Number of inductions	-0.516***	-0.591***	-0.572***
Latency to first head movement		0.619***	0.760***
Number of head movements			0.891***
All birds			
Number of inductions	-0.477***	-0.500***	-0.495***
Latency to first head movement		0.402***	0.637***
Number of head movements			0.793***

[1] * $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 2.4. Spearman rank correlations[1] between five behaviour variables measured in open field tests for each strain and for all birds. n = 49 for each strain.

Variable	Latency to first vocalisation	Number of steps	Number of vocalisations	Number of defecations
White				
Latency to first step	0.343*	-0.824***	-0.450**	-0.537***
Latency to first vocalisation		-0.261	-0.872***	-0.361*
Number of steps			0.393**	0.542***
Number of vocalisations				0.378**
Brown				
Latency to first step	0.219	-0.846***	-0.350*	-0.280
Latency to first vocalisation		-0.201	0.515**	-0.163
Number of steps			0.417*	0.323*
Number of vocalisations				-0.037
All birds				
Latency to first step	0.253*	-0.827***	-0.363**	-0.409***
Latency to first vocalisation		-0.158	-0.752***	-0.223*
Number of steps			0.332**	0.437***
Number of vocalisations				0.149

[1] * $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 2.5. Spearman rank correlations[1] between four behaviour variables measured in runway tests for each strain and for all birds. n = 49 for each strain.

Variable			
White	Latency to reach 0.5 m	Latency to reach within 0.2 m of goal box	Time spent within 0.2 m of goal box
Latency to leave start	0.877***	0.767***	-0.828***
Latency to reach 0.5 m		0.731***	-0.787***
Latency to reach within 0.2 m of goal box			-0.909***
Brown			
Latency to leave start	0.892***	0.767***	-0.760***
Latency to reach 0.5 m		0.839***	-0.837***
Latency to reach within 0.2 m of goal box			-0.997***
All birds			
Latency to leave start	0.878***	0.754***	-0.781***
Latency to reach 0.5 m		0.781***	-0.806***
Latency to reach within 0.2 m of goal box			-0.953***

[1] * $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 2.6. Loadings from principal components analysis of variables in tonic immobility, open field and runway tests combined for white and brown birds together.

Behaviour variable	Component			
	1	2	3	4
Tonic immobility				
Number of inductions	0.072	0.648	0.036	-0.130
Time to first head movement	0.232	-0.194	0.045	0.506
Number of head movements	-0.040	-0.832	-0.03	-0.132
Duration	-0.062	-0.751	0.239	0.414
Runway				
Latency to leave start box	0.876	0.072	-0.104	0.247
Time to reach 0.5 m from start box	0.905	0.144	-0.097	0.124
Time to reach 0.2 m from goal box	0.925	0.014	-0.112	-0.008
Time within 0.2 m of goal box	-0.909	-0.002	0.044	-0.009
Open field				
Time to first step	0.248	0.080	-0.664	0.400
Time to first vocalisation	-0.027	-0.001	-0.190	0.798
Number of steps	-0.404	-0.106	0.673	-0.077
Number of vocalisations	-0.076	0.068	0.104	-0.796
Number of defecations	0.103	0.065	0.841	0.003

Table 2.7. Comparison of corticosterone responses in white and brown laying hens to different stressors. Corticosterone concentrations at different times after initiation of a stressor are shown for studies of corticosterone responses in laying hens.

Stressor	Corticosterone (ng/ml)								References
	0 min		4 – 6 min		10 – 15 min		25 – 30 min		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Open field									
White	1.0	0.1			3.2	0.2			Present study
Brown	1.0	0.1			3.7	0.3			Present study
ISA Brown (chicks)	7.5	0.4			25.0	0.4			(Jones and Merry, 1988)
Handling									
White am (1 day old chick)	16.2		34.3		27.5				(Freeman and Flack, 1980)
White pm (1 day old chick)	18.5		33.3		31.5				(Freeman and Flack, 1980)
White am (21 day old chick)	7.2		18.36		8.6				(Freeman and Flack, 1980)
White pm (21 day old chick am)	15.2		38.76		21.3				(Freeman and Flack, 1980)

Table 2.7. cont Comparison of corticosterone responses in white and brown laying hens to different stressors. Corticosterone concentrations at different times after initiation of a stressor are shown for studies of corticosterone responses in laying hens.

Stressor	Corticosterone (ng/ml)								References
	0 min		4-6 min		10-15 min		25-30 min		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
White (selected for part year egg mass)					4.2				(Craig and Craig, 1985)
White					5.3				(Craig and Craig, 1985)
Brown	0.4	0.5			7.7	0.5			(Littin and Cockrem, 2001)
White	0.3	0.3			12.0	1.5			(Fraisse and Cockrem, 2006)
Brown	0.3	0.3			8.9	1.5			(Fraisse and Cockrem, 2006)
Immobilisation									
White	1.8		5.0	1.0	4.0	1.6	5.8	1.2	(Beuving and Vonder, 1978)
					5.2	1.2	9.0	2.0	
White am	0.8		2.2	0.4	3.2	0.6			(Beuving and Vonder, 1986)
White pm	0.7		2.9	0.4	3.4	0.6			(Beuving and Vonder, 1986)

Table 2.7. cont Comparison of corticosterone responses in white and brown laying hens to different stressors. Corticosterone concentrations at different times after initiation of a stressor are shown for studies of corticosterone responses in laying hens.

Stressor	Corticosterone (ng/ml)								References
	0 min		4-6 min		10-15 min		25-30 min		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
White (high feather pecking strain)	0.7	0.1	2.3	0.2					(Korte et al., 1997)
			3.3	0.2					
White (low feather pecking strain)	1.0	0.2	4.8	0.2					(Korte et al., 1997)
			6.5	0.5					

2.5 Discussion

This is the first study in chickens in which principal component analysis has been applied to the measurement of fear behaviour; and the first report of a corticosterone response to the open field test in adult chickens. There were differences between white Leghorn and brown Hyline hens in all measures of tonic immobility and two of five measures in open field tests, with no differences between strains in measures in runway tests. Principal component analyses identified components that measured fearfulness, and scores for three of four components differed between strains. The combined measures provided by principal component analysis are valuable for quantifying fearfulness in hens.

2.5.1 Tonic immobility and open field tests

The four variables of the tonic immobility test all differed between the two strains of hen, with the white hens displaying a greater degree of fear behaviour than the brown hens. Most previous studies of tonic immobility in white and brown hens also found white hens to show a longer duration of tonic immobility (Gallup et al., 1976; Jones and Faure, 1981; Jones and Mills, 1983; Jones, 1987a; Albentosa et al., 2003; Mahboub et al., 2004; Fraisse and Cockrem, 2006) fewer inductions, more head movements (Fraisse and Cockrem, 2006) and longer latency to first head movement (Jones and Faure, 1981; Jones, 1987a; Fraisse and Cockrem, 2006). There are also reports of fewer head movements in white hens (Jones and Faure, 1981; Jones, 1987a), no difference between strains in head movements (Jones and Mills, 1983), and no difference in latency to first head movement (Jones and Mills, 1983).

Two of the five variables of the open field test showed differences between the two strains of hen, with the white hens taking longer to vocalise and vocalising less than the brown hens. Fear inhibits a number of behavioural activities including vocalisation (Jones, 1987b; Jones, 1987c; Jones, 1996) so the white hens displayed a greater degree of fear behaviour than brown hens. Previous studies of open field behaviour also found that white hens displayed behaviour consistent with a greater underlying fearfulness and lower social motivation (Gallup et al., 1976; Jones, 1977; Jones and Mills, 1983) compared with brown hens. The open field test is a commonly used method to assess fear behaviour in poultry (Jones, 1977; Jones and Merry, 1988; Jones et al., 1992; Jones, 1996; Schutz et al., 2004) and other species e.g. calves (Depassille et al., 1995; Miller et al., 2005) rodents (Hirsjarvi and Valiaho, 1995; Choleris et al., 2001; Wartella et al., 2003) and sheep (Romeyer and Bouissou, 1992). It is generally thought that the latency to begin moving, amount of movement and number of vocalisations are inversely related to fear, so vocal and active individuals are thought to have a lower degree of fear than silent and inactive individuals (Jones and Merry, 1988; Jones, 1996). Another view of open field behaviour in chickens is that it represents a compromise between opposing tendencies to evade predation and to reinstate social contact with conspecifics. Active behaviour in the open field is then considered to reflect attempts to re-establish social contact (Gallup and Suarez, 1980). However, Jones (1987c) noted that fear is involved in the underlying motivational state during antipredator behaviour, and hence fear and antipredator behaviour is unlikely to be mutually exclusive. It is probable that fear and

social motivation are two of the motivations that control open field behaviour (Mills and Faure, 1991).

Corticosterone increased in white and brown hens after 10 minutes in the open field, with no difference between strains in their corticosterone response to the open field. Corticosterone responses to other stressors including handling (Craig and Craig, 1985; Littin and Cockrem, 2001; Fraisse and Cockrem, 2006) and immobilisation (Beuving and Vonder, 1986) have been measured in laying hens (for details see Table 2.7; Beuving and Vonder, 1978; Korte et al., 1997). Corticosterone responses to handling were greater and responses to immobilisation similar to the corticosterone response to the open field. Jones and Merry (1988) found that the corticosterone response to the open field in 7 day old chicks was much higher than the response of adult hens in the present study (Table.7). This difference may be age related as chicks (Freeman and Flack, 1980) have a much higher corticosterone response to handling than adults (Craig and Craig, 1985). Treatment with corticosterone (Jones et al., 1988; El-Lethey et al., 2003) or exposure to a stressor (Marin et al., 2001) before a tonic immobility test increases the duration of tonic immobility and hence the underlying fear levels in hens. This relationship between corticosterone and fear suggests that the corticosterone response to the open field test reflects an increase in fear levels of the hens, supporting the suggestion that the behaviour of hens in the open field can be used to assess fear.

2.5.2 Runway test

Runway tests are a commonly accepted method of measuring sociality in chickens and quail (Suarez and Gallup, 1983; Jones et al., 1996; Carmichael et al., 1998; Hocking et al., 2001; Marin et al., 2001; Vaisanen and Jensen, 2003). The absence of differences between white and brown hens in their performance in the runway test suggests that these strains do not differ in their sociality. This is the first report of sociality in white compared with brown hens. White hens are more fearful than brown hens, and Jones et al (2002) found that quail selected for low corticosterone responses appeared to show higher sociality than quail selected for high corticosterone responses. If there is an inverse relationship between fear and sociality in quail then brown hens might be expected to show greater sociality than white hens. Conversely, Mills and Faure (1991) found that quail selected for high and low levels of social reinstatement behaviour did not differ in their response to tonic immobility. Similar results were also found in adult hens (Hocking et al., 2001). These results combined and those of the present study suggest, in contrast to Jones et al (2002), that there is no relationship between fear and sociality in poultry. This has impact on poultry selection programmes as it means that selection for one of these traits will not necessarily cause a change in the other trait.

2.5.3 Principal components analysis

A number of studies have further examined the relationship between the variables of different behavioural tests to measure fear. This has included ranking hens for each individual behavioural measure and comparing ranks between tests (Jones and Mills, 1983; Jones, 1987a; Jones, 1988), performing correlations between each behavioural

measure (Jones et al., 1991; Mignon-Grasteau et al., 2003) and principal components analysis (Jones et al., 1991; Hocking et al., 2001; Mignon-Grasteau et al., 2003; Miller et al., 2005). In the present study principal components analysis was used to combine the results from the open field and tonic immobility tests to form an aggregate measure. Individually these tests show a difference between the two strains of hen with 6 of the 9 behavioural variables being significantly different. However the use of principal components analysis further distinguished this difference with 3 of the 4 factors being significantly different between the two strains of hens. This suggests that principal components analysis is a useful tool for distinguishing between fear responses in white and brown hens.

The present results have clearly shown that white hens have a greater underlying fearfulness than brown hens, and indicate that there is no difference in sociality between the two strains of hen. The study has also shown the value of using principal components analysis to form an aggregated measure from the two tests of fear to identify differences in fearfulness between different groups of laying hens.

Chapter 3 Effects of handling and restraint methods on corticosterone responses in white and brown hens

3.1 Abstract

Changes in plasma corticosterone concentrations during a 15 min handling stressor and 15 and 60 min restraint stressors were studied in White Leghorn and brown Hyline hens. Plasma corticosterone responses to these stressors were measured by the collection of blood samples at 0, 15, 30 and 60 min from the initiation of the stressor. Corticosterone increased from 0 to 15 min then decreased from 15 to 30 minutes in hens that experienced both procedures, but differed from 30 to 60 min with corticosterone increasing following restraint and remaining constant after handling. The handling procedure had a greater corticosterone response than the restraint procedure, with no difference in response between the strains of hen. Both restraint procedures elicited a similar corticosterone response, with white hens having a greater response than the brown hens. This study clearly shows that the corticosterone response of white and brown hens is dependent on both the strain of hen and the type of stressor.

3.2 Introduction

Corticosterone is the predominant adrenal glucocorticoid in birds (Carsia and Harvey, 2000) and is secreted following the activation of the hypothalamo-pituitary-adrenal (HPA) axis by a stressor (Cockrem et al., 2004). In chickens the corticosterone response to a stressor may differ depending on the type of stressor (Beuving and Vonder, 1978), the individual's strain (Korte et al., 1997) or due to individual variation (Littin and Cockrem, 2001). The corticosterone response of chickens to different situations can be used to indicate the degree of stress the individual is experiencing.

A number of studies have examined the corticosterone response to handling (Freeman and Flack, 1980; Craig and Craig, 1985; Beuving and Vonder, 1986; Littin and Cockrem, 2001; Fraisse and Cockrem, 2006), while only a few studies have examined the corticosterone response to restraint (Beuving and Vonder, 1978). Corticosterone is thought to be positively associated with fear in that chickens that have been treated with corticosterone (Jones et al., 1988; El-Lethey et al., 2001) show an increased fear response. Fraisse and Cockrem (2006) also found that the hens that showed a greater corticosterone response to handling had greater fear responses.

While the corticosterone response of laying hens to handling and restraint stressors has been previously examined, no previous study has compared the corticosterone response between the two stressors; therefore the aim of the present study is to compare the corticosterone response of white Leghorn and brown Hyline hens to handling and restraint stressors.

3.3 Materials and methods

3.3.1 Animals and husbandry

The study was conducted in a local poultry farm in a shed that could house 28 000 hens. The shed had eight rows of four tiered cages, with 152 cages in each tier. Two rows contained White Leghorn hens and the other six rows contained brown Hyline hens. The cages were 2.00 x 0.59 x 0.55 m (width x depth x height). Each cage was divided into three compartments of two different sizes; the first and third compartment were 0.70 x 0.59 x 0.55 m (width x depth x height) in size and contained 7 hens, whilst the middle compartment was 0.60 x 0.59 x 0.55 m (width x depth x height) in size and contained 6 hens. There was 585 cm^2 floor space per hen in all compartments. Food and water were provided automatically, and eggs and faeces were collected on conveyor belts.

The hens selected for use in this study were from two rows adjacent to one another. One row contained white Leghorn hens and the other brown Hyline hens. Both rows of hens had a row of their respective strain directly behind them. Hens were sampled along each row, with the first hens in each row selected from the first compartment of the second cage. One hen from each 7-bird cage was randomly removed and was banded with a coloured plastic leg band. 50 birds of each strain were used.

3.3.2 Experimental design

3.3.2.1 Corticosterone responses to handling in white and brown hens

Blood samples were collected following the method of Littin and Cockrem (2001) and Fraisse and Cockrem (2006). Hens were removed individually from their cages and

carried a short distance to sampling station. Blood samples (0.5 – 3 ml) were taken from the ulnar vein using a heparinised 25 g needle and 3 ml syringe. Each bird was blood sampled four times, twice on each wing. All samples were taken within three minutes of the hen being picked up. Samples were expelled into a heparinised test tube, stored on ice then transported to the laboratory where they were centrifuged and plasma frozen at -70°C. After the first blood sample was collected the hen was placed in a white plastic box with a lid (0.29 x 0.39 x 0.29 m; w x l x h). The hen was then picked up from the box, turned upside down and returned to the box repeatedly for 15 min when a second blood sample was collected. The hen was returned to the box, left undisturbed until 30 min when a third blood sample was taken, then left again until 60 min when a fourth blood sample was collected. The hen was then returned to its cage. One of the white hens died during the study and complete series of blood samples were not obtained from two white hens, so the sample sizes were 47 white hens and 50 brown hens.

3.3.2.2 Comparison of handling and restraint methods in white and brown hens

Corticosterone responses to three methods of handling and restraint were compared in 15 white and 15 brown hens chosen randomly from the birds used in the comparison between strains of corticosterone responses to our standard handling method. Data from the 15 birds of each strain birds in the first study were used for comparison with corticosterone responses of the same hens to 15 or 60 min of continuous restraint. All hens used in the comparison of handling methods were sampled at intervals of two weeks, with the order of the three handling methods randomised. The sampling method

for the restraint tests was the same as the standard handling method until the first blood sample had been collected. The hen was then put into the box and a movable wooden partition placed in the box against the flank of the bird. The bird could move its feet and head but not its wings during this period of restraint. A second blood sample was collected after 15 min, and the bird was then returned to the box and not restrained thereafter (15 min restraint), or the wooden partition remained in place during the 60 min period of sample collection (60 min restraint).

3.3.3 Plasma sample preparation and corticosterone radioimmunoassay

3.3.3.1 Plasma sample preparation

Each plasma sample was thawed, and spun for 10 min at 13 000 rpm. This process separated lipid from the plasma. The clear plasma from the lipid layer was transferred to a Sterilin RT20 tube and diluted in PBSG by a factor of 6 for the assay of corticosterone.

3.3.3.2 Radioimmunoassay of corticosterone

Corticosterone concentrations in plasma extracts were measured by radioimmunoassay following the method of Fraisse and Cockrem (2006). Samples were assayed in duplicate. 10µl of extract in PBSG was incubated with 20µl of iodinated corticosterone and 20µl of antiserum (^{125}I-corticosterone and antiserum ImmuChem™ Double Antibody Corticosterone ^{125}I RIA kit for rats and mice, MP Biomedicals, USA; 4000 cpm) for 2 hours at room temperature (22°C – 25°C). 50 µl of precipitant solution (MP Biomedicals, USA) was added and each sample was centrifuged at 3 000 rpm for 15 min at 4°C. 20 µl of starch (2.5 g/l starch in PBSG) was then added to increase adhesion of the pellet to the

tube. Samples were then centrifuged for a further 15 min at 3 000 rpm at 4°C, and the supernatant aspirated off. The pellets were counted on a LKB Wallac 1261 Multigamma gamma counter for 2 min each.

This radioimmunoassay was validated by Fraisse and Cockrem (2006) for the measurement of corticosterone in chicken plasma. The sensitivity of the corticosterone assay was the minimum hormone level that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean - 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity, expressed as ng corticosterone/ml plasma, was 0.35 ng/ml. Solutions of corticosterone in PBSG at concentrations that gave approximately 80, 50 and 20% binding on the standard curve were used as low, medium and high quality controls in every assay. The intra-assay coefficients of variation were 8.4 % (n=20), 6.0 % (n=20), and 5.3 % (n=10) and inter-assay coefficients of variation for ten assays 16.0%, 8.3% and 16.9% for low, medium and high solutions respectively.

3.3.4 Statistical analysis

Plasma corticosterone concentrations were transformed to logarithms and Levene's tests were performed to check for equality of variances before parametric analyses were performed.

Changes in plasma corticosterone concentrations were compared between strains using repeated measures two way ANOVA with time (0, 15, 30 and 60 min) and strain (white

and brown) as grouping factors. Post hoc comparisons were made between times for each strain and between strains for each time using univariate F tests. The areas under corticosterone response curves were determined in Prism using the trapezoid rule and termed integrated corticosterone responses (Cockrem and Silverin, 2002). The total area under the curve and the total area minus the area attributable to corticosterone concentrations at 0 min (corrected area) were calculated. If corticosterone concentrations after 0 min were less than those at 0 min then a corrected area was not calculated for that bird. Integrated corticosterone responses were compared between strains by t-tests.

Changes in plasma corticosterone concentrations were compared between strains and handling methods using repeated measures ANOVA with time (0, 15, 30 and 60 min), strains and handling method (standard handling, 15 min restraint and 60 min restraint) as the grouping factors. Post hoc comparisons were made using univariate F tests. Integrated corticosterone responses were compared between strains and handling methods by Kruskal-Wallis non-parametric ANOVA and Mann-Whitney U tests.

Statistical analyses were performed using Prism (GraphPad Software Inc.) and Systat (SPSS Inc.). Data are presented as mean ± S.E.

3.4 Results

3.4.1 Corticosterone responses to handling in white and brown hens

Mean corticosterone concentrations increased from approximately 1 ng/ml at 0 min to 5.5 to 6.0 ng ml at 15 min, then decreased to approximately 3 ng/ml at 30 min in white and brown hens subjected to handling for 15 min (Fig. 3.1; see Table 3.1 for statistics). Corticosterone remained constant from 30 to 60 min, and at 60 min was still elevated compared with initial corticosterone concentrations at 0 min. The pattern of changes in corticosterone was the same for white and brown hens and there were no differences between strains of hen in corticosterone concentrations at any time. Total and corrected integrated plasma corticosterone responses did not differ between white and brown hens ($t_{89} = 0.185$, P=0.854; $t_{89} = 0.371$, P=0.711).

3.4.2 Comparison of handling and restraint methods in white and brown hens

Corticosterone responses to 15 min handling in 15 white and 15 brown hens from the previous study were compared with responses of the same hens to 15 or 60 min restraint (Fig. 3.2). There were significant effects of strain of hen, time and method of handling and restraint on corticosterone concentrations, and a significant interaction between time and method (see Table 3.2 for statistics). Corticosterone increased from 0 to 15 min, decreased from 15 to 30 min then increased again at 60 min in hens subjected to 15 min restraint. Corticosterone concentrations at 60 min were higher than initial concentrations at 0 min. The corticosterone response to 15 min restraint was lower at 15 and 30 min than the corticosterone response to 15 min handling, whereas corticosterone at 60 min was the same when birds were subjected to handling or to restraint. The patterns of

changes in corticosterone concentrations were similar in white and brown hens when they experienced 60 min of restraint compared with 15 min restraint, with no differences between restraint methods in corticosterone concentrations at any time. Corticosterone was lower at 15 and 30 but not 60 min in hens restrained for 60 min compared with concentrations when the birds were subjected to 15 min handling.

Total integrated plasma corticosterone responses of white hens did not differ between handling and restraint methods, whereas corrected integrated responses were lower when birds experienced 15 or 60 min restraint than when they experienced 15 min handling (see Table 3.3 for statistics). Total and integrated responses were both lower in brown hens when they were restrained than when they were handled.

Fig. 3.3 shows corticosterone responses arranged by handling and restraint method. The pattern of changes in corticosterone was similar in white and brown hens restrained for 15 min, but corticosterone was significantly higher in white than brown hens at 15 min (2.96 ± 0.48 c.f. 1.81 ± 0.28 ng/ml). Corticosterone remained higher, but not significantly so, in white hens at 30 and 60 min (2.08 ± 0.47 c.f. 1.42 ± 0.28 ng/ml and 2.38 ± 0.27 c.f. 2.13 ± 0.47 ng/ml). Changes in corticosterone concentrations with time in hens restrained for 60 min were similar in white and brown hens. Corticosterone was significantly higher in white than brown hens at 15 min (2.75 ± 0.28 c.f. 1.82 ± 0.22 ng/ml), and did not differ between strains at 30 and 60 min. Total and corrected integrated plasma corticosterone responses to the standard handling procedure did not differ between white and brown hens (Fig. 3.4; $U = 104.00$, $P=0.528$; $U = 107.00$,

P=0.438). The total but not the corrected integrated plasma corticosterone response to 15 min of restraint was greater in white hens (U = 141.00, P=0.045; U = 136.00, P=0.076), whereas there were no differences between strains of hens in their integrated responses to 60 min of restraint (U = 115.00, P=0.435; U = 118.00, P=0.358).

Corticosterone responses of individual birds to handling and restraint generally followed the pattern of mean responses. However, 4 white and 1 brown hen (17% of all hens) had greater responses to restraint than to handling. One hen did not show any change in corticosterone after 15 min handling, and corticosterone did not change after 15 min restraint in 8 hens (27% of all hens). Corticosterone concentrations remained unchanged throughout the 60 min sampling period in one of these 8 birds. The range of corticosterone concentrations of individual hens that experienced 15 min handling was 1.05 - 18.74 ng/ml at 15 min, and was 0.54 - 7.25 ng/ml at this time in hens restrained for 15 min. After 60 min of restraint corticosterone concentrations varied from 0.33 - 7.16 ng/ml.

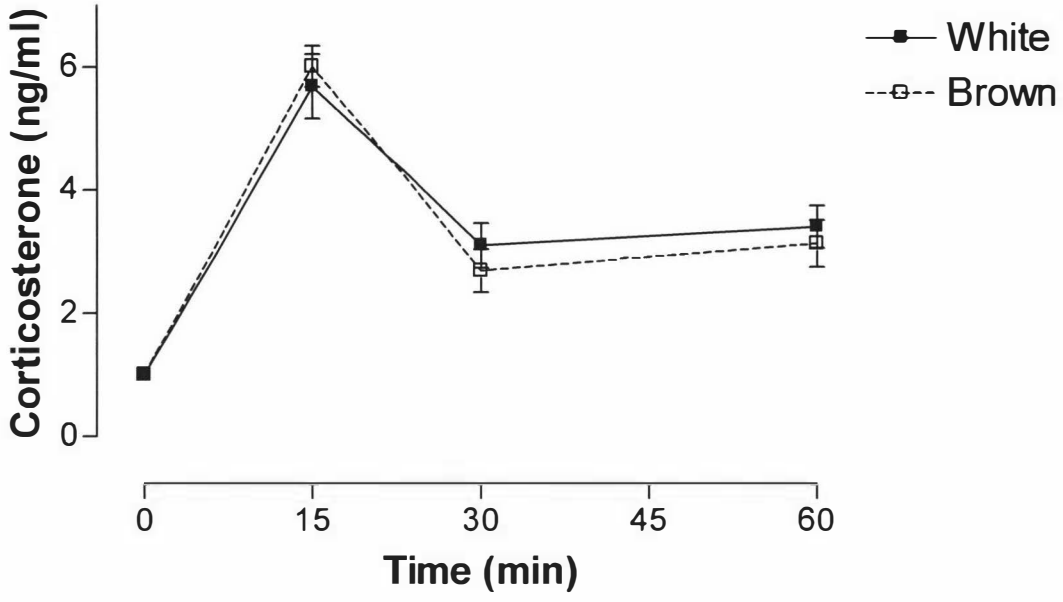


Fig. 3.1. Corticosterone responses to a standard handling procedure in White Leghorn and brown Hyline hens. The handling procedure consisted of 15 min of repeated handling followed by 45 min of social isolation. n = 47 and 50 respectively. Data are represented as means ± S.E.

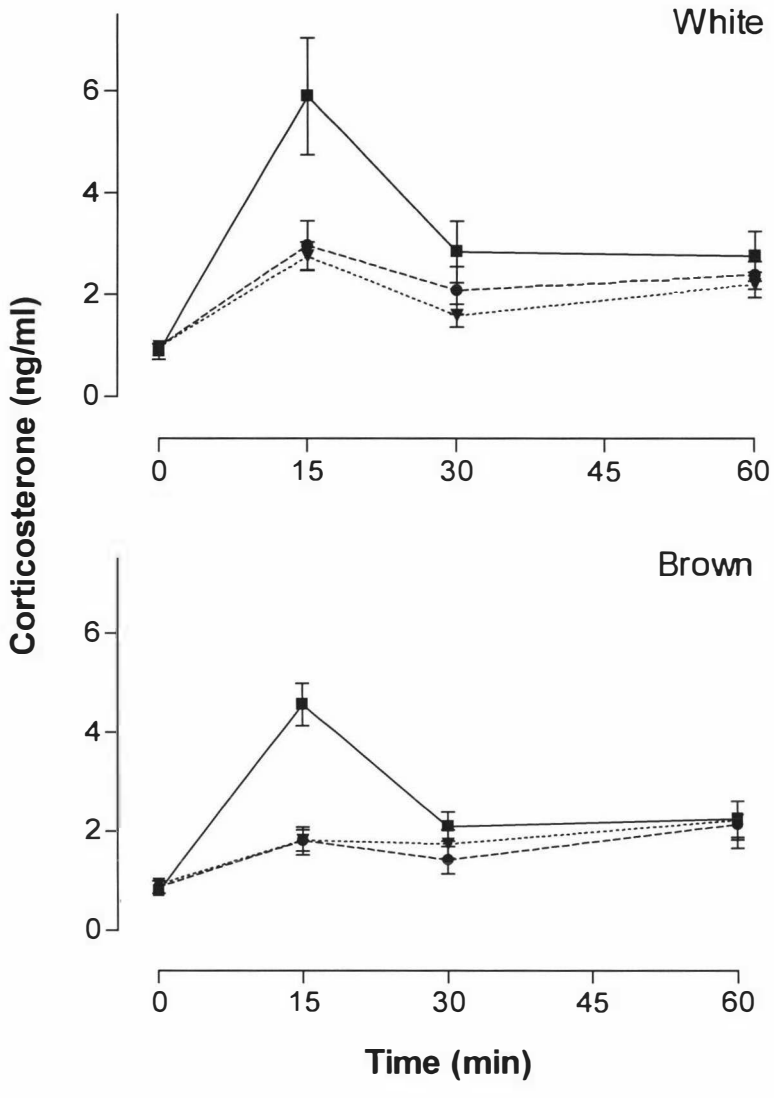
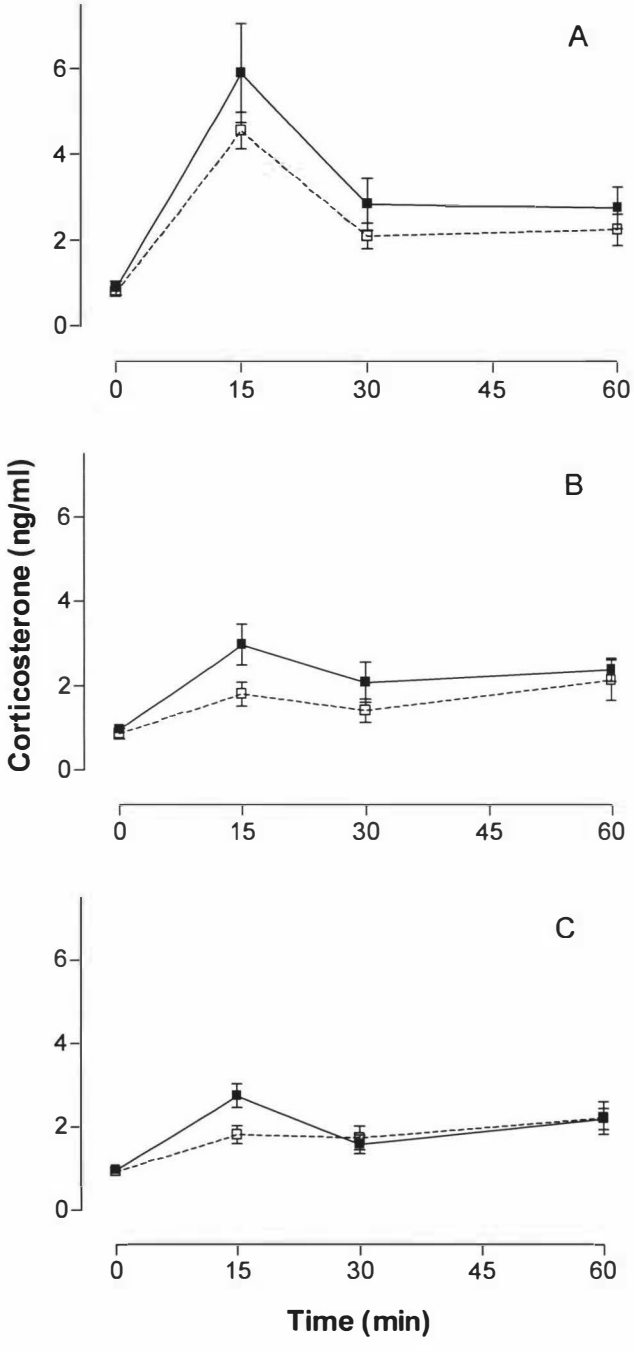


Fig. 3.2. Corticosterone responses to three handling and restraint methods in White Leghorn and brown Hyline hens. Hens were handled by a standard method (—■—) or experienced 15 (---●---) or 60 (···▼···) min of restraint. n = 15 for each group. Data are represented as means ± S.E.

Fig. 3.3. Corticosterone responses to three handling and restraint methods in White Leghorn (—■—) and brown Hyline (---□---) hens. Hens were handled by a standard method (A) or experienced 15 (B) or 60 (C) min of restraint. n = 15 for each group. Data are represented as means ± S.E.


A
B
C
Corticosterone (ng/ml)
Time (min)
0
2
4
6
0
15
30
45
60

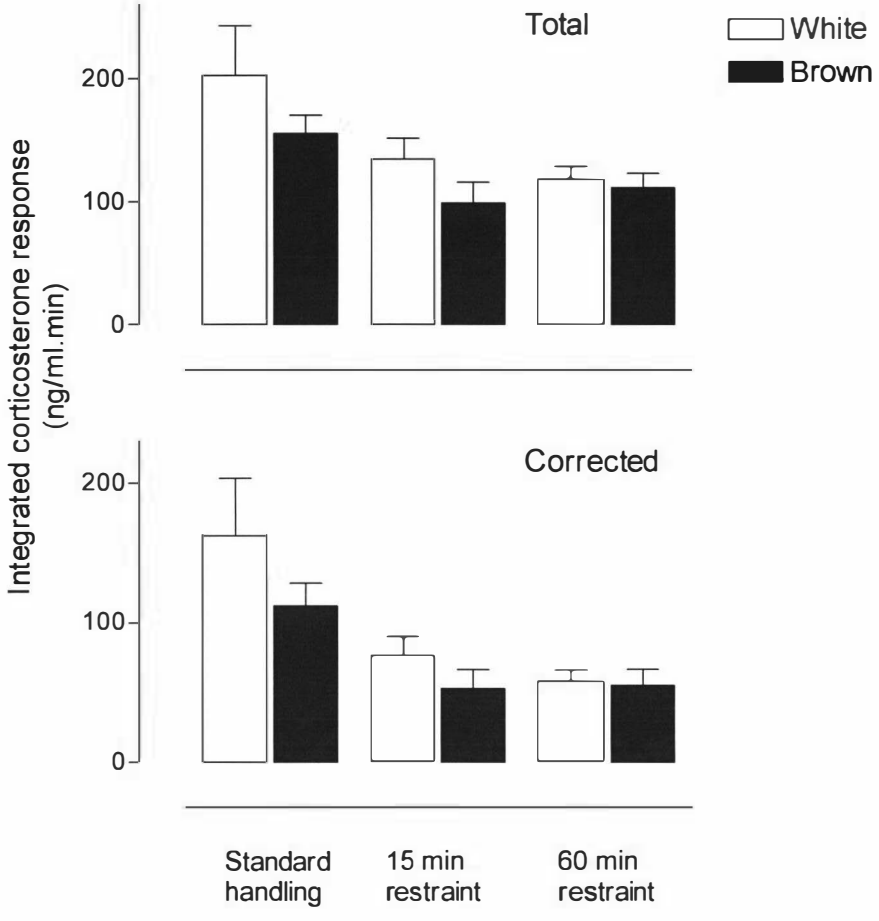


Fig. 3.4. Total and corrected integrated corticosterone responses to handling and restraint of White Leghorn and brown Hyline hens. The corrected response is the total response minus the integrated corticosterone secretion attributable to initial corticosterone concentrations at 0 min. Hens were handled by a standard method or experienced 15 or 60 min of restraint. n = 15 for each group. Data are represented as means $\pm$ S.E.

Table 3.1. Two way repeat measures ANOVA for plasma corticosterone concentrations in White Leghorn and brown Hyline hens subjected to a standard handling procedure for 15 min.

Effect	F	df	P
Strain	0.016	1,95	0.899
Time	210.896	3,285	0.000
Interaction of strain and time	1.282	3,285	0.281
Comparisons between times for each strain			
0 min vs 15 min			
White	260.704	1,95	0.000
Brown	317.691	1,95	0.000
15 min vs 30 min			
White	99.021	1,95	0.000
Brown	175.554	1,95	0.000
30 min vs 60 min			
White	2.503	1,95	0.117
Brown	1.193	1,95	0.277
0 min vs 30 min			
White	78.060	1,95	0.000
Brown	69.201	1,95	0.000
0 min vs 60 min			
White	107.924	1,95	0.000
Brown	88.736	1,95	0.000
Comparisons between strains for each time			
0 min	0.128	1,95	0.721
15 min	1.956	1,95	0.165
30 min	0.123	1,95	0.727
60 min	0.513	1,95	0.475

Note: The first three rows show the results of the ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons of times for each strain and comparisons of strains at each time.

Table 3.2. Statistical analysis for comparison of plasma concentrations of corticosterone between strains and handling and restraint methods. n = 15 for each strain.

Effect	F	df	P
Strain	4.768	1,84	0.032
Time	104.326	3,252	<0.001
Method	4.319	2,84	0.016
Interaction of strain and time	1.163	3,252	0.325
Interaction of strain and method	0.766	2,84	0.468
Interaction of time and method	8.019	6,252	<0.001
Comparisons between times for each strain			
0 vs 15 min			
White	170.157	1,84	<0.001
Brown	115.230	1,84	<0.001
15 vs 30 min			
White	45.031	1,84	<0.001
Brown	20.258	1,84	<0.001
30 vs 60 min			
White	5.804	1,84	0.018
Brown	6.574	1,84	0.012
0 vs 30 min			
White	44.562	1,84	<0.001
Brown	40.348	1,84	<0.001
0 vs 60 min			
White	78.729	1,84	<0.001
Brown	75.321	1,84	<0.001

Note: The first six rows show the results of the ANOVA for the main effects and their interactions. The remaining rows show the results of post hoc comparisons of times and strains.

Table 3.2. cont Statistical analysis for comparison of plasma concentrations of corticosterone between strains and handling and restraint methods. n = 15 for each strain.

Comparisons between strains for each time and method	F	df	P
0 min white vs brown			
Standard	0.155	1,84	0.695
15 min restraint	1.221	1,84	0.272
60 min restraint	0.117	1,84	0.733
15 min white vs brown			
Standard	0.182	1,84	0.671
15 min restraint	6.123	1,84	0.015
60 min restraint	4.135	1,84	0.045
30 min white vs brown			
Standard	0.412	1,84	0.523
15 min restraint	2.587	1,84	0.111
60 min restraint	0.124	1,84	0.726
60 min white vs brown			
Standard	0.609	1,84	0.437
15 min restraint	1.210	1,84	0.274
60 min restraint	0.000	1,84	0.994

Table 3.2. cont Statistical analysis for comparison of plasma concentrations of corticosterone between strains and handling and restraint methods. n = 15 for each strain.

Comparisons between times for each method	F	df	P
0 vs 15 min			
Standard	227.206	1,84	<0.001
15 min restraint	51.061	1,84	<0.001
60 min restraint	47.667	1,84	<0.001
15 vs 30 min			
Standard	52.839	1,84	<0.001
15 min restraint	105.55	1,84	0.002
60 min restraint	10.325	1,84	0.002
30 vs 60 min			
Standard	0.114	1,84	0.737
15 min restraint	9.994	1,84	0.002
60 min restraint	6.720	1,84	0.011
0 vs 30 min			
Standard	65.963	1,84	<0.001
15 min restraint	16.127	1,84	<0.001
60 min restraint	14.575	1,84	<0.001
0 vs 60 min			
Standard	72.994	1,84	<0.001
15 min restraint	45.566	1,84	<0.001
60 min restraint	37.562	1,84	<0.001

Table 3.2. cont Statistical analysis for comparison of plasma concentrations of corticosterone between strains and handling and restraint methods. n = 15 for each strain.

Comparisons between methods for each time	F	df	P
0 min			
Standard vs 15 min restraint	1.366	1,84	0.246
15 min vs 60 min restraint	0.389	1,84	0.534
Standard vs 60 min restraint	3.214	1,84	0.077
15 min			
Standard vs 15 min restraint	31.041	1,84	<0.001
15 min vs 60 min restraint	0.118	1,84	0.732
Standard vs 60 min restraint	27.331	1,84	<0.001
30 min			
Standard vs 15 min restraint	5.506	1,84	0.021
15 min vs 60 min restraint	0.112	1,84	0.739
Standard vs 60 min restraint	11.060	1,84	0.047
60 min			
Standard vs 15 min restraint	0.219	1,84	0.641
15 min vs 60 min restraint	0.003	1,84	0.954
Standard vs 60 min restraint	0.276	1,84	0.601

Table 3.3. Statistical analysis for comparison of integrated corticosterone responses between handling and restraint methods. n = 15 for each strain.

	Kruskal Wallis K	P
White		
Total	3.282	0.194
Corrected	7.133	0.028
Brown		
Total	10.762	0.005
Corrected	10.256	0.006

	Mann-Whitney U	P
White		
Total		
Standard vs 15 min restraint	126.00	0.189
15 min vs 60 min restraint	115.00	0.663
Standard vs 60 min restraint	127.00	0.081
Corrected		
Standard vs 15 min restraint	140.00	0.050
15 min vs 60 min restraint	118.50	0.556
Standard vs 60 min restraint	144.00	0.010
Brown		
Total		
Standard vs 15 min restraint	145.00	0.009
15 min vs 60 min restraint	51.00	0.052
Standard vs 60 min restraint	153.00	0.012
Corrected		
Standard vs 15 min restraint	145.50	0.008
15 min vs 60 min restraint	77.00	0.497
Standard vs 60 min restraint	159.00	0.005

3.5 Discussion

This is the first study in chickens in which the corticosterone response to handling and restraint stressors were compared. There was no difference between white Leghorn and brown Hyline hens in the corticosterone response of hens subjected to15 min of handling followed by 45 min of social isolation, nor was there a difference between strains in the corticosterone response to 15 or 60 min restraint. Both white and brown hens had lower corticosterone response to restraint than to handling, with white birds having a higher corticosterone response to restraint than brown birds.

3.5.1 Corticosterone responses to handling in white and brown hens

The corticosterone response to a handling stressor has been measured previously in laying hens (Beuving and Vonder, 1978; Freeman and Flack, 1980; Craig and Craig, 1985; Beuving and Vonder, 1986; Littin and Cockrem, 2001; Fraisse and Cockrem, 2006). The corticosterone response to 15 min of handling did not differ between the two strains of hen at any time and while the pattern of corticosterone response is consistent with prior studies using a similar handling procedure (Littin and Cockrem, 2001; Fraisse and Cockrem, 2006), the corticosterone concentrations for each strain were lower in the current study than that of Fraisse and Cockrem (2006) who carried out their study on the same farm using the same strains of hen (see appendix figure 12). Fraisse and Cockrem (2006) also found that the white hens had a greater corticosterone response to handling than the brown hens at both 15 and 30 min after handling. Differences between the two studies may be caused by a number factors, such as, the change in density (400cm^2 and 440cm^2 cf 585cm^2 per hen; Onbasilar and Aksoy, 2005; Mashaly et al., 1984; Craig et

440cm^2 cf 585cm^2 per hen; Onbasilar and Aksoy, 2005; Mashaly et al., 1984; Craig et al., 1986; Cunningham et al., 1987), amount of contact with humans (Hemsworth et al., 1994; Barnett et al., 1994) differences in handling (Kannan and Mench, 1996) change in genetic stock (Korte et al., 1997) and maternal exposure to corticosterone during development of the egg (Janczak et al., 2006).

There is evidence to suggest that there is a positive relationship between fear and corticosterone in chickens, as chickens that have been treated with corticosterone for several days (Jones et al., 1988; El-Lethey et al., 2001) show an increased fear response. As there was no difference in the corticosterone response to handling in white and brown hens it might be expected that both strains of hen would have similar levels of underlying fearfulness. However this was not the case as the same individuals who showed no difference in corticosterone response to handling showed differences in fear with white hens showing a greater amount of fear than brown hens (see chapter two for details). Fraisse and Cockrem (2006) also found greater fear in white than brown hens. Like corticosterone secretion, measurements of fear in chickens can be affected by a number of factors, such as strain (Gallup et al., 1976; Albentosa et al., 2003), degree of prior exposure to humans (Jones and Waddington, 1992; Jones, 1993; Jones, 1994; Hemsworth et al., 1994) and what type of fear the test is measuring e.g. underlying fearfulness or fear of humans (Jones and Faure, 1981). Thus the interaction between the underlying factors can make it difficult to clearly demonstrate relationships such as the fear and corticosterone relationship.

3.5.2 Comparison of handling and restraint methods in white and brown hens

The corticosterone response and profile differed between the restraint and handling procedures. The differences in the corticosterone response show that both strains of hen found the handling procedure to be a greater stressor than the restraint procedure. This may be due to the hens perceiving the stressors in different ways, Suarez and Gallup (1981) suggest that humans are perceived as potential predators by chickens, thus the handling procedure consisting of 15 minutes of contact with humans may represent a life threatening situation which will in turn instigate a greater corticosterone response.

Few studies have looked at the corticosterone response to restraint in chickens (Korte et al., 1997)Beuving and Vonder, 1978) and only Beuving and Vonder (1978) followed this response for 60 minutes. The pattern of corticosterone response found by Beuving and Vonder (1978) differs from the present study as it increased, rapidly for the first five min and then slowly increased for the remaining time. There are number of reasons why these differences in the corticosterone response pattern may have occurred, including differences in handling (Kannan and Mench 1996), previous contact with humans (Hemsworth et al,. 1994; Barnett et al., 1994) and strain (Korte et al., 1997). The mean corticosterone responses to the restraint stressors show a clear difference between the strains of hen, with white hens having a higher corticosterone concentration at 15 minutes than the brown hens. The individual corticosterone response to a stressor has been previously reported in chickens (Littin and Cockrem, 2001) and great tits (Cockrem and Silverin, 2002). The plasma corticosterone concentration following the handling and restraint procedures differed between birds (see appendix figs. 10 and 11) and differed

more in the restraint procedure than the handling procedure. This is consistent with the restraint procedure being a weaker stressor than the handling procedure.

The present study has shown that white and brown hens show a greater corticosterone response to handling than restraint, with the white hens having a greater corticosterone response to restraint than brown hens. The study has also shown that the corticosterone response of hens depends on both the strain of hen and the stressor, and indicates that weaker stressors may be required to identify differences in the corticosterone response between groups of hen. This identification of a weaker stressor as opposed to a stressor which causes all birds to respond maximally is of importance to poultry selection programmes, as it allows for the ability to choose methods of selection that will show actual differences in corticosterone response.

Chapter 4 Repeatability of behavioural tests of fear, and relationships between corticosterone and behaviour in white and brown hens

4.1 Abstract

Behavioural measures of fear and sociality and physiological measures of stress were taken in brown Hyline and white Leghorn hens to determine the repeatability of the tonic immobility test and relationships between corticosterone and the behavioural measures. Tonic immobility and open field tests were conducted to measure fear, and a runway test was used to measure sociality. Corticosterone responses to handling were measured by the collection of blood samples at 0, 15, 30 and 60 min after handling. Correlations, fear ranks and principal components analyses were used to examine relationships between variables. There were no differences between the mean behavioural measures in the two tests of tonic immobility for either strain of hen. Correlations and statistical repeatability calculations indicated that the test was more repeatable for brown than white hens. Correlations were found between the number of inductions, number of vocalisations and latency to leave start box and corticosterone in white hens only. Principal components analysis further distinguished relationships between behaviour variables and corticosterone, indicating a positive relationship between corticosterone and fear and a negative relationship between corticosterone and sociality.

4.2 Introduction

Fear is a basic emotion and is thought to be a defensive mechanism, allowing an individual to react quickly to a stimulus to increase its probability of survival and lessen its chance of injury (LeDoux, 1996). The subjective nature of fear makes it hard to measure directly. However, when an individual perceives a situation to be fearful, the fear response predominates and inhibits all other behavioural responses. Placing an individual in an experimental situation allows fear to be indirectly measured, with behavioural responses used to infer how much fear is experienced. There are two main methods of measuring fear in chickens; the first involves novelty, in which an individual is introduced to a novel situation, such as a new environment, object or person and their behaviour is recorded and analysed to determine fear (Jones, 1996). The second major method is to measure tonic immobility, is an innate response triggered by physical restraint in which the animal adopts an immobilised posture and has reduced response to external stimuli. The time for taken for the individual to return to its normal state is thought to reflect the level of fear it is experiencing (Jones, 1986; Jones, 1996).

Corticosterone is the predominant adrenal glucocorticoid hormone found in birds (Carsia and Harvey, 2000) and is secreted in response to a stressor (Cockrem et al., 2004). A number of studies have examined the relationship between fear and corticosterone in chickens and have found results that support the suggestion that there is a positive relationship between fear and corticosterone (Jones et al., 1988; El-Lethey et al., 2001).

Numerous studies have used the tonic immobility test to assess the underlying fearfulness in chickens E.g. Gallup (1979), Hocking et al (2001), Albentosa et al (2003), Hansen et al (2003) and Fraisse and Cockrem (2006); however few have assessed the repeatability of this method (Jones, 1988; Jones, 1989). The repeatability of this and other behavioural tests is vital, as it determines whether the test is measuring the actual underlying characteristics of the individual or if it is just measuring the reactions of the individual at one point in time.

The aims of the present study were to determine the repeatability of the tonic immobility test, and to examine relationships between the corticosterone response to handling and fear and sociality in white Leghorn and brown Hyline hens. Tonic immobility and open field tests were conducted to examine fear, whilst a runway test was used to investigate sociality. Correlations and the derived measures of fear ranks and principal components analysis were used to identify relationships between corticosterone and behaviour variables.

4.3 Materials and methods

4.3.1 Animals and husbandry

The study was conducted in a local poultry farm in a shed that could house 28 000 hens. The shed had eight rows of four tiered cages, with 152 cages in each tier. Two rows contained white Leghorn hens and the other six rows contained brown Hyline hens. The cages were 2.00 x 0.59 x 0.55 m (width x depth x height). Each cage was divided into three compartments of two different sizes; the first and third compartment were 0.70 x 0.59 x 0.55 m (width x depth x height) in size and contained 7 hens, whilst the middle compartment was 0.60 x 0.59 x 0.55 m (width x depth x height) in size and contained 6 hens. There was 585 cm^2 floor space per hen in all compartments. Food and water were provided automatically, and eggs and faeces were collected on conveyor belts.

The hens selected for use in this study were from two rows adjacent to one another. One row contained white Leghorn hens and the other brown Hyline hens. Both rows of hens had a row of their respective strain directly behind them. Hens were sampled along each row, with the first hens in each row selected from the first compartment of the second cage. One hen from each 7-bird cage was randomly removed and was banded with a coloured plastic leg band. 50 birds of each strain were used.

4.3.2 Experimental design and behavioural observations

4.3.2.1 Repeatability of tonic immobility behaviour test

All hens of each strain underwent three behavioural tests (tonic immobility, open field and runway). The order of these tests and the handler performing them were randomised for each bird, with a period of two weeks between each behavioural test. Hens were subjected to a second tonic immobility test 10 days after the first series of three behaviour tests was completed.

4.3.2.1.1 Tonic immobility tests

Hens were removed from their cage and taken to the end of the shed where they were placed on their side on several layers of cloth. Tonic immobility (TI) was induced by lightly restraining the bird with one hand held over its head and the other hand placed on its keel for 15 sec (Fraisse and Cockrem, 2006). The handler then slowly removed their hands and moved back from the bird. If the bird righted itself within 15 sec then the induction process was repeated up to 5 times. The duration of TI, the latency from induction to the first head movement and the number of head movements were recorded. If TI was not induced after 5 attempts the hen was given a score of 0 for both duration and latency to first head movement. When the bird remained in TI a maximum 20 min test period was used and the bird was given a score of 20 min for the duration of TI.

4.3.2.2 Relationships between corticosterone and behaviour

Corticosterone responses to handling were measured and hens subjected to three behavioural tests (tonic immobility, open field and runway) so that relationships between corticosterone responses and behavioural measures could be examined.

Blood samples for corticosterone responses were collected following the method of Littin and Cockrem (2001) and Fraisse and Cockrem (2006). Hens were removed individually from their cages and carried a short distance to sampling station. Blood samples (0.5 – 3 ml) were taken from the ulnar vein using a heparinised 25 g needle and 3 ml syringe. Each bird was blood sampled four times, twice on each wing. All samples were taken within three minutes of the hen being picked up. Samples were expelled into a heparinised test tube, stored on ice then transported to the laboratory where they were centrifuged and plasma frozen at -70°C. After the first blood sample was collected the hen was placed in a white plastic box with a lid (0.29 x 0.39 x 0.29 m; w x l x h). The hen was then picked up from the box, turned upside down and returned to the box repeatedly for 15 min when a second blood sample was collected. The hen was returned to the box, left undisturbed until 30 min when a third blood sample was taken, then left again until 60 min when a fourth blood sample was collected. The hen was then returned to its cage. One of the white hens died during the study and complete series of blood samples were not obtained from two white hens, so the sample sizes were 47 white hens and 50 brown hens.

4.2.3.3.Open field tests

Hens were removed from their cages and taken to one end of the shed in which they were placed in a square arena (2.00 x 2.00 m) for open field tests. The arena was barren and had unpainted concrete floors and walls of strong, plain cardboard 0.85 m high. After the hen was placed in the arena the observer sat close by and watched the behaviour of the bird. The latency to first step, the number of steps, latency to first vocalisation, number of vocalisations and number of defecations were recorded in a 10 min period. If the bird did not step or vocalise in this time a score of 10 min was given for the latency to first step or latency to first vocalisation. At 10 min the bird was picked up and a single blood sample (0.5 – 3 ml) taken from the ulnar vein using a heparinised 25 g needle and 3 ml syringe. All samples were taken within three min of the hen being picked up. In addition, blood samples had been collected from the hens up to 8 weeks before or after the open field test to allow the comparison of corticosterone concentrations between undisturbed hens and hens that after 10 min in the open field test. Samples were collected from undisturbed hens by removing a hen from its cage and collecting a blood sample within three min by the same method used for sampling hens after the open field test. All blood samples were expelled into heparinised test tubes, stored on ice then transported to the laboratory where they were centrifuged and plasma frozen at -70°C.

4.2.3.4 Runway tests

The runway was 2.40 m long x 0.60 m wide and 0.85 m high. A wire partition 0.4 m from one end created an area was designated as the start box. At the opposite end of the runway there was a plastic chicken crate (0.54 x 0.31 x 0.74 m width x depth x height).

Two hens from the same cage as the test bird were placed in the chicken crate and this was designated the goal box. Test hens were individually removed from their cage, carried to the end of the shed and placed in the start box of the runway. After a hen had been in the start box for 2 min the partition was raised. The latency for the bird to leave the start box, time to reach 0.5 m from the start box, time to reach within 0.2 m of the goal box and total time spent within 0.2 m of the goal box were recorded during a 10 min test period.

4.3.3 Plasma sample preparation and corticosterone radioimmunoassay

4.3.3.1 Plasma sample preparation

Each plasma sample was thawed, and spun for 10 min at 13 000 rpm. This process separated lipid from the plasma. The clear plasma from the lipid layer was transferred to a Sterilin RT20 tube and diluted in PBSG by a factor of 6 for the assay of corticosterone.

4.3.3.2 Radioimmunoassay of corticosterone

Corticosterone concentrations in plasma extracts were measured by radioimmunoassay following the method of Fraisse and Cockrem (2006). Samples were assayed in duplicate. 10µl of extract in PBSG was incubated with 20µl of iodinated corticosterone and 20µl of antiserum (^{125}I-corticosterone and antiserum ImmuChem^{TM} Double Antibody Corticosterone ^{125}I RIA kit for rats and mice, MP Biomedicals, USA; 4000 cpm) for 2 hours at room temperature (22°C – 25°C). 50 µl of precipitant solution (MP Biomedicals, USA) was added and each sample was centrifuged at 3 000 rpm for 15 min at 4°C. 20 µl

of starch (2.5 g/l starch in PBSG) was then added to increase adhesion of the pellet to the tube. Samples were then centrifuged for a further 15 min at 3 000 rpm at 4°C, and the supernatant aspirated off. The pellets were counted on a LKB Wallac 1261 Multigamma gamma counter for 2 min each.

This radioimmunoassay was validated by Fraisse and Cockrem (2006) for the measurement of corticosterone in chicken plasma. The sensitivity of the corticosterone assay was the minimum hormone level that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean - 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity, expressed as ng corticosterone/ml plasma, was 0.35 ng/ml. Solutions of corticosterone in PBSG at concentrations that gave approximately 80, 50 and 20% binding on the standard curve were used as low, medium and high quality controls in every assay. The intra-assay coefficients of variation were 8.4 % (n=20), 6.0 % (n=20), and 5.3 % (n=10) and inter-assay coefficients of variation for ten assays 16.0%, 8.3% and 16.9% for low, medium and high solutions respectively.

4.3.4 Statistical analysis

4.3.4.1 Fear score ranks

Fear score ranks were calculated for the tonic immobility and open field tests following methods used for chickens (Jones and Mills, 1983; Jones, 1987a; Jones, 1988) and quail (Jones and Mills, 1983). The ranks are calculated by ranking birds from the least fearful to most fearful for each variable within each test (Jones and Mills, 1983). Values of each

variable are ranked from low to high or from high to low depending on the predicted relationship of each variable with fear. The ranks are added to get a fear score for each bird for each behaviour test, so a low score corresponds to relatively low fearfulness and a high score to high fearfulness. The fear scores are then ranked from low to high to give fear score ranks (least to most fearful) for birds in each behaviour test.

The relationships with fear of each variable in the tonic immobility test were assigned as follows (Jones, 1988): number of inductions negative relationship so birds were arranged in order from high to low numbers of inductions; time to first head movement positive relationship so birds were arranged in order from low to high times; number of head movements negative relationship; duration of tonic immobility positive relationship. The relationships with fear of each variable in the open field test were assigned as follows (Gallup et al., 1976; Jones and Mills, 1983; Mills and Faure, 1986): time to first step positive relationship so birds were arranged in order from low to high times; time to first vocalisation positive relationship; number of steps negative relationship so birds were arranged in order from high to low numbers; number of vocalisations negative relationship; number of defaecations negative relationship. The relationship between the number of defaecations and fear was taken to be negative (Gallup et al., 1976; Faure et al., 1983; Suarez and Gallup, 1983), although (Jones and Merry, 1988) considered the relationship to be positive.

4.3.4.2 Principal components analyses

Principal components analysis (PCA) was used to examine relationships between variables in the tonic immobility, open field and runway tests. This approach has been used in studies of feather pecking behaviour in hens (Van Hierden et al., 2002), tonic immobility and open field tests in Japanese quail (Jones et al., 1991), and open field and novel object tests in calves (Van Reenen et al., 2005). Principal components are linear combinations of the original variables, and principal components analysis can summarise many variables into a few components. The loading of each variable for a principal component indicates the importance of each variable for the component. Principal components analyses were performed on the Pearson correlation matrix for each combination of variables, with principal components with eigenvalues greater than or equal to one retained for further analyses. Components were rotated after the initial analysis, and component loadings and the percentage of total variance explained by each component were calculated. Principal components were identified in analyses for white and brown birds separately. Analyses were conducted for each behaviour test, and for tonic immobility and open field tests together.

A PCA analysis identifies the proportion of the total variance that is accounted for by each component, and the loadings of the original behaviour variables on each component. An analysis can also include for each component the calculation of coefficients for each behaviour variable. These coefficients can then be applied to the original data and used to calculate PCA scores for each bird. These scores form a new variable, derived from the individual behaviour variables, whose mean values can be compared between groups

of birds in the same way that mean values of individual variables can be compared between groups. For example, the PCA for tonic immobility in white birds in their first immobility test produced two components that accounted for 38.5 and 28.0% of the total variance respectively. The loadings of individual behaviour variables on the first component were -0.371 for number of inductions, -0.072 for time to first head movement, 0.848 for number of head movements and 0.822 for duration. These loadings indicate that the number of inductions had a moderate and negative influence on component one whilst the number of head movements and duration of immobility had high positive influences on the component. The time to first head movement had no influence on the component. The equation with coefficients to calculate component one scores was:

Factor 1 score = (-0.223 x number of inductions) + (-0.092 x time to first head movement) + (0.567 x number of head movements) + (0.523 x duration)

Coefficients derived from analyses of data from the first tonic immobility test were used to calculate component scores for each bird in both the first and second tests. Relationships between these component scores from the two tests were then determined with Pearson correlations.

4.3.4.3 Comparisons between the first and second tonic immobility tests

Comparisons between mean values of behaviour variables in the first and second tonic immobility tests were made using two tailed t-tests. Relationships between behaviour variables and fear score ranks in the first and second tonic immobility tests were determined with Pearson correlations.

4.3.4.4 Statistical repeatability

Statistical repeatability is a measure that describes the proportion of variance in a variable that occurs among rather than within individuals. Repeatability for a variable can be calculated from a one way analysis of variance in which repeatability, r, is given by the formula: $r = s^2_A/(s^2 + s^2_A)$ where s^2_A is the among-groups variance component and s^2 is the within-group variance component. These variance components are calculated from the mean squares in the analysis of variance as $s^2 = MS_W$ and $s^2_A = (MS_A - MS_W)/n_0$ where $n_0$is a coefficient related to the sample size per group in the analysis of variance. Statistical repeatabilities of behaviour variables in the first and second tonic immobility tests were calculated by the method of (Lessells and Boag, 1987). This method has been used to calculate repeatabilities for exploratory and risk-taking behaviour in great tits (Parus major; Dingemanse et al., 2002; Van Oers et al., 2004).

4.3.4.5 Relationships between corticosterone and behaviour

The areas under corticosterone response curves were determined in Prism using the trapezoid rule and termed integrated corticosterone responses (Cockrem and Silverin, 2002). The total area under the curve and the total area minus the area attributable to corticosterone concentrations at 0 min (corrected area) were calculated. If corticosterone concentrations after 0 min were less than those at 0 min then a corrected area was not calculated for that bird. Integrated corticosterone responses were compared between strains by t-tests. Relationships between corticosterone response variables (corticosterone at 15 min, and total and integrated corticosterone responses) and

behaviour variables, and between corticosterone concentrations at the end of the open field test and behaviour variables, were determined with Spearman correlations. Relationships between corticosterone response variables and fear score ranks, and between corticosterone variables and PCA component scores, were also determined with Spearman correlations.

Statistical analyses were performed using Prism (GraphPad Software Inc.) and Systat (SPSS Inc.). Data are presented as individual points or as mean $\pm$ S.E.

4.4 Results

4.4.1 Repeatability of tonic immobility behaviour test

The numbers of inductions, duration of tonic immobility, time to first head movement and numbers of head movements in white and brown hens in the tonic immobility test differed between white and brown hens (see chapter 2 for statistics), and did not change when birds were retested after 10 days (Table 4.1). The correlation between numbers of inductions in the first and second tests approached significance for white hens, whereas there were no significant correlations between the two tests for other behaviour variables, in white hens (Table 4.2). Correlations between first and second tonic immobility tests were significant for all behaviour variables other than numbers of inductions for brown birds (Table 4.2). Correlations between the two tests were higher in brown than white birds for the behaviour variables with significant correlations.

A principal components analysis (PCA) using the four measures of tonic immobility in white birds produced two principal components that accounted for 66.5% of the total variance. A PCA for brown birds also produced two components which in these birds accounted for 71.4% of the total variance. The duration of tonic immobility and the number of head movements had high positive loadings and the number of inductions had a moderate negative loading on component one for both strains of birds. There was thus no clear relationship with fear for component one. Loadings of behaviour variables on component two differed from loadings on component one. There was a high negative loading of time to first head movement on component two and a moderate positive loading of number of inductions in white birds. These loadings are consistent with an

inverse relationship between component two and fear in white birds. Loadings on component two for brown birds were moderate or high and positive for duration and time to first head movement, indicating a positive relationship between this component and fear in brown birds.

There were no correlations between the two tests for the PCA component scores in white birds, whereas there was a significant correlation for the component one score in brown birds (Table 4.2). Fear score ranks were not correlated between tests for either strain of hen (Table 4.2). Statistical repeatabilities were markedly higher in brown than white birds for three of the four behaviour variables (Table 4.3), the exception being duration of tonic immobility.

4.4.2 Relationships between corticosterone and behaviour

Corticosterone responses to handling of white and brown birds and corticosterone responses to 10 min in the open field test were described in detail in chapter 3. Mean corticosterone concentrations in birds handled for 15 min increased from approximately 1 ng/ml at 0 min to 5.5 to 6.0 ng ml at 15 min, decreased to approximately 3 ng/ml at 30 min and remained constant from 30 to 60 min. Corticosterone responses to handling did not differ between white and brown hens. Corticosterone concentrations after 10 min in the open field test were elevated in white and brown hens compared with corticosterone in undisturbed hens (see chapter 3 for statistics), with no difference between strains in the corticosterone response to the open field. Corticosterone concentrations at the end of the open field test were not correlated with any of the five behaviours measured during

the test or with PCA scores for component one (white birds) or components one and two (brown birds; Spearman correlations; see Appendix Table 11).

There were five significant Spearman correlations in white hens between behaviour variables in the tonic immobility, open field and runway tests and corticosterone response variables (corticosterone at 15 min, total integrated corticosterone response and corrected integrated corticosterone response), and no significant correlations for brown hens (see Appendix Tables 12 and 13). The significant correlations in white hens were between the number of inductions in the tonic immobility test and corticosterone at 15 min ($r = 0.306$, $p = 0.037$), the number of open field vocalisations and corticosterone at 15 min ($r = -0.321$, $p = 0.028$), and between latency to leave the start box in the runway test and the three corticosterone response variables ($r = 0.294$, $p = 0.045$; $r = 0.329$, $p = 0.029$; $r = 0.36$, $p = 0.026$ respectively).

Principal components analyses using the five measures of open field behaviour produced one principal component that accounted for 56.5% of the total variance in white birds and two components that accounted for 65.4% of the variance in brown birds. There were high negative loadings of time to first step and time to first vocalisation on component one in white birds, and high positive loadings of number of steps, number of vocalisations and number of defaecations. These loadings suggest a strong inverse relationship between component one of the open field test and fear in white birds. Loadings on component one for brown birds showed a different pattern and a positive relationship with fear, with moderate or high positive loadings for time to first step and time to first vocalisation and a high negative loading for number of vocalisations.

Component two in brown birds had a moderate positive loadings for time to first step, and high negative loadings for number of steps and number of defaecations. Loadings in brown birds are consistent with strong positive relationships with fear for both components.

Principal components analyses using the four measures of tonic immobility and the five measures of open field behaviour combined in a single analysis for white birds and in a single analysis for brown birds produced four principal components that accounted for 75.8% of the total variance in white birds and 73.2% in brown birds. There were high loadings of three of the nine individual behaviours on two components for white birds and three components for brown birds. The loadings were consistent with a weak positive relationship with fear for components one and four and a negative relationship for component three in white birds, and with weak positive relationships with fear for components one and four and a negative relationship for component three in brown birds.

There were significant positive correlations for white but not brown hens between fear score ranks and corticosterone variables. Tonic immobility fear score ranks in white hens were correlated with the three corticosterone response variables, and open field fear score ranks were correlated with corticosterone at 15 min (Table 4.4).

Runway component one scores in white but not brown hens were positively correlated with two of the corticosterone variables. This component had a strong negative relationship with sociality, so the positive correlations of the component scores with

corticosterone indicate that birds that show higher degrees of sociality have relatively lower corticosterone responses to a stressor.

PCA scores in white hens had both significant positive and significant negative correlations with some corticosterone variables in white hens (Table 44), whereas there were no significant correlations in brown hens (see Appendix Table 14). Tonic immobility component two scores and open field component one scores in white hens were negatively correlated with a corticosterone variable. These components were considered to be negatively related to fear, so the negative correlations of the component scores with corticosterone are consistent with an association between fear and corticosterone in white hens. Component scores one and three from the combined tonic immobility and open field PCA analysis were negatively correlated with all three corticosterone variables and component four scores were positively correlated with the corticosterone variables. Component three was considered to be negatively related to fear and component four to be positively related to fear, so for these two components the correlations with corticosterone support an association of fear and corticosterone in white hens. The correspondence between relationships of components derived from PCA analyses of fear behaviour with significant correlations of component scores with corticosterone variables for four of the five components provide strong support for a relationship between fear and corticosterone in white hens. White hens that display greater fearfulness have higher corticosterone responses to a handling stressor than hens that display lower fearfulness.

Table 4.1.Mean values and statistics for behaviour variables from first and second tonic immobility tests. First test n=50; second test white hens n=48, brown hens n=46.

	First test		Second test		t	P
	Mean	SEM	Mean	SEM		
White hens						
Number of inductions	1.64	0.16	2.10	0.19	-1.863	0.066
Duration	9.53	1.00	9.73	1.04	-1.531	0.129
Time to first head movement	1.03	0.19	1.52	0.26	0.097	0.923
Number of head movements	27.92	6.38	27.04	6.49	-0.139	0.889
Brown hens						
Number of inductions	3.32	0.23	3.17	0.24	0.444	0.658
Duration	2.85	0.62	2.71	0.66	-0.804	0.423
Time to first head movement	0.27	0.10	0.39	0.11	-0.188	0.851
Number of head movements	14.22	3.23	15.41	5.58	0.152	0.880

Table 4.2. Pearson correlations between variables in first and second tonic immobility tests.

	R	df	P
White			
Number of inductions	0.274	46	0.060
Duration	0.187	46	0.203
Time to first head movement	-0.038	45	0.801
Number of head movements	-0.026	46	0.859
Fear score rank[1]	0.127	45	0.396
PCA component 1 score[2]	0.008	46	0.959
PCA component 2 score[2]	-0.062	46	0.677
Brown			
Number of inductions	-0.107	44	0.477
Duration	0.416	44	0.004
Time to first head movement	0.348	44	0.018
Number of head movements	0.320	44	0.030
Fear score rank[1]	0.068	44	0.652
PCA component 1 score[2]	0.353	44	0.016
PCA component 2 score[2]	0.044	44	0.773

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

[2]Coefficients derived from PCA analyses of data from the first tonic immobility test were used to calculate component scores for each bird in both the first and second tests.

Table 4.3. Repeatabilities and statistics for behaviour variables from first and second tonic immobility tests. White hens n=48, brown hens n=46.

	R	SEM	Lower 95% CI	Upper 95% CI
White				
Number of inductions	0.197	0.089	-0.565	0.777
Duration	0.242	0.086	-0.039	0.487
Time to first head movement	-0.042	0.091	-0.317	0.239
Number of head movements	-0.016	0.092	-0.293	0.263
Brown				
Number of inductions	0.422	0.074	0.164	0.627
Duration	-0.099	0.090	-0.367	0.184
Time to first head movement	0.345	0.080	0.074	0.569
Number of head movements	0.298	0.083	0.022	0.532

Table 4.4. Spearman correlations between corticosterone variables and fear score ranks, and between corticosterone variables and PCA behaviour scores in white hens. Significant r and P values are indicated in bold. n = 46.

	Corticosterone at 15 min (ng/ml)		Total integrated corticosterone response (ng/ml.min)		Corrected integrated corticosterone response (ng/ml.min)	
	R	P	r	P	r	P
Fear score rank						
Tonic immobility	0.320	**0.032**	0.343	**0.026**	0.393	**0.010**
Open field	0.307	**0.038**	0.227	0.143	0.234	0.131
PCA scores for individual behaviour variables						
Tonic immobility component 1	-0.149	0.091	-0.167	0.277	-0.193	0.210
Tonic immobility component 2	-0.352	0.318	-0.293	0.054	-0.335	**0.026**
Open field component 1	**-0.303**	**0.015**	-0.249	0.103	-0.237	0.121
Runway component 1	0.260	0.078	0.305	**0.044**	0.299	**0.048**
PCA scores for tonic immobility and open field combined						
Component 1	-0.376	**0.009**	-0.302	**0.046**	-0.338	**0.025**
Component 2	0.153	0.303	0.169	0.272	0.197	0.199
Component 3	-0.389	**0.007**	-0.313	**0.039**	-0.355	**0.018**
Component 4	0.407	**0.005**	0.344	**0.022**	0.385	**0.010**

4.5 Discussion

The behavioural measures of tonic immobility test did not differ between the two tests of tonic immobility for either white Leghorn or brown Hyline hens. Correlations between individuals and statistical repeatability indicated that the tonic immobility test is more repeatable for brown than white hens. The corticosterone response to handling correlated with behavioural measures from the tonic immobility, open field and runway tests in white but not brown hens. Principal components analysis and fear rank scores allowed greater correlations between corticosterone and behavioural variables to be identified indicating a positive relationship between corticosterone and fear and a negative relationship between corticosterone and sociality.

4.5.1 Repeatability of tonic immobility behaviour test

Mean values if the four variables of the tonic immobility test did not differ between the first and second tests of tonic immobility for either strain of hen. These results are consistent with Jones (1988) and Jones (1989) who also found no change in duration following repeated testing. There are also reports of an increase in the number of inductions required (Jones, 1989) and a decrease in duration (Hocking et al., 2001). A number of studies have investigated the consistency of results in behavioural tests of fear. This has included ranking individuals for each test and comparing ranks between tests (Jones and Mills, 1983; Mills and Faure, 1986; Jones, 1988; Jones, 1989; Hocking et al., 2001; Janczak et al., 2003; Van Reenen et al., 2005), assigning fear scores to individuals based on behaviour in a test and comparing scores between tests (Rekila et al., 1997), assigning trait scores and comparing these scores (Svartberg et al., 2005), performing

partial correlations between each behavioural measure (Miller et al., 2005; Miller et al., 2006) and performing correlations between each behavioural measure (Webster and Hurnik, 1990). In the present study correlations between each behavioural measure and fear ranks were used to determine consistency between individuals. Statistical repeatability was also used to determine the variance among individuals. This is the first report in which statistical repeatability has been used for fear behaviour in birds or mammals. These calculations are important as they allow for the identification of which methods are accurately assessing the underlying fearfulness in an individual. The differences between the two strains in correlations of behavioural measures and statistical repeatability indicate that the tonic immobility test is more repeatable in brown hens than white hens. This indicates that the tonic immobility test is providing more accurate information about the overall underlying fearfulness in individual brown hens than in white hens. Previous studies of repeated tonic immobility in brown hens also found duration (Jones, 1988; Jones, 1989), and latency to first head movement (Jones, 1988) to be stable across individuals. In contrast, Jones (1988) found number of inductions but not number of head movements to be similar across individuals.

4.5.2 Relationships between corticosterone and behaviour

The relationship between fear and corticosterone in chickens (Jones et al., 1988; El-Lethey et al., 2001) and quail (Jones et al., 1994a; Jones et al., 2000) has been examined in a number of studies, with results indicating a positive association between the two variables It has also been reported that following tonic immobility quail have increased plasma corticosterone concentration (Jones et al., 2005). The open field test is commonly

used to assess fear behaviour in poultry (Jones, 1977; Jones and Merry, 1988; Jones et al., 1992; Jones, 1996). Therefore, it may be expected that the behavioural measures would be correlated with the plasma corticosterone concentration following the open field test but this was not the case for either strain of hen. However both the open field and tonic immobility tests showed correlations between behavioural measures and the corticosterone response to handling in white but not brown hens. These results are in contrast to Mignon-Grasteau et al (2003) who found no correlation between corticosterone response to restraint and open field and tonic immobility test behavioural measures in quail. White hens show a greater corticosterone response to restraint than brown hens (see chapter 3 for details), and Jones et al (1994b) reported results consistent with the present study as they found that quail selected for high corticosterone had a significant relationship between corticosterone response to restraint and fear test scores, but those selected for low corticosterone response did not.

Few studies have investigated the relationship between sociality and corticosterone in poultry (Mills et al., 1993; Jones et al., 2002; Marin, 2003). This is the first report in which corticosterone response to a handling stressor has been compared to behavioural measures of sociality in birds. There was one correlation between the runway behavioural measure and corticosterone in white birds. No correlations were found in brown birds.

While the correlations between corticosterone and the behavioural measures show a relationship between these two variables in white hens, the use of the derived measures of

fear ranks and principal components analysis further distinguished these relationships, with corticosterone appearing to have a positive and negative relationship with fear and sociality respectively.

The present study clearly shows that the tonic immobility test is more repeatable in brown than white hens. This study has also shown that the relationship between corticosterone and behavioural measures from tests of fear and sociality is more evident in white hens than brown hens, and that the use of derived behaviour variables is useful for further distinguishing the relationships between corticosterone and fear and sociality in laying hens.

Chapter 5 General discussion

5.1 General discussion

White Leghorn and brown Hyline hens are two commonly used strains in the commercial egg laying industry. Public concern over the welfare of caged hens compared to hens kept in other housing systems has contributed to an increased number of studies in this area. Strains of hen differ in their temperament (Gallup et al., 1976; Murphy, 1977; Jones, 1987a; Keerkeer et al., 1996) and white Leghorn and brown Hyline hens are considered to be flighty and docile respectively. Differences in temperament can result in contrasting behavioural and physiological responses to stimuli that the hens encounter on a daily basis including the physical environment, other hens and humans. It is likely that some strains will be more successful at interacting with certain environments than others, so studies of corticosterone responses, fear and sociality of commercially used laying hens can provide valuable information about strains of hen.

5.2 Major conclusions

The main aims of this research were to compare fear, sociality and corticosterone responses to a stressor between white Leghorn and brown Hyline hens and to investigate relationships between corticosterone and fear and sociality. The major conclusions are as follows:

1. The two strains of hen showed distinct differences in fear behaviour, with white hens displaying a greater underlying fearfulness than brown hens. This difference was apparent from behaviour tests, and could be further distinguished by the use of principal components analysis.

2. The two strains of hen differed in the individual repeatability of the tonic immobility test, with the test more repeatable in brown than white hens.

3. There were no differences in sociality between the two strains of hen.

4. The corticosterone response of the hens was dependent on the type of stressor and the strain of hen. A 15 min handling stressor elicited a greater corticosterone response than either a 15 min or 60 min restraint stressor in both strains of hen. There was no difference in corticosterone response between the two strains of hen to the handling stressor, but the white hens had a greater corticosterone response to the restraint stressor than brown hens.

5. Relationships between corticosterone and fear and sociality differed between the two strains of hen. No relationship was found between the behavioural variables and corticosterone in brown hens. However, in white hens there was a positive relationship between fear and corticosterone and a negative relationship between sociality and corticosterone.

5.3 Future directions

The findings of the present study indicate that further work could be undertaken to measure fear, sociality and corticosterone responses in white and brown hens, other strains of hen or other species of poultry. The results described in this thesis demonstrate

that principal components analysis can be used to identify differences in underlying fearfulness between different strains of laying hen. This may be useful in the selection of appropriate traits or to determine which individuals would be suitable for a particular environment in different strains of laying hen and other commercially used species of poultry. The corticosterone responses to the different stressors provide a basis for examining differences in the perception of stressors in these two strains of hen. Further measurement of corticosterone responses to a variety of stressors (for example, a change in the environment or exposure to different novel objects) could provide useful information about how these birds perceive these situations in comparison to previously identified stressors. In order to further investigate sociality in white and brown hens, it would be useful to run a social proximity experiment as described in Jones et al (2002). This would allow for the assessment of sociality in the familiar environment of their home cage thus providing additional information about the social characteristics of these strains. In summary, the experiments described in this thesis have provided physiological and behavioural information about white Leghorn and brown Hyline hens and provide a basis for further work in both laying hens and other species of poultry.

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Appendix

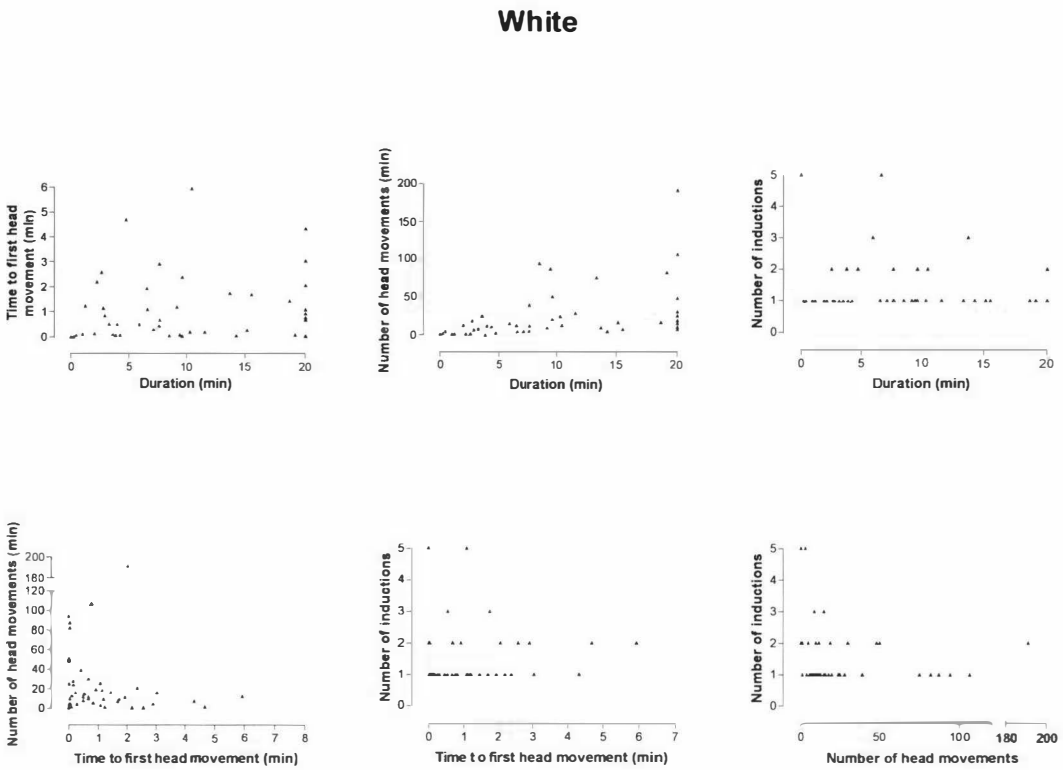


Fig. 1. Correlations between the four behavioural variables of tonic immobility in white Leghorn hens. n = 50. Spearman rank correlations of number of inductions and time to first head movement r = -0.0843, p = 0.5646; number of inductions and number of head movements r = -0.2233, p = 0.1191; number of inductions and duration of tonic immobility r = -0.0253, p = 0.8617; latency to first head movement and number of head movements r = -0.0203, p = 0.8900; latency to first head movement and duration of tonic immobility r = 0.2880, p = 0.0448; and number of head movements and duration of tonic immobility r = 0.6623, p = <0.0001.

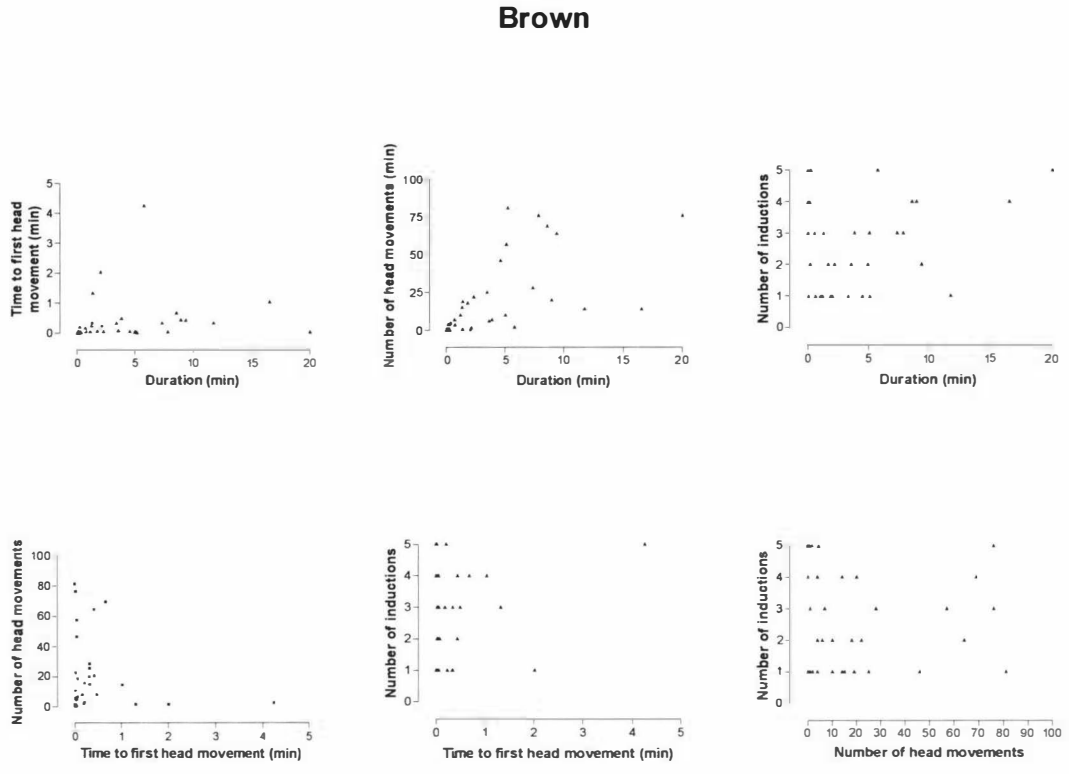


Fig. 2. Correlations between the four behavioural variables of tonic immobility in brown Hyline hens. n = 50. Spearman rank correlations of number of inductions and time to first head movement r = -0.5163, p = 0.0001; number of inductions and number of head movements r = -0.5909, p = <0.0001; number of inductions and duration of tonic immobility r = -0.5721, p = <0.0001; latency to first head movement and number of head movements r = 0.6189, p = <0.0001; latency to first head movement and duration of tonic immobility r = 0.7601, p = <0.0001; and number of head movements and duration of tonic immobility r = 0.7925, p = <0.0001.

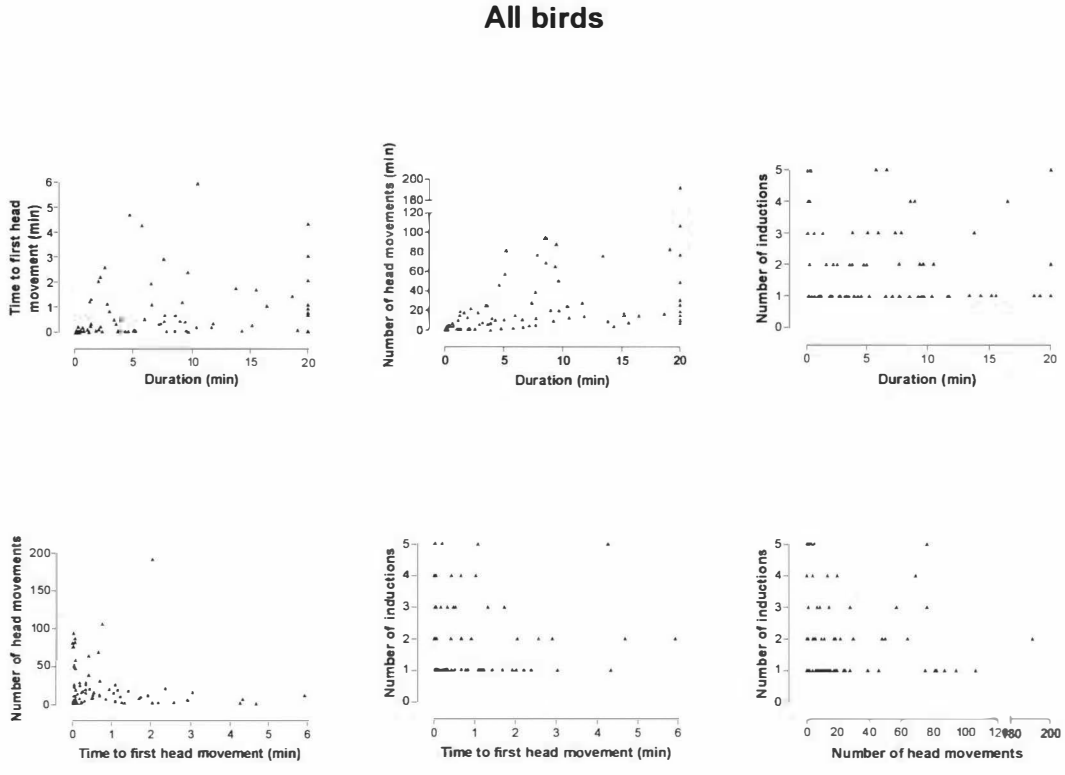
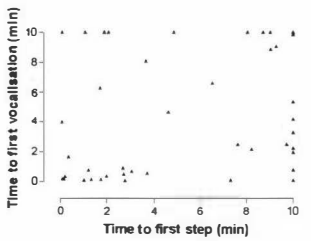
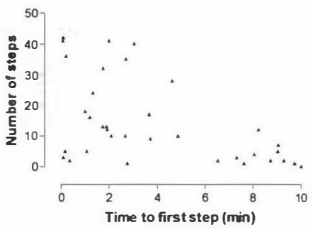


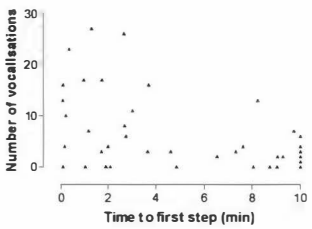
Fig. 3. Correlations between the four behavioural variables of tonic immobility in both white Leghorn and brown Hyline hens. n = 100. Spearman rank correlations of number of inductions and time to first head movement r = -0.4765, p = <0.0001; number of inductions and number of head movements r = -0.5001, p = <0.0001; number of inductions and duration of tonic immobility r = -0.4949, p = <0.0001; latency to first head movement and number of head movements r = 0.4015 , p = <0.0001; latency to first head movement and duration of tonic immobility r = 0.6371, p = <0.0001; and number of head movements and duration of tonic immobility r = 0.7925, p = <0.0001.

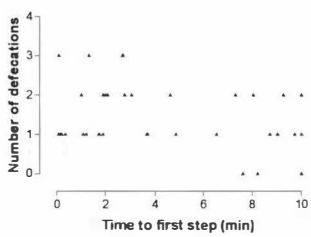
Fig. 4. Correlations between the five behavioural variables of the open field test in white Leghorn hens. n = 49. Spearman rank correlations of latency to first step and latency to first vocalisation r = 0.3430, p = 0.0160; latency to first step and number of steps r = -0.8240, p = <0.0001; latency to first step and number of vocalisations r = -0.4500, p = 0.0012; latency to first step and number of defecations r = 0.5370, p = <0.0001; latency to first vocalisation and number of steps r = -0.2610, p = 0.0696; latency to first vocalisation and number of vocalisations r = -0.8720, p = <0.0001; latency to first vocalisation and number of defecations r = -0.3610, p = 0.0108; number of steps and number of vocalisations r = 0.3930, p = 0.0053; number of steps and number of defecations r = 0.5420, p = <0.0001; number of vocalisations and number of defecations r = 0.378, p = 0.0074.

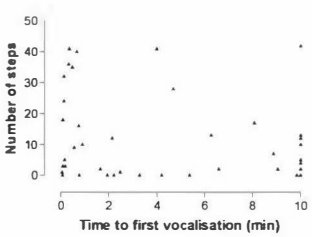
White

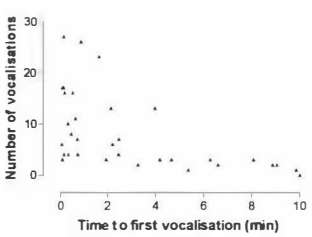

Time to first vocalisation (min)
Time to first step (min)

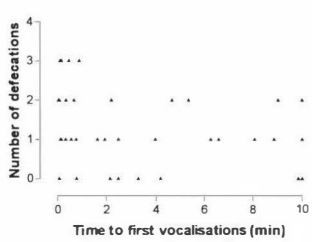

Number of steps
Time to first step (min)

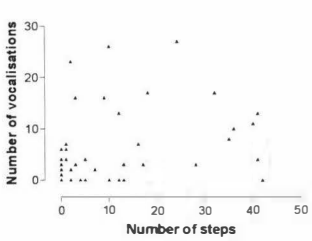

Number of vocalisations
Time to first step (min)

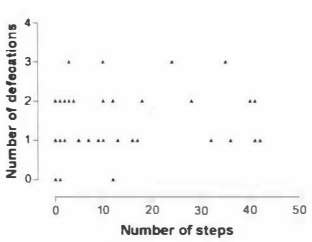

Number of defecations
Time to first step (min)


Number of steps
Time to first vocalisation (min)


Number of vocalisations
Time to first vocalisation (min)


Number of defecations
Time to first vocalisations (min)


Number of vocalisations
Number of steps


Number of defecations
Number of steps

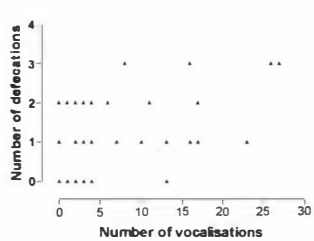
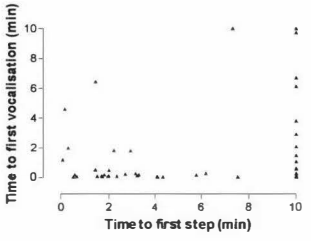
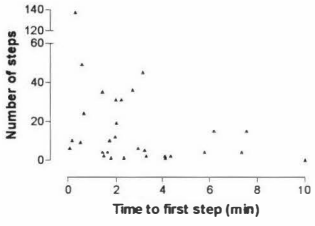
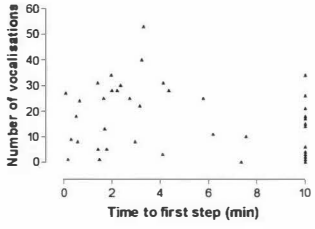

Number of defecations
Number of vocalisations

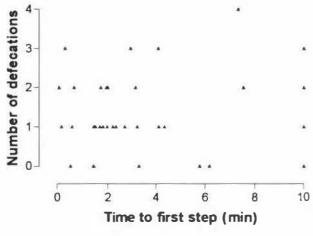
Fig.5. Correlations between the five behavioural variables of the open field test in brown Hyline hens. n = 49. Spearman rank correlations of latency to first step and latency to first vocalisation r = 0.2190, p = 0.1310; latency to first step and number of steps r = -0.8460, p = <0.0001; latency to first step and number of vocalisations r = -0.3500, p = 0.0137; latency to first step and number of defecations r = -0.2800, p = 0.0517; latency to first vocalisation and number of steps r = -0.2010, p = 0.1670; latency to first vocalisation and number of vocalisations r = 0.5150, p = 0.0002; latency to first vocalisation and number of defecations r = -0.1630, p = 0.2617; number of steps and number of vocalisations r = 0.4170, p = 0.0029; number of steps and number of defecations r = 0.3230, p = 0.0236; number of vocalisations and number of defecations r = -0.0370, p = 0.7991.

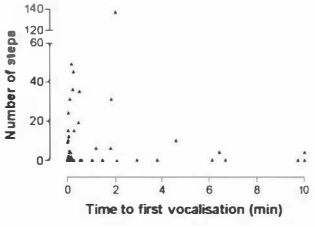
Brown

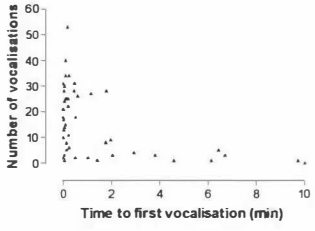

Time to first vocalisation (min)
Time to first step (min)

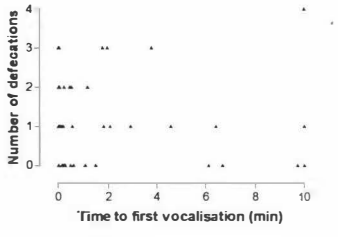

Number of steps
Time to first step (min)

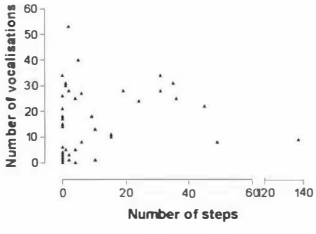

Number of vocalisations
Time to first step (min)

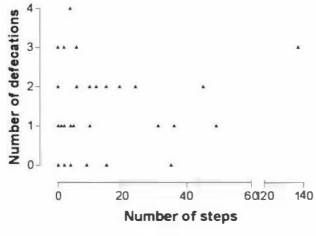

Number of defecations
Time to first step (min)


Number of steps
Time to first vocalisation (min)


Number of vocalisations
Time to first vocalisation (min)


Number of defecations
Time to first vocalisation (min)


Number of vocalisations
Number of steps


Number of defecations
Number of steps

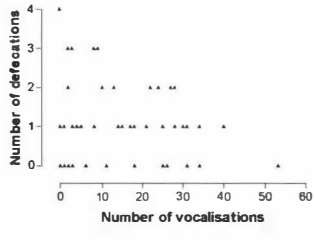
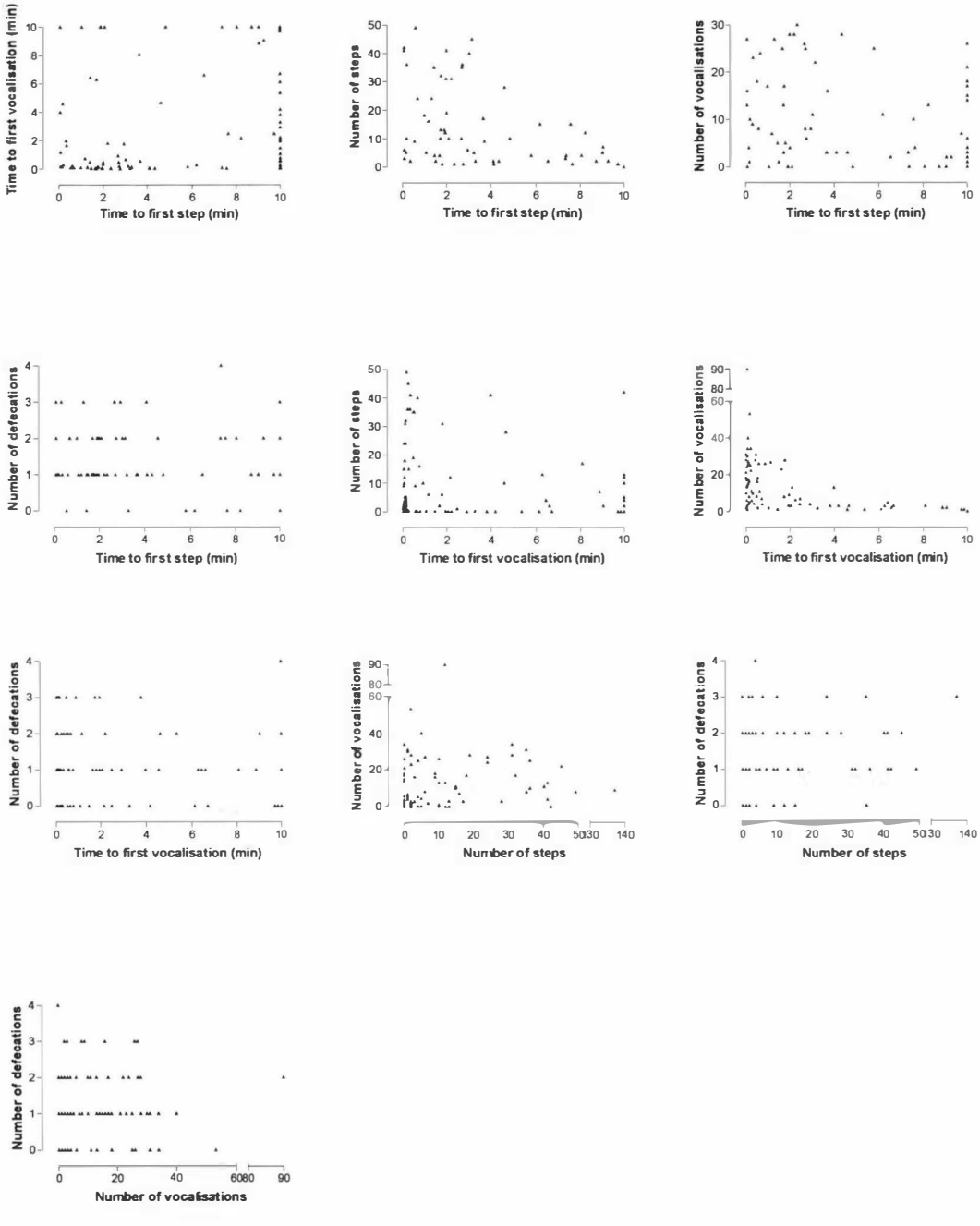

Number of defecations
Number of vocalisations

Fig. 6. Correlation between the five behavioural variables of the open field test in both white Leghorn and brown Hyline hens. n = 98. Spearman rank correlations of latency to first step and latency to first vocalisation r = 0.2530, p = 0.0121; latency to first step and number of steps r = -0.8270, p = <0.0001; latency to first step and number of vocalisations r = -0.3630, p = 0.0002; latency to first step and number of defecations r = -0.4090, p = <0.0001; latency to first vocalisation and number of steps r = -0.1580, p = 0.1207; latency to first vocalisation and number of vocalisations r = -0.7520, p = <0.0001; latency to first vocalisation and number of defecations r = -0.2230, p = 0.0276; number of steps and number of vocalisations r = 0.3320, p = 0.0008; number of steps and number of defecations r = 0.4370, p = <0.0001; number of vocalisations and number of defecations r = 0.1490 , p = 0.1419.

All birds


Time to first vocalisation (min)
Time to first step (min)
Number of steps
Time to first step (min)
Number of vocalisations
Time to first step (min)
Number of defecations
Time to first step (min)
Number of steps
Time to first vocalisation (min)
Number of vocalisations
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Number of defecations
Number of vocalisations

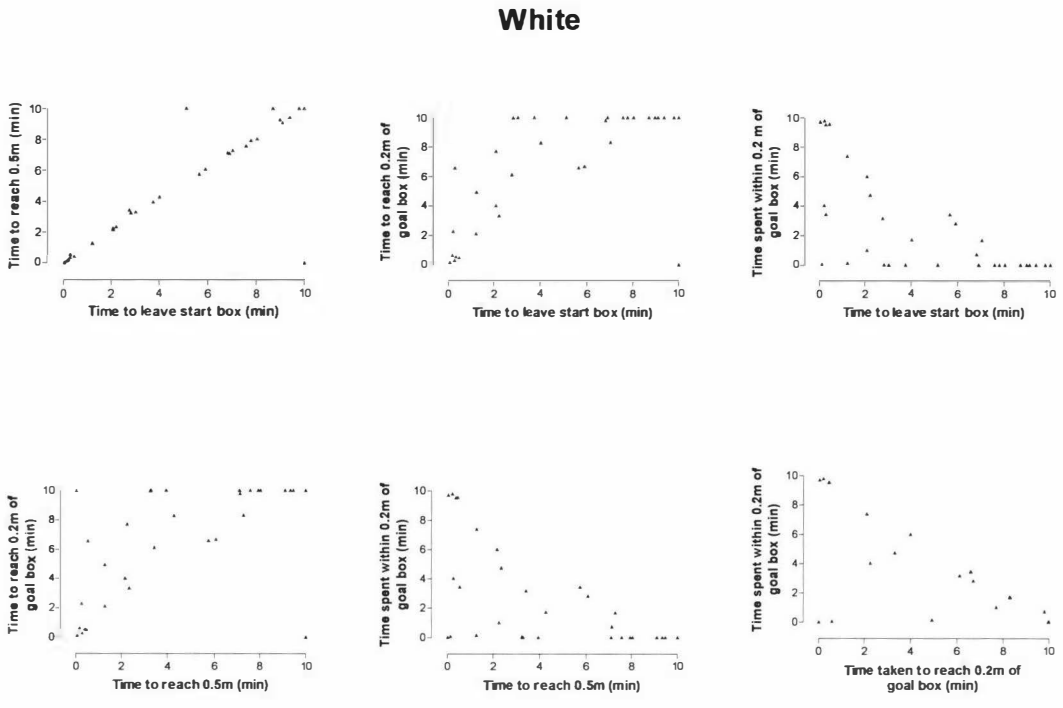

Fig. 7. Correlations between the four behavioural variables of the runway test in white Leghorn hens. n = 50. Spearman rank correlations of latency to leave start and latency to reach 0.5 m r = 0.8770, p = <0.001; latency to leave start and latency to reach 0.2 m of goal box r = 0.7670, p = <0.001; latency to leave start and time spent within 0.2 m of goal box r = -0.8280, p = <0.001; latency to reach 0.5 m and latency to reach within 0.2 m of goal box r = 0.7310, p = <0.001; latency to reach 0.5 m and time spent within 0.2 m of goal box r = -0.7870, p = <0.001; and latency to reach 0.2 m of goal box and time spent within 0.2 m of goal box r = -0.9090, p = <0.001.

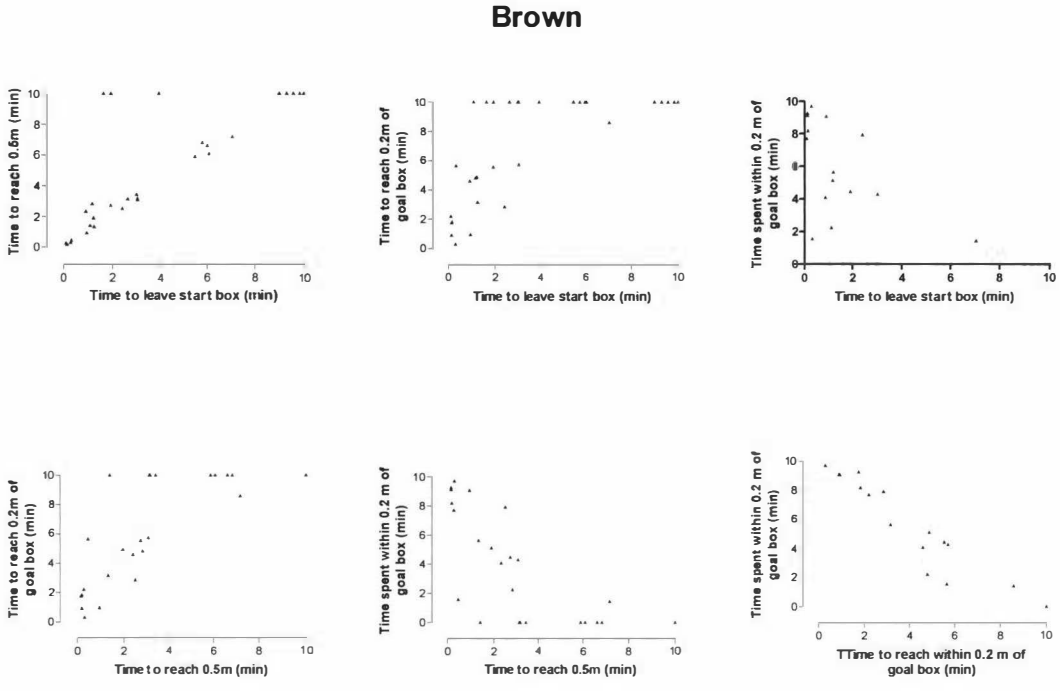

Fig. 8. Correlations between the four behavioural variables of the runway test in brown Hyline hens. n = 50. Spearman rank correlations of latency to leave start and latency to reach 0.5 m r = 0.8920, p = <0.001; latency to leave start and latency to reach 0.2 m of goal box r = 0.7670, p = <0.001; latency to leave start and time spent within 0.2 m of goal box r = -0.7600, p = <0.001; latency to reach 0.5 m and latency to reach within 0.2 m of goal box r = 0.8390, p = <0.001 ;latency to reach 0.5 m and time spent within 0.2 m of goal box r = -0.8370, p = <0.001; and latency to reach 0.2 m of goal box and time spent within 0.2 m of goal box r = -0.9970, p = <0.001.

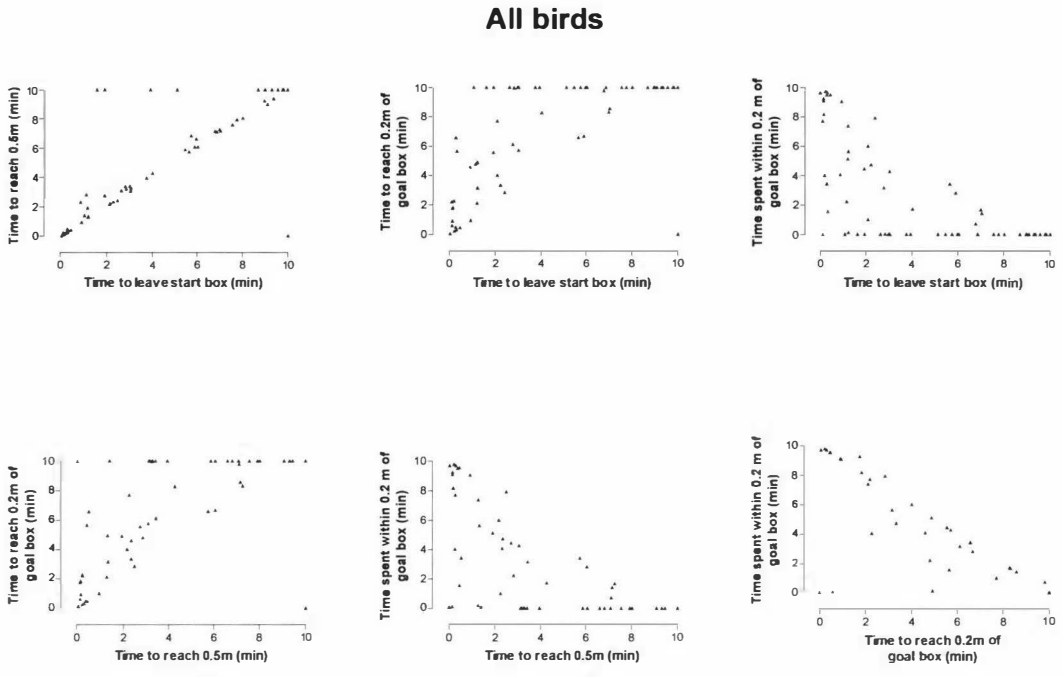

Fig. 9. Correlations between the four behavioural variables of the runway test in both white Leghorn and brown Hyline hens. n = 100. Spearman rank correlations of latency to leave start and latency to reach 0.5 m r = 0.8780, p = <0.001; latency to leave start and latency to reach 0.2 m of goal box r = 0.7540, p = <0.001; latency to leave start and time spent within 0.2 m of goal box r = -0.7810, p = <0.001; latency to reach 0.5 m and latency to reach within 0.2 m of goal box r = 0.7810, p = <0.001 ;latency to reach 0.5 m and time spent within 0.2 m of goal box r = -0.8060, p = <0.001; and latency to reach 0.2 m of goal box and time spent within 0.2 m of goal box r = -0.9530, p = <0.001.

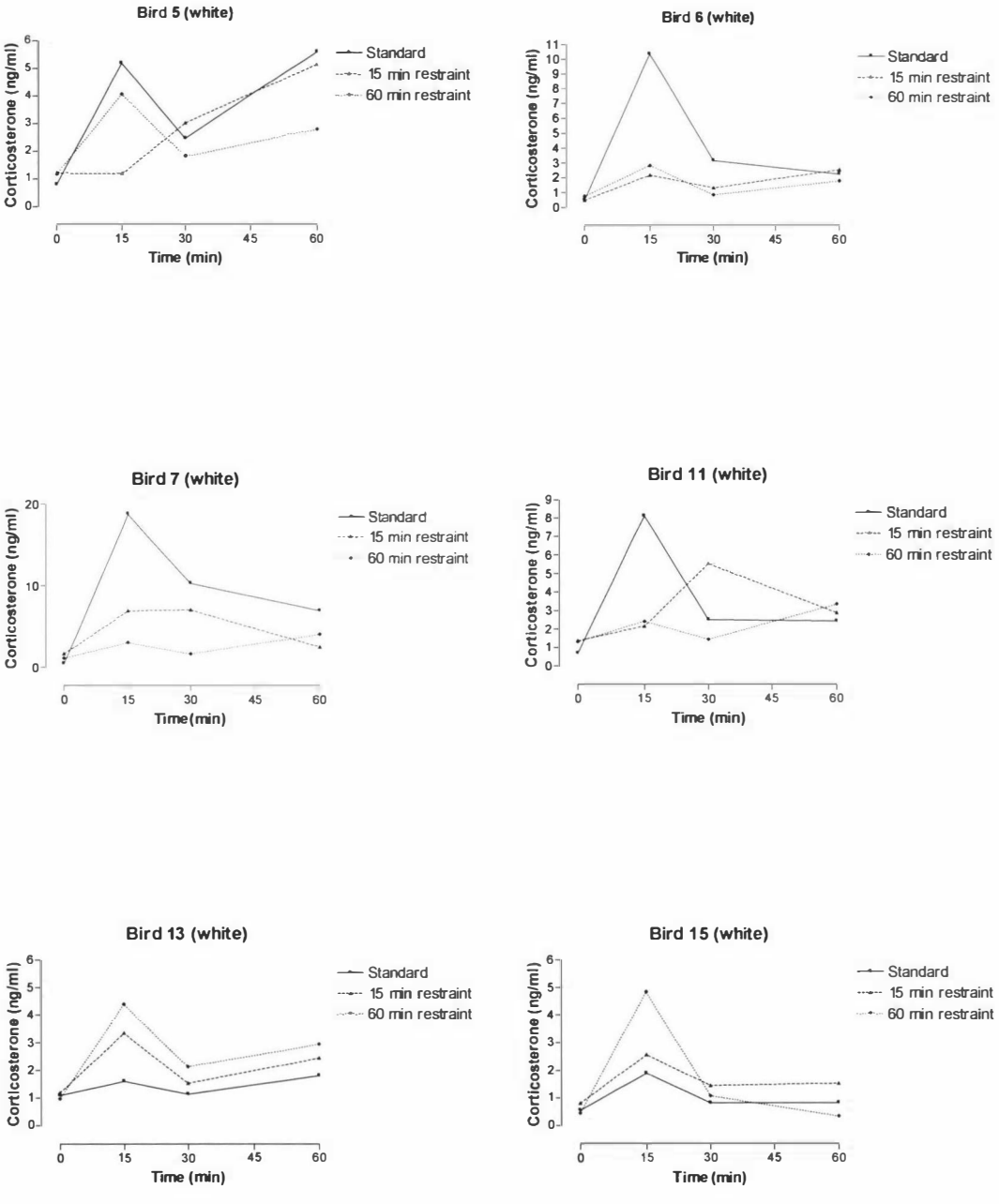

Fig 10. Corticosterone responses of individual White Leghorn hens to standard handling procedure and to 15 or 60 min of restraint.

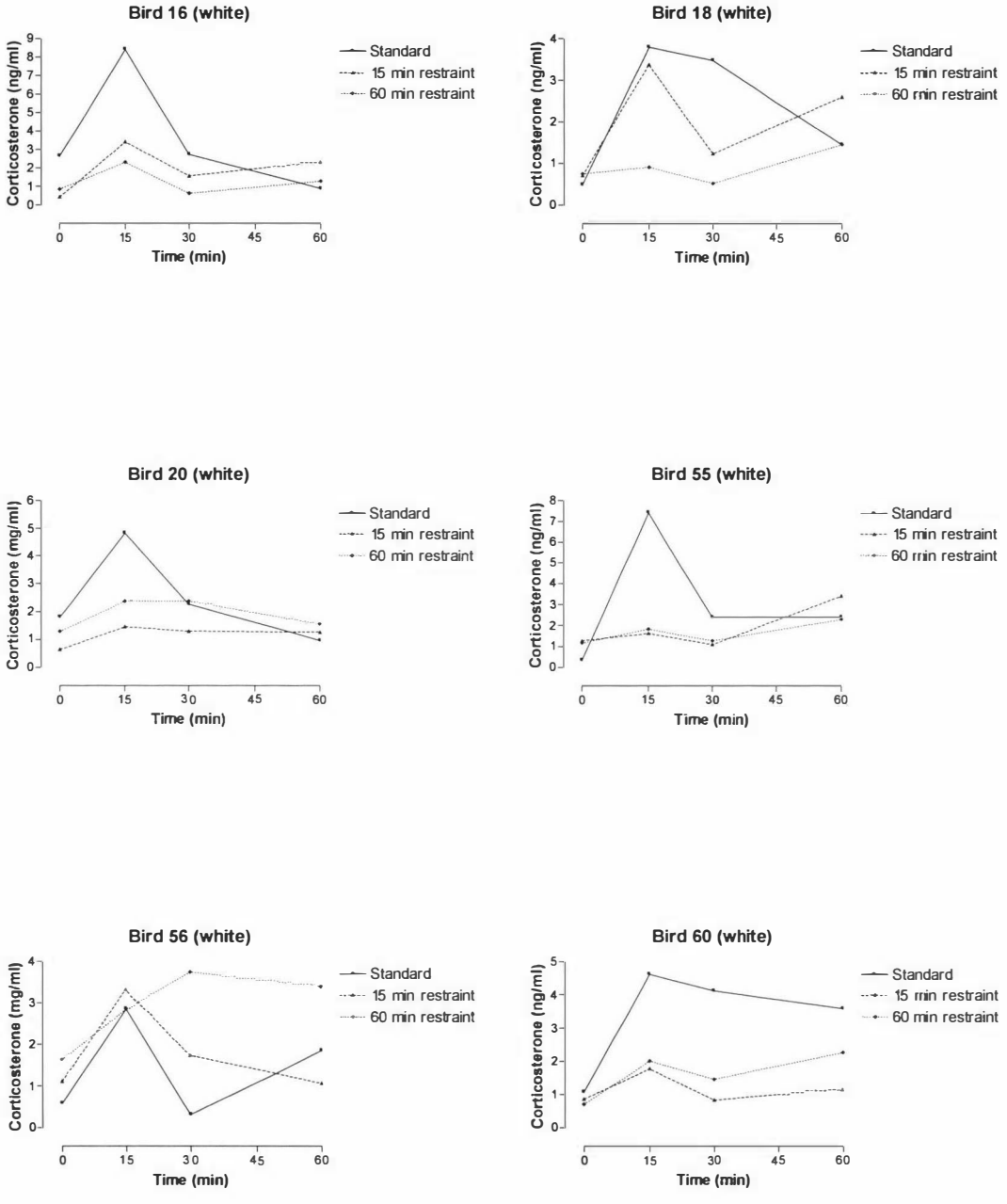

Fig. 10 cont. Corticosterone responses of individual White Leghorn hens to standard handling procedure and to 15 or 60 min of restraint.

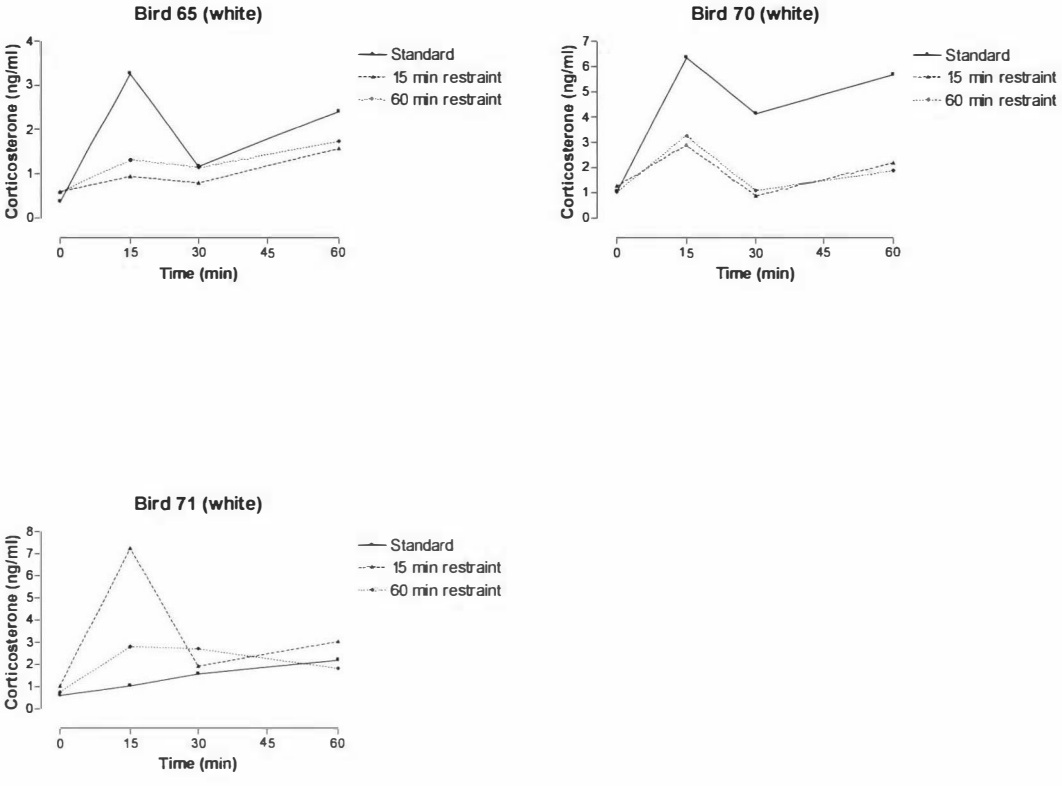

Fig. 10 cont. Corticosterone responses of individual White Leghorn hens to standard handling procedure and to 15 or 60 min of restraint.

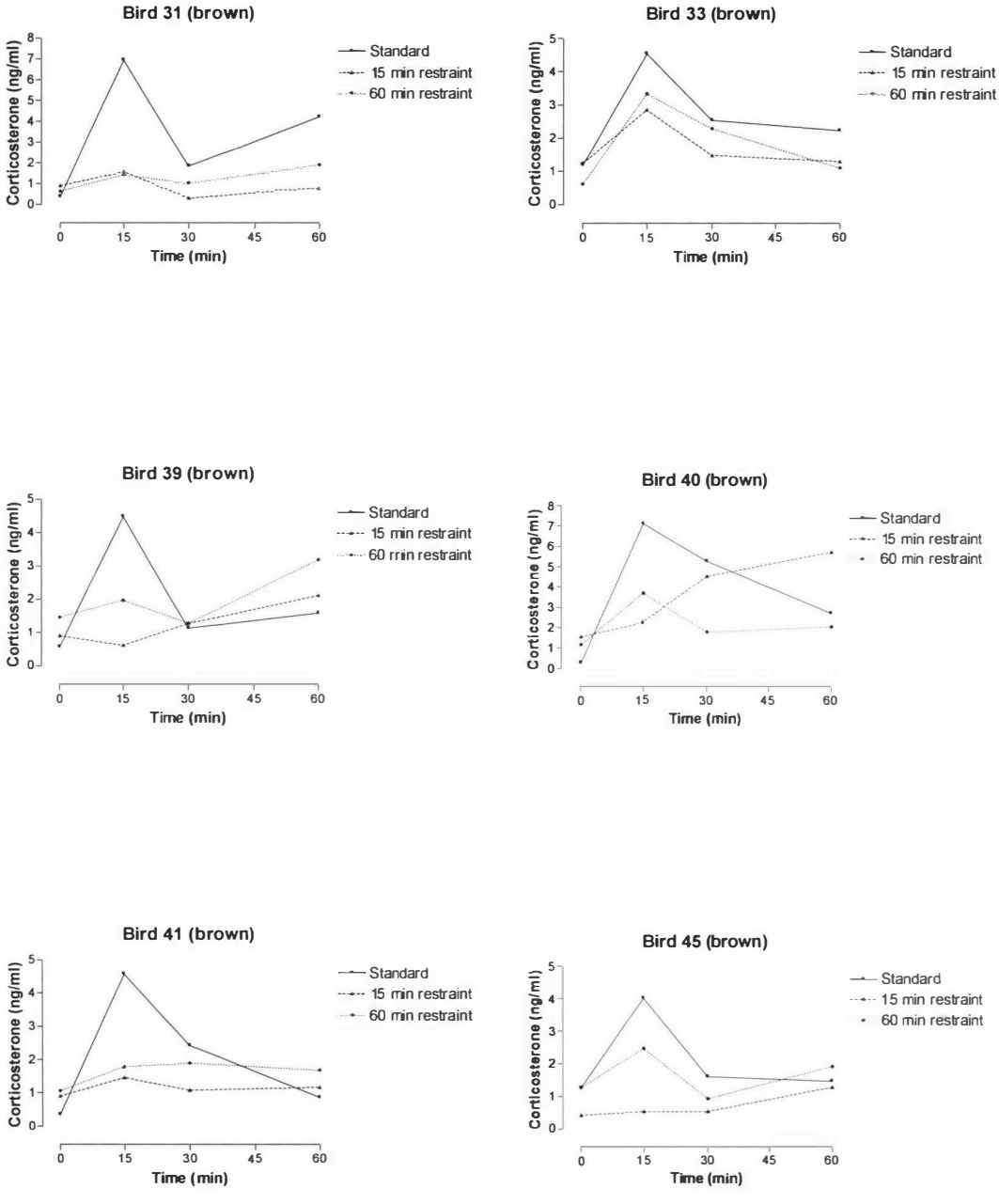

Fig. 11. Corticosterone responses of individual brown Hyline hens to standard handling procedure and to 15 or 60 min of restraint.

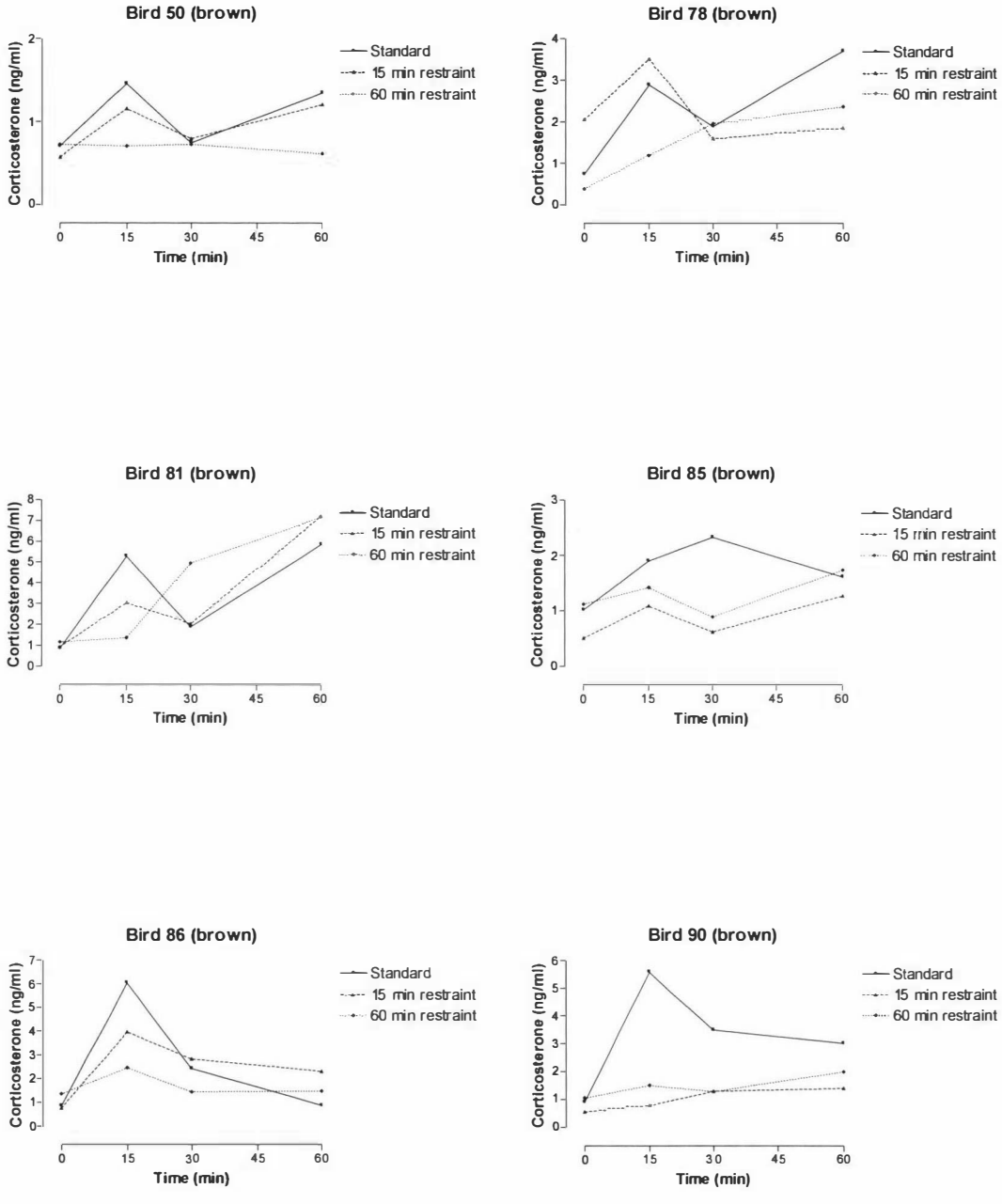

Fig. 11. cont Corticosterone responses of individual brown Hyline hens to standard handling procedure and to 15 or 60 min of restraint.

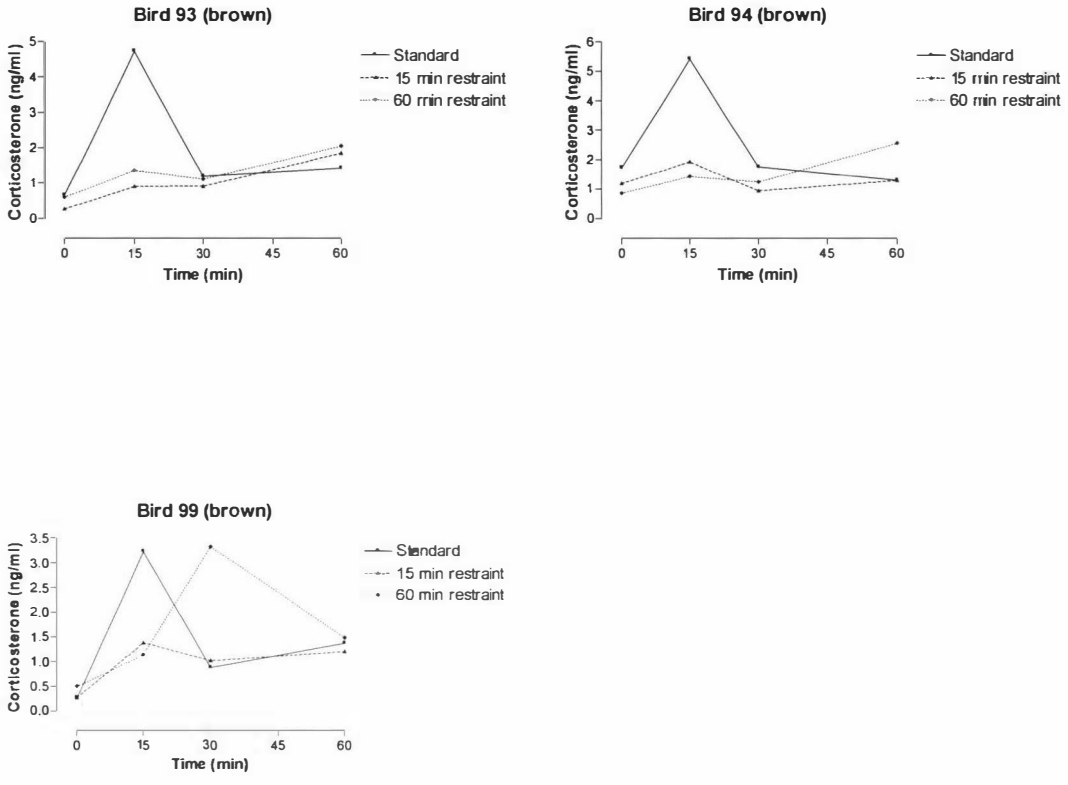

Fig. 11. cont Corticosterone responses of individual brown Hyline hens to standard handling procedure and to 15 or 60 min of restraint.

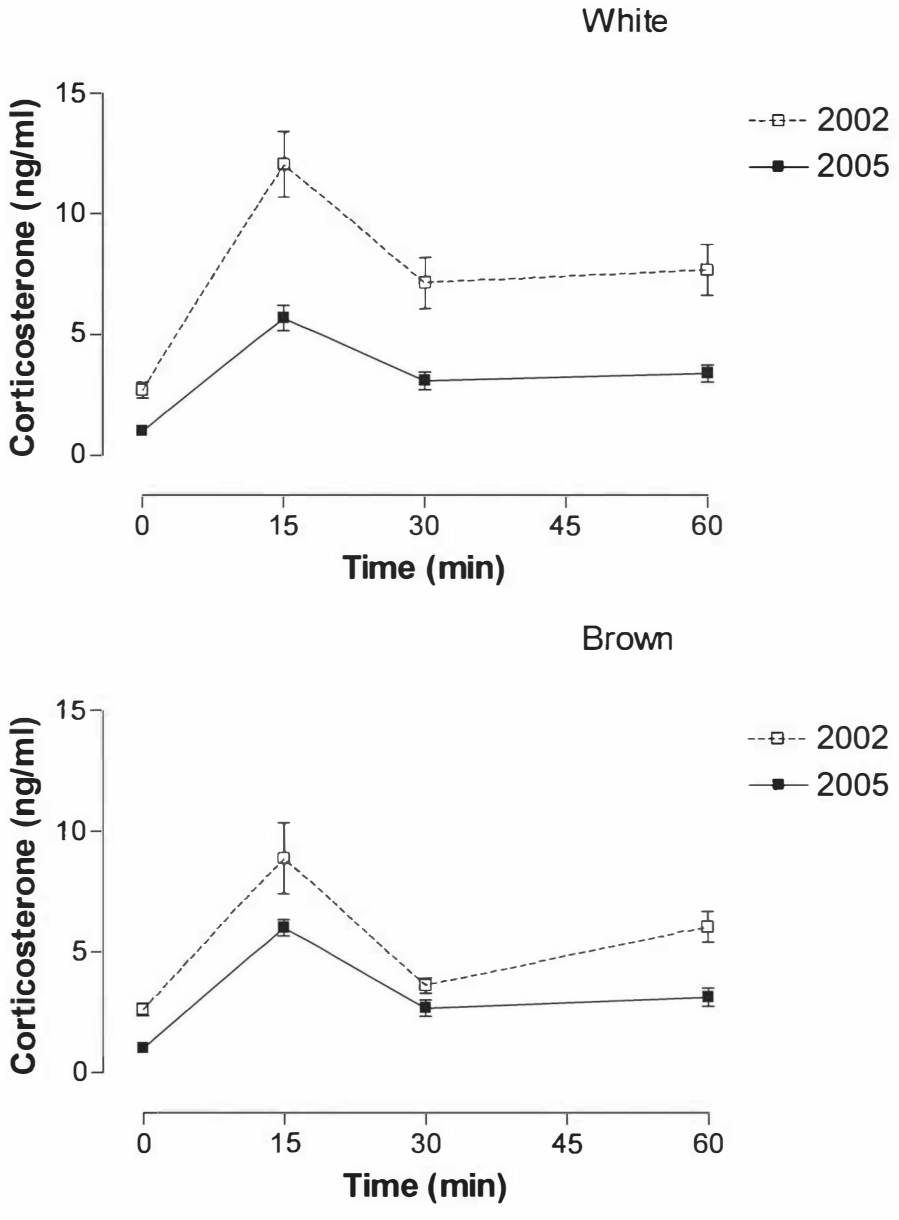

Fig. 12. Corticosterone responses of white and brown hens to 15 min handling and 45 min social isolation in the current study (2005) and in a previous study at the same farm (2002).

Table 1. Kruskal-Wallis one way ANOVA analyses of the effect of the number of times the hen had been picked up on the tonic immobility test variables at the time of their tonic immobility test for white Leghorn and brown Hyline hens. n = 50 for both strains in the first test. n = 48 for white hens and n = 46 for brown hens in the second test.

Variable	K	df	P
White			
Duration	2.887	6	0.823
Latency	8.016	6	0.237
Number of inductions	9.122	6	0.167
Number of head movements	1.266	6	0.974
Brown			
Duration	7.289	6	0.295
Latency	4.893	6	0.558
Number of inductions	5.121	6	0.528
Number of head movements	4.872	6	0.560

Table 2. Kruskal-Wallis one way ANOVA analyses of the effect of the number of times the hen had been picked up on the open field test variables at the time of their open field test for white Leghorn and brown Hyline hens.

n = 49 for both strains.

Variable	K	df	P
White			
Latency to first step	10.125	5	0.072
Latency to first vocalisation	8.086	5	0.152
Number of steps	7.069	5	0.216
Number of vocalisations	4.562	5	0.472
Number of defecations	7.544	5	0.183
Brown			
Latency to first step	5.918	4	0.205
Latency to first vocalisation	2.605	4	0.626
Number of steps	4.713	4	0.318
Number of vocalisations	6.651	4	0.156
Number of defecations	2.137	4	0.711

Table 3. Kruskal-Wallis one way ANOVA analyses of the effect of the number of times the hen had been picked up on the run way test variables at the time of their run way test for white Leghorn and brown Hyline hens. n = 50 for both strains.

Variable	K	df	P
White			
Latency to leave start box	2.792	4	0.593
Latency to reach 0.5 m	0.766	4	0.943
Latency to reach 0.2 m of goal box	6.275	4	0.180
Total time spent within 0.2 m of goal box	3.623	4	0.459
Brown			
Latency to leave start box	6.315	5	0.277
Latency to reach 0.5 m	6.915	5	0.227
Latency to reach 0.2 m of goal box	4.581	5	0.469
Total time spent within 0.2 m of goal box	5.035	5	0.412

Table 4. Kruskal-Wallis one way ANOVA analyses and Mann Whitney U tests for the effect of handler on the results of tonic immobility for white leghorn and brown Hyline hens. n = 50 for both strains in the first test. n = 48 for white hens and n = 46 for brown hens in the second test.

Variable	K	df	P
White			
Duration	0.104	2	0.949
Latency	22.479	2	<0.001
Number of inductions	6.395	2	0.041
Number of head movements	17.097	2	<0.001
Brown			
Duration	0.274	2	0.872
Latency	9.162	2	0.010
Number of inductions	5.254	2	0.072
Number of head movements	1.915	2	0.384
Comparisons between people for white birds			
		U	P
Duration			
First and second person		617.50	0.945
First and third person		23.50	0.660
Second and third person		380.00	0.960
Latency			
First and second person		950.0	<0.001
First and third person		468.50	0.262
Second and third person		128.50	<0.001
Number of inductions			
First and second person		429.00	0.013
First and third person		458.00	0.155
Second and third person		433.00	0.309
Number of head movements			
First and second person		299.00	<0.001
First and third person		570.00	0.891
Second and third person		582.50	0.001

Table 4. cont Kruskal-Wallis one way ANOVA analyses and Mann Whitney U tests for the effect of handler on the results of tonic immobility for white leghorn and brown Hyline hens. n = 50 for both strains in the first test. n = 48 for white hens and n = 46 for brown hens in the second test.

Comparisons between people for brown birds	U	P
Duration		
First and second person	543.50	0.731
First and third person	553.00	0.799
Second and third person	402.00	0.622
Latency		
First and second person	687.00	0.022
First and third person	480.50	0.246
Second and third person	256.00	0.005
Number of inductions		
First and second person	364.50	0.032
First and third person	472.50	0.200
Second and third person	518.50	0.180
Number of head movements		
First and second person	456.00	0.731
First and third person	640.00	0.799
Second and third person	515.00	0.622

Table 5. Mann Whitney U test comparing the effect of handler on the results of open field tests for white leghorn and brown Hyline hens. n = 49 for both strains.

Variable	U	P
White		
Latency to first step	331.50	0.092
Latency to first vocalisation	239.00	0.724
Number of steps	169.50	0.060
Number of vocalisations	290.00	0.439
Number of defecations	241.00	0.102
Brown		
Latency to first step	367.50	0.022
Latency to first vocalisation	381.00	0.013
Number of steps	152.00	0.013
Number of vocalisations	228.00	0.442
Number of defecations	258.50	0.901

Table 6. Kruskal-Wallis one way ANOVA analyses for effect of handler on the results of the runway test for white leghorn and brown Hyline hens. n = 50 for both strains.

Variable	K	df	P
White			
Latency to leave start box	1.056	2	0.590
Latency to reach 0.5 m	1.197	2	0.550
Latency to reach 0.2 m of goal box	2.212	2	0.331
Total time spent within 0.2 m of goal box	1.627	2	0.443
Brown			
Latency to leave start box	0.617	2	0.735
Latency to reach 0.5 m	0.164	2	0.921
Latency to reach 0.2 m of goal box	0.648	2	0.723
Total time spent within 0.2 m of goal box	0.586	2	0.746

Table 7. Mann Whitney U test comparing the first and second tests of tonic immobility for white leghorn and brown Hyline hens. n = 48 for white hens and n = 46 for brown hens.

Variable	U	P
White		
Duration	1197.50	0.986
Latency	971.00	0.139
Number of inductions	904.00	0.021
Number of head movements	1318.00	0.401
Brown		
Duration	1176.00	0.847
Latency	1059.50	0.498
Number of inductions	1199.50	0.706
Number of head movements	1177.50	0.847

Table 8. Principal components analyses for four measures of tonic immobility for white leghorn and brown Hyline hens. n = 50 for both strains in the first test. n = 48 for white hens and n = 46 for brown hens in the second test.

Measure	Factor 1	Factor 2	Factors 1 and 2
	Percent of total variance explained		
White birds first test	38.455	28.017	66.472
Brown birds first test	43.804	27.560	71.364
All birds first test	41.421	31.409	72.830
All birds both tests	38.612	36.442	75.054
	Factor 1	Factor 2	
	Coefficients for standardised factor scores		
White birds first test			
Inductions	-0.223	0.329	
Latency to first head movement	-0.092	-0.816	
Number of head movements	0.567	0.278	
Duration	0.523	-0.210	
Brown birds first test			
Inductions	-0.339	0.289	
Latency to first head movement	-0.094	0.860	
Number of head movements	0.526	-0.085	
Duration	0.424	0.304	
All birds first test			
Inductions	-0.224	0.317	
Latency to first head movement	-0.177	-0.782	
Number of head movements	0.607	0.314	
Duration	0.436	-0.187	
All bird both tests			
Inductions	-0.073	0.473	
Latency to first head movement	-0.204	-0.636	
Number of head movements	0.646	0.227	
Duration	0.463	-0.198	

Table 9. Principal components analyses for five measures of open field for white leghorn and brown Hyline hens. n = 49 for both strains.

Measure	Factor 1	Factor 2	Factors 1 and 2
	Percent of total variance explained		
White birds	56.479		56.479
Brown birds	34.017	31.417	65.434
All birds	35.369	32.571	67.940
	Factor 1	Factor 2	
	Coefficients for standardised factor scores		
White birds			
Latency to first step	-0.291		
Latency to first vocalisation	-0.249		
Number of steps	0.250		
Number of vocalisations	0.282		
Number of defecations	0.256		
Brown birds			
Latency to first step	0.247	0.350	
Latency to first vocalisation	0.476	-0.072	
Number of steps	0.008	-0.503	
Number of vocalisations	-0.526	0.125	
Number of defecations	0.223	-0.518	
All birds			
Latency to first step	-0.403	-0.091	
Latency to first vocalisation	0.073	-0.540	
Number of steps	0.488	-0.113	
Number of vocalisations	-0.128	0.586	
Number of defecations	0.445	-0.134	

Table 10. Principal components analyses for four measures of the runway test for white leghorn and brown Hyline hens. n = 50 for both strains.

Coefficients for standardised factor scores	Factor 1
White birds	
Latency to leave the start box	0.283
Latency to reach 0.5 m along runway	0.278
Latency to reach 0.2 m of goal box	0.273
Time spent within0.2 m of goal box	-0.268
Brown birds	
Latency to leave the start box	0.258
Latency to reach 0.5 m along runway	0.271
Latency to reach 0. 2m of goal box	0.275
Time spent within0.2 m of goal box	-0.268
All birds	
Latency to leave the start box	0.270
Latency to reach 0.5 m along runway	0.275
Latency to reach 0.2 m of goal box	0.274
Time spent within0.2 m of goal box	-0.269

Table 11. Spearman correlations between corticosterone concentrations after 10 min in the open field test and behaviour variables measured during the test.

	r	df	P
White			
Latency to first step	-0.007	47	0.960
Number of steps	0.034	47	0.816
Latency to first vocalisation	0.006	47	0.968
Number of vocalisations	-0.057	47	0.699
Number of defecations	0.057	47	0.695
PCA scores component 1	0.103	47	0.482
Brown			
Latency to first step	0.160	47	0.273
Number of steps	-0.168	47	0.249
Latency to first vocalisation	0.244	47	0.091
Number of vocalisations	-0.132	47	0.367
Number of defecations	0.098	47	0.502
PCA scores component 1	0.158	47	0.280
PCA scores component 2	0.236	47	0.102

Table 12. Spearman correlations between corticosterone variables and behaviour variables in white hens. Significant r and P values are indicated in bold. n = 46.

	Corticosterone at 15 min (ng/ml)		Total integrated corticosterone response (ng/ml.min)		Corrected integrated corticosterone response (ng/ml.min)	
	r	P	r	P	r	P
Tonic immobility						
Number of inductions	**0.306**	**0.037**	0.177	0.250	0.214	0.163
Duration	0.060	0.689	0.025	0.874	0.032	0.836
Time to first head movement	0.217	0.148	0.203	0.193	0.258	0.094
Number of head movements	-0.242	0.102	-0.255	0.094	-0.281	0.065
Open field						
Latency to first step	0.175	0.239	0.119	0.443	0.120	0.439
Number of steps	-0.224	0.131	-0.190	0.217	-0.194	0.208
Latency to first vocalisation	0.205	0.167	0.104	0.501	0.118	0.446
Number of vocalisations	**-0.321**	**0.028**	-0.265	0.082	-0.263	0.085
Number of defecations	-0.250	0.091	-0.250	0.091	-0.182	0.239
Runway						
Latency to leave start box	**0.294**	**0.045**	**0.329**	**0.029**	**0.336**	**0.026**
Time to reach 0.5 m from start box	0.250	0.090	0.270	0.070	0.260	0.090
Time to reach 0.2 m from goal box	0.059	0.693	0.128	0.406	0.144	0.352
Time spent within 0.2 m of goal box	-0.170	0.260	-0.220	0.150	-0.240	0.110

Table 13. Spearman correlations between corticosterone variables and behaviour variables in brown hens. Significant r and P values are indicated in bold. n = 49.

	Corticosterone at 15 min (ng/ml)		Total integrated corticosterone response (ng/ml.min)		Corrected integrated corticosterone response (ng/ml.min)	
	r	P	r	P	r	P
Tonic immobility						
Number of inductions	-0.115	0.426	-0.198	0.183	-0.069	0.645
Duration	0.141	0.328	0.068	0.652	0.031	0.837
Time to first head movement	0.224	0.118	0.167	0.261	0.149	0.319
Number of head movements	0.148	0.304	0.124	0.406	0.041	0.787
Open field						
Latency to first step	-0.007	0.961	-0.123	0.415	-0.045	0.766
Number of steps	-0.006	0.968	0.010	0.509	0.069	0.648
Latency to first vocalisation	-0.155	0.288	-0.238	0.112	-0.227	0.129
Number of vocalisations	0.053	0.720	0.152	0.315	0.205	0.172
Number of defecations	0.101	0.489	0.064	0.672	0.042	0.784
Runway						
Latency to leave start box	0.059	0.682	0.053	0.723	0.096	0.522
Time to reach 0.5 m from start box	0.000	0.990	0.030	0.850	0.040	0.780
Time to reach 0.2 m from goal box	-0.077	0.593	-0.069	0.645	-0.031	0.837
Time spent within 0.2 m of goal box	0.070	0.630	0.050	0.720	0.020	0.910

Table 14. Spearman correlations between corticosterone variables and fear score ranks, and between corticosterone variables and PCA behaviour scores in brown hens. Significant r and P values are indicated in bold. n = 49.

	Corticosterone at 15 min (ng/ml)		Total integrated corticosterone response (ng/ml.min)		Corrected integrated corticosterone response (ng/ml.min)	
	r	P	r	P	r	P
Fear score rank						
Tonic immobility	0.219	0.143	0.220	0.156	0.166	0.288
Open field	-0.105	0.473	-0.226	0.132	-0.201	0.181
PCA scores for individual behaviour variables						
Tonic immobility component 1	0.146	0.311	0.116	0.437	0.040	0.789
Tonic immobility component 2	-0.012	0.934	-0.110	0.464	0.004	0.980
Open field component 1	-0.053	0.717	-0.179	0.233	-0.149	0.322
Open field component 2	-0.059	0.687	-0.148	0.327	-0.177	0.239
Runway component 1	0.038	0.794	0.049	0.744	0.098	0.512
PCA scores for tonic immobility and open field combined						
Component 1	0.071	0.629	0.056	0.711	-0.019	0.901
Component 2	-0.069	0.633	-0.123	0.414	-0.144	0.340
Component 3	-0.039	0.789	-0.079	0.600	0.028	0.851
Component 4	0.096	0.512	-0.059	0.697	-0.011	0.942