THE IDENTIFICATION, RATIONALIZATION AND CORRELATION OF PHYSIOLOGICAL AND BEHAVIOURAL RESPONSES TO STRESS

By

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DEDICATION

I wish to dedicate this thesis to my family and my late grandparents Mr. Joseph Dunn and Mrs. Ruth Dunn who were guiding forces in my life.

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SUMMARY

This thesis describes a multidisciplinary approach to the measurement of bird welfare.

In chapter 2 commercial and traditional lines of layer birds were compared to determine time of onset of lay and the occurrence of superficial shell abnormalities. The commercial lines laid earlier, a feature consistent with the demand for high egg production in these strains and these eggs also displayed an increased incidence of surface defects at that time. Following the move to cages, shell quality was similar across all lines.

Experiment 2 permitted an evaluation of specific stress responses, in terms of eggshell quality, plasma corticosterone levels and prior simple tests of avoidance across all breed types. The results were inconsistent suggesting that each measure was assessing a different aspect of the bird's response.

Chapter 3 describes an in-house trial constructed to detect the immediacy or otherwise of the bird's response to stress. The more in-depth investigation applied in this work facilitated comment to be made on the intra-shell location of defects associated with the stress response. In terms of shell quality, the response was not immediate and was more pronounced in birds moved into pens with a variety of enrichments. The complex variables associated with this experiment were too diverse to dissect out and dissociate from each other. In the second experiment more defined acute and chronic stressors were applied and it was clear that the time delay, in terms of bird response, was real but was only elicited in those birds which had experienced acute stress.

Shell quality defects are not the prerogative of the layer bird and in chapter 4 the effect of rearing enrichment on subsequent broiler breeder performance illustrated improvements in both the physical / material and structural quality of the eggshell from mid lay onwards.

Previous chapters have highlighted the fact that the eggshell can be used as a non-invasive indicator of welfare. In Chapter 5 this methodology was applied to a multi-tier system to determine tier effect with respect to product quality under normal commercial conditions. Eggs from the top and bottom tiers demonstrated a variety of shell and internal quality

variables which correlated with specific environmental parameters viz lighting, temperature and ammonia concentration.

Chapter 6 describes the consolidation of the measurements of eggshell quality currently available. Resonance frequency testing is a simple, cost-effective, non-destructive measure of egg quality with the potential to distinguish not only cracked eggs but those predisposed to crack at some point during routine handling.

CHAPTER 1

WELFARE AND STRESS IN THE DOMESTIC FOWL

1.1: WELFARE CONCERNS

1.1.1: GENERAL

Many authors have written extensive and detailed reviews of stress and welfare research (Dawkins, 1980; Broom and Johnson, 1993; Balm, 1999) and so, with reference to the literature review, only research which is directly applicable to the work contained within this thesis will be discussed.

The intensive methods of keeping and farming animals has generated widespread criticism from the general public (Dawkins 1980). Public attention to animal welfare within intensive husbandry systems was encouraged by the publication of Ruth Harrison's book 'Animal Machines' in 1964 (Harrison, 1964; cited by Duncan, 1981). This controversial book increased public awareness to such an extent that the government were forced to form the Brambell committee, a specialist working group, to investigate and report on animal welfare within these systems. The Farm Animal Welfare Council (FAWC) was subsequently established in 1979 to keep under review the welfare of farm animals on agricultural land, at market, in transit and at place of slaughter and to advise the Ministers of any legislative or other changes considered necessary. FAWC expressed concerns over animal welfare and in a press statement on 5th December 1979, it was recommended that a UK Welfare Code should be structured to permit farm animals five basic rights or freedoms (FAWC, 1979 cited by Jones 1996b).

Viz:

1. Freedom from Hunger and Thirst
2. Freedom from Discomfort
3. Freedom from Pain, Injury or Disease
4. Freedom to Express Normal Behaviour
5. Freedom from Fear and Distress

These were described as ideals which anyone with a responsibility for animals should aim to provide. FAWC also suggested that these freedoms would be better provided for if those who have care of livestock practice:

1. caring and responsible planning and management
2. skilled, knowledgeable and conscientious stockmanship
3. appropriate environmental design
4. considerate handling and transport
5. humane slaughter. (FAWC, 2001)

In order to understand the subject of animal welfare there are a few definitions which need to be discussed and defined:

1.1.2: Definition of Welfare

A variety of definitions exist within the literature and to date there is still no agreement on a precise all encompassing description. The Brambell Committee (1965) defined welfare as 'a wide term that embraces both the physical and mental well-being of the animal'. Hughes (1976) augmented this by describing welfare as 'a state of complete mental and physical health, where the animal is in harmony with its environment'. Following these descriptions Duncan and Dawkins (1983) suggested that in order to tell if an animal was suffering one should know if it was experiencing mental trauma and thus was conscious of what was happening. Following this hypothesis, many definitions of welfare have been considered in terms of animal cognition (Duncan and Petherick, 1991; Dawkins, 1988) and Baxter (1989) suggested that physical well-being was only important if the animal has a conscious awareness of it. An alternative adaptatory viewpoint was proposed by Carpenter (1980) (cited by Duncan and Dawkins, 1983) who defined the welfare of managed animals in terms of 'the degree to which they can adapt, without suffering, to the environments designed by man.' Thus as long as a species remains within the limits to which it can adapt, its well-being is assured. More recently Broom (1986) followed this view and expressed welfare as 'the state of an individual as regards its attempts to cope with its environment'. Following these definitions and for the purpose of this thesis it will be assumed that an animal's mental well-being as well as its ability to adapt are the most important factors when considering its welfare.

1.1.3: Definition of Suffering

Dawkins (1980) and Duncan and Dawkins (1983) defined suffering as 'a wide range of intense and unpleasant emotional states' and it is now generally agreed that fear is a state of suffering (Jones, 1996b).

1.1.4: DEFINITION OF STRESS

The term 'stress' has also been defined in many ways and again there is currently no general consensus within the literature. Selye (1973) described stress as ' the non-specific response of the body to any demand made upon it'. Freeman (1985) criticised Selye's definition as it can equally be applied to the effects of pleasant as well as noxious stimuli. More recently Broom and Johnson (1993) classified a stressor as 'an environmental effect on an individual which overtaxes its control system and reduces its fitness or appears likely to do so'. This definition is unusual as it does not involve a conscious thought process and therefore can be equally applied to plants and micro-organisms.

1.1.5: THE STRESS RESPONSE

When an animal faces a stressful situation both neurological and endocrinological pathways participate (Maxwell, 1993):

1. Alarm Reaction (Freeman, 1985): This stage involves pre-ganglionic neurone feedback to the adrenal medullary tissue which results in the release of catecholamines (adrenaline and nor-adrenaline). The sudden concentration change in the peripheral circulation leads to, among other things, an increased release of energy and thus prepares the animal for 'fight or flight' (Cannon, 1929, cited by Dawkins, 1980).

If this does not result in the danger being removed or evaded then the animal moves on to a second stage:

2. The General Adaptation Syndrome (Selye, 1950, 1973): This stage involves a hypothalamic-pituitary-adrenal cortical axis (HPA) reaction which is responsible for adaptation (Freeman, 1985). Although this reaction is not immediate, it does occur within a matter of minutes (Beuving and Vonder, 1978). Adrenocorticotropin (ACTH) is released which stimulates the adrenal gland to produce a number of steroid hormones including corticosterone, which is the predominant active glucocorticoid in birds. This can have effects on the immune system, the circulating white blood cell numbers (Siegel, 1985), and can cause an increase in adrenal weight, cholesterol depletion, increased blood citric acid and result in a reduced growth rate (Beck, 1991).

Selye (1973) goes on to add a third stage in this reaction which will occur if that animal fails to adapt:

3. Exhaustion: At this stage adrenal insufficiency becomes evident and the animal will deteriorate and die.

A few reservations have been raised about Selye's theory. Firstly the biological response to adversity is not as coherent as proposed and so should not be taken as an assumption (Broom and Johnson, 1993). It has also been shown that similar patterns of response can be seen during stressful and non-stressful stimuli (Broom, 1988). More recently detailed analysis of the hormonal response has revealed that the physiological reaction is much more variable than Selye contended.

1.1.6: Concern Relating to Bird Welfare

Concern for the welfare of laying hens began as the traditional, low density, deep-litter systems were replaced by high intensity battery cage and broiler systems (Blockhuis, 1994). By keeping the birds in a controlled environment the risk of disease and parasite infection was reduced, feed and land requirements were lower and the quality of the product more predictable. Later, mechanisation of the routine operation reduced labour costs and further enhanced efficiency and profitability. For the birds however this resulted in increased stocking densities, reduced space allocation, increased production pressures and, in the case of laying hens in cages, lack of access to facilities such as perches, nest-boxes and litter. The increased vocalisation of welfare issues, and in particular, with reference to the battery cages, along with the perceived demand by the public for eggs to be derived from alternative systems, has now led to a proposed ban of conventional cages by the year 2012 (EU Directive 1999/74/EC).

1.2: How can Welfare be Measured?

Baxter (1994) stated that 'the welfare of animals is an intrinsically difficult subject to study scientifically'. The central issue is to determine what the animal itself is experiencing (Duncan and Dawkins, 1983) and this cannot presently (and will probably never) be measured directly (Baxter, 1994; Duncan and Dawkins, 1983). Currently welfare can only be calculated indirectly by measuring changes which occur during the stress response, therefore demands exist for scientific theory to explain and justify the relationship between these indicators and the animals' subjective experiences (Baxter, 1989).

There are several established techniques available to measure the stress response in the domestic fowl. These techniques can be broadly split into two types, invasive and non-invasive. Invasive techniques include sampling blood by means of venipuncture, to allow the measurement of hormone levels and white blood cell counts, tonic immobility tests, by means of bird restraint, and measures of heart rates and blood pressure. These are termed invasive as each involves handling and consequently disturbing the birds in some way in order to obtain the measurement. Non-invasive measures include productivity, mortality levels, behavioural observations, eggshell quality analyses, measures of heart rate by means of radio-telemetry and more recently the measurement of hormone levels present within the urine and faeces. These techniques do not involve any physical contact with the animals and thus should not compromise the results.

1.2.1: INVASIVE MEASURES

1.2.1.1: Direct Physiological Measures of Welfare

Physiological indices of animal welfare include the measurement of cardiac acceleration and body temperature (Jones, 1987b). Traditionally these measurements require bird restraint or the attachment of probes, both of which are likely to be stressful (Duncan and Filshie, 1979). Duncan and Filshie (1979) introduced a technique to allow recording of these measures using radio telemetry methods by attaching remote transmitters to the birds, however this procedure involved inserting the radio-transmitters sub-cutaneously prior to the study. The need for this initial invasive procedure means that this measurement technique can only be considered non-invasive if the experimental design allows for a sufficient recovery period.

1.2.1.2: Hormone Levels within the Blood Plasma

Anticipation that physiological measurements of stress will provide a truly objective measure of welfare has existed since Selye (1932 cited by Terlouw *et al.*, 1997) first described the General Adaptation System.

Catecholamines (adrenaline and nor-adrenaline): These hormones are rapidly secreted in response to a challenge and thus any change is transient (Freeman, 1985). It is also very difficult to obtain adequate volumes of blood and the act of obtaining blood can elicit a further medullary response. For this reason these hormones tend not to be used to measure stress.

Corticosterone: Corticosterone, the primary glucocorticoid in birds, is secreted from cortical cells in the adrenal glands following activation of the HPA reaction. These are secreted in the second phase of the stress response a few minutes after the initial stress stimuli have occurred (Siegel, 1971). This is the most commonly measured hormone as the response time of the cortex is slower than that of the medulla and yet a response can still be recorded within a few minutes (Beuving and Vonder, 1978). This reaction is also longer lasting and thus easier to detect (Freeman, 1985). Physiological stress responses are dependant on the type, intensity and duration of the stressor and the sensitivity of the animal concerned (Terlouw *et al.*, 1997). It is thought that the levels of circulating plasma corticosterone indicate the degree of a reaction to a stressor. Variation in the amount of steroid has been reported to affect an animals' susceptibility to infection (Gross and Colmano, 1970; Siegel, 1971) as well as its ability to adapt the body to stressors by suppressing unnecessary activities and allowing behaviours which will promote survival (Sapolsky, 1987). The cellular action of corticosterone in steroid mediated immuno-suppression was reviewed by Baxter and Harris (1975). This hormone is transported to all body cells by the circulatory system and is specifically bound to protein (Maxwell, 1993). The traditional method for obtaining an accurate indication of circulating corticosteroid levels in birds is by obtaining a blood sample and measuring the amount of the steroid within the blood plasma by means of radio-immunoassay (RIA) techniques. Methods of housing and handling birds can increase the activity of the HPA resulting in elevated levels of corticosterone (Craig and Craig, 1985). Chronic elevation of plasma corticosterone is associated with impaired growth, reduced egg production and immuno-suppression (Gross and Siegel, 1985; Saadoun *et al.*, 1987). Jones *et al.* (1988) suggested that a chronic increase in circulating corticosterone not only altered an animal's cellular response but also predisposed birds to exhibit a more fearful reaction which had serious welfare implications as the animals were more likely to sustain, as well as inflict injury. However the use of corticosterone levels, as an indicator of welfare, has received many criticisms throughout the literature.

There is still a problem in deciding how much of a physiological change an animal can tolerate before it can be classed as suffering (Duncan and Dawkins, 1983). Also changes in biochemistry occur in animals as a daily function viz, during the ovulatory cycle, with time of day, time since stress, time since exercise, time since feeding and during sexual activity. Furthermore these systems have evolved to help the animal adapt and in which case changes may simply be indicating that the animal is coping with the situation rather than suffering in any way (Duncan and Dawkins, 1983). Freeman (1985) suggested that Selye's hypothesis was

too simple for the domestic fowl and quoted many examples of inconsistencies in the literature regarding the physiological stress response and finally stated that not all increases in adrenal activity can be ascribed to stress. Rushen (1986) also concluded that it was very difficult to equate stress with a simple physiological response such as corticosterone release and went on to conclude that these changes may not necessarily indicate suffering. Dawkins (pers. comm.) noted that similar results were also obtained following pleasure and anticipation which further complicates the interpretation of these data. Finally the most common criticisms revolve around the fact that the method itself is invasive and therefore can be considered as a stressor (Duncan, 1981). The use of small indwelling cannulae allowing blood samples to be withdrawn without disturbing the animal (Beuving and Vonder, 1977, 1978), still involves use of an invasive procedure to insert the cannulae prior to the experimentation. For this reason this sampling technique can only be considered non-invasive if the animal is allowed ample time to recover fully following the insertion of the cannulae.

1.2.1.3: White Blood Cell Counts

The relationship between stress in birds and blood leukocyte numbers was first reported by Gross and Siegel (1983) and Davison *et al.* (1983) and has been proposed as a sensitive index of chronic stress in the chicken. A change in the heterophil / lymphocyte ratio has been reported due to heat stress (Chancellor and Glick, 1960), social disruptions (Anthony *et al.*, 1988), restriction of feed (Maxwell *et al.*, 1990), increasing circulating corticosterone (Gross *et al.*, 1980) and a change in the proportion of basophils has been demonstrated following an induced moult (Brake *et al.,* 1982) and during heat stress (Maxwell *et al.*, 1992; Maxwell and Robertson, 1995).

As with plasma corticosterone levels this technique involves blood sampling via venipuncture, which can itself initiate a medullary response in the bird (Freeman, 1985). These changes also take slightly longer to occur, in relation to any stressor, as white blood cell numbers are modified in response to corticosterone action on the lymphatic tissue (Dougherty, 1952, cited by Siegel, 1995) making the results slightly more difficult to interpret.

1.2.1.4: Tonic Immobility

Tonic immobility, although a behavioural test of fear, is an invasive measure as it involves restraint of the bird. This test involves putting the birds into a fear-potentiated catatonic-like state of reduced responsiveness to external stimulation and is induced by physical restraint. It

is considered a useful behavioural index of fear with the more fearful birds remaining in a state of immobility for longer periods (Gallup, 1979; Jones, 1986).

1.2.2: NON-INVASIVE MEASURES

1.2.2.1: Productivity

A common argument is that the high productivity of managed farm animals is evidence that they cannot be suffering. Productivity analysis can be useful in small-scale housing systems or studies where the all the animals within the house will be affected in a similar way. Duncan and Dawkins (1983) cautioned that there were many pitfalls in using productivity as an indicator of welfare, most notably the fact that the productivity of a unit was not the same as the productivity of an individual and farmers will only ever be able to view the productivity of the chicken shed as a whole (Dawkins, pers. comm.). In this way a problem, for example with the ventilation system in one specific area which affects only a few individual animals, will not be detected as it will result in a very small change in the productivity of the complete shed.

1.2.2.2: Mortality Levels

This is very easy to measure within the commercial system and is obviously an indicator of poor welfare. Dawkins (pers. comm.) suggested that although this is a good indicator it should not be used in isolation.

1.2.2.3: Injury, Disease and Deformities

Duncan and Dawkins (1983) stated that any factor that reduced health would also reduce welfare. However these authors caution that although ill health denotes suffering, it's absence should not be taken as proof of well-being. Baxter (1989) also stated that suffering did not vary purely in proportion to the severity of an injury and thus assuming that all animals with clinical signs of disease or injury were suffering does not provide a sensitive measure of welfare. For example any type of mental stress or boredom will not be detected by this measurement type but may represent an equally important welfare problem.

1.2.2.4: Behavioural Analyses

Dawkins (1989) suggested that in order to ask questions about whether an animal is suffering as a result of being deprived of certain behaviours, a baseline was required for comparison. This author suggested using the Red Jungle Fowl to set behavioural baselines as domestic chickens are descended from this species and the behaviour of this species has been

extensively studied and described (Johnson, 1963; Kruijt, 1964; Collias and Collias, 1967; cited by Dawkins, 1989). In order to measure behaviour in an animal, the behavioural patterns must be broken down into units which can be quantified and examined in combination with other welfare indices to provide a clearer idea of welfare (Mills and Wood-Gush, 1985). Different types of behavioural analysis have been defined, each with its own advantages and disadvantages, and are described as follows:

Abnormal Behaviour: If an animal performs a particular abnormal behaviour when suffering then its welfare can be assessed by simple observations (Duncan and Dawkins, 1983). These authors defined abnormal behaviour as 'a persistent undesirable action, shown by a minority of the population which is not due to any obvious neurological lesion and which is not confined to the situation which originally elicited it' (Broadhurst, 1960, Fox, 1968). Others have stated that, in addition, the abnormal behaviour should also be 'damaging and maladaptive to the animal' (Fraser, 1968). Duncan and Dawkins, (1983) cautioned that it was difficult to know how much abnormality must be shown to indicate suffering and Baxter (1989) reported that it was difficult to judge which behaviours are abnormal as the circumstance under which these behaviours are displayed must also be taken into account. Some authors have also stated that genetic selection for certain characteristics has led to a greater deviation from normal behaviour (Kretchmer and Fox, 1965).

Comparison of Behaviour: Many authors have suggested comparing the behaviour of animals in two systems, one of which is more artificial and intensive than the other (Hughes, 1976; Duncan and Dawkins, 1983). Duncan (1981) described the difficulty in deciding which behaviours were normal or ideal. This technique also has limitations in that chickens behave very differently in different environments and thus behaviour differences may simply prove how adaptable these animals are (Duncan, 1981; Duncan and Dawkins, 1983).

Behavioural Preference: The simplest form of indirect measurement of welfare is to give an animal a choice between two environments. Techniques have been developed for running these types of tests (Dawkins, 1976, 1977; Hughes and Black, 1973). However, in practice, these tests frequently do not look for a simple preference and Baxter (1989) used the example of an animal 'choosing' to use a pen with bedding instead of a bare pen. The pen with the bedding will be warmer, more interesting, more comfortable and allow nesting and dust-bathing. The bare pen however provides a less dusty environment and is abrasive enough to prevent excessive claw growth. The different advantages of each environment in this

preference test will only be obvious to the hen after a certain period of time in the test pens (Baxter, 1989) and it is true that animals do not always choose what is best for their physical health (Duncan and Dawkins, 1983). Dawkins (1977) also proved that the choice made by each individual was strongly influenced by prior experience. There can also be problems interpreting these results as a choice only tells us something about the relative properties of two environments and not their absolute properties (Duncan, 1978b).

Operant Techniques: This is an advancement of the 'behavioural preference test' and makes use of the fact that animals will learn to perform a response to gain a reward or avoid a punishment. This technique can therefore be used to find out how much effort or 'work' an animal will put into avoiding something or gaining something and thus estimate how adverse or rewarding they find the object (Dawkins, 1980, Duncan, 1981). Again there are interpretation problems in that an animal may choose to 'work' for something which is not necessarily good for its physical health.

Fear Tests: As fear is described as a state of suffering (Jones, 1996b) many authors have used simple behavioural tests to estimate the fearfulness of birds often in relation to strain type (Hughes and Black, 1974a; Barnett *et al.*, 1992; Keer-Keer *et al.*, 1996). These tests include approach of a looming human and reaction to a novel object and can be conducted without removing the bird from the home cage. It is presumed that tests such as these will elicit an increased behavioural response in the birds which are most fearful.

Behavioural Indexing following a Stress: A final technique is to stress an animal by subjecting them to noxious stimuli or frustration and draw up a catalogue of the behaviour patterns displayed. (Duncan and Wood-Gush, 1971, 1972; Jones *et al.*, 1981). Duncan (1981) and Duncan and Dawkins (1983) described the process of frustrating a hen and observing the behavioural responses shown and then using these results to decide whether birds in a commercial situation were eliciting similar behavioural patterns (Duncan and Dawkins, 1983).

Most of the above methods of examining behaviour have received some criticism. Barnett and Hemsworth (1990) claimed that behavioural indicators were equally as problematic as physiological measures and results should be treated with some caution. For example it has been reported that battery cages reduce the incidence of wing-flapping (Hughes, 1973) and although Duncan (1981) demonstrated that it was true that a commercial battery cage was not

large enough to allow a full behavioural pattern to take place, it could also be said that birds in battery cages were not motivated to wing-flap.

1.2.2.5: Faecal Corticosterone Levels

Recent work carried out by Wasser *et al.* (1997) suggests that faecal levels of corticosterone can be used to indicate an animal's response to unpredictable events. Sampling blood requires restraining the animal whereas urine and faeces can often be collected without disrupting the animal's activities or intruding on behavioural or endocrinological states (Whitten *et al.*, 1998). These authors also stated that non-invasive methods allow a continuous monitoring that can be used to assess differences in day to day changes in cumulative stress. Steroid levels can be measured by means of either a radio-immunoassay (RIA) or an enzyme-linked immunosorbant assay (ELISA). It has been suggested that excreted corticosterone may provide a better picture of overall stress than plasma levels, as the excreted levels are cumulative over a number of hours. In parallel to this thesis, Lord (2001) has successfully applied this technique to studies on domestic fowl.

There are however some disadvantages with this method in that plasma corticosterone levels reflect endocrine responses at the moment of sampling whereas faecal and urine collections reflect events occurring hours or days before (Whitten *et al.*, 1998). Knowing these lag times is very important if attempting to test the relation to particular events and this prolonged time lag along with the re-circulation of steroids from the gut may make faecal steroids less-sensitive indices of the stress following a single event (Whitten *et al.*, 1998).

1.2.2.6: Egg and Eggshell Quality (ESQ)

Observations by Hughes *et al.* (1986) suggest that disturbances to the laying hen are associated with changes in eggshell quality. Disruption to a hen's environment can induce the formation of abnormal eggshells and birds subjected to a handling stress produce a higher proportion of body-checked eggs (Hughes and Black, 1976). The work of Watt (1989) drew attention to the changes which occur in the oviduct in response to environmental stress. This laboratory based investigation demonstrated the ability of the oviduct to undergo both breakdown and recovery within a 30 day period and highlighted the fact that one of the birds' natural responses to stress is the retention of the egg beyond the anticipated time of oviposition. On farm, calcium splashed eggs account for a considerable financial loss. Solomon *et al.* (1987) noted structural disorganisation at the level of the mammillary layer following adrenaline injection and Watt (1989) noted that this disruption continued for many

days after the superficial quality of the egg had returned to normal. More recently Walker and Hughes (1998) showed that caged hens lacking access to an enclosed nest-site laid an increased number of calcium dusted eggs suggestive of increased stress in these birds. Many authors have suggested that eggshell quality parameters may provide a quantifiable non-invasive indicator of stress in laying hens (Hughes *et al.*, 1986; Mills *et al.*, 1987, 1991). Evidence linking this measurement to other techniques for evaluating bird welfare is however lacking and so work is required to determine the relationship between eggshell quality measures and other indicators of stress.

1.2.2.7: Multidisciplinary Approach to Welfare

Duncan (1978a, 1981) and Duncan and Dawkins (1983) recommended that in order to make the best estimate of welfare or suffering, it is essential to look at all available evidence. These authors suggested that the problem with using indirect indicators was that of calibration and deciding how much of a change indicated suffering. Baxter (1989) stated that the best way to have confidence that welfare is being effectively measured was to combine different types of indicator and Broom and Johnson (1993) also suggested using several measures of welfare if possible. Finally Rushen (1986) recommended attempting to quantify animal welfare in order to provide 'sober professional judgments based on scientific data as a counter to emotionally based anthropomorphic judgments'.

1.3: THE FORMATION OF THE EGG

Before fully understanding the effects of stress on eggshell quality the dynamic processes involved in egg formation must be appreciated.

The cleidoic (self-contained) egg provides the growing embryo with nutrition and protection, both from mechanical damage and microbial infection. The commercial breeds of chicken found in industry today lay on almost a daily basis. This level of production is a far cry from the egg production of the Jungle fowl (*Gallus ferrugineus*), the ancestral breed of fowl from which modern chicken breeds are derived, which tends to lay between 22 to 26 eggs per year (Buckner *et al.*, 1922 cited by Watt, 1989).

1.3.1: THE FEMALE REPRODUCTIVE SYSTEM

In female domestic fowl only the left ovary and oviduct become functional, the right ovary regresses prior to hatching and exists only as a rudimentary organ throughout life.

Ovary (OV): In the mature hen the ovary develops a hierarchy of follicles, many of which will never be ovulated but will simply regress and be reabsorbed (Johnson, 2000; Plate 1.1).

Oviduct: This is split into six functionally distinct regions (Plate 1.1), viz the infundibulum (IN), magnum (MA), isthmus (IS), tubular shell gland (TSG), shell gland pouch (SGP) and vagina (VA) (Simkiss, 1968; Solomon, 1991).

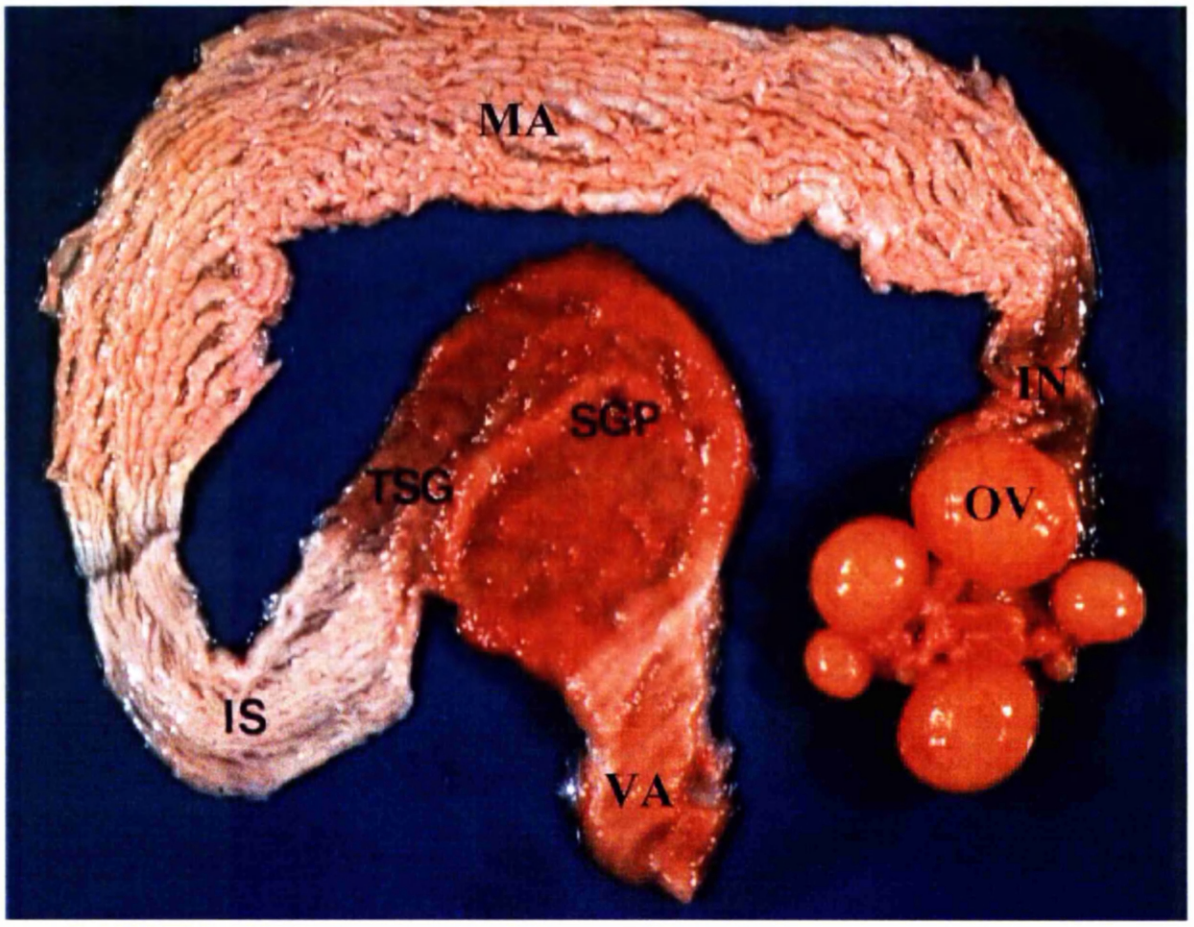


Plate 1.1: The reproductive system of the domestic fowl: *OV – ovary; IN – infundibulum; MA – magnum; IS – Isthmus; TSG – tubular shell gland; SGP – shell gland pouch; VA – vagina.*

Following ovulation the ovum enters the infundibulum which is the area of production of the perivitelline membrane and chalazae (Solomon, 1991). This region is also the site of fertilization of the ovum and it is here that the first layers of albumen are secreted (Johnson, 2000). The next region is the magnum which is the longest section of the oviduct and secretes most of the albumen proteins. Albumen serves a nutritive function for the growing embryo, acts to block invasion of microorganisms and provides a buffer for the yolk against mechanical damage (Solomon, 1991). Following this the forming egg enters the isthmus region and the inner and outer shell membranes are secreted over the albumen layers (Tullett, 1987). The egg subsequently passes quickly through the tubular shell gland region where the initial seeds of calcification are formed around chemically distinct regions on the shell membranes called 'organic or mammillary cores' (Cooke and Balch, 1970). The forming egg then enters the shell gland pouch which performs several functions including the addition of 'plumping fluid' (Roberts and Brackpool, 1994; Rose, 1997) to distend the egg and so expose the mammillary cores (Solomon, 1991), formation of the organic shell matrix, calcification of the shell via deposition of calcium salts onto the mammillary cores, secretion of the pigment porphyrin and finally, immediately preceding oviposition, the secretion of a surface cuticular layer. The egg then exits via the vagina. The complete process from ovulation to oviposition takes approximately 24 hours.

1.3.2: THE EGGSHELL

The morphology of the shell has been well defined (Bain, 1990; Solomon, 1991; Brackpool, 1995) and the regions responsible for the production of individual constituents identified at both light and ultrastructural level (Solomon, 1973a, 1973b, 1975, 1991). Histo-chemical analysis of the oviduct has revealed the nature and chemical composition of the functional units within the egg and evidence has accumulated to prove that the oviduct can no longer be considered as a systematic production-line in which specific regions contribute only to one process.

The eggshell consists of both organic and inorganic fractions.

1.3.2.1: Organic Fraction

Surrounding the albumen there are two shell membranes which are closely adhered except at the air space (Petersen, 1965). These membranes consist of cross-linked protein fibres and (Johnson, 2000) and are semi-permeable to allow the passage of both gas and water but not albumen. Nucleation sites, termed mammillary cores, exist as projections on the outer shell

membrane surface and provide the initial sites for mineralisation (Solomon, 1991). Changes in shell quality can be related to changes in these membranes. The organic shell matrix is composed of proteins, glycoproteins, and proteoglycans (Tullett, 1987; Arias *et al.*, 1993 cited by Gautron *et al.*, 2001). The matrix is believed to modify the calcification process and influence mechanical properties of the egg (Nys *et al.*, 1999 cited by Gautron *et al.*, 2001). Immediately prior to oviposition an uneven covering of thin waxy cuticle, consisting of protein, polysaccharide and lipid, is normally secreted on the outer surface of the eggshell and functions to protect the egg from water loss and microbial infection.

1.3.2.2: Inorganic (Calcified) Fraction

The calcified shell consists of calcium carbonate in its calcitic form and enables the egg to withstand considerable biological and mechanical abuse while still maintaining its structural integrity (Parsons, 1982) and has been described in detail by many authors (Watt, 1989; Solomon, 1991; Darnell-Middleton, 1999). Beginning on the inner surface of the shell, outward crystallisation from the mammillary cores results in the formation of the mammillary layer which exists in close association with the outer shell membrane. The palisade (spongy) layer begins above the mammillary layer and ends at the vertical crystal layer (Parsons, 1982) and represents most of the thickness of the shell (Plate 1.2). The final calcified layer is the vertical crystal layer, which lies above the palisade layer and below the cuticle (Simons, 1971) and consists of short narrow crystals, of varying thickness, which are aligned perpendicular to the surface.

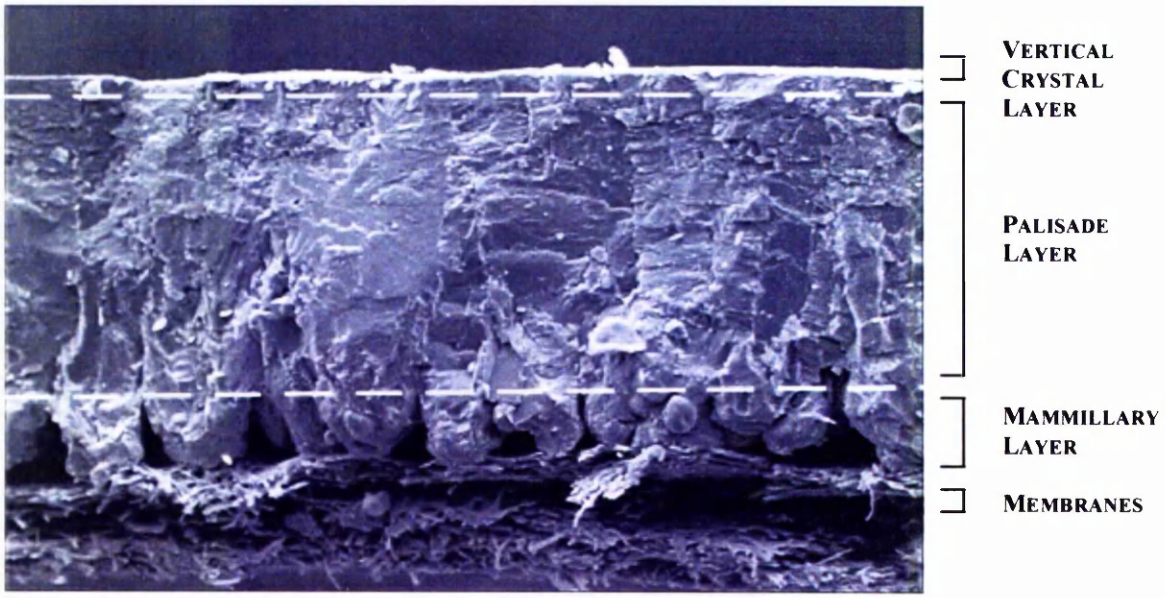


Plate 1.2: SEM of transverse section through the eggshell showing the various layers. The cuticle (outermost layer) is absent in this section.

1.4: EGG AND EGGSHELL QUALITY

Eggs laid by commercial strains today are different in composition to those laid by unselected strains in the past (Akbar *et al.*, 1983) and quality is normally considered in the context of consumer requirements (Overfield, 1995). Many techniques have been developed to measure the quality of various components of the egg although most of these have concentrated on measuring the quality of the shell. Belyavin (1986), Hunton (1987) and Overfield (1995) all provide comprehensive reviews of the available methods for evaluating egg quality.

1.4.1: SUPERFICIAL EGGSHELL QUALITY

During egg packing cracked, misshapen and abnormal shelled eggs are removed for further processing. Thus the consumer rarely sees the diversity of form that an egg can exhibit at oviposition. Plates 1.3, 1.4 and 1.5 illustrate this diversity which has been documented in layer and to a lesser extent breeder eggs. A description of these abnormalities and underlying factors involved in their generation are discussed in detail throughout the literature (Hughes and Black, 1976; Hughes and Gilbert, 1984; Hughes *et al.*, 1986; Solomon, 1991). In breeder eggs these abnormalities can result in the prevention of embryonic growth as eggshell porosity is often reduced (van Middelkoop, 1972). The resulting poor eggshell quality also has a direct effect on the ability of bacteria to penetrate the eggshell and this can have serious consequences on hatchability and the keeping quality of eggs (Nascimento, 1992).

1.4.2: INTERNAL QUALITY

The internal quality of the egg cannot be judged by its external appearance. Thus eggs selected on the basis of price, colour and size must await breakout before judgment can be given on yolk colour and albumen quality. With reference to the former, consumer preference varies and to accommodate these preferences dietary manipulation in terms of pigment concentration can be effected. The consistency and height of the albumen not only reflects bird age (Noles and Tindell, 1967; Roberts *et al.*, 1997), but also provides information as to storage conditions and length of time in storage (Jay *et al.*, 1992). The inclusion of blood and meat spots within either of these aspects of the egg contents also reflects the structural and functional status of the oviduct (Solomon, 2000).

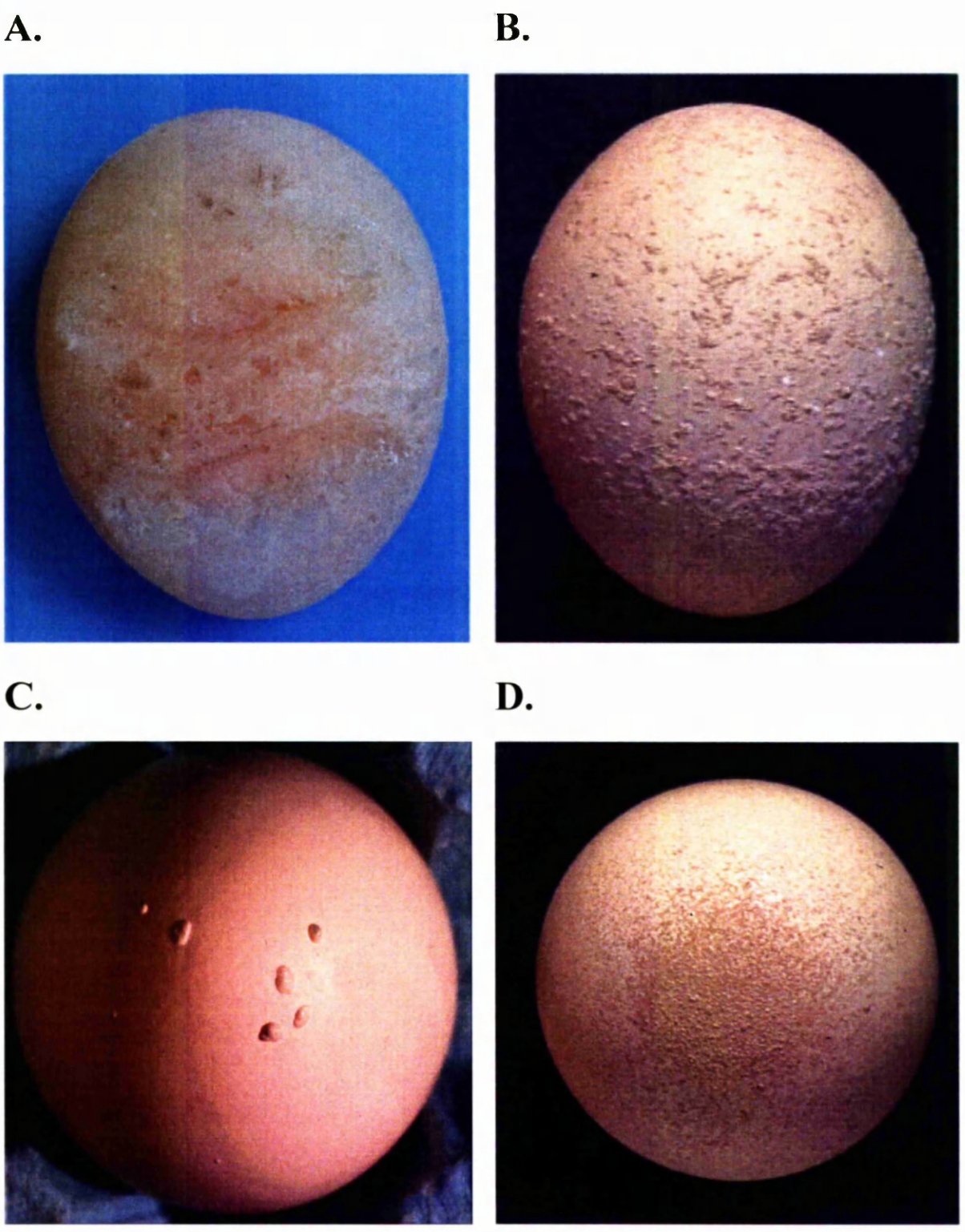


Plate 1.3: Diversity of superficial abnormalities: A – Soft Shelled Egg; B – Light / widespread surface accretions; C – Heavy / localised surface accretions (pimpled); D – Sugared pole.

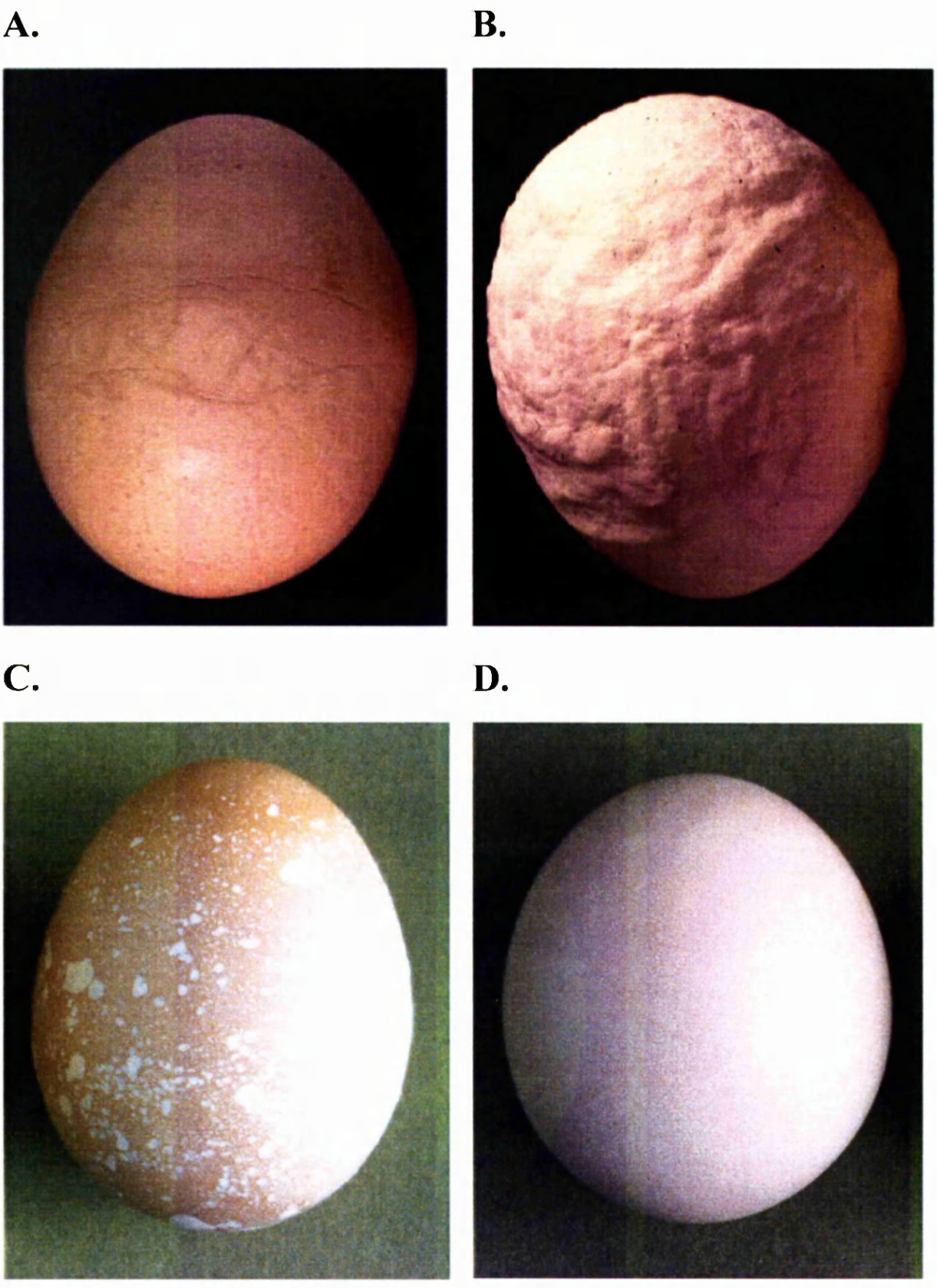


Plate 1.4: Diversity of superficial abnormalities: A – Equatorial bulge (body checked); B – Wrinkled shell; C – Calcium splash; D – Calcium dusting.

A. **B.**

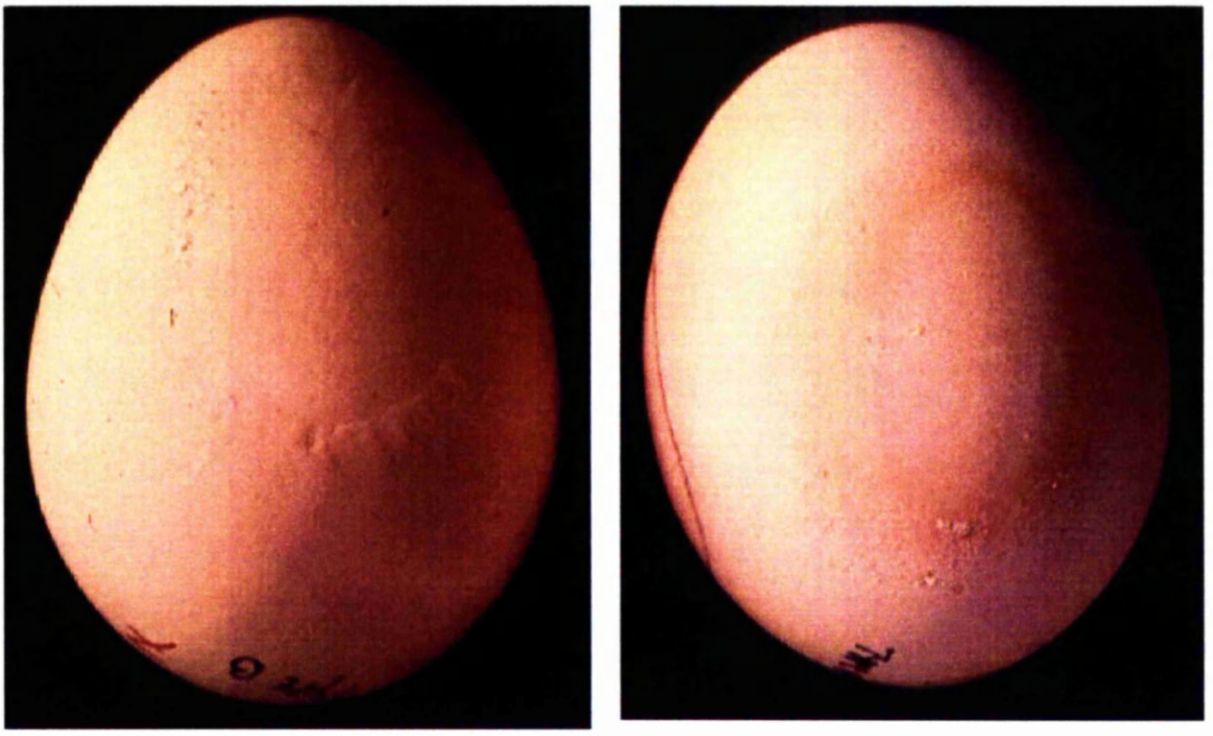


Plate 1.5: Diversity of superficial abnormalities: A – Slab sided; B – Target egg.

1.4.3: PHYSICAL AND MATERIAL PROPERTIES OF THE EGGSHELL

Cracks account for the largest percentage of downgrades (Bain, 1990). These losses are carried mainly by the farmer since they occur during the laying (Carter, 1971; Anderson and Carter, 1972), collection (Eggleton and Ross, 1971), and transportation process (Anderson *et al.*, 1969). As the eggshell is a complex structure, the measurement of its strength has been estimated using a wide range of techniques, which are reviewed, by Voisey and Hunt (1974) and Hunton (1995). Shell thickness has been found to be significantly correlated with strength and varies throughout the shell (Tyler, 1961) tending to be thinnest and most uniform at the equator (Tyler and Geake, 1965). Until relatively recently only total shell thickness has been measured, however Bain (1990) suggested that the most important measurement pertaining to shell strength was the effective shell thickness (T. Effective). This is the distance between the point of fusion of the mammillary cones and the cuticular surface of the shell and can only be measured by scanning electron microscopy. The shape of an egg also has a role to play in its strength with more elongate eggs showing a higher propensity to crack formation. Having determined an egg's breaking strength, shell thickness and shape, its elastic modulus and fracture toughness can be calculated by use of formulae developed by Bain (1990). The latter indices provide a measure of the material properties of the shell.

1.4.4: ULTRASTRUCTURAL ASSESSMENT OF THE MAMMILLARY LAYER

Solomon (1991) stated that 'the perfect eggshell does not exist' and suggested that the dynamic process of calcification witnesses the formation of many aberrant crystal forms. Within the mammillary layer of the hen's eggshell 12 ultrastructural variations have been documented and are described in detail by others (Reid, 1983; Watt, 1985, 1989; Bain 1990; Solomon 1991). Many authors have reported increases in these ultrastructural defects with increasing bird age (Bain, 1990; Nascimento, 1992; Brackpool, 1995), following environmental stress (Watt, 1989) and following adrenaline injection (Solomon *et al.*, 1987).

1.4.5: CUTICLE COVERING

The cuticle can be accurately examined under the scanning electron microscope and quantified by means of a scoring system devised by Cranstoun (1992). More than 98% of eggshells have abnormal (patchy or absent) cuticle covering (Nascimento, 1992) and Alls *et al.* (1964) showed that cuticle removal increased bacterial contamination from 20 to 60%.

1.4.6: FACTORS KNOWN TO INFLUENCE EGGSHELL QUALITY

Various factors are known to affect eggshell quality. These include age, strain, housing type, nutrition, ambient temperature, noise / disturbance and disease.

Many facets of eggshell quality deteriorate with increasing bird age including shell thickness, breaking strength and ultrastructure (Solomon *et al.*, 1987; Watt, 1989; Bain, 1990; Belyavin *et al.*, 1991). The strain of bird (genetics) is also significantly related to eggshell quality (Arad and Marder, 1982; Buss, 1982) with some strains being recognised to have a superior quality of shell (Roberts *et al.*, 1997). Hughes *et al.* (1985) also demonstrated that eggs laid in free-range systems are thicker than those laid in cages and that the incidence of cracks is greater in eggs laid in cages.

In addition dietary regimes affect different aspects of the egg for example Vitamin D_3 is thought to influence the efficiency of calcium uptake and a phosphorus deficiency has been suggested to result in reduced egg production (Hurwitz, 1987). High environmental temperature causes a reduction in shell thickness and egg weight (Wolford and Tanaka, 1970; Nordstrom, 1973; Brackpool, 1995), a decline in shell strength (Zimmerman *et al.*, 1972) and an increase in percentage of cracked eggs (Wolford and Tanaka, 1970). Brackpool (1995) also found a reduction in shell thickness following the exposure of birds to continuous lighting and noise, which was attributed to a reduction in the amount of time the egg spends in the SGP. Many poultry diseases including Infectious Bronchitis and Newcastles disease also adversely affect egg production, shell quality and shell appearance (Spackman, 1987; Tullett, 1987). Finally attention has already been drawn to the work of Watt (1989) who established that experimentally increasing the number of birds within a cage resulted in an inferior quality of egg. It is therefore clear that the eggshell provides a very sensitive measure of a variety of factors, many of which have obvious implications in terms of bird welfare.

1.5: METHODS OF IMPROVING WELFARE

Given all the available techniques and with knowledge of many of the factors which adversely affect bird well-being, it is not surprising that many methods for improving bird welfare have been attempted with varying degrees of success.

1.5.1: ENVIRONMENTAL ENRICHMENT

Jones (1996b) suggested that the barren nature of many poultry houses was likely to encourage boredom which could lead to other problems and suggested that increasing stimuli may reduce this boredom. Some authors however have cautioned that novelty is a potent fear elicitor (Jones, 1987c; Boissey, 1995) but it has been noted that animals will seek out novel stimuli even if they are frightened of them (Chamove, 1989). Many benefits for environmental enrichment have been documented, including improved growth and feed conversion (Jones, 1985b; Gvaryahu *et al.*, 1989; Nicol, 1992) and reduced aggressiveness and mortality (Gvaryahu *et al.*, 1994).

Hens appear to be very highly motivated to perch (Baxter, 1994) and several advantages of perches have been documented including refuge for subordinate hens (Mankovich and Banks, 1982) and a route for avoiding aggression. Robertson, *et al.* (1989) reported that both plumage and foot condition were improved in modified cages with perches. Perches have however been criticised for causing certain welfare problems including keel deformation and bumblefoot, but it has since been shown that these deformities are usually due to the perch being the wrong shape or in the wrong position within a cage (Duncan *et al.*, 1992; Engstrom and Schaller, 1993). Duncan *et al.* (1992) related the increase in incidence of cracked eggs to perch provision which although not a welfare concern, is a concern for the farmer and to date has prevented the successful introduction of perches to all cage systems.

The introduction of pecking devices as an enrichment has been suggested to reduce feather pecking and mortality. Gvaryahu *et al.* (1994) reported reduced aggressive head-pecking and mortality levels following the introduction of coloured pecking devices within layer cages while Jones *et al.* (2000) demonstrated that birds had a clear preference for certain pecking devices over others.

Nesting and dust-bathing motivation in the hen has also been shown to be very strong (Baxter, 1994) with birds willing to use trap-nests even when, as a consequence, it results in them being deprived of food and water for 6 hours (Duncan, 1978b). Sherwin and Nicol (1992) concluded that laying hens suffer when deprived of suitable nest sites. Van Liere *et al.* (1989) demonstrated that hens housed in deep litter systems will carry out incomplete dust-bathing behaviour if litter is of poor quality and thus have suggested that incomplete dust-bathing is a conflict behaviour.

1.5.2: Rearing Enrichment

There is increasing evidence that the environment in which birds are reared will play a role in their response and adaptation at a later age (Reed *et al.*, 1993). Trials by Schaller and Emlen (1962) and Broom (1969) suggested that the response demonstrated by birds, as a reaction to an environmental change, were reduced if the complexity of the rearing conditions was greater.

1.5.3: Genetic Manipulation

Mench (1992) proposed that genetic selection could be a powerful tool for decreasing the incidence of behaviours associated with poor welfare. Modern laying hybrids have been selected for increased feed conversion efficiency and increased egg production. Domestication has also produced animals which are more placid and less aggressive (Craig, 1981)

1.5.4: Stocking Density

A reduction in stocking density has been reported to reduce the incidence of lameness and tonic immobility in broilers (Sanotra *et al.*, 2001). Mortality levels have been found to be lower (Hall, 2001) and behavioural repertoires increased in smaller group sizes (Carmichael *et al.*, 1999). However Nicol *et al.* (1999) demonstrated an increase in aggressive pecking in birds kept at a lower stocking density, and commented that this was due to the establishment of social hierarchies.

1.5.5: Disease Control

Improved vaccination, housing and management procedures have led to a reduction in many disease outbreaks and hence improved welfare. However, some of the vaccination procedures, although reducing mortality, have been shown to have adverse effects on eggshell quality (Spackman, 1987).

1.6: Aims of Thesis

This thesis adopts a multidisciplinary approach to the subject of welfare and product quality. The textural condition of the eggshell has been used as a non-invasive measure of welfare under controlled experimental conditions and the occurrence of superficial abnormalities correlated with egg retention. The work on the response, in terms of superficial shell quality,

has been repeated and the results confirmed by many authors but as the shell is a multi-layered structure, each layer of which contributes to shell performance, it is of fundamental importance to assess whether stress can influence individual shell layers.

The first aim of this thesis is to determine, in the absence of any knowledge of the position of the egg in the oviduct, whether eggshell abnormalities will occur regardless or, as evidenced by others, will be confined only to those situations where a hard-shelled egg is in the shell gland pouch when the bird is stressed.

The thesis also sets out to determine the immediacy of the spatial and temporal response to stress in terms of eggshell quality i.e. are defects observed within the deeper layers of the shell or confined to the shell surface and do they manifest themselves immediately or is there a delay in their occurrence.

The experimental work described in chapter 4 applies ultrastructural analysis in conjunction with a series of specialised and non-specialised measures of shell quality to determine whether environmental enrichment during the rearing phase has any effect on subsequent reproductive performance of broiler breeders.

Similar technology is used in chapter 5 to provide profiles of shell quality throughout the laying period in layer birds housed in a multi-tier system. The object of this exercise is to assess whether in making use of the full vertical height within the poultry shed, environmental changes, if any, have an influence on shell quality and internal egg quality.

Throughout this work parallel investigations are carried out on a variety of aspects of bird welfare including behaviour and corticosterone levels, in both blood and faeces, in order to correlate these results with the quantifiable measures of shell quality.

In the final chapter the application of non-destructive resonance frequency testing is used to rationalize the varied measures of eggshell quality previously applied and to determine the relationships between this measurement and the other parameters described in earlier chapters.

CHAPTER 2

THE ASSESSMENT OF STRESS IN A VARIETY OF BREED TYPES

This chapter is divided into two experimental sections.

2.1: EXPERIMENT 1

Modern laying hybrids have been selected for increased feed conversion efficiency and increased egg production (Schutz *et al.*, 2001). These authors suggested that genetic selection for production traits has resulted in a bird which displays less active responses to fearful situations and it has been shown that certain unselected breeds are more social and more active than these hybrids. Jones (1996a) reported that there is mounting evidence of negative relationships between underlying fearfulness and many other measures of reproductive performance and growth.

Inhibitory effects such as prolonged tonic immobility (TI), avoidance of a novel object, depressed vocalisation and reduced exploration have all been associated with the fear response (Jones, 1985b). TI is considered a particularly useful and reliable index of fearfulness in the domestic fowl (Gallup, 1979; Jones, 1986, 1987b). Jones and Hughes (1986) and Mills *et al.* (1991) found that birds with a history of laying eggs with abnormal shells were also more fearful than 'normal' layers.

It has been suggested that stress and the release of adrenaline can depress oviduct motility and cause egg retention (Jones and Hughes, 1986). Both environmental stress (Hughes *et al.*, 1986) and administration of adrenaline (Hughes and Gilbert, 1984; Watt, 1989) can result in surface defects on many of the eggs laid subsequently. Ouart and Adams (1982) found a correlation between nervousness and the number of cracked and body checked eggs produced. Hughes *et al.* (1986) showed that even slight disturbances to the hens, which did not depress production levels, were enough to have a quantifiable effect on external eggshell quality and so suggested that this type of analysis could provide a non-invasive method of assessing stress in laying flocks.

Although the use of non-invasive indicators of stress and fear appear to be the ideal solution for welfare studies it is important to compare the results from these measures with those obtained from traditional invasive methods in order to validate the results generated.

2.2: AIMS

Given that stress can cause shell surface defects (Hughes *et al.*, 1986) this experiment was designed to assess whether the onset of lay and the experience of relocation was comparable or whether bird breed type influenced the formation of specific shell patterns. Thus commercial lines, selected for high egg production, were compared with traditional lines at two periods of lay. The investigations reported in this chapter formed part of a larger behavioural study, which afforded the opportunity to examine correlations between shell quality and several tests of fear and restraint.

2.3: MATERIALS AND METHODS

2.3.1: ANIMALS USED

Day old chicks from 25 lines of domestic fowl were obtained from 5 sources (Table 2.1). The chicks were from 12 commercial pure lines and 13 traditional lines. The birds were sexed by DNA analysis and the females from each line were divided equally between two pens at 5 weeks of age.

Breed	Type[1]	Source	Number
Auracana	T	1	11
Brown Leghorn	T	1	16
Barnvelder	T	1	7
Buff Orpington	T	1	9
Corn Game	T	2	9
Friesien Fowl	T	1	17
Ixworth	T	2	11
Jersey Giant	T	1	8
J-Line	T	3	30
Light Sussex	T	2	11
Maran	T	1	19
White Dorking	T	2	11
White Sussex	T	1	6
Commercial 1	C	4	18
Commercial 2	C	4	27
Commercial 3	C	4	28
Commercial 4	C	4	31
Commercial 5	C	4	16
Commercial 6	C	4	29
Commercial 7	C	5	31
Commercial 8	C	5	32
Commercial 9	C	5	31
Commercial 10	C	5	32
Commercial 11	C	5	26
Commercial 12	C	5	32

Table 2.1: Breeds, breed type, source, number of birds at the start of the experiment: *[1]Traditional (T) or Commercial (C).*

2.3.2: HOUSING

From 5 weeks of age until 30 weeks, classified as Phase 1, all birds were housed in 48 litter pens measuring 2.4 x 1.5 metres (depth x width) within a poultry shed. The pens were allocated according to a blocking design to control for possible environmental effects. The birds from each strain were housed in two groups in each of two blocks in a randomised block design, with the exception of the White Sussex and the Barnvelder which only had sufficient numbers to fill one pen each. The number of birds in each pen varied from 4 to 12 for the traditional lines and 8 to 16 for the commercial lines.

At the onset of lay the first 10 eggs were collected from each pen as described in section 2.3.5.

2.3.3: OBSERVATIONS AND ANALYSES DURING PHASE 1

Many tests were carried out on these birds throughout Phase1 and these are described in detail in Hocking *et al.* (In Press). These are summarised below but did not form the main part of this thesis.

2.3.3.1: Fear Tests

Novel Object: At 7 weeks of age birds in each pen were tested for their approach to and avoidance of a novel object (traffic cone). Immediately after the addition of the novel object the bird's responses were scored every 10 seconds for a total of 3 minutes. The scores ranged from 1 (birds in closest proximity) to 4 (birds more than 1.5 metres from object. A mean proximity score was calculated for each pen.

Tonic Immobility (TI): The birds were tested for TI at both 13 and 25 weeks of age. TI was induced by restraining the birds on their backs in a wooden cradle. Time taken for the bird to right itself was recorded.

2.3.3.2: Response to a mechanical restraint

Crush Cage (CC): The bird was placed in a metal and wire crush cage and secured for 5 minutes. The number of vocalisations and struggling bouts were recorded for each bird. The test was repeated at 14 and 28 weeks of age.

Plasma corticosterone levels: Immediately after completion of the test at 28 weeks of age a blood sample was obtained and plasma corticosterone levels were determined using a radioimmunoassay as described in Hocking *et al.* (In Press).

2.3.3.3: Skin Score

At 30 weeks of age birds were visually assessed for skin lesions. Lesions were classified as mild (scab of less that 5 mm) or moderate (more than 5 mm usually with associated bleeding). The lesions of birds that had died or were culled as a consequence of feather pecking and cannibalism were classified as severe.

2.3.4: MOVEMENT TO CAGES (PHASE 2)

At 30 weeks of age, classified as Phase 2, 100 birds (4 from each line) were transferred from the floor pens into 4 blocks of individual cages measuring 45 x 40 x 80cm (depth x width x height). The cages were again allocated according to a blocking design to control for possible environmental effects. One bird from each strain was housed randomly within each of the four blocks.

2.3.5: EGG COLLECTION AND ANALYSIS

The first 10 eggs laid within each pen were collected for a comparison of shell quality at the onset of lay (Phase 1). The bird's age (in days) at which each pen came into lay was also recorded. After the movement of the birds from the floor pens to the cages (Phase 2) the first 10 eggs laid by each bird were again collected. All the eggs collected for analysis were labeled on the pole with the date laid and identified by the pen or cage number. The eggs were then visually examined for shell surface abnormalities as described below.

2.3.5.1: Shell Surface Abnormalities

Several abnormalities of the shell surface have been identified and described (Hughes and Black, 1976; Hughes and Gilbert, 1984; Hughes *et al.*, 1986). The shells in this experiment were classified as either normal or abnormal according to a modified version of the methods used by Hughes *et al.* (1986) (Table 2.2).

Classification	Specific Fault	Description:
Normal		Egg which is regular in shape, with an even colouring and smooth surface.
Misshapen	*Equatorial Bulge*	Appears as a bulge around the equator of the egg.
	Wrinkled Shell	Appears as distorted or wrinkled shell surface. This can be limited to certain areas of the egg or can be found all over.
Accretions	*Light Accretions*	Appears as a roughened texture across the shell surface.
	Heavy Accretions	Appear as large rough pimples on the shell surface.
	Sugared Pole	A roughened area found at the sharp pole of the egg.
Extraneous Deposits	*Fine Dusting*	Shells have a light dusting or lilacing across the shell surface.
	Heavy Dusting / Splashing	Shells have a heavy chalky covering of calcium or heavy splashes across the surface.
	Targeting	Eggs with a white band of calcium which has a ringed appearance.
Shells	*Slab Sided*	Shell with a thin flattened side.
	Soft Shells	Non-calcified shell.

Table 2.2: Classification of shell surface abnormalities - a modified version of the classification system developed by Hughes et al. (1986).

2.3.6: STATISTICAL ANALYSES

The proportion of the first 10 eggs with abnormal shells for each pen and bird respectively was calculated at the onset of lay (Phase 1) and after the move to cages (Phase 2). A Generalised Linear Mixed Model (GLMM) analysis was conducted using the GLMM procedure in Genstat version 4. GLMM produces ANOVA-like analyses for binary and binomial data in balanced and unbalanced designs. The model used for the 10 eggs at the onset of lay and after the move to cages was:

Response = Block (random) + Breed (random) + Type (fixed)

Position of the egg within the series or sequence: In an attempt to investigate the probability of an egg having an abnormal shell with increasing position within the series of eggs collected, a stepwise regression analysis was used with the model:

Response = Block (random) + Type (fixed) + Eggs position within the series/sequence (fixed)

When factors were found to be significant, multiple regression analyses were used to obtain probabilities and R^2 values.

Relationships with prior behavioural data: Breed means were calculated using the data from the prior tests of fear (Phase 1), restraint and skin scores, as recorded by Hocking *et al.* (In Press). Breed means were also calculated for the proportion of eggs with abnormal shells both during the onset of lay (Phase 1) and following the move to cages (Phase 2). Stepwise regressions were then used to look for any significant relationships between these data sets. Minitab release 12.1 (© Minitab Inc., 1998) was used to carry out stepwise regressions in order to identify factors which accounted for a significant proportion of variability seen in the resulting eggshell quality. Again when factors were found to be significant, multiple regression analyses were used to obtain probabilities and R^2 values.

2.4: RESULTS

2.4.1: ONSET OF LAY:

Proportion of abnormal eggs laid at the onset of lay (Phase 1):

The proportion of eggs with abnormal shells laid at the onset of lay was similar in both types of bird (λ^2 (1df) = 3.07; Figure 2.1). The back-transformed means for commercial and traditional lines respectively were 0.46 and 0.19 (-0.15 and –1.43 sed 0.305).

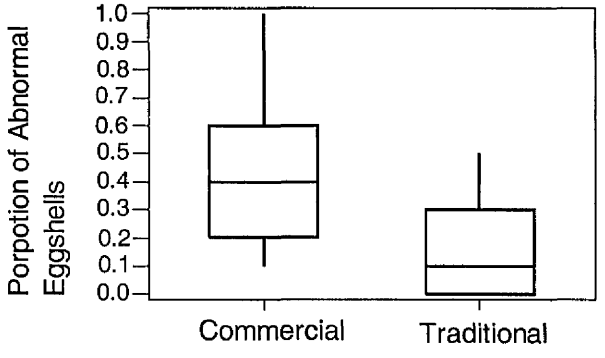


Figure 2.1: Box-plot showing the proportion of abnormal eggshells laid within the first 10 eggs during the onset of lay for commercial and traditional breeds of laying hen.

Probability of abnormal shell with increasing position in series at the onset of lay:

Figure 2.2 shows that the proportion of eggs with abnormal shells decreases as the egg number in the series advances in both commercial and traditional lines. A multiple regression revealed that both breed type (t=6.51, $P<0.001$) and position within the first 10 eggs (t=-3.32, $P=0.001$) explained a significant part of the variation ($R^2=10.5\%$), implying that a correlation exists between these two factors and the proportion of abnormal eggs laid. There was no interaction found between results from the breed types (t=1.58, $P=0.114$).

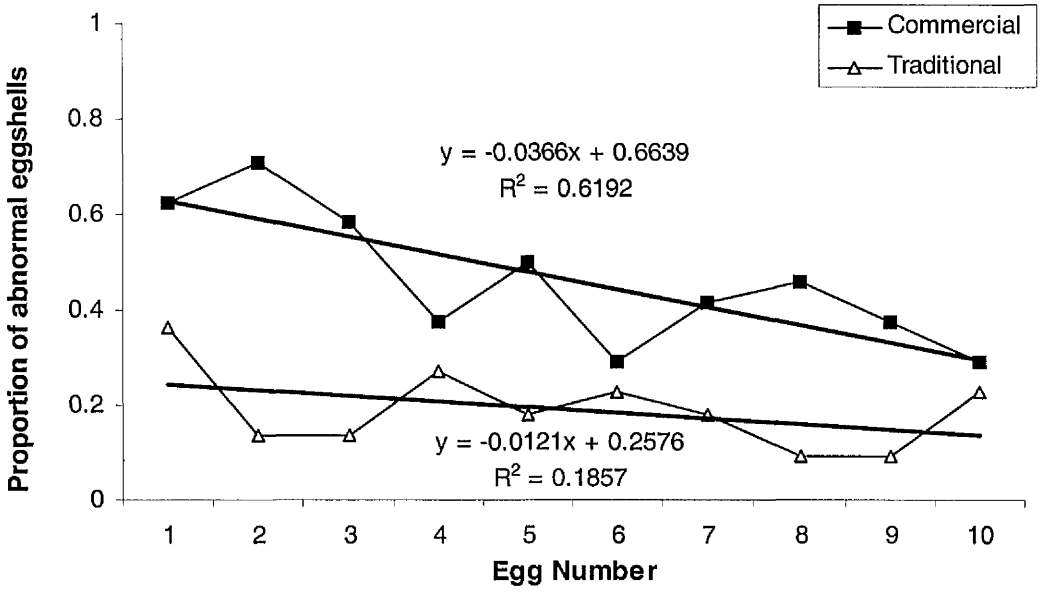


Figure 2.2: Mean proportion of eggs with abnormal shells (S.E. bars not shown) within a series of 10 eggs collected at onset of lay for commercial and traditional breeds of laying hen.

The onset of lay occurred significantly earlier in the commercial lines (Figure 2.3). The mean bird age at the onset of lay for commercial and traditional lines respectively was 140 and 168 days (sed 3.27, $P<0.001$).

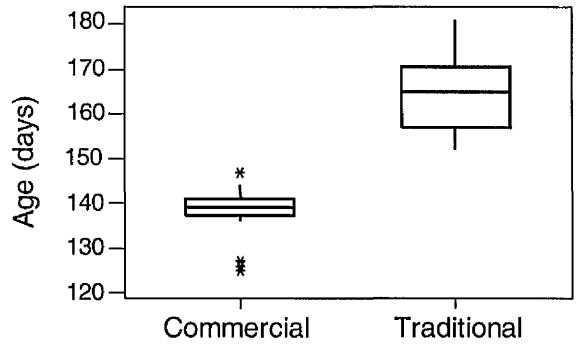


Figure 2.3: Box-plot showing the bird age (days) at onset of lay for commercial and traditional breeds of laying hen (* represents outliers).

A regression analysis revealed that the age at onset of lay explained a significant part of the variation in the proportion of abnormal eggshells (t=-4.05, $P<0.001$, $R^2=27.6\%$; Figure 2.4). This result implies that a correlation exists between the bird's age and the proportion of abnormal eggs being laid. Breed type was not significant when included in the model (t=-1.25, $P=0.218$).

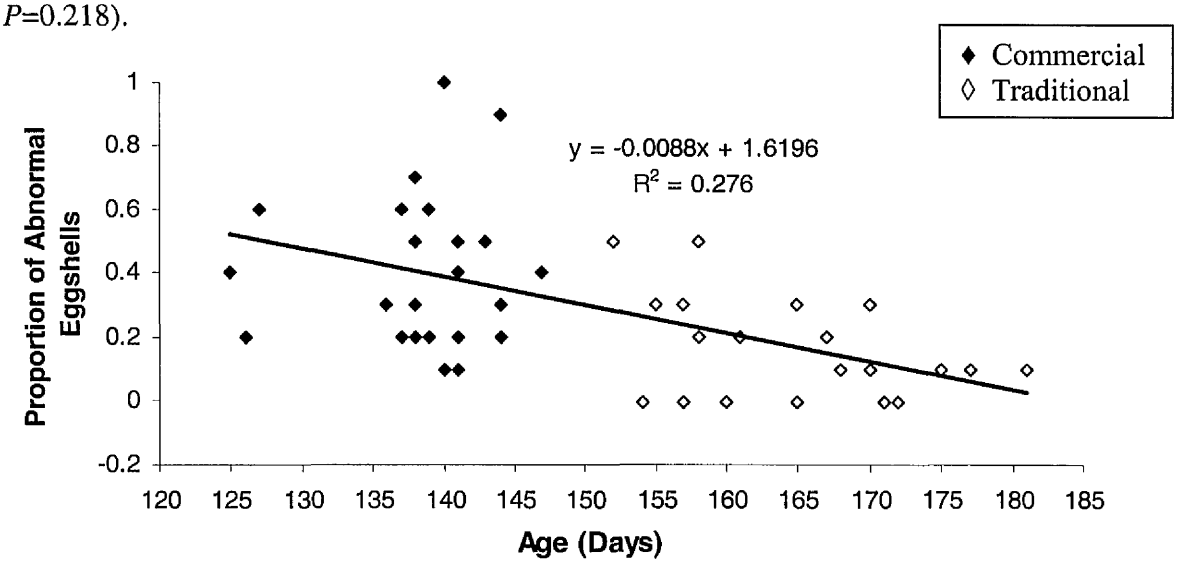


Figure 2.4: Proportion of abnormal eggshells laid associated with bird age (days) at coming into lay (pen means).

Proportion of abnormal eggshells laid at the onset of lay related to breed type and tests of fear measured during Phase 1:

A multiple regression revealed that both breed type (t=-5.57, $P<0.001$) and corticosterone concentration after restraint (t=-3.22, $P=0.004$; Hocking *et al.*, In Press) explained a significant part of the variation ($R^2=60.4\%$) in the proportion of abnormal eggshells at the onset of lay (Figure 2.5). This result suggests that a correlation exists between these two factors and the proportion of abnormal eggs laid. The mean plasma corticosterone concentration following the crush cage restraint for commercial and traditional lines respectively was 5.31 (±0.75) and 3.48 (±0.36) ng/ml. There were no interactions with breed types (t=0.57, P=0.874).

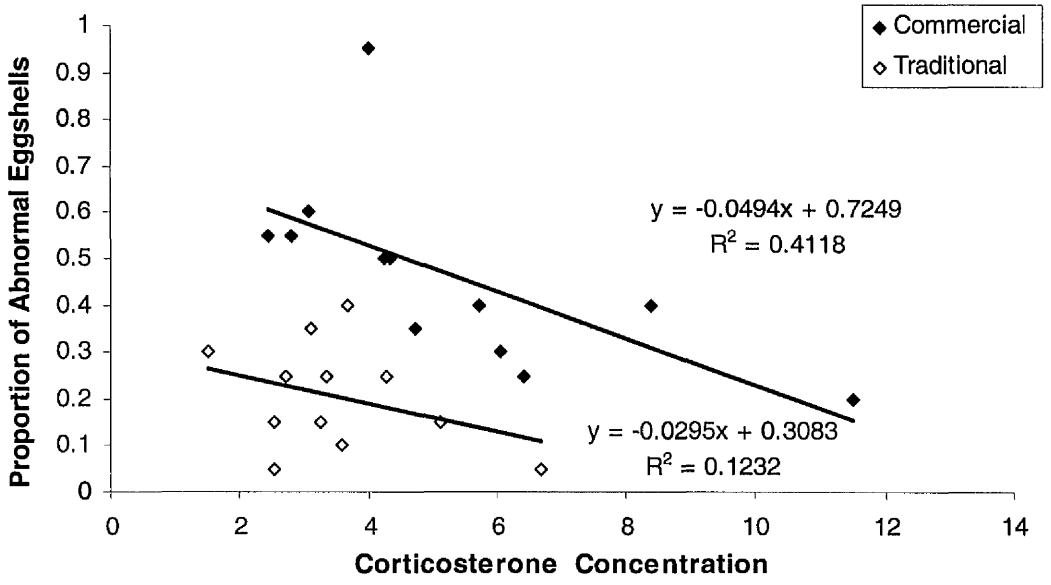


Figure 2.5: Pen means (separated into breed type) for proportion of abnormal eggshells laid at the onset of lay in relation to corticosterone concentration following crush cage restraint.

2.4.2: MOVE TO CAGES

Proportion of eggs with abnormal shells following the move to cages (Phase 2):

The percentage of eggs with abnormal shells laid after the move to cages was not significantly different between breed types (λ^2 (1df) = 1.50, Figure 2.6). The back-transformed means for commercial and traditional lines respectively were 0.15 and 0.19 (-1.733 and –1.465, sed 0.345).

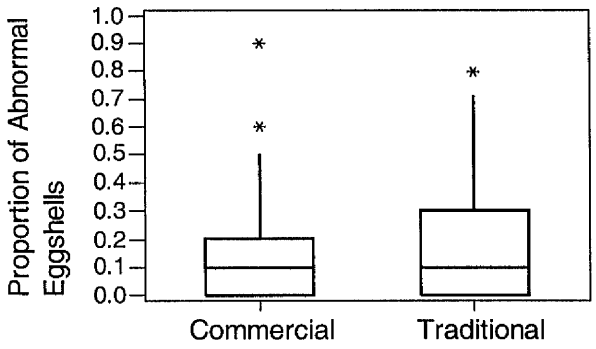


Figure 2.6: Box-plot showing the proportion of abnormal eggshells laid within the first 10 eggs collected following the move to cages (* represents outliers).

Probability of abnormal shells with increasing position in sequence after the move to cages: A regression analysis revealed that the position of an egg in the first 10 eggs laid after the move to cages explained a significant part of the variation in proportion of eggs abnormal eggshells (t=-6.49, $P<0.001$, $R^2=4.6\%$; Figure 2.7), implying that there was a correlation between these two measures. Breed type was not a significant factor in this case (t=1.40, $P=0.162$).

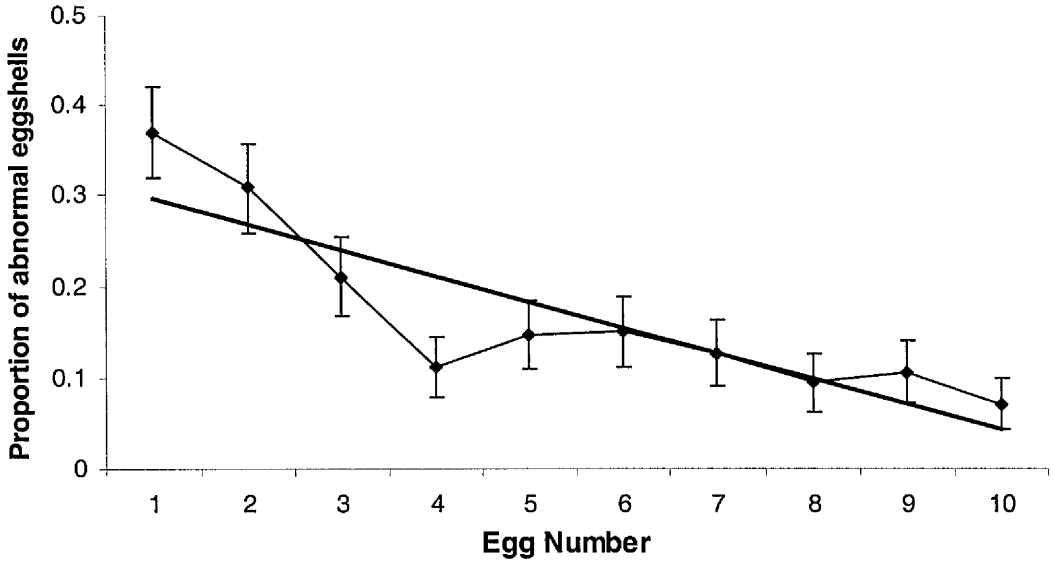


Figure 2.7: Mean proportion (± S.E.) of eggs with abnormal shells in a series of 10 eggs collected following the move to cages (traditional and commercial breeds combined).

Proportion of eggs with abnormal shells laid after the move to cages related to breed type and tests of fear measured during Phase 1:

A multiple regression analysis revealed that a breed's response in the first crush cage test (at 14 weeks of age) explained a significant part of the variation in proportion of eggs with abnormal shells (t=2.63, P=0.015, R^2=23.1%, Figure 2.8), implying a correlation exists between these two measures. Breed type had no effect.

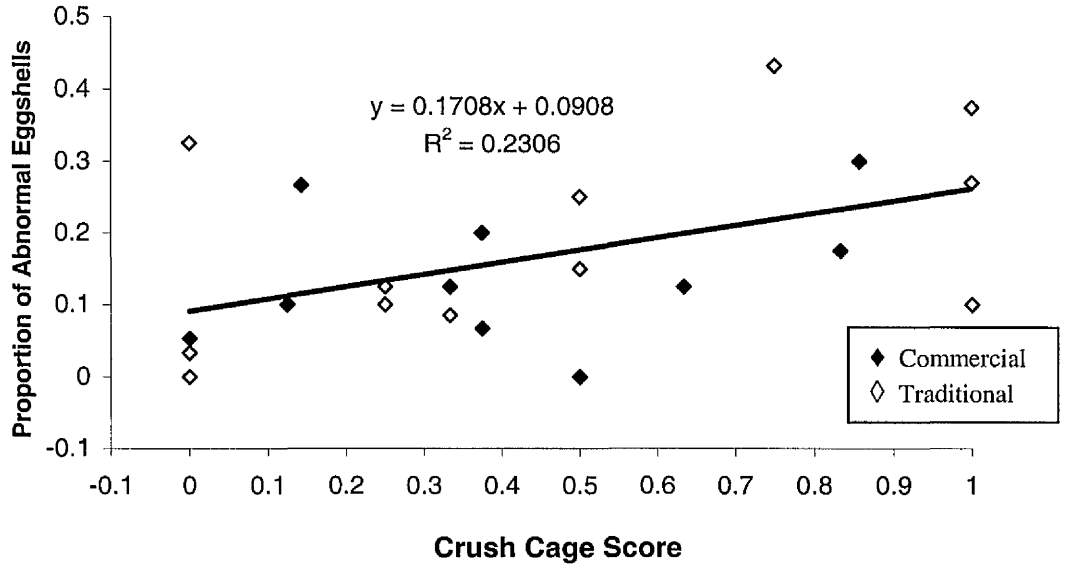


Figure 2.8: Pen means for the proportion of eggs with abnormal shells plotted against the pen mean crush cage score at 14 weeks of age.

2.5: DISCUSSION

2.5.1: Onset of Lay (Phase 1)

Although there was no significant difference in the incidence of surface defects associated with the first 10 eggs laid by the commercial and traditional breeds at the onset of lay (Figure 2.1), when position of the egg within the series was taken into account both breed type and position within the series were shown to explain a significant part of the variation in proportion of abnormal eggshells (Figure 2.2). The commercial breeds showed a consistently higher proportion of eggs with surface defects throughout the onset of lay period, however in both breeds, as the egg number in the series advanced the proportion of eggs with external abnormalities decreased. It must be emphasised however that these eggs were not in sequence with regard to any specific bird as there was no method of identifying eggs from individual

birds. Therefore it is possible that a collection of ten eggs from one pen may be comprised of first eggs from many individuals and those from another may be a mixture of a series of eggs from one or two individuals. It is also worth remembering that one of the shortfalls in the design of this study was that the number of birds within each pen varied from 4 to 16 and this too would have influenced the number and sequence of eggs collected. For these reasons these results must be treated with caution and methods for labelling the eggs from individual birds further investigated.

The age at coming into lay was also significantly earlier in the commercial breeds (Figure 2.3). This result was expected as the commercial hybrids have been selected for higher reproductive performance. Age at coming into lay could therefore explain a significant part of the variation in the proportion of eggs with abnormal shells (Figure 2.4), perhaps reflecting the immature status of the reproductive tract in the commercial hybrids at this time. Mills *et al.* (1991) suggested that the premature termination of pigmentation is the most likely cause of the whitening of eggs laid at the onset of lay, whereas stress-related egg retention has been cited as having more of a role to play in the production of calcium dusted eggs later in lay. Within this study both pale and dusted shells were recorded at the beginning of lay suggesting that both premature oviposition and egg retention were occurring during this time.

When these eggshell quality results were analysed in relation to the tests of fearfulness, conducted during Phase 1, it was revealed that both breed type and corticosterone concentration following crush cage restraint explained a significant part of the variation in proportion of eggs with poor shell quality at the onset of lay, implying these measures were correlated. The commercial breeds displayed higher corticosterone levels and consistently laid poor quality eggs at this stage (Figure 2.5) while both breed types demonstrated a negative relationship between corticosteroid levels and proportion of eggs with abnormal shells i.e. the strains exhibiting lower corticosterone levels were the strains which laid an increased proportion of eggs with abnormal shells. It would appear from this result that the crush cage test elicited a different type of stress response within the bird than the physiological stress experienced by the bird at the onset of lay.

2.5.2: Move to Cages (Phase 2)

There was no significant difference in the shell quality of eggs laid by the commercial or traditional lines following their transfer into cages (Figure 2.6). The position of the egg within the sequence of ten eggs collected after the move was found to explain a significant part of the

variation in proportion of abnormal shells observed. Breed type was not found to be a significant factor, suggesting that all birds reacted to the move in a very similar way. The data showed that as the egg numbers in the sequence advanced, the proportion of eggs with abnormal shells decreased (Figure 2.7). This result was consistent with the findings of Watt (1989) who reported that following environmental stress only the first few eggs laid had surface shell abnormalities. This author however subsequently found that although surface defects decreased after a few days, microscopic structural variations within the eggshell continued to exist for many days thereafter. Unfortunately within this study it was not possible to measure the baseline eggshell quality by collecting eggs from each pen prior to the move to cages as many of the breeds were still in the process of laying the first ten eggs following the onset of lay. This fact would have biased any results from a collection taken prior to the move. As there is no information regarding the proportion of abnormal eggshells being laid prior to the move then no comment can be made on the change in incidence of abnormal shells as a results of this move. It is also possible that the observed reduction in abnormal eggshells, throughout the sequence following the relocation, was due to a preference, by the birds, for this new environment.

When the tests of fearfulness, measured during Phase 1, were analysed together with the above eggshell quality results, it became apparent that the strains which laid a higher proportion of eggs with abnormal shells were also those which had struggled the most in the crush cage test (Figure 2.8). The struggling reaction is generally considered to be a fear response but could also be an expression of active coping rather than fear (Hocking, pers. comm.). Duncan and Filshie (1979), for example, measured the behavioural response of several strains of bird subjected to frightening stimuli and found that the light-hybrid strain reacted in a more violent manner than the medium or heavy-hybrid strains, suggesting the latter lines were less fearful. However when these authors compared the heart rates of the birds in question, they found that whilst the rate in the light-hybrid was higher following the stimulus, the heart rate in the medium and heavy-hybrids took longer to return to pre-stress levels.

It is perhaps pertinent at this time to point out that the time delay in the onset of lay between the traditional and commercial breeds may have distorted the results obtained following the move to cages. In the next experiment (2) and following acclimatisation to the cage environment the birds were subjected to a series of stress events to determine whether the effects were similarly experienced across breeds.

2.6: CONCLUSIONS

At the onset of lay the proportion of eggs with abnormal shells was consistently higher in the commercial breeds. The commercial birds also came into lay significantly earlier than the traditional breeds suggesting that an immature reproductive tract may be responsible for this increased number of abnormal eggs. These results were found to have a negative relationship with plasma corticosterone levels recorded following the crush cage experiment implying that this test of restraint elicited a different type of stress response then the physiological stress experienced at the onset of lay.

There was no significant difference in the shell quality of eggs laid by the traditional or commercial lines following the transfer into cages. As the egg numbers in the sequence advanced, following the move, the proportion of eggs with abnormal shells decreased. Finally there was a positive relationship between the degree of struggling recorded during the crush cage test and the proportion of eggs with abnormal shells laid following the move to cages.

2.7: EXPERIMENT 2

Fear is regarded as an adaptive psychophysiological response to perceived danger and is therefore an important component of stress (Jones, 1987a, Jones *et al.,* 1988). Fear is recognised by behavioural changes which include diverse forms of defensive behaviour such as passive avoidance, freezing, tonic immobility, withdrawal and vigorous escape (Duncan, 1985; Jones, 1987b).

Many authors have suggested that the best way to have confidence that welfare or fear is being effectively measured is to combine different types of indicator (Duncan, 1981; Duncan and Dawkins, 1983; Koelkebeck *et al.*, 1987; Baxter, 1989; Broom and Johnson, 1993). As previously mentioned one of the criticisms about the assessment of stress is that many of the currently used indicators are invasive in nature and can themselves disturb the animal and therefore compromise the measurement being taken (Freeman, 1985). For this reason there has been a move towards measuring many indicators of stress in order to get a better picture of the changes which are occurring.

Jones and Hughes (1986) stated that birds with a history of laying eggs with certain shell defects were more fearful than normal layers. As previously stated many authors have suggested the use of superficial eggshell quality as a possible means of assessing information about the physiological status of the domestic fowl (Hughes and Black, 1976; Hughes *et al.*, 1986; Solomon *et al.*, 1987; Watt, 1989). Mills and Wood-Gush (1985) reported that behaviour could be quantified and examined in combination with other welfare indices to provide a clearer idea of welfare. For example the adrenocortical response to catching and transport was found to be highest in hens which showed the greatest home-cage avoidance of the experimenter (Broom *et al.*, 1986). More recently Keer-Keer *et al.* (1996) used a series of simple behavioural tests to assess fearfulness in different strains of bird.

2.8: AIMS

Experiment 2 was designed in the first instance to evaluate fear levels, in commercial and traditional breeds of hen, via simple tests of avoidance and thereafter to assess whether more overt stressors such as blood sampling and restraint affected all breeds to the same degree. There was also an opportunity to examine relationships between these measures and the results from Experiment 1. This allowed comment to be made on the association between measures of fear prior to the onset of lay and indicators of fear and stress at 45 weeks of age.

2.9: MATERIALS AND METHODS

2.9.1: ANIMALS AND HOUSING

Between 30 and 45 weeks of age the birds from Experiment 1 were kept under standard husbandry conditions within the cage system as described in section 2.3.4. This experiment commenced when the birds were 45 weeks of age.

2.9.2: BEHAVIOURAL OBSERVATIONS AT 45 WEEKS OF AGE

The following behavioural observations were carried out to test whether the bird's reaction to a minor stress event could be used as an indicator of its response to a more severe stress experience.

2.9.2.1: Response to a Novel Object

This test was based on the protocol devised and described by Hughes and Black (1976) and modified by Keer-Keer *et al.* (1996). It measures the bird's response to a novel object placed in the food trough in front of its home cage. The novel object in this case was a wooden rod (34cm x 2.5cm) mounted vertically on a wooden base (10cm x 15cm) which was coated with five different colours of 2cm wide plastic tape. During each test the rod was placed vertically in the centre of the food trough in front of the cage (Plate 2.1) with the observer standing 3 metres to the side of the cage. Immediately following the introduction of rod the bird's response was scored at ten second intervals over a three minute period. The orientation of the bird was recorded on a five-point scale as described in Table 2.3 and the total score for each bird was a mean of 18 scores. Cage blocks and birds within each block were tested at random and all tests were completed on the same day.

Plate 2.1: Novel rod placed in feed trough in front of individual cages.

SCORE	CORRESPONDING BEHAVIOUR
0	Head out of front of cage
1	Head within cage and bird orientated towards front
2	Bird facing sideways
3	Bird facing back of cage
4	Bird exhibiting escape behaviour

Table 2.3: Scoring system used for the behavioural observations.

2.9.2.2: Response to a Looming Human

This was a modification of the test described by Barnett *et al.* (1992) and Keer-Keer *et al.* (1996). It measures the response of the bird to the approach of a human. Each bird was tested individually within the home cage. All tests were completed on the same day. The observer, dressed in a yellow boiler suit and wearing respiration equipment, stood at a distance of 1 metre directly opposite the cage of the bird being tested, with their face at cage level, then approached in four stages:

1. Stood motionless with arms by the side at 1 metre distance for 10 seconds.
2. Moved forward to 0.5 metres distance from the cage and stood for 10 seconds (Plate 2.2).
3. Moved forward to be in front of the cage (0 metres) and stood for 10 seconds.
4. Placed the face up against the cage bars and placed a gloved hand on the bars. Remained in this position for 10 seconds (Plate 2.3).

The orientation of the bird was scored at 5 second intervals using the same scoring system as used for the novel object experiment. The total score for each bird was a mean of 8 scores.

Plate 2.2: Experimenter at stage 2: standing 0.5 metres from the cage front.

Plate 2.3: Experimenter at stage 4: face up against the cage and a gloved hand on the bars.

2.9.3: Subjecting the Birds to an Overt Stress Event

The severe stress event consisted of restraining the bird in three stages.

Stage 1

Each bird was removed from its home cage and taken to another room where it was restrained while a blood sample was taken. Approximately 1ml of blood was removed by superficial venopuncture of the Brachial vein and collected in heparinised syringes.

Stage 2

Immediately after blood sampling the birds were inverted in a metal cone and the legs were restrained in two slots with thick rubber tubing to protect the skin. The birds remained in the cone for 10 minutes.

Stage 3

The birds were blood sampled a second time, after the restraining period, as described above and then replaced in their home cage.

2.9.4: Measuring the Bird's Response to the Overt Stress Event

2.9.4.1: Plasma Corticosterone Levels Pre and Post Stress

Blood samples were transferred into heparin-coated tubes and gently shaken. Each tube was labelled with the bird number before being centrifuged at 1500g for 10 minutes in a Sanyo Minstrel refrigerated bench centrifuge. The plasma was then removed and placed in 0.5 ml eppendorf tubes and stored at –20°C. Plasma corticosterone concentrations were determined at a later date using a radio-immunoassay (Cambridge Diagnostics Kit, Biogenesis Ltd., Bournemouth) after extraction in dichloromethane as described by Mitchell *et al.* (1986) (Full Method detailed in Appendix 1).

2.9.4.2: Egg Collection Pre and Post Stress

Eggs were collected from each cage for a total of 21days (7 days before the test and 14 days after the test). Collection was divided into three periods for preliminary analyses. Period 1 (P1) corresponded to eggs collected during one week prior to any experimentation. Period 2 (P2) corresponded to eggs collected during the seven days immediately after the experimentation. Period 3 (P3) corresponded to eggs collected between 7 to 14 days after experimentation. Each egg was assessed using the method described in section 2.3.5.1,

however, for this experiment, the proportion of shells with individual types of surface defect was also taken into consideration.

2.9.5: STATISTICAL ANALYSES

Residual maximum likelihood (REML) analysis (equivalent to ANOVA) for unbalanced split plot design (Patterson and Thomson 1971) was performed on all the measurements. Block and breed were fitted as random factors to account for any differences due to variations in house conditions and breed. Block x Breed was used as the residual term. The significance of fixed effects was tested by using the 'sub model' feature within Genstat. The significance of breed type (Traditional or Commercial) was evaluated by comparing the deviance difference from fitting models with and without the effect. Effects were assessed using Wald tests (a generalisation of the Student's t-test) which are compared against a chi-squared distribution for large data sets. The Generalised Linear Mixed Model (GLMM) was used to analyse binary ESQ data. Any data found to be skewed were logarithmically transformed to create a normal distribution. All statistical analysis was carried out on Genstat version 4.

Relationships between all measures obtained from Experiments 1 and 2: Breed means from the prior tests of fear, recorded by Hocking *et al.* (In Press), and ESQ results during the onset of lay and following the move to cages (Experiment 1) were considered along with the breed means for the behavioural tests conducted at 45 weeks of age, corticosterone response and eggshell quality found pre and post stress (Experiment 2). Stepwise regressions were then used to look for any significant relationships between these data sets. Minitab release 12.1 (© Minitab Inc., 1998) was used to carry out stepwise regressions in order to identify factors which accounted for a significant proportion of variability seen in the resulting eggshell quality. Again when factors were found to be significant, multiple regression analyses were used to obtain probabilities and R^2 values.

2.10: RESULTS

2.10.1: BEHAVIOURAL OBSERVATIONS AT 45 WEEKS OF AGE

2.10.1.1: Novel Object Test

Mean novel object scores were similar for commercial and traditional breeds. No significant differences were found for breed type in relation to mean novel object score (W=0.4, df=1, P>0.5; Figure 2.9).

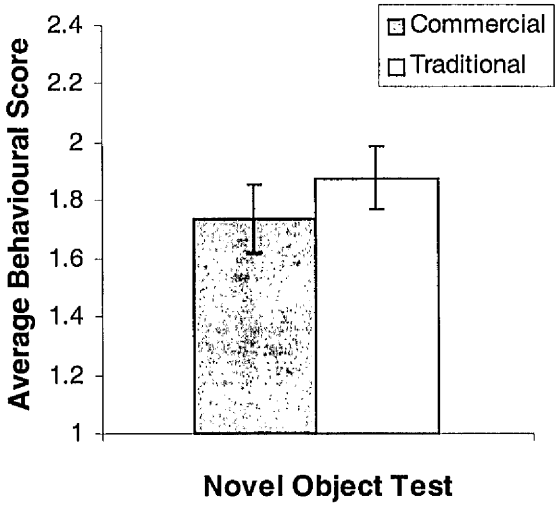


Figure 2.9: Mean behavioural scores for novel object behavioural tests.

Novel object score related to breed type and all other measures:

A multiple regression analysis revealed that a bird's response in the second crush cage test (at 28 weeks old) explained a significant part of the variation in response to the novel object test carried out at 45 weeks (t=-2.92, P=0.008, R^2=27%, Figure 2.10), implying a correlation existed between these scores.

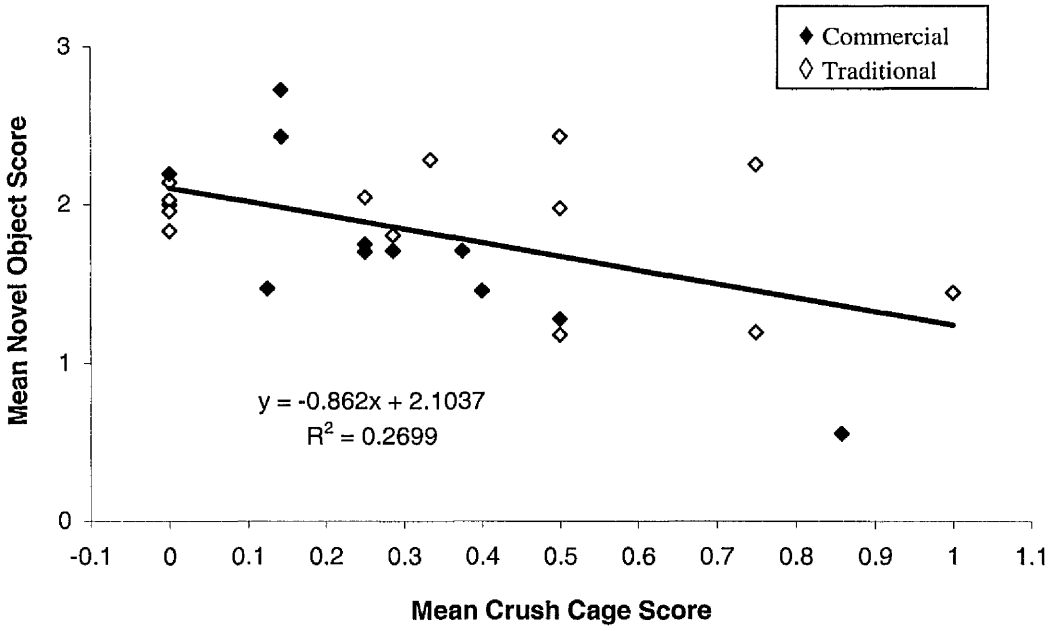


Figure 2.10: Breed means for mean novel object score plotted against the crush cage score carried out at 28 weeks of age.

2.10.1.2: Looming Human Test

Mean looming human scores were also similar for commercial and traditional breeds. Again no significant differences were found for breed type in relation to mean looming human score (W=2.7, df=1, $P>0.1$; Figure 2.11).

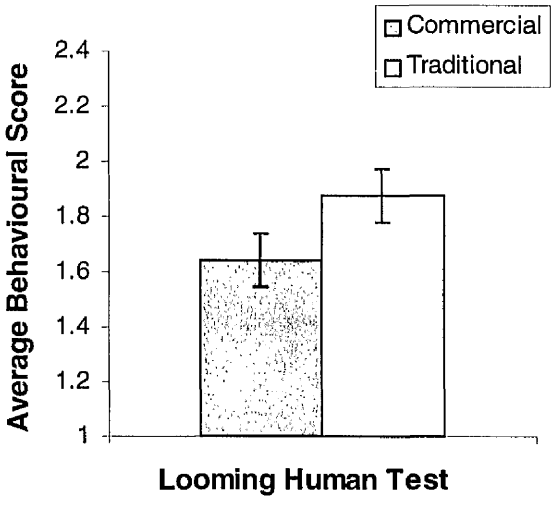


Figure 2.11: Mean behavioural scores for looming human behavioural tests.

Looming human score related to breed type and all other measures:

A multiple regression analysis revealed that a bird's breed type (t=3.28, *P*=0.003) and corticosterone concentration following crush cage restraint at 28 weeks (t=3.32, *P*=0.003) explained a significant part of the variation in the response to the looming human test carried out at 45 weeks (R^2=41%, Figure 2.12). This result implies that a correlation exists between these two factors and the subsequent looming human score. There were no interactions with breed type (t=0.00, *P*=0.998).

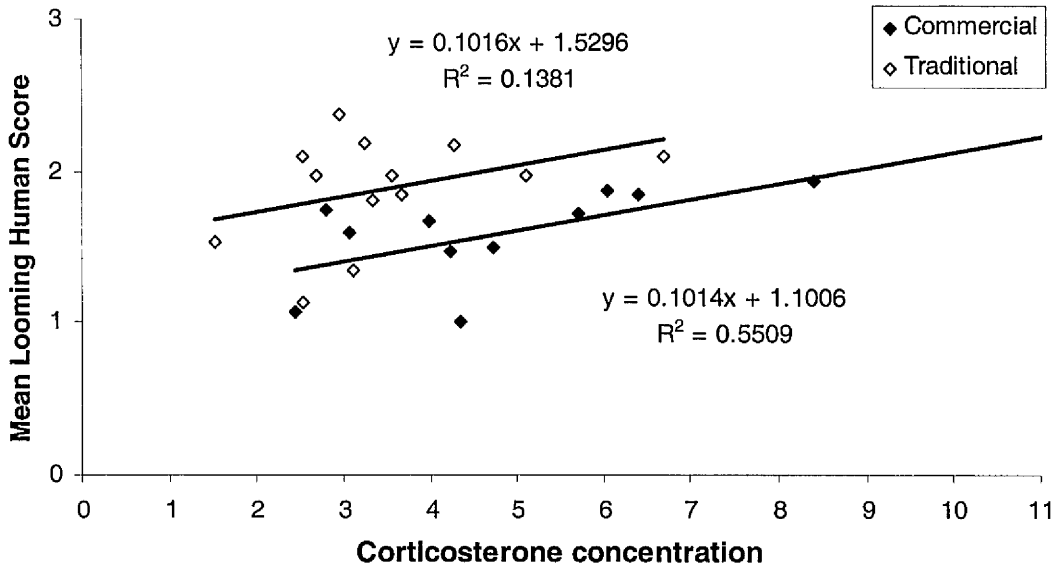


Figure 2.12: Breed means for looming human score, at 45 weeks, in relation to corticosterone concentration following crush cage restraint at 28 weeks (separated into breed type).

A stepwise regression analysis showed that the mean novel object score explained a significant part of the variation in response to looming human (t=2.58, *P*=0.017, R^2=22.5%, Figure 2.13), implying that a correlation exists between these two scores. Breed type was found not to be significant.

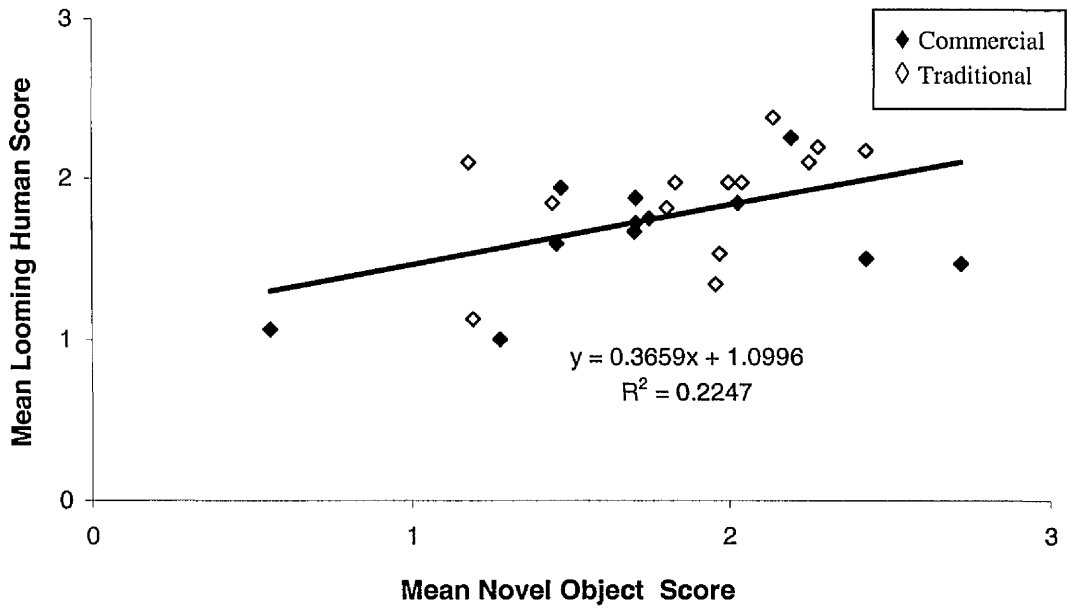


Figure 2.13: Breed means for mean novel object score plotted against the breed mean for mean looming human score.

2.10.2: Plasma Corticosterone Levels Pre and Post Stress

Mean plasma corticosterone levels before stress were similar for commercial and traditional breeds. No significant differences were found between pre plasma corticosterone levels for each breed type (W=0.2; $P>0.6$). Figure 2.14 shows that the plasma corticosterone concentrations in both breed types increased following the stress event. Differences in pre and post corticosterone levels in relation to breed type were found to be significant (W=10.8, df=1, $P<0.005$).

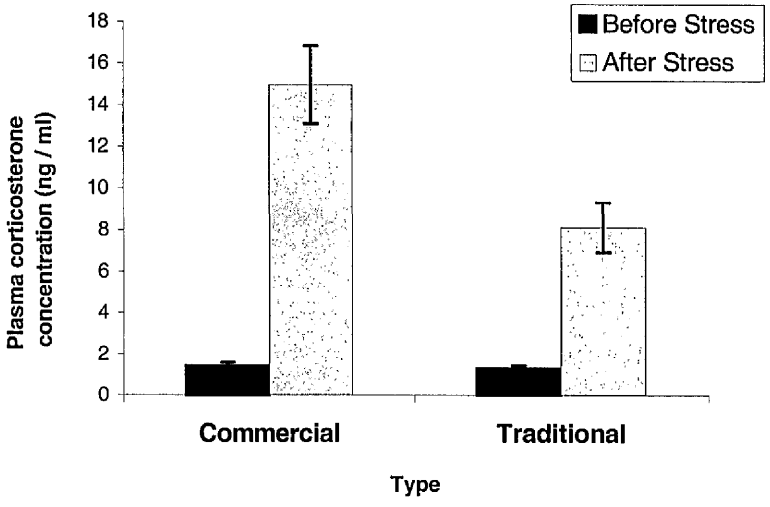


Figure 2.14: Plasma corticosterone levels before and after the stress event.

Change in corticosterone levels related to breed type and all other measures:

A stepwise regression analysis revealed that breed type alone explained a significant part of the variation in corticosterone response following stress (t=-3.78, P=0.001, R^2=38.3%), suggesting that a correlation exists between these two factors. The second most significant relationship was with the corticosterone concentration found following crush cage restraint at 28 weeks (t=2.76, P=0.011, R^2=24.8%). In the traditional breeds there was also a positive correlation between corticosterone levels following this crush cage restraint and the change in corticosterone levels following the stress (R^2=0.7613, P<0.001; Figure 2.15)

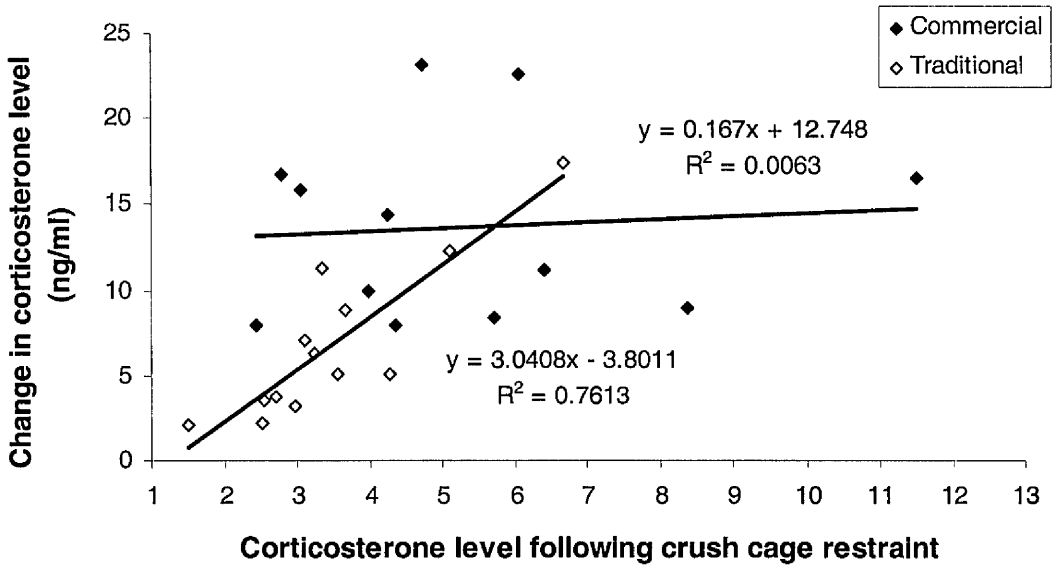


Figure 2.15: Mean change in corticosterone concentration plotted against the corticosterone concentration following crush cage restraint at 28 weeks (separated into breed type).

2.10.3: EGGSHELL QUALITY PRE AND POST STRESS

Probability of obtaining eggs with abnormal shells following stress:

A regression analysis revealed that eggs collected after the stress event (P2) displayed a small but significant increase in surface defects (t=2.48, P=0.013, R^2=0.7%; Figure 2.16). Breed type had no effect on the incidence of shell abnormalities during P1 and P2 (sed 0.0910; W=0.36, P=0.548).

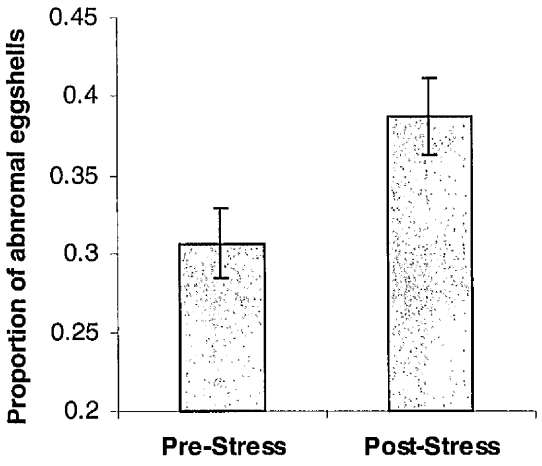


Figure 2.16: Mean proportion of eggs with abnormal shells before and after the stress event.

Probability of eggs with abnormal shells with increasing position in sequence:

A regression analysis revealed that day of collection explained a small but significant part of the variation in proportion of eggs with superficial defects (t=2.08, P=0.038, R^2=0.5%; Figure 2.17), implying that a slight correlation exists between these two factors. There was no significant effect of breed type (t=0.58, P=0.564).

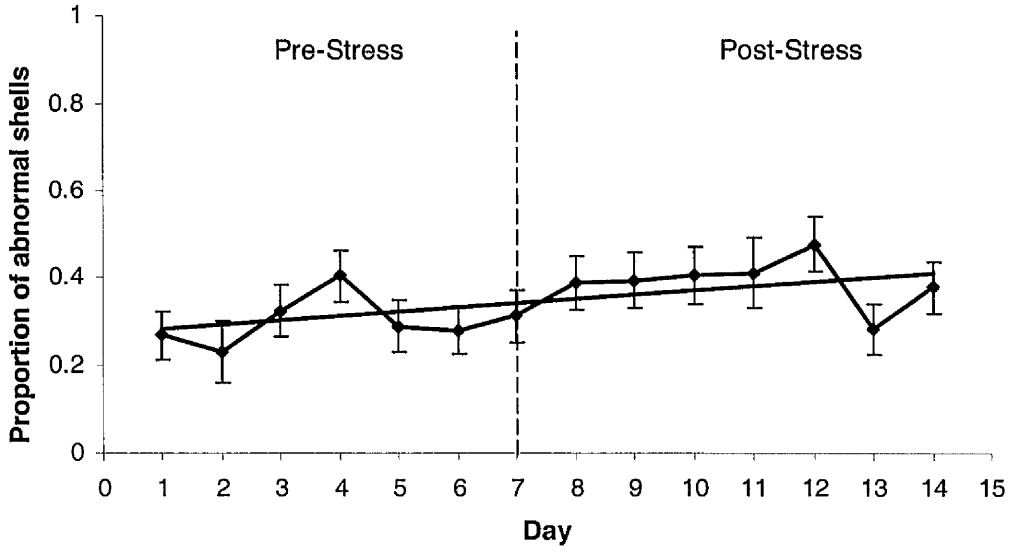


Figure 2.17: Mean proportion of eggs with abnormal shells during the 7 days prior to (P1) and following (P2) stress event (traditional and commercial breeds combined).

Effect of breed type on type of faults seen following stress:

Regression analysis revealed no relationships between breed type and any specific type of eggshell fault (Table 2.4).

Fault	Relationship of Breed Type
Mis-shapen	t=0.12; *P*=0.902
Accretions	t=-0.72, *P*=0.478
Extraneous Deposits	t=0.15, *P*=0.879
Shell faults	t=0.96, *P*=0.347

Table 2.4: Regression analysis results between specific eggshell faults using breed type as a predictor.

Proportion of eggs with abnormal shells found in the seven days following stress (P2) related to breed type and all other factors previously measured:

A stepwise regression analysis showed that the quality of eggs laid in the seven days prior to stress (P1) explained a significant part of the variation in proportion of eggs with abnormal shells in the eggs laid in the seven days post stress (P2) (t=2.83, *P*=0.009, R^2=25.8%; Figure 2.18), suggesting that these two measures may be correlated. The best alternative indicator was with the proportion of eggs with abnormal shells laid following the move to cages (t=2.06, *P*=0.051, R^2=15.6%).

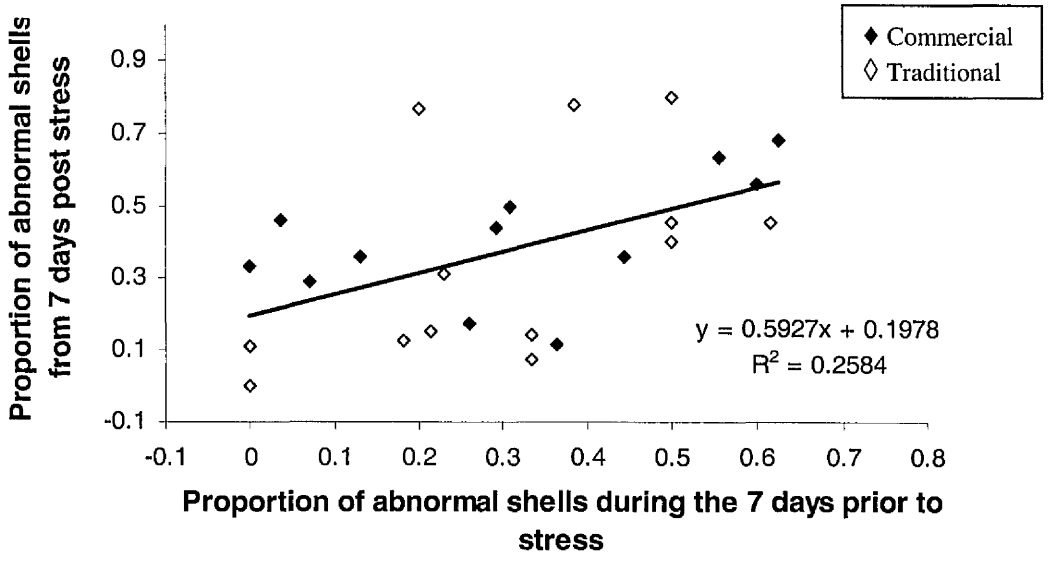


Figure 2.18: Breed means for proportion of eggs with abnormal shells in 7 days prior to stress (P1) plotted against the proportion of abnormal shells in 7 days post stress (P2).

Proportion of eggs with abnormal shells found during P1, P2 and P3 for each breed type:

This trial was not designed to look for differences between individual breeds *per se*, but graphs looking at differences in ESQ over the three time periods revealed that 14 of the breeds showed some increase in the proportion of eggs with superficial defects following stress (Figure 2.19) whereas the other 11 breeds followed a different and, to date, unexplained pattern (Figure 2.20).

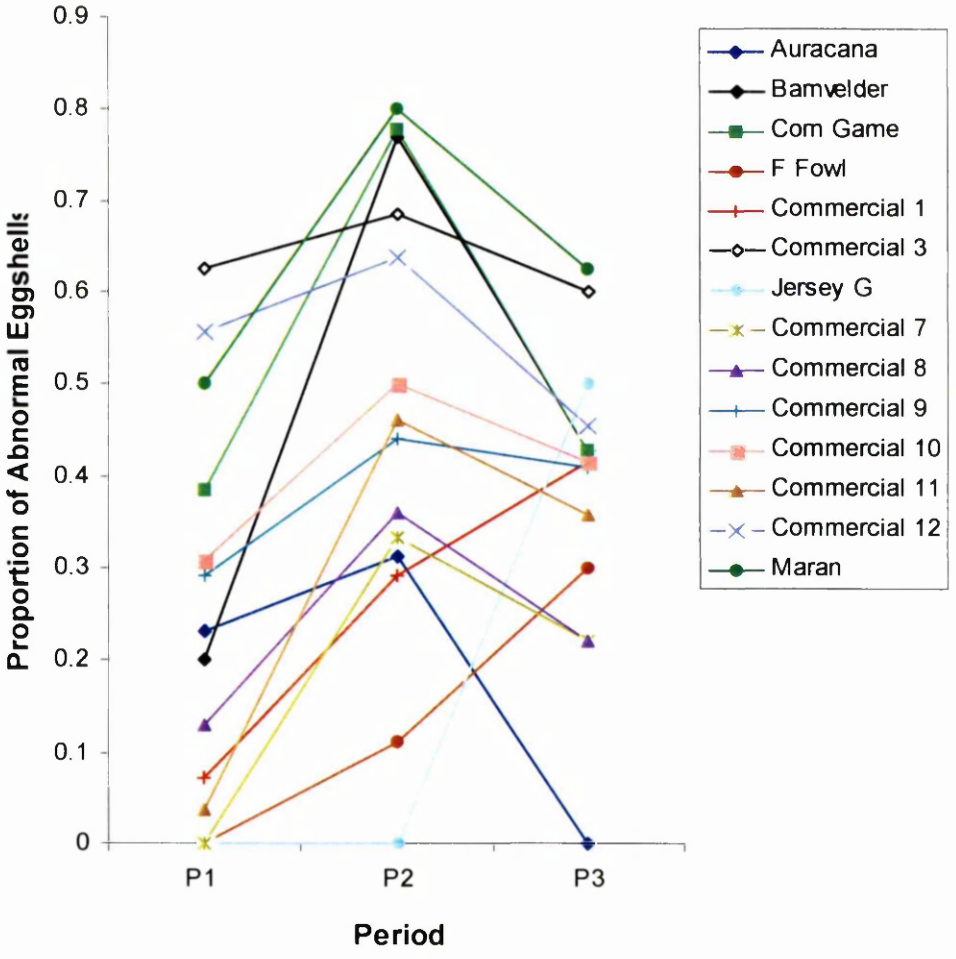


Figure 2.19: Individual breed means for proportion of eggs with abnormal eggshells during the three time periods – breeds showing a rise in the proportion of abnormal eggshells following stress.

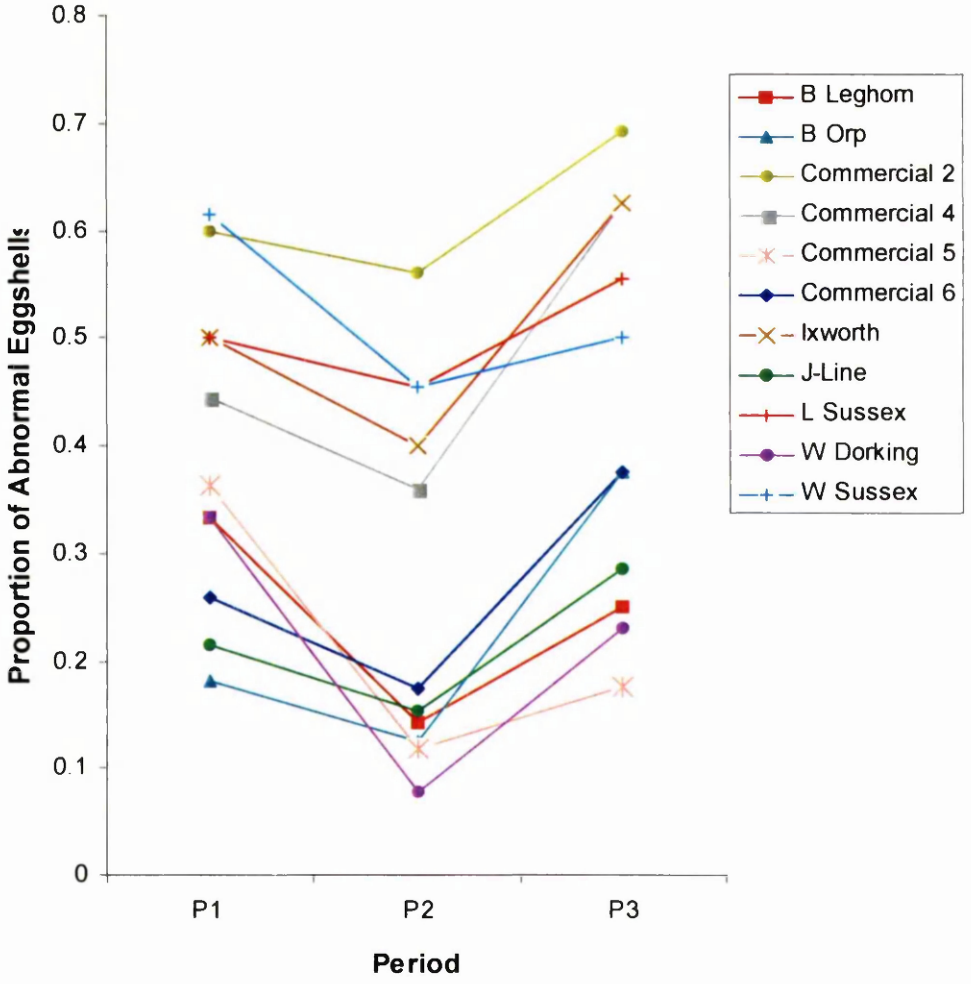


Figure 2.20: Individual breed means for proportion of eggs with abnormal eggshells during the three time periods – breeds showing a decrease in proportion of abnormal eggshells following stress.

2.11: DISCUSSION

The results discussed in this section are considered in relation to the observations recorded by Hocking *et al.* (In Press) and also with reference to the shell quality results reported in experiment 1 (Sections 2.4.1 and 2.4.2).

2.11.1: Novel Object Test

Both breed types responded similarly to the presence of a novel object (Figure 2.9). The findings are in accordance with earlier observations of Keer-Keer *et al.* (1996) who conducted an experiment on White Leghorn and broiler breeders. The birds' response in the second crush

cage experiment (28 weeks), conducted by Hocking *et al.* (In Press), accounts for a significant part of the variation seen in a bird's response to the presence of a novel object (Figure 2.10). This was a negative correlation demonstrating that the higher a breed's score in the second crush cage experiment the lower the novel object score. The lack of consistency between these results suggests that these tests are either measuring different aspects of the fear response or that the birds are displaying different coping mechanisms in each case (Duncan and Fishlie, 1979; Duncan and Dawkins, 1983). Lack of movement within the crush cage is generally considered to be the less fearful reaction, however it could be argued that this lack of response is in fact a fear reaction somewhat akin to the tonic immobility response.

2.11.2: Looming Human Test

Both breed types responded similarly to the presence of a looming human (Figure 2.11). Breed type and corticosterone concentration following crush cage restraint at 28 weeks (Hocking *et al.*, In Press) explained a significant part of the variation in the looming human score (Figure 2.12), with the traditional breeds demonstrating an elevated response to the test. The scores from the novel object test were also found to be correlated with this score, but this relationship was not as strong (Figure 2.13). Although both of these behavioural tests are intended as indicators of the same fear response, it would appear from these results that this is not the case. It should also be highlighted that the results obtained during the looming human test may reflect some adaptation by the birds following their prior experience of the novel object test.

The design of this experiment did not allow for observations to be carried out on control birds which had not been subject to either fear test. Had control observations been used, it may have been possible to confirm that these standard fear tests induced a response from the birds, however the lack of controls was acceptable, in this specific study, as the objects of this phase was to compare the response of birds of different breed types.

2.11.3: Plasma corticosterone – pre and post stress

Plasma corticosterone levels before the stress event (blood sampling and restraint) were similar in both breed types (Figure 2.14). The mean basal level measured was 1.36 ng/ml. This is in accordance with most of the literature which reports basal levels of corticosterone as being approximately 2 ng/mL (Beuving and Vonder, 1978; Craig and Craig, 1985; Lagadic *et al.*, 1990). Obviously the levels of plasma corticosterone vary with time of day, time since stress, time since exercise and time since feeding and so it would be virtually impossible to keep all these variables constant (Broom, 1988; Wingfield *et al.*, 1992). The mean level of

plasma corticosterone following the stress event was 14.95 ng/ml and 8.12ng/ml for commercial and traditional lines respectively. Again these results are consistent with those recorded in the literature. Littin and Cockrem (2001) found peak corticosterone levels following handling to be around 4 to 8 ng/ml and Beuving and Vonder (1978, 1986), using a similar procedure, recorded levels of between 4 and 16 ng/ml. The change in corticosterone levels following the stress event was significantly different between traditional and commercial strains with commercial strains showing a more marked response (Figure 2.14). The fact that the commercial breeds showed an increased concentration suggests that these birds elicited a stronger HPA response and, following the suggestions of Jones *et al.* (1988), that they were consequently more fearful. This result is not in accordance with the conclusions of Schutz *et al.* (2001) who suggested that the selected breeds would display a less active fear response. These authors, however studied the fear response in terms of bird behaviour and did not measure plasma corticosterone levels. Following the results of Duncan and Filshie (1979) it therefore seems plausible that different coping strategies are adopted by each breed type. The change in corticosterone levels following stress was also positively correlated with corticosterone levels subsequent to crush cage restraint at 28 weeks in the traditional breeds (Figure 2.15), but there was no such relationship in the commercial breeds. It appears from this result that the commercial breeds did not respond in the same way, in terms of plasma corticosterone levels, to these two events while the tradi6tional breeds did. It can only be suggested that the apparent inconsistency in these results may be due to age-related changes in the commercial breeds but at this time no further conclusions can be made.

2.11.4: Eggshell Quality

In general, categorisation of the shell abnormalities proved slightly problematic and quite subjective as previously reported by Hughes *et al.* (1986). The diversity of egg colour, texture and size presented by the various breeds complicated the classification.

Breed type had no effect on the probability of obtaining eggs with abnormal shells either before or after the stress event. Day of collection explained a small but significant part of the variation in eggshell quality (Figure 2.17), implying that a correlation exists between these two factors. As there is no clear change in the proportion of abnormal eggs collected between days 7 and 8 then it is possible that this correlation simply reflects a trend occurring over time. A regression analysis also revealed that the proportion of eggs with abnormal shells prior to stress explained a significant part of the proportion of eggs with abnormal shells laid following stress (Figure 2.18). This implies that the high pre-stress score was associated with a

high post stress score and is in accordance with the conclusion by Jones and Hughes (1986) that birds that generally laid poor quality eggs were more fearful. Mills *et al.* (1991) also found high fearfulness to be associated with poor eggshell quality.

Following the results of previous studies by Hughes *et al.* (1986) and Watt (1989) it was surprising that there was not a stronger effect of day or time period on the proportion of eggs with abnormal shells. Watt (1989) found an increase in visually abnormal eggs from 7 – 21% following adrenaline injection and 31-50% following environmental stress. Such a change in proportion of poor quality shells was not detected within this study. It is perhaps pertinent to point out that the protocol followed by Watt (1989) was essentially social stress based whereas in the present situation the tests were of a procedural and handling nature. This author also palpated the birds prior to the experiment to ensure the presence of a hard-shelled egg *in utero*. Within this study the testing of birds was random and occurred throughout the day with no regard to the position of the egg in the oviduct. Many of the superficial shell defects reflect a change in muscle tone within the oviduct, however the data presented herein give no reason to believe that the experimental regime was of sufficient strength to elicit such a response. Likewise the light dusting on the surface of the eggshells, although indicative of egg retention, was not of a magnitude comparable to that observed by Watt (1989). It was of interest to note however, that many strains consistently laid eggs with a dusting of calcium.

The results discussed in this experiment emphasise the fact that the presence of superficial abnormalities alone is not an adequate indicator of stress in domestic fowl, as the variations observed are dependant on the position of the egg within the oviduct at the time of the stress event. Watt (1989) reached a similar conclusion and found that examination of the ultrastructure of the mammillary layer of the eggshell revealed disorganisation following a stress event, even when the eggshell surface appeared normal. In the following chapters, a more detailed assessment of eggshell quality is applied.

2.12: CONCLUSIONS

Both breed types responded similarly to the presence of a novel object and looming human but although the responses recorded during each test were correlated this was not as significant as would be expected. The looming human test appeared to elicit a stronger fear reaction in the birds.

The plasma corticosterone levels were similar in both breed types prior to restraint and increased significantly following restraint. The change in corticosterone concentration was significantly greater in the commercial breeds suggestive of a stronger HPA response in these birds.

The relationship between the results recorded by Hocking *et al.* (In Press) during Phase 1 and those carried out in Experiment 2 were inconsistent highlighting the diverse responses exhibited by each breed type to the variety of tests used.

The proportion of eggs with abnormal shells recorded prior to the stress event was positively correlated with the proportion of abnormal eggs following the event suggesting that birds that display a tendency to lay abnormal eggs react more fearfully.

CHAPTER 3

THE EFFECT OF CHRONIC AND ACUTE STRESSORS ON EGGSHELL QUALITY

This chapter is divided into two experimental sections.

3.1: EXPERIMENT 1

One problem associated with the assessment of welfare in poultry is the measurement of stress (Duncan, 1980). An animals health, productivity, physiology, biochemistry and behaviour can all be monitored in order to assess its well-being, and there has been increasing support for a multidisciplinary approach in order to obtain the best estimate of welfare (Duncan and Dawkins, 1983). However, several of the methods currently employed have received criticism as they are invasive and involve disturbing the animal and so compromise the measurement being taken (Freeman, 1985). Traditionally behavioural testing has provided the definitive non-invasive measure but more recently there has been some progress in the application of alternative non-invasive methods.

The recent work, carried out by Wasser *et al.* (1997) has suggested that faecal levels of corticosterone could also be used to indicate an animal's response to unpredictable events. Whitten *et al.* (1998) reported that this non-invasive method was advantageous, in that it allows continuous monitoring over a period of time and as a result could provide a better picture of the long-term effects of stress. Wasser *et al.* (2000) agree that faecal glucocortical analyses have great potential as a means of assessing the stress associated with environmental disturbance. One shortfall of this technique, however, is the existence of a time-lag between the stress event and resulting increase in steroid in the faeces (Wasser *et al.*, 1994) which can make interpretation of the results difficult. In addition non-aversive stimuli may also result in an increased corticosterone concentration (Rushen, 1986; Broom, 1988).

Given that eggshell quality has been shown to deteriorate following stress (Hughes *et al.*, 1986; Watt, 1989), the degree of eggshell abnormality has also been suggested as a possible non-invasive means of assessing stress in poultry (Mills *et al.*, 1987, 1991). According to Hughes and Black (1976) any disruption to the hen's usual environment can induce the

formation of abnormal eggshells. Application of stressors such as transfer from pens to cages, catching and crating, exclusion from nest-boxes, and administration of adrenaline have also been shown to reduce eggshell quality without depressing egg production (Jones and Hughes, 1986). As previously discussed Hughes *et al.* (1986) reported that the type of shell abnormality produced depended on the position the developing egg had reached within the oviduct at the time of disturbance. Furthermore Solomon *et al.* (1987) illustrated severe structural disorganisation of the mammillary layer following adrenaline injection demonstrating that the abnormalities following disturbance extended throughout the shell. The latter would suggest that any stimulation (adverse or non-adverse) which results in a HPA response will also have an effect on the quality of subsequent eggs.

Many benefits of environmental enrichment have been documented, including improved growth and feed conversion rates (Gvaryahu *et al.*, 1989; Nicol, 1992) and reduced aggressiveness and mortality (Gvaryahu *et al.*, 1994; Jones, 1996b). However others have cautioned that "any novelty is a potent fear elicitor" in birds (Jones, 1987a; Boissy, 1995). Other factors known to induce fearfulness in domestic fowl include separation from con-specifics, exposure to a novel environment and approach or proximity of a human being (Keer-Keer *et al.*, 1996).

3.2: AIMS

In this study the effect of environment on a commercial layer strain was considered. Thus birds were moved from a control pen into a novel experimental pen, which was either enriched or impoverished, and a wider range of eggshell quality traits were assessed as a means of monitoring the bird's response. Given that Solomon *et al.* (1987) reported a time delay in the occurrence of shell defects following a stress event, this experiment was designed to establish the immediacy of response or any delay in its occurrence. Jones (1996b) also reported that birds will react fearfully to any novelty and so this experiment also examined if the bird's response, in terms of eggshell quality, depended on the type of experimental pen into which the birds were placed.

In addition to changes in eggshell quality, bird behaviour and faecal glucocorticoid levels were monitored simultaneously as part of another PhD programme. Lord (2001) describes the results of these other non-invasive measurements of bird welfare in detail and has kindly given permission for their inclusion in the context of this chapter.

3.3: MATERIALS AND METHODS

3.3.1: BIRDS AND HOUSING

The birds used were ISA Brown commercial layers. From 15 weeks of age the birds were housed in three control pens A, B and C of differing dimensions (A and B 2.3 x 1 metres and C 2.4 x 0.9 metres). Each pen had wood-shavings as a floor substrate and each contained eight birds. Lighting was provided at approximately 200 lux for 14 hours per day with 15 minutes of fading to simulate dusk. Coloured leg rings were used to identify individuals and all birds were fitted with beak 'bits' to protect against feather-pecking.

3.3.2: EXPERIMENTAL SET-UP

The experiment was run by means of two replicate trials conducted at different times of year with two different batches of birds. The birds were 29 weeks old at the start of each experiment. Both trials consisted of four experimental periods. Trial 1 consisted of experimental periods 1 – 4 and Trial 2 consisted of experimental periods 5 – 8 (Table 3.1).

Experimental Period	*Start Date*	*Bird Age (weeks)*	*Bird Batch*
1	25/01/98	29	1
2	01/02/98	30	1
3	08/02/98	31	1
4	15/02/98	32	1
5	12/07/98	29	2
6	19/07/98	30	2
7	16/08/98	33	2
8	23/08/98	34	2

Table 3.1: Details of start date, bird age and bird batch for each experimental period.

3.3.3: PROCEDURE

Each experimental pen was divided into two sections and separated by a doorway measuring 60 x 30cm (width x height). Both pens had an identical 'home' area (86 x 86cm) containing a feeder, drinking dish and nest-box with a flooring of heavy brown card. The second section was the 'experimental' area (79 x 95cm). In the first pen, termed 'Enriched', the experimental area consisted of a thick layer of bark chips and a dish (23 x 37cm) of growing sprouted wheat. In the second pen, termed 'Barren', the experimental area consisted of wire mesh (3 x 3.5cm) underlain with card.

At the beginning of each experimental period two birds were placed in each pen. Each pair of birds came from the same control pen and were placed in the experimental pen at 0900 each Monday (Day 1). The birds were removed at 1400 the following Friday (Day 5) and placed back in their original control pen. Pairs of birds were used rather than single birds, as isolation is in itself a stressor (Keer-Keer *et al.*, 1996). Each pair of birds was used only once.

3.3.4: EGG COLLECTION

Eggs were collected from the control pens during the Tuesday, Wednesday and Thursday of the week prior to the first experimental period and used as controls (Day 0). All eggs laid were collected from the experimental pens daily throughout each experimental period.

3.3.5: EGGSHELL QUALITY ANALYSIS

Within this chapter more detailed analyses of the shell were applied in order to provide additional information on intra-shell changes.

3.3.5.1: Superficial Shell Abnormalities

The eggs were initially examined to look for any superficial eggshell abnormality which would result in them being classed as seconds within the commercial environment. The shells in this experiment were classified as either normal or abnormal according to a modified version of the methods used by Hughes *et al.* (1986) as described in section 2.3.5.1 (Table 2.2). These records were used to calculate a total proportion of eggs with abnormal shells laid on each day.

3.3.5.2: Physical Measures of Eggshell Quality

Fresh Weight: Intact eggs were weighed and measured to an accuracy of 10mg.

Shell Thickness Profiles:

Shell thickness can be measured by using screw callipers modified with rounded tips to correct for the curvature of the egg. This technique, although widely used, only provides a measure of the total eggshell thickness. Bain (1990) showed that the most important measurement pertaining to shell strength was the effective thickness (T. Effective). This is the distance between the point of fusion of the palisade columns to the cuticular surface (Figure 3.1). In this thesis T. Effective was derived using the scanning electron microscope after preparing the eggs as follows; first the contents of each egg were emptied out and a circular

saw was used to cut a small section of shell, approximately $10mm^2$, from the equator of each egg. This was then snapped in two and one piece was mounted vertically, using silver paint, on a grooved aluminium stub with the snapped edge uppermost. This stub was then placed in an Emscope sputter coated Sc 500 (Ashford, Kent, England) and coated with gold / palladium for four minutes.

Each transverse section of shell was viewed using a Phillips 501B scanning electron microscope at 15 Kv, at a magnification of x 160 and working distance of 13 units. The sections were tilted until neither the mammillary nor the cuticular surface were visible.

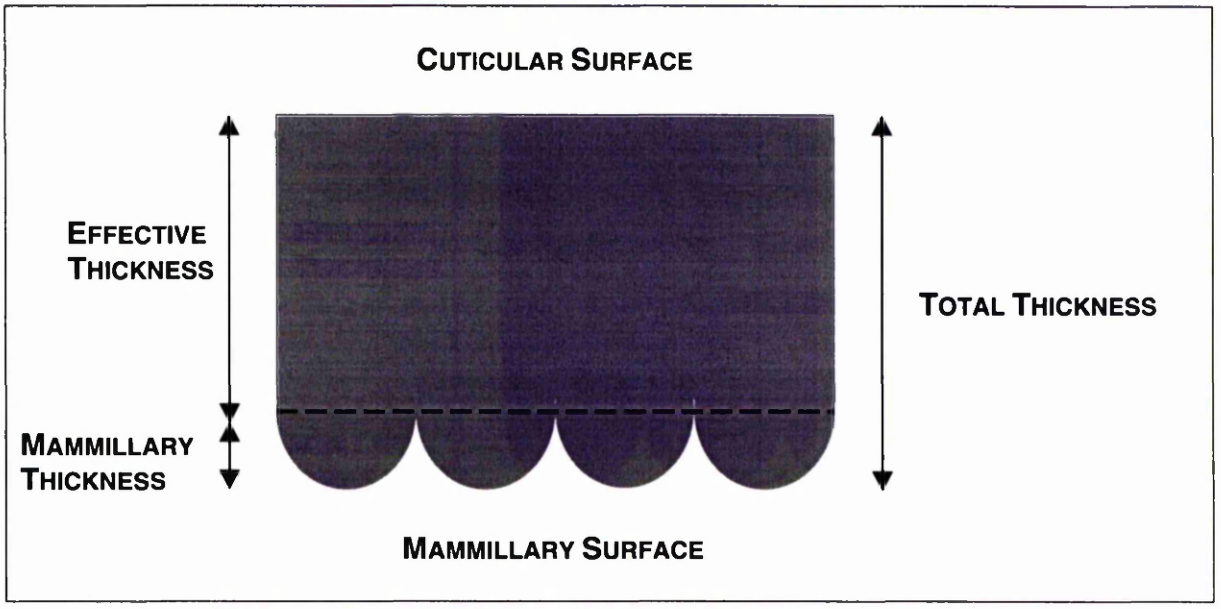


Figure 3.1: Diagram of T.S. of eggshell demonstrating total and effective thickness.

Measurements, to the nearest 1/100mm, were taken of total shell thickness, the distance between the tip of the mammillary bodies to the cuticular surface (Figure 3.1), and mammillary thickness (T.Mamm.), the distance between tip of mammillary bodies to fusion of the palisade columns. Three separate points across each sample were measured in this way to derive the mean total and T.Mamm. value for each egg. From these two values the effective thickness (T. Effective) was calculated by subtraction as described by Bain (1990).

3.3.5.3: ULTRASTRUCTURAL EGGSHELL QUALITY ASSESSMENT

Ultrastructural Assessment of the Mammillary Layer

The mammillary layer ultrastructure was also assessed using scanning electron microscopy. A circular saw was used to cut another small piece of shell, approximately $10mm^2$, from the equator of each egg. This section of shell was then soaked in distilled water for approximately 1 minute, which allowed the inner shell membrane to be peeled off by hand. The sections were then placed on a glass plate and dried in an oven, at 60°C, for approximately 30 minutes. In order to remove the outer shell membrane the sections were subsequently placed, membrane side uppermost, in a Nanotech 100 plasma chemistry unit and subjected to the non-destructive technique of plasma etching for four hours (Reid, 1983). During this treatment the specimens are surrounded by an atmosphere of oxygen gas at 133.3 Pascals, which is then made reactive by applying a radio frequency of 100 ohms. The outer shell membrane is thus removed by volatilisation leaving the inorganic, crystalline shell intact. Any remaining ash on the mammillary surface can be removed by lightly applying a pressurised aerosol.

Following plasma etching the shell samples were mounted with silver paint, mammillary side upwards, on aluminium stubs and left to dry, then placed in an Emscope sputter coater Sc 500 (Ashford, Kent, England) and coated with gold / palladium for four minutes. Each section of shell was viewed in a Phillips 501B scanning electron microscope at 15 Kv and a spot size of 1000. Ultrastructural analysis of the samples was subsequently carried out blind, in terms of day and treatment.

Within the mammillary layer of the hen's eggshell 12 ultrastructural variations have been documented and described in detail (Reid, 1984; Watt, 1989; Bain 1990; Solomon 1991). These 12 faults are illustrated in Appendix 2 (Plates A2.1, A2.2, A2.3 and A2.4). The ultrastructural organisation of the mammillary layer is scored using a weighted scoring system. This methodology was originally developed by Bain (1990) for quantifying the incidence of structural variation in the eggs from commercial layer breeds. The original scoring system has since been modified (Fraser, 1996) and is shown in Table 3.2. According to Bain (1990), the total score provides the simplest way of summerising the ultrastructural data. The total score is calculated for each egg by adding together the individual scores for each of the 12 structural variations assessed. In this thesis the total scores and the individual scores for each structural variant were considered for each egg.

CONFLUENCE	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
CAPS	GOOD 1	AVERAGE 3	BELOW AVG. 5	POOR 7	
FUSION	EARLY 1	MAINLY EARLY 3	50:50 5	MAINLY LATE 7	LATE 10
ALIGNMENT	RANDOM 1	MOD. ORGAN. 3	FISSURED 5		
TYPE B	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
DEPRESSION	NONE 1	LIMITED 2	MODERATE 4	DETRIMENTAL 6	
EROSION	NONE 1	LIMITED 3	MODERATE 5	DETRIMENTAL 8	
ARAGONITE	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
TYPE A	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
RHOMBOHEDRAL CALCITE	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
CUFFING	NONE /ISOL. 7	ISOL./MOD. 3	MOD. EXT. 1		
CHANGED MEMBRANE	NONE /ISOL. 1	ISOL./MOD. 4	MOD. EXT. 7		

Table 3.2: Ultrastructural weighted scoring system for table eggs (cited from Fraser, 1996).

Ultrastructural Assessment of Cuticle

The cuticle is the final layer laid down on the egg surface prior to oviposition and premature oviposition can lead to an incomplete or absent cuticular layer. In order to determine if the eggs produced during the experimental periods had an adequate covering of cuticle, an additional section of eggshell was cut and mounted on an aluminium stub with conductive silver paint, cuticle side uppermost. Each section was then viewed in the Phillips 501B scanning electron microscope using the same settings as described previously. The cuticle covering of each egg was assessed using a modified version of the scoring system developed by Cranstoun (1992) (Table 3.3) as illustrated in Appendix 2 (Plate A2.5).

GOOD COVERING **1**	ALL OVER THE SHELL
GOOD - PATCHY **2**	PORES VISIBLE; SOME BARE AREAS
POOR COVERING **3**	PATCHES OF CUTICLE
CUTICLE-LESS **4**	VERY LITTLE OR NO CUTICLE

Table 3.3: Cuticle scoring system modified from Cranstoun (1992).

3.3.6: ADDITIONAL NON-INVASIVE MEASUREMENTS (carried out by Lord, 2001)

3.3.6.1: Faecal Collection and Analysis

This part of the study was completed by Lord (2001). Faeces were collected from birds in home pens during one day of the week prior to transfer to the experimental enclosures. Following transfer only the birds in the barren environment were observed during day 1 and faecal collection occurred continuously between 09.00 - 12.00 and 13.00 – 17.00. Observations and collections from this environment were also made between 09.00 and 12.00 on day 5. In addition daily faecal collections were taken from both pens at 08.00, 12.00 and 17.00. Selected faecal samples (afternoon samples from the control pen and experimental days one and four from both environment types and morning samples from days one and five from the barren environment only) were processed and used for glucocorticoid analysis following the protocol as described by Lord (2001). As it is likely that corticosterone exists in an altered form following excretion, this author specifically used the term glucocorticoid when reporting the results.

3.3.6.2: Behavioural Time Budget / Preference Tests

This part of the study was also completed by Lord (2001). Video recordings were taken between 09.00 and 15.00 on days 1 to 5 and the average percentage of time spent by each pair of birds in the experimental (barren or enriched) compartments was determined. This evaluated the degree of preference for the test compartment. All results were analysed and are fully detailed in Lord (2001).

3.3.7: STATISTICAL ANALYSIS OF EGGSHELL QUALITY DATA

The data from both replicate trials were collated and data from all experimental periods were treated as a repeated measure design with 8 repeated experimental periods. The results from the control pens during the week prior to each set of experimental periods was also collated and used as Day 0 (Control) for each analysis. Day averages were calculated for all measures and used within the statistical models described.

3.3.7.1: Superficial Eggshell Abnormalities

DAY EFFECT *– To establish if the effect of moving birds into the novel experimental pen changed with time after the event:*

These data were highly non-parametric and so Friedman's test (non-parametric equivalent of the randomised block design for parametric data) was used with Day fitted as Treatment and Pair fitted as Block. In order to use this test the model was simplified with no random or interaction terms.

ENVIRONMENT EFFECT *– To establish if the bird's response to the move was affected by the environment type (Enriched or Barren) of the novel pen:*

These data again did not fit the assumptions required by a parametric test and therefore data were analysed separately for each day using a Mann-Whitney Test.

3.3.7.2: Physical Eggshell Quality Data

DAY EFFECT:

These data were parametric but unbalanced due to missing values and therefore General Linear Models (GLM) were used. Each pair of birds was treated as an independent unit throughout the statistical analyses as eggs were collected on a pen basis and so each bird cannot be treated as independent from the other bird within a pair. The following model was fitted to all the physical parameters measured:

*Response = Day + Environment + Day*Environment + Pair (Environment)*

Random factor: Pair

Fixed factors: Day and Environment

Pair and environment were both used as fixed factors. Pair was nested within environment as each pair of birds only experienced one environment type during the course of each trial. Pair was also fitted as a random factor as these birds are representative of the population and are not of particular individual interest. An interaction term between Day and Environment (Day*Environment) was included to check for any significant differences in patterns seen between each pen type with day. When the interaction term was found to be non-significant then this was dropped from the model and the GLM was re-run using Bonferroni pair-wise comparisons to find the source of any significant differences. Adjusted critical *P*-values were used to counter the problem of multiple comparisons between the treatment levels (days) and the inherent increased likelihood of a type 1 error.

ENVIRONMENT EFFECT:

The environment factor in the full model may be used to detect differences in eggshell data between the enriched and barren environments. However in order to test more specifically whether the bird's response to the move was affected by a particular environment, and if so on which days, the wire and litter pen data was analysed separately using a GLM with the simplified model: *Response = Day + Pair*

Random Factor: Pair

Fixed Factor: Day

Bonferroni pair-wise comparisons, with adjusted critical *P*-values, were used on the significant data to find the source of these differences.

3.3.7.3: Ultrastructural Data

DAY EFFECT:

All ultrastructural data were found in general to be highly non-parametric and so did not lend themselves to being transformed into parametric form. For this reason Friedman's test (non-parametric counterpart to the randomised block design for parametric data) was used on Enriched and Barren ultrastructural data separately. In order to use this test the model was simplified with no interaction term and pair was not considered as a random factor as the general model could not be developed for use with non-parametric data.

Model: *Response = Day + Pair*

With Day fitted as treatment and Pair fitted as block

A Wilcoxon Signed-Rank test was used, on differences between control (day 0) data and day means, to carry out post-hoc comparisons where required, and Bonferroni adjustments were again made.

ENVIRONMENT EFFECT:

The ultrastructural data, as previously mentioned, were highly non-parametric and so did not lend themselves to being transformed into parametric form. For this reason a Mann-Whitney test comparing enriched and barren data was used on results from each experimental day separately.

All statistical analyses were carried out using Minitab release 12.1 (© Minitab Inc., 1998).

3.4: EGGSHELL QUALITY RESULTS

3.4.1: SUPERFICIAL EGGSHELL ABNORMALITIES

No significant day or environment effect was found in the proportion of abnormal shells produced.

3.4.2: PHYSICAL MEASURES OF EGGSHELL QUALITY

No interactions were detected within the physical data for day and environment and so the interaction term was dropped from the model.

3.4.2.1: Day Effect

A significant effect of day was found with regard to total shell thickness, T. Mamm. and T. Effective (Table 3.4). Fresh weight revealed no significant differences and so no further analyses were required.

	Fresh Weight	**Total Thickness**	**T. Mamm.**	**T. Effective**
Day	F=1.61 df = 5 *P*=0.167	**F=6.31** **df = 5** ***P*<0.001**	**F=3.62** **df = 5** ***P*=0.006**	**F=3.14** **df = 5** ***P*=0.013**

Table 3.4: Test statistics and P-values for effect of day on physical data.

Total Shell Thickness: This measure decreased significantly in eggs laid on day 3 (Figure 3.2). Further analysis revealed significant differences between day 3 and all other days apart from day 2 ($P \leq 0.015$; *Appendix 3 Table A3.1*). Day 3 corresponds to the Wednesday after the birds were moved into the experimental pens.

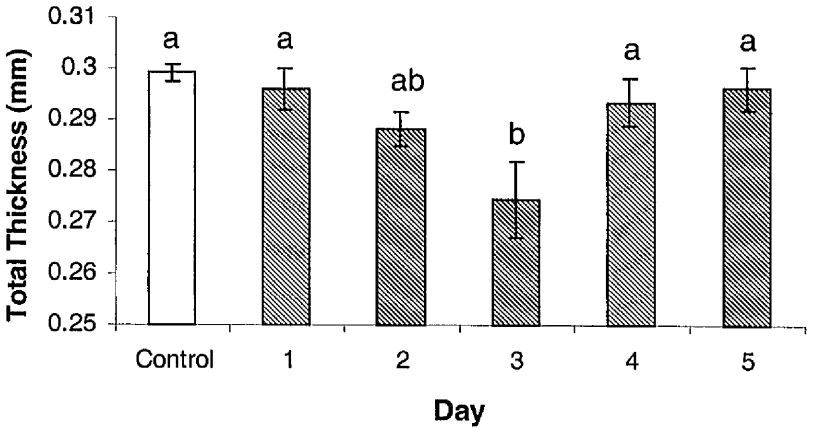


Figure 3.2: Pen means (± S.E.) for total shell thickness with day. Columns sharing letters are not significantly different (P>0.05).

Mammillary Thickness: This measure decreased significantly between day 1 and day 3 (*P*=0.031; Figure 3.3; *Appendix 3 Table A3.2*).

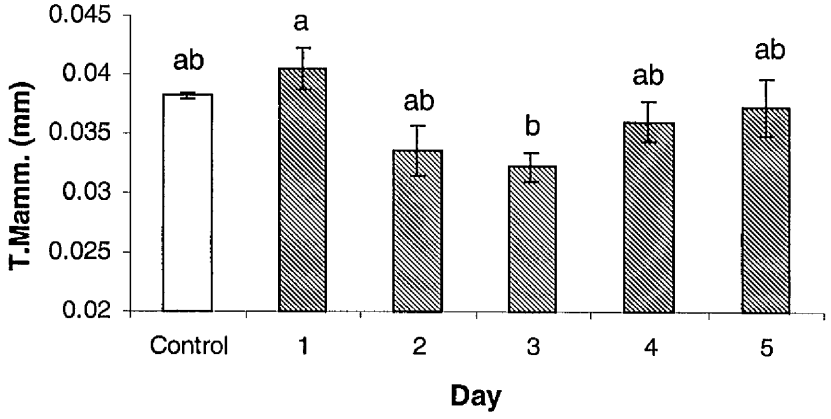


Figure 3.3: Pen means (± S.E.) for mammillary thickness with day. Columns sharing letters are not significantly different (P>0.05).

Effective Thickness: This measure followed a similar pattern to total shell thickness with a significant decrease between the control and day 3 (*P*=0.014) and a significant increase between day 3 and day 5 (*P*=0.044; Figure 3.4; *Appendix 3 Table A3.3*).

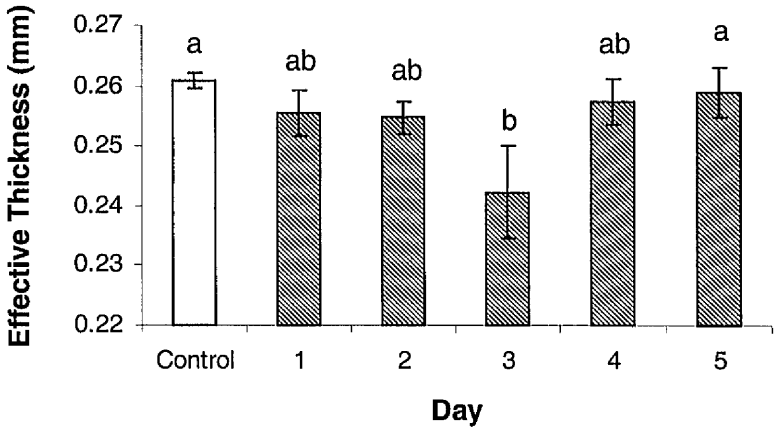


Figure 3.4: Pen means (± S.E.) for effective thickness with day. Columns sharing letters are not significantly different (P>0.05).

3.4.2.2: Environment Effect

No significant effect of environment (enriched vs. barren) was found for any of the physical measurements within the full model (Table 3.5).

	Fresh Weight	Total Thickness	T. Mamm.	T. Effective
Environment	F=0.37 df = 1 *P*=0.550	F=1.98 df = 1 *P*=0.182	F=0.82 df = 1 *P*=0.380	F=1.62 df = 1 *P*=0.224

Table 3.5: Test statistics and P-values for environment (enriched or barren) effects on physical data.

When enriched and barren data were analysed separately however a significant day response was found for total shell thickness in eggs from the enriched pen (Table 3.6). The results from this pen also showed a difference in T.Effective with day but this was only at the $P<0.1$ level. The data from the barren environment revealed significant differences in total shell thickness and mammillary thickness.

	Total Thickness	*T. Mamm.*	*T. Effective*
Enriched Pen	**F=4.01** **df = 5** ***P*=0.005**	F=1.35 df = 5 *P*=0.268	*F=2.21* *df = 5* *P=0.075*
Barren Pen	**F=2.74** **df = 5** ***P*=0.035**	**F=2.70** **df = 5** ***P*=0.037**	F=1.16 df = 5 *P*=0.351

Table 3.6: Test statistics and P-values for day effects on physical data from both environment types.

Total Shell Thickness: Significant changes in this measure were found in eggs from both enriched and barren pens. However the differences were more pronounced in the enriched pen with the total thickness decreasing significantly between control and day 3 (*P*=0.002; Figure 3.5; *Appendix 3 Table A3.4*).

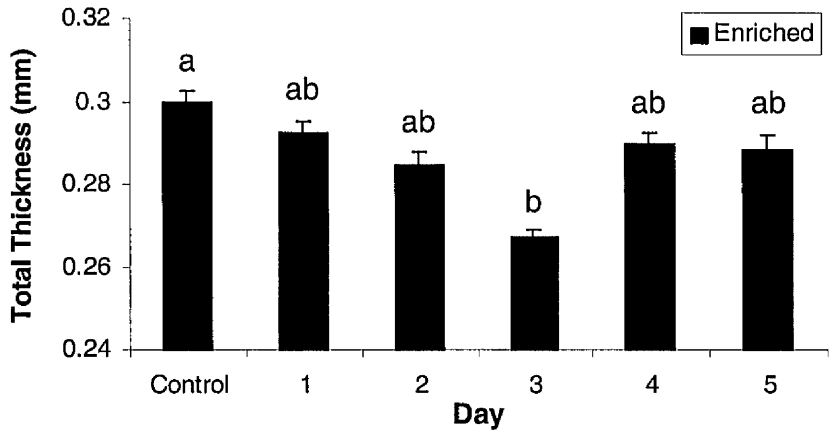


Figure 3.5: Pen means (± S.E.) for total thickness in relation to day for enriched data only. Columns sharing letters are not significantly different (P>0.05).

The data from the barren pen showed a similar but less pronounced pattern (Figure 3.6). Further analysis on the barren pen results however revealed no significant differences between any of the days with respect to this measure.

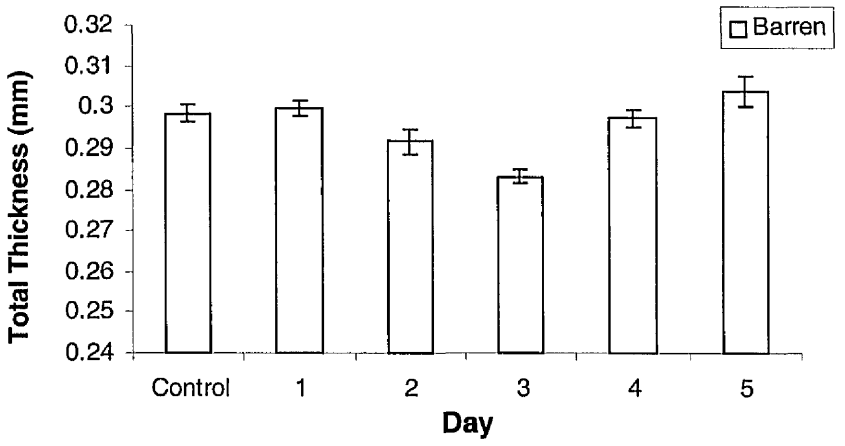


Figure 3.6: Pen means (± S.E.) for total thickness in relation to day for barren data only.

Mammillary Thickness: The results from the barren environment also showed significant differences with respect to T. Mamm. It appeared that there was a decrease in T.Mamm. in eggs from days 2 and 3 (Figure 3.7) however further analysis revealed no significant differences between any of the days with respect to this measurement.

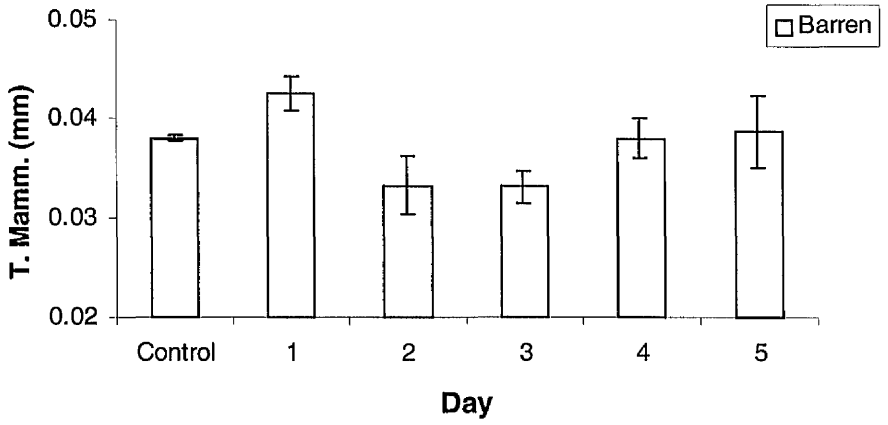


Figure 3.7: Pen means (± S.E.) for mammillary thickness in relation to day for barren data only.

3.4.3: Ultrastructural Measures of Eggshell Quality

3.4.3.1: Day Effect

In these analyses data from each pen type were analysed separately. The data from the Enriched pen showed significant differences in relation to total score, cap score and the cuticle score. In contrast the data from the barren pen only showed significant differences in relation to the cuticle score (Table 3.7).

	Enriched	Barren
Total Score	**S=10.74 df=4 *P*=0.030**	S=1.85 df=4 *P*=0.764
Confluence	S=5.84 df=4 *P*=0.212	S=6.20 df=4 *P*=0.185
Caps	**S=11.48 df=4 *P*=0.022**	S=0.98 df=4 *P*=0.912
Fusion	S=7.76 df=4 *P*=0.101	S=5.71 df=4 *P*=0.222
Alignment	S=4.00 df=4 *P*=0.406	S=2.92 df=4 *P*=0.572
Type B	S=3.42 df=4 *P*=0.490	S=1.29 df=4 *P*=0.862
Depression	S=2.58 df=4 *P*=0.630	S=2.93 df=4 *P*=0.569
Erosion	S=2.26 df=4 *P*=0.688	S=2.43 df=4 *P*=0.657
Aragonite	S=4.56 df=4 *P*=0.336	S=1.00 df=4 *P*=1.000
Type A	S=6.95 df=4 *P*=0.139	S=2.62 df=4 *P*=0.623
Rhombohedral Calcite	S=4.43 df=4 *P*=0.350	S=1.18 df=4 *P*=0.881
Cuffing	S=2.90 df=4 *P*=0.575	S=5.47 df=4 *P*=0.242
C. Membrane	S=3.44 df=4 *P*=0.486	S=2.15 df=4 *P*=0.707
Cuticle	**S=22.68 df=4 *P*<0.000**	**S=17.79 df=4 *P*=0.001**

Table 3.7: Adjusted P-values and Friedman test statistics (S) for data from each environment.

Enriched Pen

In the enriched pens the total score significantly decreased between the control and day 2 (P=0.014) and then displayed a significant increase again between day 2 and day 5 (P=0.042; Figure 3.8; *Appendix 3 Table A3.5*).

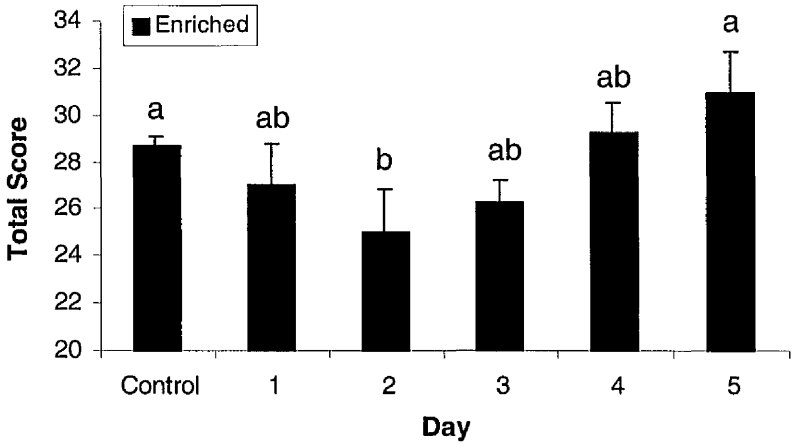


Figure 3.8: Pen means (± S.E) for total score in relation to day for enriched data only. Columns sharing letters are not significantly different (P>0.05).

The data from the Enriched pens showed a decrease in cap score between control and day 2 (Figure 3.9) but further analysis only revealed significant differences between day 2 and day 3 (*P*=0.036; *Appendix 3 Table A3.6*).

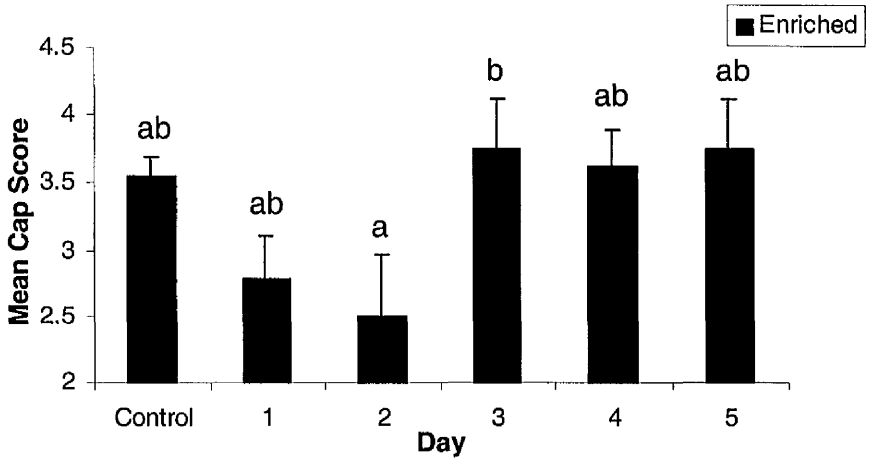


Figure 3.9: Pen means (± S.E) for cap score in relation to day for enriched data only. Columns sharing letters are not significantly different (P>0.05).

The Enriched pen revealed a significant decrease in cuticle score between day 1 and day 3 (*P*=0.30) and then a significant increase between day 3 and day 5 (*P*=0.014; Figure 3.10). Further analysis demonstrated significant differences between most of the days (*Appendix 3 Table A3.7*).

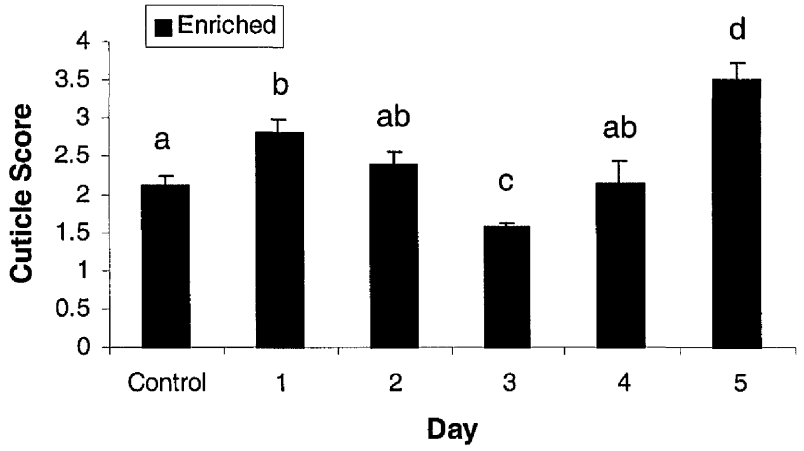


Figure 3.10: Pen means (± S.E) for cuticle score in relation to day for enriched data only. Columns sharing letters are not significantly different (P>0.05).

Barren Pen

The cuticle scores from the barren pens varied throughout the week but did not follow any obvious pattern (Figure 3.11). Further analysis again revealed that significant differences existed between most of the days ($P \leq 0.044$; *Appendix 3 Table A3.8*).

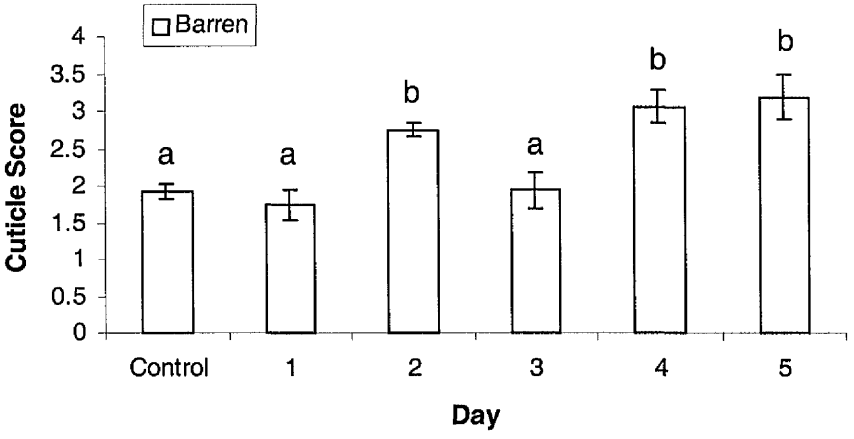


Figure 3.11: Pen means (± S.E) for cuticle score in relation to day for barren data only. Columns sharing letters are not significantly different (P>0.05).

3.4.3.2: Environment Effect

The data for each pen type were compared on each day. Significant differences were found in relation to the cuticle scores on Day 1, Type A Bodies on Day 2 and the cuticle scores on Day 4 (Table 3.8).

	Day 1	Day 2	Day 3	Day 4	Day 5
Confluence	W=34 *P*=0.438	W=65 *P*=0.770	W=64 *P*=1.000	W=70 *P*=0.836	W=79.5 *P*=0.225
Caps	W=33.5 *P*=0.371	W=63 *P*=0.627	W=75 *P*=0.200	W=73.5 *P*=0.568	W=77 *P*=0.316
Fusion	W=37 *P*=0.800	W=55.5 *P*=0.169	W=68 *P*=0.662	W=80.5 *P*=0.175	W=67 *P*=0.945
Alignment	W=38 *P*=1.000	W=60.5 *P*=0.396	W=52 *P*=0.157	W=61.5 *P*=0.500	W=51.5 *P*=0.0612
Type B	W=43 *P*=0.504	W=63 *P*=0.561	W=62 *P*=0.843	W=71 *P*=0.762	W=77.5 *P*=0.277
Depression	W=34 *P*=0.418	W=53.5 *P*=0.112	W=62.5 *P*=0.901	W=61.5 *P*=0.497	W=75.5 *P*=0.428
Erosion	W=39 *P*=1.000	W=68 *P*=1.000	W=64 *P*=1.000	W=68.5 *P*=1.000	W=69 *P*=1.000
Aragonite	W=38.5 *P*=1.000	W=67 *P*=1.000	W=63.5 *P*=1.000	W=68 *P*=1.000	W=68.5 *P*=1.000
Type A	W=44 *P*=0.422	**W=48.5** ***P*=0.027**	W=53 *P*=0.206	W=72 *P*=0.707	W=62 *P*=0.543
Rhombohedral Calcite	W=39.5 *P*=1.000	W=68.5 *P*=1.000	W=63 *P*=1.000	W=67.5 *P*=1.000	W=73 *P*=0.488
Cuffing	W=37 *P*=0.803	W=5835 *P*=0.335	W=54 *P*=0.253	W=74 *P*=0.552	W=76 *P*=0.408
C. Membrane	W=39 *P*=1.000	W=67.5 *P*=1.000	W=64.5 *P*=1.000	W=68.5 *P*=1.000	W=69 *P*=1.000
Total Score	W=30.5 *P*=0.198	W=56.5 *P*=0.246	W=50 *P*=0.118	W=81.5 *P*=0.171	W=77 *P*=0.371
Cuticle	**W=55** ***P*=0.012**	W=54 *P*=0.127	W=53.5 *P*=0.217	**W=49** ***P*=0.047**	W=75 *P*=0.474

Table 3.8: Mann-Whitney test statistics (W) and adjusted P-values for ultrastructural data from each day.

Day 1:

The mean score for cuticle was significantly higher in the enriched pen on day 1 (*P*=0.012; Figure 3.12).

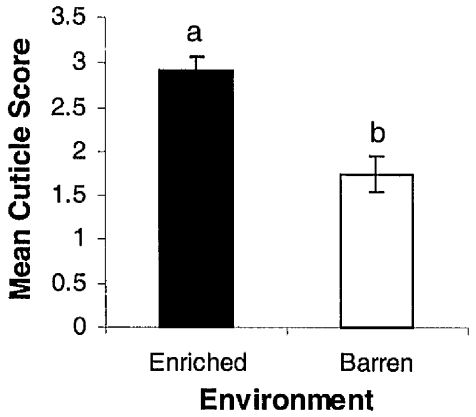


Figure 3.12: Comparison of scores for cuticle in enriched vs. barren pens during day 1. Columns sharing letters are not significantly different (P>0.05).

Day 2:

The mean score for type A bodies was significantly higher in the barren pen during day 2 (P=0.027; Figure 3.13). There were no significant differences with this score on any other day.

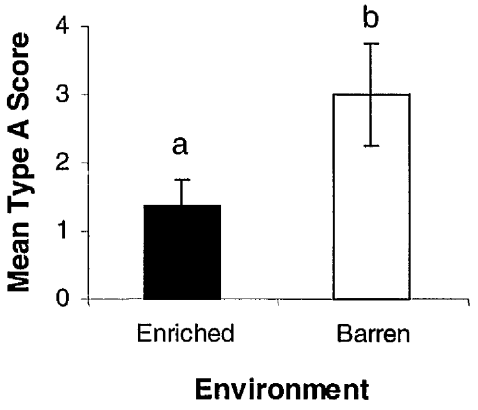


Figure 3.13: Comparison of scores for type A's in enriched vs. barren pens during day 2. Columns sharing letters are not significantly different (P>0.05).

Day 4:

The mean score for cuticle was significantly higher in the barren pen on day 4 (P=0.047; Figure 3.14) which is opposite to the effect recorded on day 1.

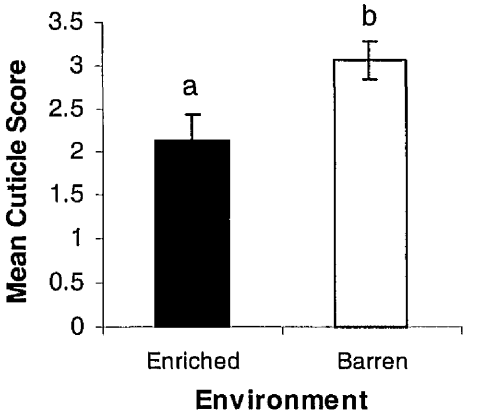


Figure 3.14: Comparison of scores for cuticle in enriched vs. barren pens during day 4. Columns sharing letters are not significantly different (P>0.05).

3.5: Results from Additional Non-Invasive Measures

3.5.1: Faecal Glucocorticoid Levels (as reported by Lord, 2001)

Afternoon Glucocorticoid Levels:

The faecal glucocorticoid (GC) levels from both environments differed significantly between days (Table 3.9) with levels on day 1 being higher than on day 4 (Figure 3.15). Environment did not quite reach statistical significance although Figure 3.15 shows a slightly higher level of glucocorticoid in the enriched environment. There was no interaction between day and environment.

Variable	F, df	*P*-value
Pair	0.80, 14	0.660
Day	7.98, 1	0.014*
Environment	3.52, 1	0.080
Day * Environment	0.07, 1	0.790

Table 3.9: ANOVA table of faecal GC comparisons between days and environments (Table taken from Lord, 2001).

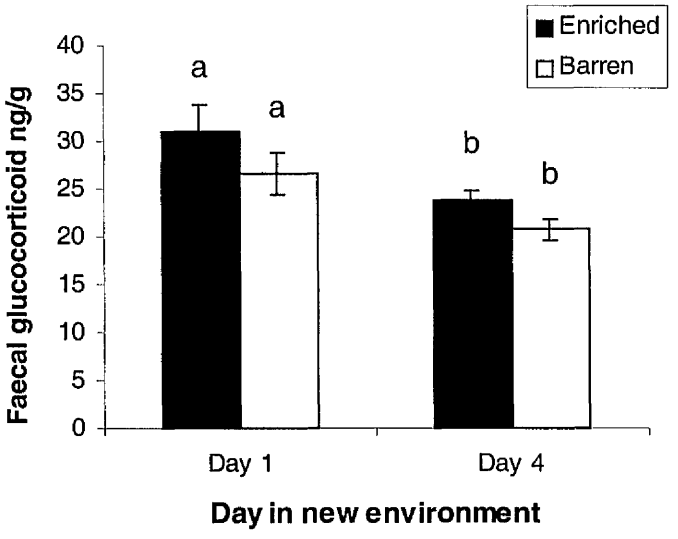


Figure 3.15: Faecal GC (mean ± S.E.) of enriched and barren birds on days 1 and 4 of transfer. Columns sharing letters are not significantly different (P>0.05) (Graph taken from Lord, 2001).

There was a significant effect of day on the pre vs. post-transfer comparison of the birds transferred to the barren environment (Table 3.10). On day one glucocorticoid levels were significantly higher than on the pre-transfer day (Figure 3.16).

	Mornings (11.00 – 12.00)		Afternoons (12.00 – 17.00)	
Variable	F, df	*P-value*	F, df	*P-value*
Day	1.46, 1	0.272	6.28, 2	0.011*
Pair	0.70, 6	0.676	2.70, 7	0.054

Table 3.10: ANOVA table of GLM results for birds transferred to the barren environment (Table taken from Lord, 2001).

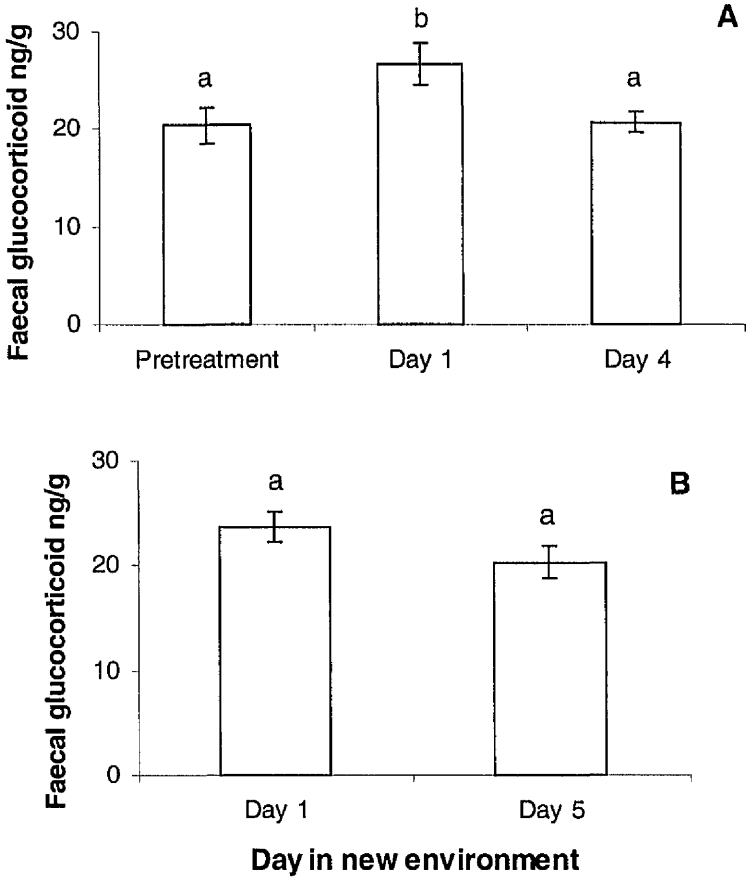


Figure 3.16: Faecal GC (mean ± S.E.) of barren birds from (A) 12.00-17.00 and (B) 11.00-12.00 on different days. Columns sharing letters are not significantly different (P>0.05) (Graph taken from Lord, 2001).

Morning Glucocorticoid Levels:

In the morning the faecal glucocorticoid levels in birds kept in the barren environment were not significantly different between day 1 and day 5 (Figure 3.16).

3.5.2: BEHAVIOURAL OBSERVATIONS (as reported by Lord, 2001)

The birds with access to an enriched area spent significantly more time in the latter than birds with a barren environment ($F=10.68$, $P=0.002$). The amount of time spent in the experimental compartment increased significantly over the five experimental days.

3.6: DISCUSSION

Handling and relocation have both been shown to be potent fear elicitors in birds (Craig and Craig, 1985; Jones, 1996b) and within this study the experimental treatments were found to have significant effects on several of the measures recorded.

The behavioural results reported by Lord (2001) demonstrated that the birds placed into the enriched pens spent a significantly higher proportion of time within the experimental area compared to the birds placed into the barren environment. This can be crudely interpreted as a preference for an enriched environment (Dawkins, 1981; 1983b). Dawkins (1983a) found that birds, when given the choice between litter flooring and wire flooring, tended to show a preference for the litter flooring.

The HPA response, traditionally detected by calculating the levels of circulating corticosterone within the blood plasma, was, for the purpose of this study, measured non-invasively by means of glucocorticoid levels present within the faeces. The faecal analysis, conducted by Lord (2001), revealed a significant increase in faecal glucocorticoid concentrations following the move to an experimental pen and then a decrease in levels between days one and four (Figures 3.15, 3.16). This author also reported no significant effect in terms of the environment into which the bird was transferred, although there was a trend for increased levels in birds moved into the enriched pens. The latter observation supports the views of both Rushen (1986) and Broom (1988) who demonstrated that corticosterone elevation is often caused by non-aversive stimuli. The increased glucocorticoid levels could

also be explained by increased activity in these birds as they were able to engage in dust-bathing, pecking and scratching behaviours. Lord (2001) however commented that this was unlikely as the birds in this experiment were relatively inactive on the morning following the transfer.

Within this study shell thickness measurements were affected following the move from the control pen to an experimental pen viz thickness was compromised and reached a minimum on day 3 (48 hours following the move) (Figures 3.2, 3.4). If the elevated faecal glucocorticoid, reported by Lord (2001), is influencing the shell formation process, it is possible that it may be doing so via a mechanism involving the mobilisation of glycogen stores at the expense of lipid and protein production. Shell matrix proteins are produced in variety of tissues including the liver and the oviduct itself (Hincke *et al.*, 1992). These proteins have a fundamental role to play in the shell formation process in so far as they appear to direct crystal growth. Any impairment in their production and or release will ultimately manifest itself in terms of a change in a range of shell parameters such as thickness. Sahin and Forbes (1999) reported that the feeding of birds with corticosterone over a four week period resulted in reduced egg production and reduced protein efficiency for egg production accompanied by a higher rate of fat deposition. These authors hypothesised that this may be due to the increased demand for protein used for fat anabolism. Obviously these authors were demonstrating a chronic effect of increased corticosterone levels whereas the current study only elicited a short-term effect and as such was unlikely to have caused such severe results. Nevertheless a slight change in the protein balance would be sufficient to alter the shell formation process.

Jones (1987a) demonstrated that all novelty is a fear elicitor but, within this experiment, the type of environment (barren or enriched) into which a bird was moved did not have any significant effect on the physical measures of egg quality assessed. When data from each pen were analysed separately however it was shown that birds placed in the enriched pen exhibited greater changes in shell thickness following the move compared to the birds placed in the barren environment. This difference can perhaps be explained by a stronger reaction from these birds to the increased novelty in this pen compared to the barren pen.

Many authors have reported changes in the ultrastructure of the eggshell following a stress event (Solomon *et al.*, 1987; Watt, 1989; Brackpool, 1995). Thus, shells tend to display a variety of adverse intra-shell defects immediately following a stress experience. Within the

current study ultrastructural analyses revealed a significant effect of day in relation to specific shell inclusions but only within the eggs from the enriched pen. Thus the cap score and total score values were reduced 24 hours after the birds were placed in the enriched pen (Figures 3.8, 3.9) indicating improved eggshell quality. In the absence of supporting evidence relating to fracture toughness and breaking strength, the effect of these ultrastructural changes on shell performance must remain unqualified. Nevertheless it would appear that there has been a compensatory mechanism, at the ultrastructural level, in response to the decline in shell thickness which became significant on day 3 in the birds placed into the enriched environment. Brackpool *et al.* (1993) reported a similar compensation effect, at the level of the mammillary layer, during a time of reduced shell thickness following heat stress. Differences were also found in relation to cuticle score as on day 1 they were significantly higher in the enriched pen (i.e. a poorer covering) but by day 4 the cuticle covering was poorer in the barren pen. These conflicting results cannot at present be explained but it is worth noting that the results from both pens are not suggestive of premature oviposition as no cuticle-less eggs were observed.

Jones (1996b) cautioned that it was important to avoid applying too much environmental enrichment at any one time or to suddenly transfer an animal from a barren home cage into an unfamiliar enriched one as novelty is a potent fear elicitor (Hogan, 1965; Jones *et al.*, 1987a; Boissy, 1995). The present results substantiate this hypothesis in so far as the enriched environment, with its diversity of inclusions, triggered an elevated faecal glucocorticoid response together with undesirable changes in shell quality.

Within this study there were many potential stress factors involved in the move from control pen to experimental pen, including removal from con-specifics, handling and introduction to a novel environment. Lord (2001) noted increased aggression between the experimental pairs on day 2, and this appeared to be equally common in both experimental environments, an effect previously reported by Choudary and Craig (1972) who found that relocating hens results in an increase in agonistic behaviour. To substantiate these findings Lord (2001) acknowledged the requirement for increased behavioural observations in future studies. It is likely that these variables have compounded the stress response recorded and for this reason experiment 2 was designed to eliminate some of the confounding variables by simplifying the stressors applied.

3.7: CONCLUSIONS

The birds provided with an enriched area within the experimental pen spent significantly more time in that area than those with a barren area indicating a preference for the enriched pen type.

Faecal glucocorticoid levels were found to increase significantly on the day of the move from control pen into experimental pen, irrespective of the pen type into which the birds were transferred. This result suggests that the glucocorticoid response is occurring as a reaction to the move and novel surroundings rather than the specific features of each environment. Faecal levels of glucocorticoid returned to pre-stress levels by four days after the move.

With respect to physical eggshell quality, total thickness and effective thickness decreased significantly between the control pen and day 3 following the move, and returned to pre-stress levels by day 5. This change in shell thickness was more pronounced in the birds moved into the enriched pen suggesting that the increased novelty of the pen may be sufficient to induce a stronger fear reaction.

3.8: EXPERIMENT 2

Stressors can be broadly split into two types viz acute (short-term), such as handling or restraint, and chronic (long-term), such as environmental stress within a home pen (Terlouw *et al.*, 1997). The effect these stressors have on the animal differs and in the case of chronic stressors, following an initial increase in glucocorticoid secretion, physiological habituation to the condition occurs. Such differences complicate the interpretation of results from welfare studies.

Partial beak amputation (beak-trimming), which is often performed on commercially reared poultry in order to control feather pecking and cannibalism, has been shown to produce a number of significant alterations to bird behaviour (Gentle *et al.*, 1990). For example birds were found to peck less at their environment, and there was a reduction in head shaking and beak wiping, two activities associated with removing food particles from the mouth area. In another experiment looking at beak-trimmed birds it was observed that food consumption was significantly reduced in the first few days after trimming (Struwe *et al.*, 1992). This procedure is considered as an acute stressor as it involves both handling and short-term restraint and Mench (1992) has reported that this process probably also causes pain to the birds.

Many authors have commented on the existence of certain behavioural "needs" in poultry (Meijsser and Hughes, 1989; Vestergaard, 1981, 1982). For example Duncan (1970) and Kite (1986) reported that hens deprived of a nest site delay egg laying and retain their eggs. A nesting response is triggered by ovulation followed by the release of oestrogen and progesterone from the follicle (Wood-gush and Gilbert, 1973). Nesting motivation in the hen is very strong (Baxter, 1994) with hens willing to use trap-nests even when it, as a consequence, results in them being deprived of food and water (Duncan, 1978b). In order to gain access to a nest-box hens will also learn to push through weighted doors and run a gauntlet of footbaths or blasts of air (Duncan and Kite, 1987). These studies imply that nest-boxes are a behavioural requirement of hens.

Vestergaard (1989) reported that birds from both rich and poor environments developed dust-bathing behaviour but the ones from the poor environment showed a reduced behavioural repertoire. The author suggested that these birds also tended to be the ones which were more fearful. Mench (1992) stated that both concealment and a suitable substrate appear to be important for laying.

3.9: AIMS

Beuving and Vonder (1978) reported that acute stress, involving handling, induced elevated corticosterone secretion. The effects of such treatment on non-invasive measures including eggshell quality and faecal glucocorticoid levels have not been documented. The following experiment set out to establish if there was a stress-induced change in eggshell quality following an acute stressor (beak and claw trimming procedure). Also given that many authors have commented on the need for nest-boxes in laying hens (Duncan, 1978b; Baxter, 1994), the effect of moving birds from an enriched environment containing a nest-box and litter into a barren environment or vice-versa was also investigated. As these procedures consisted of both an acute stressor (beak and claw trim) and a chronic stressor (environment change), this experiment was also intended to determine if the bird's response, in terms of eggshell quality, depended on the type of stressor experienced. In addition to changes in eggshell quality, bird behaviour and faecal glucocorticoid levels were monitored simultaneously by Lord (2001).

3.10: MATERIALS AND METHODS

3.10.1: BIRDS AND HOUSING

The birds used were ISA Brown commercial layers, which were aged 18 weeks when they were moved into the experimental pens. The birds were placed in pairs into one of twelve pens (eight sized 74 x 52.5cm and four sized 72.5 x 55cm; Plate 3.1). Six of the pens were 'Enriched' and contained a litter substrate, nest-box and pecker block. The other six pens were 'Barren' and contained only a wire mesh floor (2.5 x 2.5cm, 19 gauge) suspended 7cm off the ground and underlined with newspaper (Plate 3.2). Pen walls extended up to the ceiling thus visual contact with the neighbouring birds was prevented. Both pen types contained identical feeders and drinkers and were cleaned twice a day. The dark: light regime was 14h : 10h

Plate 3.1: The experimental pens.

Plate 3.2: An example of a barren and enriched pen. LEFT: The barren pen consisting of a wire flooring. RIGHT: The enriched pen consisting of a litter flooring, pecker block and nest-box.

3.10.2: EXPERIMENTAL PROCEDURES

3.10.2.1: Beak and Claw Trimming Procedure (Acute Stress)

At 23 weeks of age all the birds were beak-trimmed in a pre-determined order of events. The procedure began at 10:15. Both birds were removed from each consecutive pen and each was placed in a ventilated cardboard box for 20 seconds. One of the birds was then placed in an opaque nylon sack and weighed by suspending the sack from a balance. Once the weight had been recorded, the bird was fed with a colour capsule by forcing the beak open and inserting the capsule to back of the throat with a finger[1]. The bird's beak was then lightly trimmed using a pair of scissors. This involved the removal of the tip of the upper and lower mandible and care was taken to remove only the non-innervated beak tip. A semi-permanent plastic 'bit', designed to prevent feather pecking, was then fitted between the upper and lower mandibles by placing the ends in the nostrils. Following this the bird's claws were clipped. It was then returned to its box and the process repeated with the second bird. After these procedures each pair of birds was returned to their original pen.

3.10.2.2: Swap Procedure (Chronic Stress)

At 28 weeks of age each pair of birds was transferred from their original pen type between 11.30am and 12.15 pm. The swap order was pre-determined and ensured that each pair moved from either a 'Barren' pen to an 'Enriched' pen or vice-versa. Hence there were two possible experimental treatments existing within this swap i.e.:

Treatment 1 – Movement from 'Barren' to 'Enriched'

Treatment 2 – Movement from ' Enriched' to 'Barren'

3.10.3: EGG COLLECTION

The eggs were collected from each pen for one week prior to and one week after the beak and claw trim procedure, and one week prior to and one week post the swap procedure. The eggs were identified according to pen number and date collected.

[1] This procedure was used as part of a pilot study attempting to dye eggs and faeces using a lipid soluble marker to facilitate individual bird identification. The dye was delivered orally in a gelatine capsule and results are presented in Appendix 4.

3.10.4: EGGSHELL QUALITY ANALYSIS

3.10.4.1: Superficial Shell Abnormalities

The eggs were examined as detailed in section 2.3.5.1.

3.10.4.2: Physical Measures of Eggshell Quality

During this experiment an additional method of eggshell quality was utilised viz a non-destructive measure of deformation was used to establish if any observed change in shell thickness could be related to changes in shell stiffness.

Non-destructive Deformation: This measure was calculated for each intact egg by using the commercial apparatus manufactured by Marius N.V. (Hollantlenn 18, Utrecht, Netherlands). Non-destructive deformation was measured at three points around the equator of each egg and a mean value calculated.

Egg Weight: Eggs were weighed as described in section 3.3.5.2. of this chapter.

Shell Thickness Profiles: Preparation and eggshell analysis was carried out as described in section 3.3.5.2. of this chapter.

3.10.4.3: Ultrastructural Eggshell Quality Assessment

Ultrastructural Assessment of the Mammillary Layer

Mammillary ultrastructural analysis was conducted on eight eggs from each pen (last four eggs prior to and first four eggs following each stress event) as described in section 3.3.5.3. of this chapter.

Ultrastructural Assessment of Cuticle

Ultrastructural analysis of the cuticle was conducted on eight eggs from each pen (four eggs pre and four eggs post each stress event) as described in section 3.3.5.3. of this chapter.

3.10.5: Additional Non-Invasive Measurements (carried out by Lord, 2001)

3.10.5.1: Faecal Collection and Analysis

This part of the study was completed by Lord (2001). Separate faeces were combined into one sample as the identity of individual bird faeces was not possible. Faecal samples were collected at 15.00 for three days prior to each stress event (days 4, 6 and 7) and three days following each event (days 8, 9 and 11). All faecal samples were processed and used for glucocorticoid analysis following the protocol described by Lord (2001).

3.10.5.2: Behavioural Observations

This part of the study was also completed by Lord (2001). Behaviour was recorded during three 30 minute sessions per day (morning, afternoon and evening) pre and post stress. Video recordings were made in real time and switched from camera to camera, recording from each for 20 seconds. For every session 10 instantaneous observations per pen were obtained from each bird. All results were analysed and are described in detail in Lord (2001).

3.10.6: Egg Production

The number of eggs produced within each pen was recorded each day throughout this experiment. All production data were analysed and are described in detail in Lord (2001).

3.10.7: Statistical Analysis of Eggshell Quality Data

The data from each stress event (Beak/Claw trimming and swap procedure) were analysed separately. Days 1 to 7 corresponded to the 7 days before the stress event and days 8 to 14 corresponded to the 7 days following. Day averages were calculated for all measures and used within the statistical models described.

3.10.7.1: Superficial Eggshell Abnormalities

DAY EFFECT *– To establish if the effect of stress (Acute or chronic) changes with time after the event:*

The binomial data were used to calculate the proportions of abnormal eggs laid for each day. These data were highly non-parametric and so Friedman's test (non-parametric counterpart to the randomised block design for parametric data) was used with Day fitted as treatment and

Pair fitted as Block. In order to use this test the model was simplified with no random or interaction term.

ENVIRONMENT EFFECT *– to establish if the bird's response to the stress event was affected by the environment (Barren or Enriched) from which the bird originated or, in the case of the swap, which treatment the birds experienced (move from Barren into Enriched or Enriched into Barren):*

These data again did not fit the assumptions required by a parametric test and therefore data were analysed separately for each day using a Mann-Whitney Test.

3.10.7.2: Physical Measures of Eggshell Quality

DAY EFFECT:

In view of the fact that some results were missing, the parametric physical data were analysed by using general linear models (GLM) for unbalanced data. Each pair (pen) of birds was treated as independent units throughout the statistics as eggs were collected on a pen basis and one bird could not be considered independent from the other bird within the pen. The following model was fitted to all physical data from each stress event:

*Response = Day + Environment + Day*Environment+ Pair (Environment)*

Random factor: Pair

In the data from the 'swap environments' test the term environment was replaced by the term treatment but the model remained the same. Pair was nested within Environment as each pair of birds experienced only one environment (or treatment) type during the course of each trial. Pair was also fitted as a random factor as these birds are representative of the population and are not of particular individual interest. An interaction term between day and environment *(Day*Environment)* was included to check for any significant differences in patterns seen between each pen type with day. This, if significant, would suggest that something different was occurring, with respect to the response, depending on which environment the bird came from or was placed into. When the interaction term was found to be non-significant then this was dropped from the model and the GLM was re-run using Bonferroni pair-wise comparisons to find the source of any significant differences. Adjusted critical *P*-values were used to counter the problem of multiple comparisons between the treatment levels (days) thereby avoiding the increased likelihood of making a type 1 error.

ENVIRONMENT EFFECT:

Environment was included within the main model to check for any significant differences between results obtained from the barren pen (or barren to enriched treatment) and those obtained from the enriched pen (or enriched to barren treatment), pooled over all the days. However in order to look for any significant differences in the data collected from each pen type across the experimental days, barren (barren to enriched treatment) and enriched (enriched to barren treatment) data were analysed separately using a GLM with the simplified model: *Response = Day + Pair*

Random Factor: Pair

Bonferroni pair-wise comparisons, with adjusted critical *P*-values, were used on the significant data to find the source of these differences.

3.10.7.3: Ultrastructural Data

DAY EFFECT:

The ultrastructural data was found in general to be highly non-parametric and so Friedman's test was used on enriched and barren ultrastructural data separately with Day fitted as treatment and Pair fitted as Block.

Model: *Response = Day + Pair*

With Day fitted as treatment and Pair fitted as block

The Wilcoxon Signed-Rank test was used, to carry out post-hoc comparisons where required, and Bonferroni adjustments were again made.

ENVIRONMENT EFFECT:

The ultrastructural data, as previously mentioned, were highly non-parametric and so did not lend themselves to being transformed into parametric form. For this reason a Mann-Whitney test, comparing enriched and barren data, was used on results from each experimental day separately.

All statistical analyses were carried out using Minitab release 12.1 (© Minitab Inc., 1998).

3.11: EGGSHELL QUALITY RESULTS DURING BEAK / CLAW TRIM PROCEDURE

3.11.1: SUPERFICIAL EGGSHELL ABNORMALITIES

3.11.1.1: Day Effect

Eggs laid on days 1 – 4 appeared to exhibit more superficial shell abnormalities than those laid on days 5 – 14 (Figure 3.17), however analysis revealed that these differences were only at the $P<0.1$ level and so no further analyses were carried out.

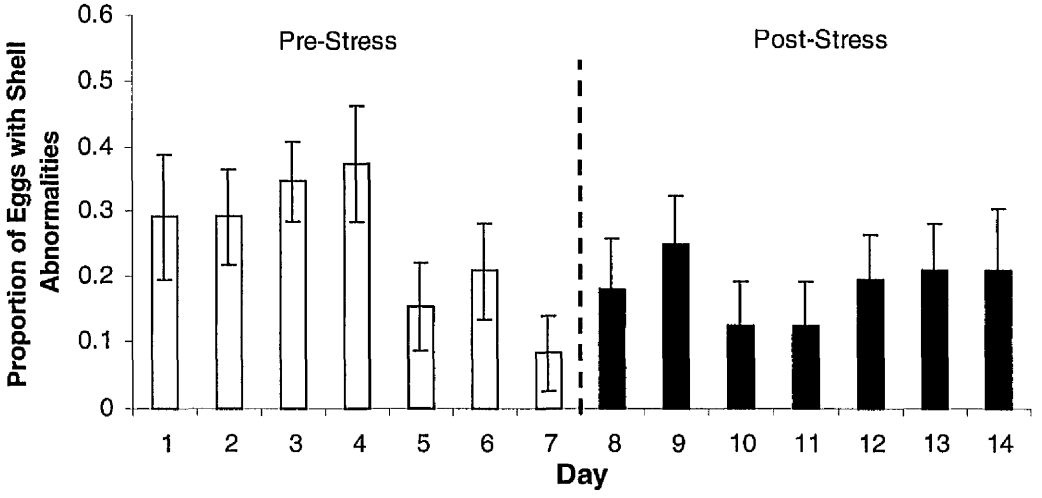


Figure 3.17: Pooled means (±S.E.) for proportion of eggs with superficial shell abnormalities for each day prior to and following the beak and claw trimming event.

3.11.1.2: Environment Effect

When data from each day were analysed separately in terms of environment, significant differences were found only during day 9 (Table 3.11). On day 9 the birds from the enriched pen laid a significantly higher proportion of eggs with abnormal shells than the birds in the barren pens (Figure 3.18).

Day	
1	W=37.5 *P*=0.859
2	W=42 *P*=0.640
3	W=38 *P*=0.928
4	W=35.5 *P*=0.584
5	W=44.5 *P*=0.336
6	W=42 *P*=0.640
7	W=33 *P*=0.174
8	W=40 *P*=0.923
9	**W=27** ***P*=0.034**
10	W=42 *P*=0.595
11	W=36 *P*=0.595
12	W=37 *P*=0.784
13	W=30 *P*=0.112
14	W=38 *P*=0.923

Table 3.11: Adjusted P-values and Mann-Whitney test statistics (W) for differences between environments for each day.

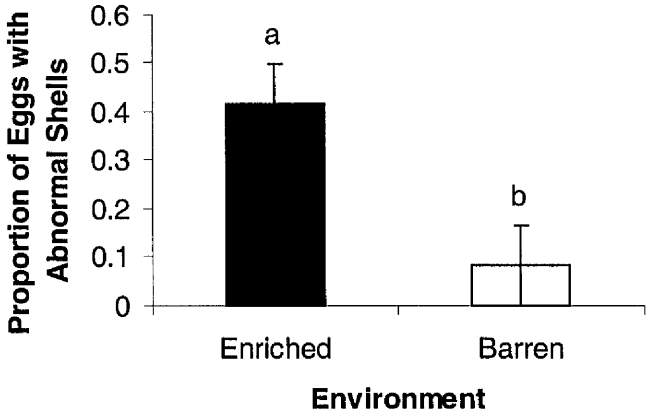


Figure 3.18: Pooled means (±S.E.) for proportion of abnormal shells with environment type during day 9. Columns sharing letters are not significantly different (P>0.05).

3.11.2: PHYSICAL EGGSHELL QUALITY

3.11.2.1: Day Effect

No interactions were detected within the physical data with regard to day and environment and so the interaction term was dropped from the model. A significant effect of day was found with regard to non-destructive deformation, total shell thickness and T. Effective (Table 3.12). Fresh weight revealed no significant differences and so no further analyses were required.

	Fresh Weight	*Non-Destructive Deformation*	*Total Thickness*	*T. Mamm.*	*T. Effective*
Day	*F=1.74* *df = 13* *P=0.058*	**F=3.00** **df = 13** ***P*=0.001**	**F=1.83** **df = 13** ***P*=0.044**	F=1.32 df = 13 *P*=0.205	**F=2.08** **df = 13** ***P*=0.018**

Table 3.12: Test statistics and P-values for day effect on physical eggshell quality data.

Non-Destructive Deformation: There was a significant increase in the measure between eggs from days 2, 5 and 6 and eggs from day 9 ($P \leq 0.017$; Figure 3.19; *Appendix 3 Table A3.9*). This measure then decreased between day 9 and days 12 ($P=0.001$) and 14 ($P=0.018$).

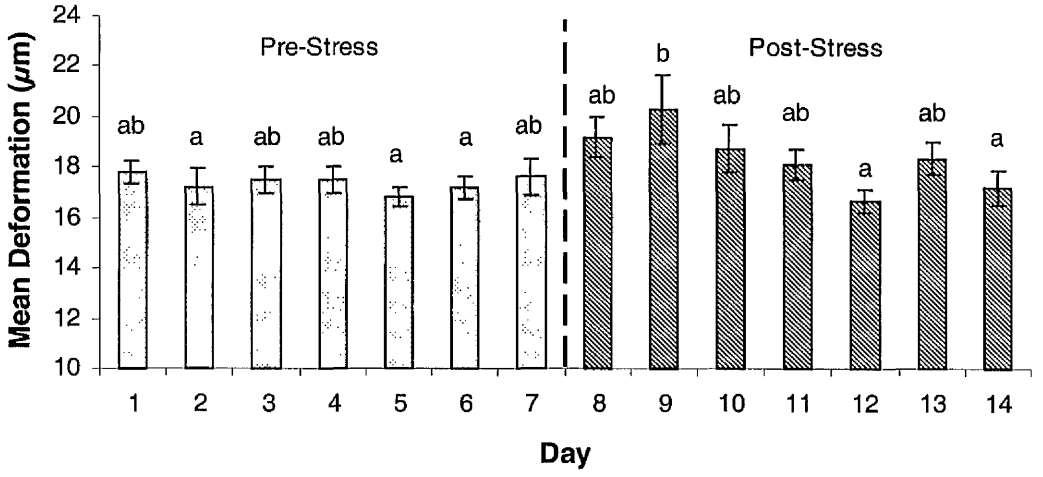


Figure 3.19: Pooled pen means (±S.E.) for non-destructive deformation measurement with day (prior to and following the beak and claw trimming event). Columns sharing letters are not significantly different (P>0.05).

Total Shell Thickness: This measure was consistent over the pre-stress days (1 to 7) and then appeared to decrease at day 9 before increasing again between day 9 and day 12 (Figure 3.20). Further analysis revealed that the only significant difference occurred between day 9 and day 12 ($P=0.05$; *Appendix 3 Table 3.10*).

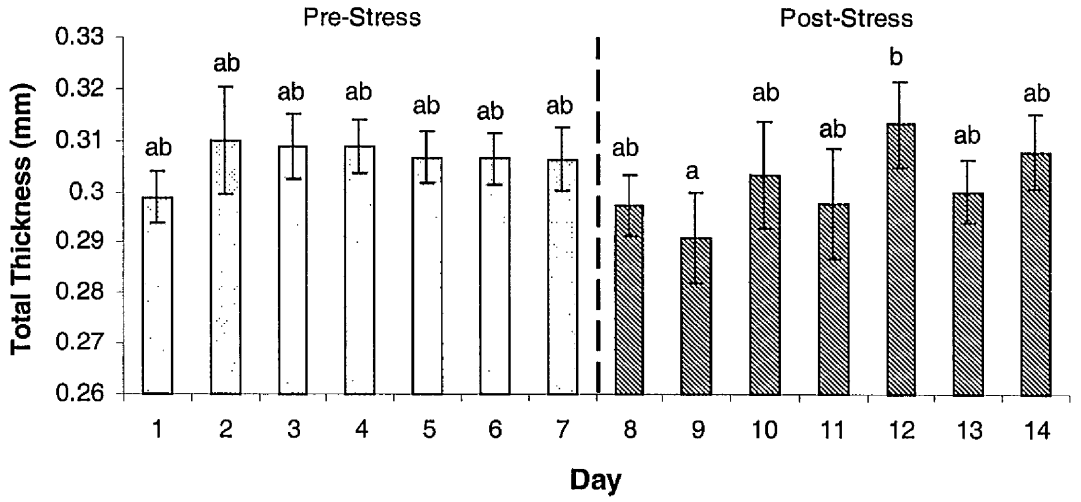


Figure 3.20: Pooled pen means (±S.E.) for total shell thickness with day (prior to and following the beak and claw trimming event). Columns sharing letters are not significantly different (P>0.05).

Effective Thickness: This measure was again consistent over the pre-stress days (1-7), thereafter regular fluctuations were detected (Figure 3.21). Further analysis also revealed a significant difference between day 9 and day 12 (*P*=0.028; *Appendix 3 Table 3.11*).

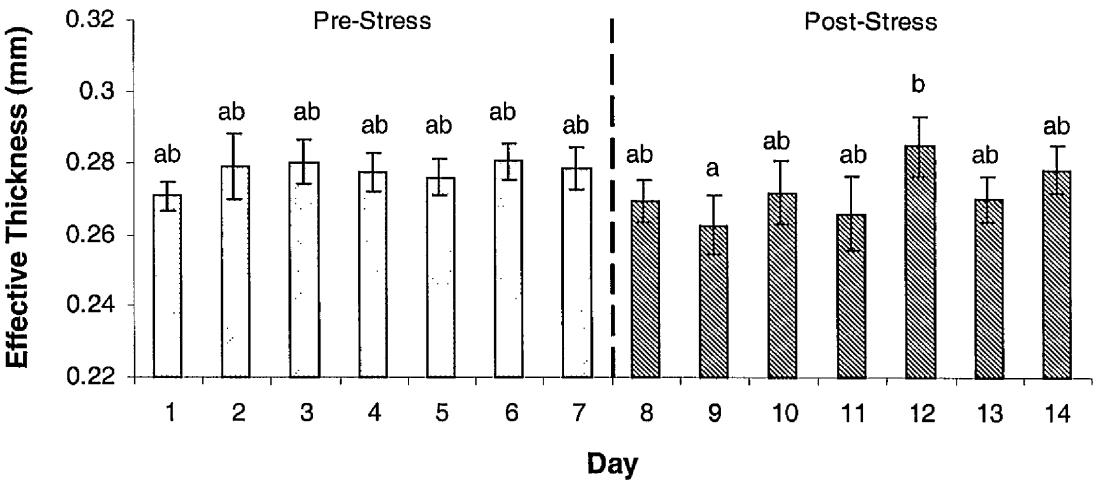


Figure 3.21: Pooled pen means (±S.E.) for effective thickness with day (prior to and following the beak and claw trimming event). Columns sharing letters are not significantly different (P>0.05).

3.11.2.2: Environment Effect

An environment effect was found in relation to non-destructive deformation and T. effective (Table 3.13). However when data from both environments were analysed separately it is clear that the birds in the enriched environment show the most significant effect with regard to these measures (Table 3.14).

	Fresh Weight	*Non-Destructive Deformation*	*Total Thickness*	*T. Mamm.*	*T. Effective*
Environment	F=0.72 df = 1 *P*=0.415	**F=7.09** **df = 1** ***P*=0.024**	*F=4.36* *df = 1* *P=0.063*	F=0.03 df = 1 *P*=0.865	**F=5.65** **df = 1** ***P*=0.039**

Table 3.13: Test statistics and P-values for environment effect on physical eggshell quality data.

	Non-Dest. Deformation	*Total Thickness*	*T. Mamm.*	*T. Effective*
Barren	F=1.51 df = 13 *P*=0.140	F=0.61 df = 13 *P*=0.835	F=1.25 df = 13 *P*=0.269	F=0.64 df = 13 *P*=0.814
Enriched	**F=2.44** **df = 13** ***P*=0.009**	*F=1.75* *df = 13* *P=0.071*	F=1.32 df = 13 *P*=0.228	**F=1.94** **df = 13** ***P*=0.041**

Table 3.14: Test statistics and P-values for day effects on physical eggshell quality data from each environment.

Non-Destructive Deformation: This measure was significantly higher in the eggs laid within the 'Barren' pens (Figure 3.22).

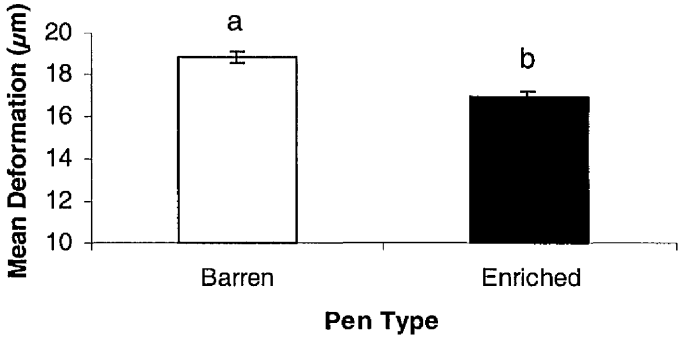


Figure 3.22 Mean non-destructive deformation (±S.E.) (pooled for all experimental days) for birds in each pen type. Columns sharing letters are not significantly different (P>0.05).

Eggs laid in the enriched pens demonstrated a significant change in non-destructive deformation with day (Table 3.14). This measure was relatively constant over the pre-stress days (1 – 7) and appeared to increase following the stress event (Figure 3.23). Further analysis revealed a significant difference between day 7 and day 9 (P=0.042; *Appendix 3 Table A3.12*).

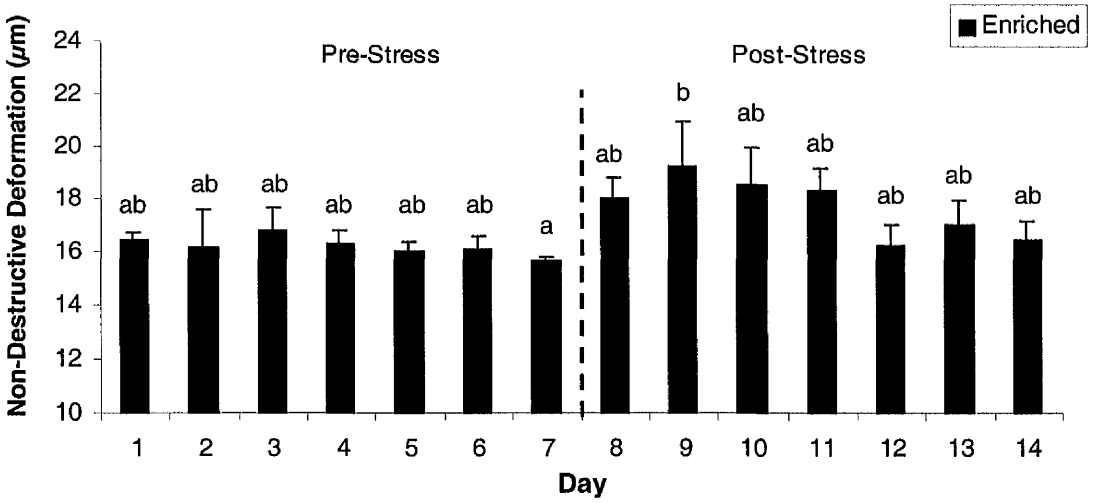


Figure 3.23: Pooled pen means (±S.E.) for non-destructive deformation with day (prior to and following the beak and claw trimming event) for enriched birds only. Columns sharing letters are not significantly different (P>0.05).

T. Effective: This measure was significantly higher in the eggs laid within the enriched pens (P=0.039; Figure 3.24).

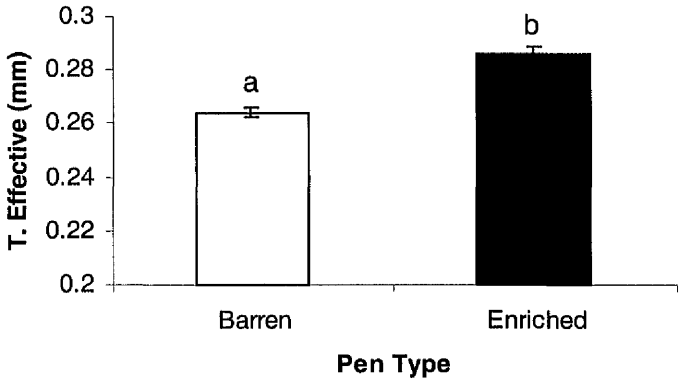


Figure 3.24: Mean T.Effective (±S.E.) (pooled for all experimental days) for birds in each pen type. Columns sharing letters are not significantly different (P>0.05).

Eggs laid in the enriched pens also demonstrated a significant change in effective thickness with day (Table 3.14). There was a slight decrease in this measure following the procedure but

further analysis failed to reveal any significant differences, indicating that the effect could not be attributed to a particular day (Figure 3.25).

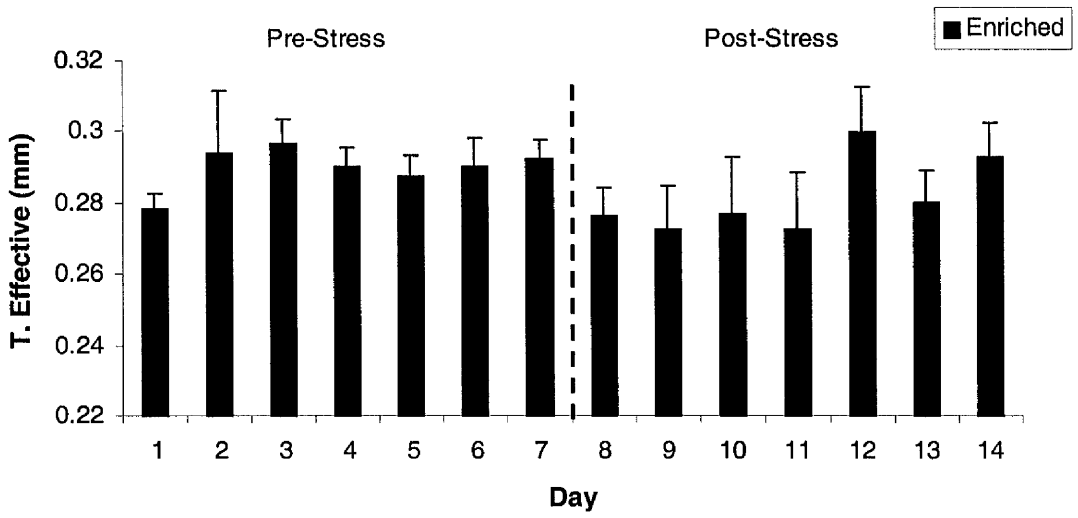


Figure 3.25: Pooled pen means (±S.E.) for T. Effective with day (prior to and following the beak and claw trimming event) for enriched birds only.

3.11.3: Ultrastructural Assessment Results

3.11.3.1: Day Effect

The data from the barren pens showed significant differences in relation to the score for depression. In comparison the data from the enriched pens showed significant differences in relation to the alignment score (Table 3.15).

	Barren	Enriched
Total Score	S=0.67 df=3 *P*=0.880	S=1.86 df=3 *P*=0.602
Confluence	S=0.71 df=3 *P*=0.870	S=4.03 df=3 *P*=0.258
Caps	S=3.32 df=3 *P*=0.345	S=1.53 df=3 *P*=0.675
Fusion	S=2.61 df=3 *P*=0.456	S=0.43 df=3 *P*=0.934
Alignment	S=6.07 df=3 *P*=0.108	**S=8.08** **df=3** ***P*=0.044**
Type B	S=3.00 df=3 *P*=0.392	S=3.00 df=3 *P*=0.392
Depression	**S=10.13** **df=3** ***P*=0.018**	S=1.29 df=3 *P*=0.733
Erosion	S=3.00 df=3 *P*=0.392	S=4.71 df=3 *P*=0.194
Aragonite	S=0.00 df=3 *P*=1.000	S=0.00 df=3 *P*=1.000
Type A	S=3.67 df=3 *P*=0.300	S=2.13 df=3 *P*=0.546
Rhombohedral Calcite	S=3.00 df=3 *P*=0.392	S=0.00 df=3 *P*=1.000
Cuffing	S=5.47 df=3 *P*=0.140	S=2.35 df=3 *P*=0.502
C. Membrane	S=0.52 df=3 *P*=0.915	S=2.29 df=3 *P*=0.514
Cuticle	S=3.39 df=3 *P*=0.335	S=4.56 df=3 *P*=0.207

Table 3.15: Adjusted P-values and Friedman test statistics (S) for data from each environment.

Barren Environment

The data from the barren pens revealed a significant change in the score for depression with respect to day (Figure 3.26), but further analysis revealed no significant differences suggesting that the effect could not be attributed to any particular day.

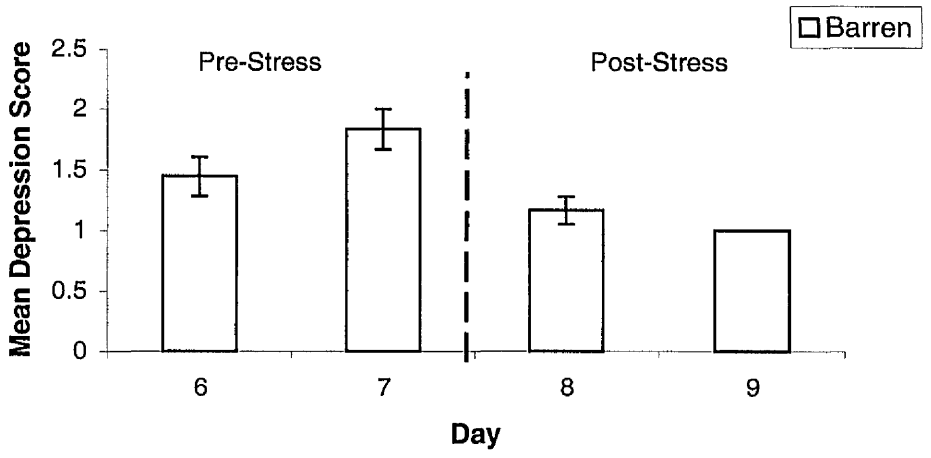


Figure 3.26: Pooled pen means (±S.E.) for mean depression score with day (prior to and following the beak and claw trimming event) for barren data only.

Enriched Environment

The data from the enriched pen revealed a significant change in the score for alignment with respect to day (Figure 3.27). Further analysis however revealed no significant differences between any of the days again suggesting that the effect could not be attributed to a particular day.

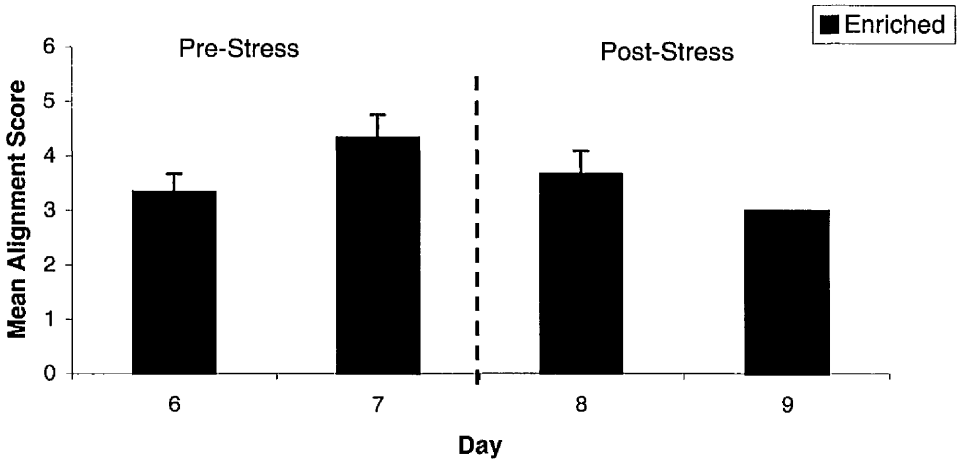


Figure 3.27: Pooled pen means (±S.E.) for mean alignment score with day (prior to and following the beak and claw trimming event) for enriched data only.

3.11.3.2: Environment Effect

When data from each day were analysed in terms of environment, no significant differences were found in terms of any of the ultrastructural variables (Table 3.16).

	Pre Stress		Post Stress	
Days	**6**	**7**	**8**	**9**
Total Score	W=41.5 *P*=0.748	W=46 *P*=0.295	W=32 *P*=0.295	W=37 *P*=0.807
Confluence	W=39.5 *P*=1.00	W=42 *P*=0.599	W=43.5 *P*=0.498	W=46.5 *P*=0.232
Caps	W=37.5 *P*=0.849	W=42 *P*=0.653	W=31.5 *P*=0.210	W=36.5 *P*=0.744
Fusion	W=29 *P*=0.101	W=39 *P*=1.00	W=35.5 *P*=0.621	W=36 *P*=0.673
Alignment	W=47 *P*=0.176	W=37 *P*=0.793	W=35 *P*=0.461	W=38.5 *P*=1.00
Type B	W=39 *P*=1.000	W=39.5 *P*=1.00	W=39 *P*=1.00	W=39 *P*=1.00
Depression	W=41 *P*=0.787	W=46.5 *P*=0.235	W=32 *P*=0.263	W=38.5 *P*=1.00
Erosion	W=35 *P*=0.462	W=38.5 *P*=1.00	W=38.5 *P*=1.00	W=39 *P*=1.00
Aragonite	W=39 *P*=1.00	W=39 *P*=1.00	W=39 *P*=1.00	W=39 *P*=1.00
Type A	W=37 *P*=0.774	W=38.5 *P*=1.00	W=39 *P*=1.00	W=38.5 *P*=1.00
Rhombohedral Calcite	W=40 *P*=1.00	W=39 *P*=1.00	W=38.5 *P*=1.00	W=39 *P*=1.00
Cuffing	W=37 *P*=0.804	W=35.5 *P*=0.615	W=40 *P*=0.933	W=38 *P*=0.932
C. Membrane	W=43.5 *P*=0.446	W=40 *P*=1.00	W=37 *P*=0.752	W=43 *P*=0.461
Cuticle	W=38.5 *P*=1.00	W=29 *P*=0.111	W=42 *P*=0.685	W=37 *P*=0.807

Table 3.16: Adjusted P-values and Mann-Whitney test statistics (W) for differences between environments for each day.

3.12: EGGSHELL QUALITY RESULTS DURING SWAP PROCEDURE

3.12.1: SUPERFICIAL EGGSHELL ABNORMALITIES

3.12.1.1: Day Effect

Eggs laid prior to the swap (days 1 – 7) in general exhibited fewer superficial shell abnormalities than those laid post swap (days 8 – 14) (Figure 3.28) however analysis revealed these differences were only at the $P<0.1$ level and so no further analyses were carried out. The simple statistical model did not allow differences between treatments to be identified, nevertheless visual assessment of eggs suggested that the increase in proportion of abnormal shells was more marked in the birds which were moved from the enriched environment into the barren.

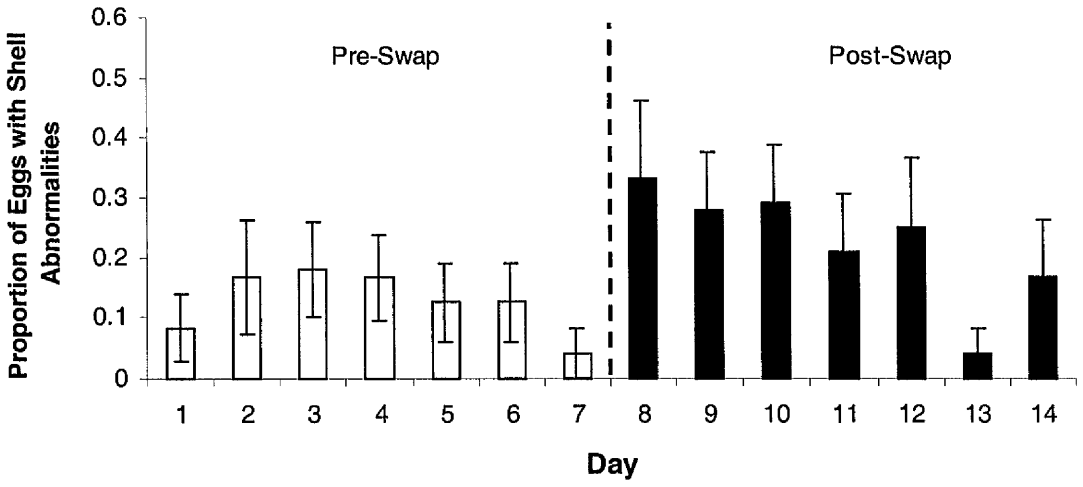


Figure 3.28: Pooled means (±S.E.) for proportion of eggs with superficial shell abnormalities for each day (prior to and following the swap procedure).

3.12.1.2: Treatment Effect

When data from each day were analysed separately in terms of treatment, no significant differences were found (Table 3.17).

Day	
1	W=39 *P*=1.00
2	W=42.5 *P*=0.527
3	W=45.5 *P*=0.248
4	W=39 *P*=1.00
5	W=42 *P*=0.595
6	W=42 *P*=0.595
7	W=36 *P*=0.405
8	W=38 *P*=0.928
9	W=42.5 *P*=0.607
10	W=40.5 *P*=0.859
11	W=40 *P*=0.923
12	W=39 *P*=1.00
13	W=36 *P*=0.405
14	W=42.5 *P*=0.527

Table 3.17: Adjusted P-values and Mann-Whitney test statistics (W) for differences between environments for each day.

3.12.2: PHYSICAL EGGSHELL QUALITY RESULTS

3.12.2.1: Day Effect

No interactions were detected within the physical data with regard to day and environment and so the interaction term was dropped from the model. A significant effect of day was found with regard to non-destructive deformation only (Table 3.18).

	Fresh Weight	*Non-Dest. Deformation*	*Total Thickness*	*T. Mamm.*	*T. Effective*
Day	F=1.20 df = 13 *P*=0.287	**F=1.90** **df = 13** ***P*=0.035**	F=1.48 df = 13 *P*=0.131	F=0.79 df = 13 *P*=0.665	F=1.12 df = 13 *P*=0.345

Table 3.18: Test statistics and P-values for day effect on physical eggshell quality data.

Non-Destructive Deformation: This measure remained reasonably constant throughout, although there did appear to be an increase during day 2 (Figure 3.29), however further analysis revealed no significant differences between individual days. Although there was some variation between day 1 and day 2 this was only at the $P<0.1$ level.

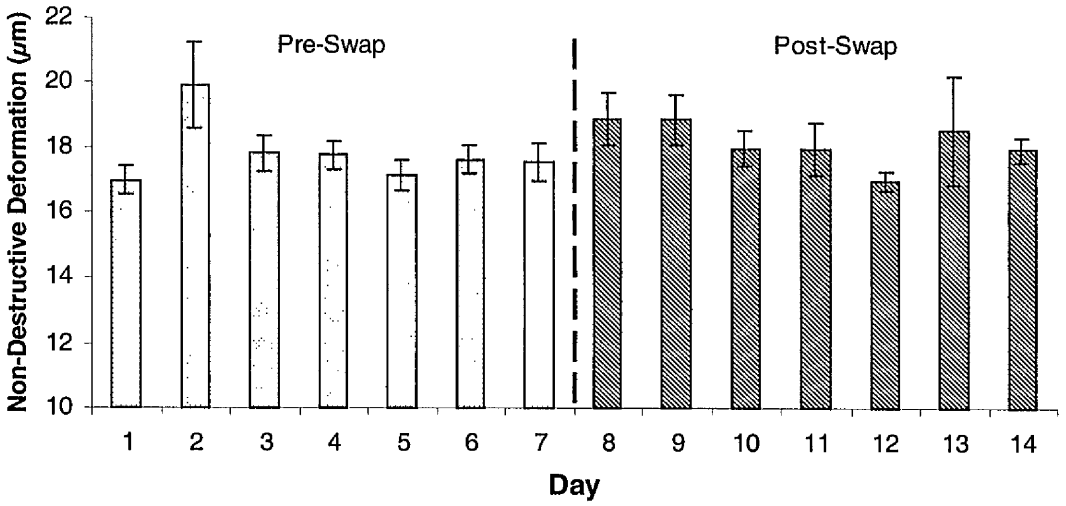


Figure 3.29: Pooled means (±S.E.) for non-destructive deformation with day (prior to and following the swap procedure).

3.12.2.2: Treatment Effect

A significant effect of environment was found with respect to non-destructive deformation, total shell thickness and T. Effective (Table 3.19).

	Fresh Weight	*Non-Dest. Deformation*	*Total Thickness*	*T. Mamm.*	*T. Effective*
Environment	F=0.96 df = 1 *P*=0.351	**F=11.91** **df = 1** ***P*=0.006**	**F=7.86** **df = 1** ***P*=0.019**	F=0.00 df = 1 *P*=0.977	**F=10.89** **df = 1** ***P*=0.008**

Table 3.19: Test statistics and P-values for treatment effect on physical eggshell quality data.

Non-Destructive Deformation: This measure was significantly higher in the birds which experienced the 'Barren to Enriched' treatment (Figure 3.30). When this measure was analysed for each treatment separately, no significant effect of day was found in eggs from either treatment.

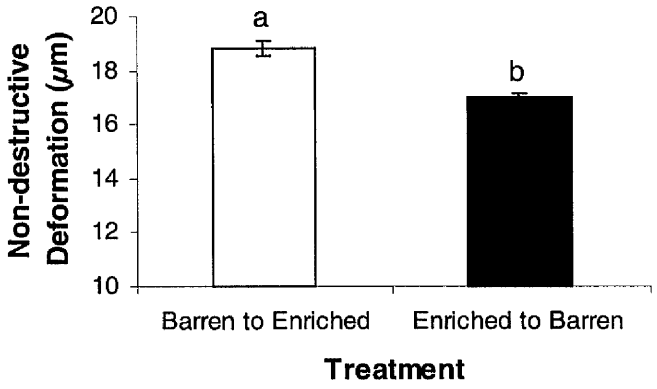


Figure 3.30: Mean non-destructive deformation (±S.E.) (pooled for all experimental days) for birds in each treatment type. Columns sharing letters are not significantly different (P>0.05).

Shell Thickness: This measure was significantly higher in the 'Enriched to Barren' treatment birds (Figure 3.31). When this measure was analysed, for each treatment separately, no significant effect of day was found in eggs from either treatment.

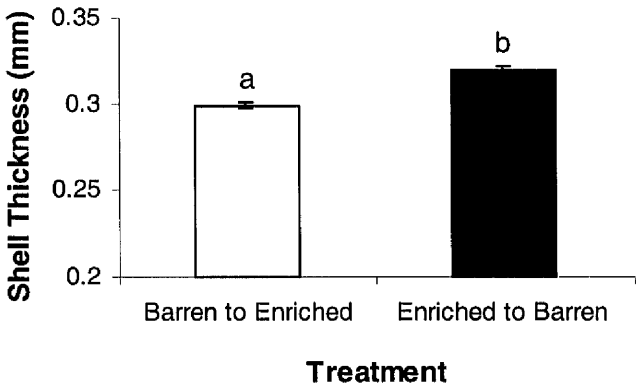


Figure 3.31: Mean total shell thickness (±S.E.) (pooled for all experimental days) for birds in each treatment type. Columns sharing letters are not significantly different (P>0.05).

Effective Thickness: This measure was significantly higher in the 'Enriched to Barren' treatment birds (Figure 3.32). When this measure was analysed, for each treatment separately, no significant effect of day was found in eggs from either treatment.

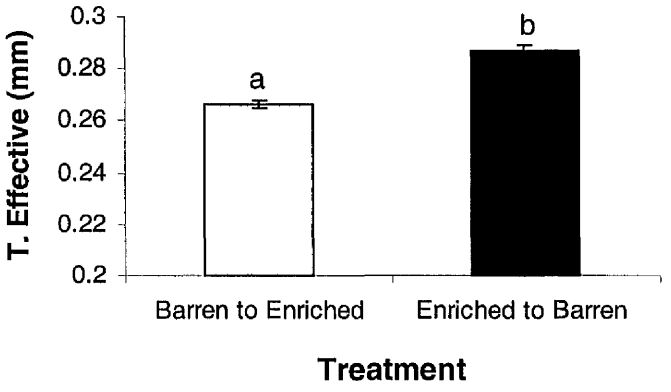


Figure 3.32: Mean T. Effective (±S.E.) (pooled for all experimental days) for birds in each treatment type. Columns sharing letters are not significantly different (P>0.05).

3.12.3: Ultrastructural Results

3.12.3.1: Day Effect

No significant differences were found in the ultrastructural data in terms of differences with day (Table 3.20).

	Barren to Enriched	Enriched to Barren
Total Score	S=7.07 df=3 *P*=0.070	S=3.41 df=3 *P*=0.332
Confluence	S=2.19 df=3 *P*=0.534	S=1.65 df=3 *P*=0.647
Caps	S=1.79 df=3 *P*=0.617	S=0.93 df=3 *P*=0.819
Fusion	S=1.27 df=3 *P*=0.735	S=2.51 df=3 *P*=0.473
Alignment	S=3.25 df=3 *P*=0.355	S=3.73 df=3 *P*=0.292
Type B	S=0.00 df=3 *P*=1.000	S=2.54 df=3 *P*=0.468
Depression	S=1.82 df=3 *P*=0.612	S=1.45 df=3 *P*=0.693
Erosion	S=3.67 df=3 *P*=0.300	S=2.54 df=3 *P*=0.468
Aragonite	S=3.00 df=3 *P*=0.392	S=3.00 df=3 *P*=0.392
Type A	S=3.73 df=3 *P*=0.292	S=3.65 df=3 *P*=0.302
Rhombohedral Calcite	S=3.00 df=3 *P*=0.392	S=3.00 df=3 *P*=0.392
Cuffing	S=4.29 df=3 *P*=0.232	S=4.03 df=3 *P*=0.258
C. Membrane	S=7.36 df=3 *P*=0.061	S=3.00 df=3 *P*=0.392
Cuticle	S=3.53 df=3 *P*=0.317	S=4.85 df=3 *P*=0.183

Table 3.20: Adjusted P-values and Friedman test statistics (S) for data from 'barren to enriched' and 'enriched to barren' treatments separately.

3.12.3.2: Treatment Effect

When data from each day were analysed in terms of environment, no significant differences were found in terms of any ultrastructural measurement (Table 3.21).

	Pre Swap		Post Swap	
Days	**6**	**7**	**8**	**9**
Total Score	W=42 *P*=0.689	W=36.5 *P*=0.746	W=35 *P*=0.568	W=38 *P*=0.936
Confluence	W=44 *P*=0.438	W=36.5 *P*=0.733	W=37 *P*=0.775	W=39 *P*=1.00
Caps	W=44.5 *P*=0.393	W=33.5 *P*=0.341	W=29.5 *P*=0.120	W=34.5 *P*=0.514
Fusion	W=30.5 *P*=0.151	W=36 *P*=0.599	W=40 *P*=0.931	W=42 *P*=0.681
Alignment	W=44.5 *P*=0.218	W=39 *P*=1.00	W=45 *P*=0.294	W=47 *P*=0.115
Type B	W=40 *P*=1.000	W=40 *P*=1.00	W=39.5 *P*=1.00	W=40 *P*=1.00
Depression	W=43.5 *P*=0.498	W=32 *P*=0.2353	W=40.5 *P*=0.862	W=40.5 *P*=0.863
Erosion	W=38 *P*=1.00	W=38.5 *P*=1.00	W=38 *P*=1.00	W=35 *P*=0.462
Aragonite	W=39 *P*=1.00	W=39 *P*=1.00	W=39 *P*=1.00	W=39 *P*=1.00
Type A	W=35 *P*=0.461	W=38.5 *P*=1.00	W=39.5 *P*=1.00	W=33 *P*=0.294
Rhombohedral Calcite	W=38.5 *P*=1.00	W=40 *P*=1.00	W=38.5 *P*=1.00	W=38.5 *P*=1.00
Cuffing	W=39 *P*=1.00	W=33 *P*=0.342	W=36.5 *P*=0.730	W=44 *P*=0.445
C. Membrane	W=38 *P*=1.00	W=45 *P*=0.282	W=39 *P*=1.00	W=39 *P*=1.00
Cuticle	W=33.5 *P*=0.413	W=44.5 *P*=0.417	W=40.5 *P*=0.869	W=32.5 *P*=0.329

Table 3.21: Adjusted P-values and Mann-Whitney test statistics (W) for differences between treatments for each day.

3.13: Results from Additional Non-Invasive Measurements

3.13.1: Faecal Glucocorticoid Levels

3.13.1.1: Beak and Claw Trim Procedure

Faecal glucocorticoid levels did not differ significantly between environments before the beak and claw trim procedure. There was however a significant effect of day caused by this stressor (Table 3.22). As there was no treatment effect statistics were carried out on the pooled results for both treatments although Figure 3.33 shows the data for each treatment separately. Faecal glucocorticoid levels on the day of the beak and claw trim (day 8) were significantly elevated ($T \geq 3.589$, $P \leq 0.008$) above all other days except day 11 ($T=0.030$, $P=1.000$; Figure 3.33). Faecal glucocorticoid levels on day 11 were significantly higher than on all other days ($T \geq 3.317$, $P \leq 0.019$).

	Beak Trim		Swap	
Variable	F, df	*P*-value	F, df	*P*-value
Pen	5.16, 10	0.000	2.13,10	0.040*
Day	10.32, 5	0.000	6.84, 5	0.000***
Environment	1.80, 1	0.209	3.76, 1	0.081
Day*Environment	0.66, 5	0.658	2.80, 5	0.026*

Table 3.22: Results of faecal GC analysis for both beak-trim and swap events (Table taken from Lord, 2001).

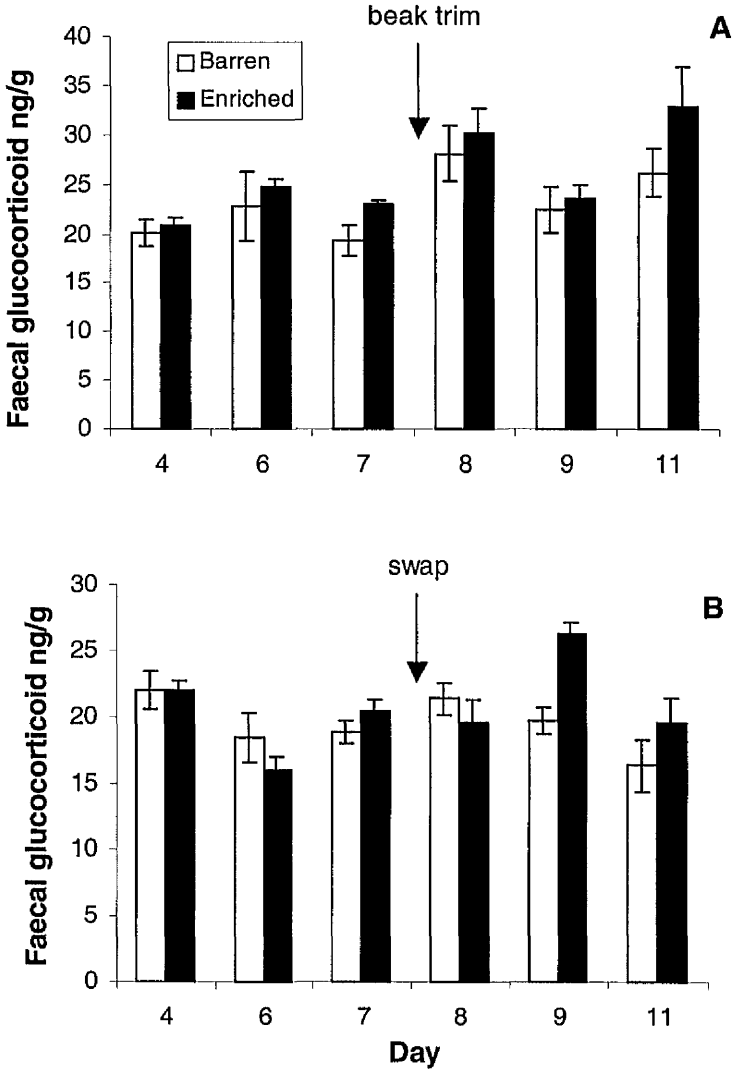


Figure 3.33: Faecal GC levels (mean ±S.E. of untransformed data) before and after (A) the beak trim, and (B) the swap. Legend indicates the environment birds were in at the time of sampling (Graph taken from Lord, 2001). Arrows indicate the occurrence of the event on the morning of day 8.

3.13.1.2: Swap Procedure

Following the swap event there was a significant interaction between environment and day (Table 3.22). A post hoc test revealed that in the birds swapped from barren to enriched environments, the glucocorticoid levels were significantly elevated on the day after the swap (T=26.04, *P*=0.000; Lord, 2001).

3.13.2: Behavioural Observations

Following the beak trim, drinking behaviour increased and maintenance behaviour decreased significantly (Lord, 2001).

Following the swap there was a significant interaction between day and environment for substrate pecking and scratching and this was due to an increase in the behaviour of birds moved from the barren to the enriched environment (Lord, 2001). Birds moved from the enriched to barren environments showed no alteration in behaviour.

3.13.3: Egg Production Data

The environment in which birds were living did not affect egg production either at the onset of lay (weeks 17-20) or thereafter (weeks 21-31) although significant age effects were found during both periods (Table. 3.23; Figure 3.34).

A *post hoc* test, on the post-21 week data, revealed that in the week following the beak trim, egg production was significantly lower than prior to the event (T=3.475, *P*=0.029), however there was no significant decline immediately following the swap or between any other weeks (T=≤3.258, P≥0.0544).

	Weeks 17 – 20		Weeks 21 -31	
Variable	F, df	*P*-value	F, df	*P*-value
Pen	5.59, 10	0.000***	1.49,10	0.156
Environment	0.14, 1	0.712	0.01, 1	0.940
Age (weeks)	46.88, 3	0.000***	3.19, 10	0.001**
Age*Environment	0.62, 3	0.608	1.33, 10	0.225

** Significant at *P*<0.01, *** Significant at *P*<0.001

Table 3.23: ANOVA table for egg production (Table taken from Lord, 2001).

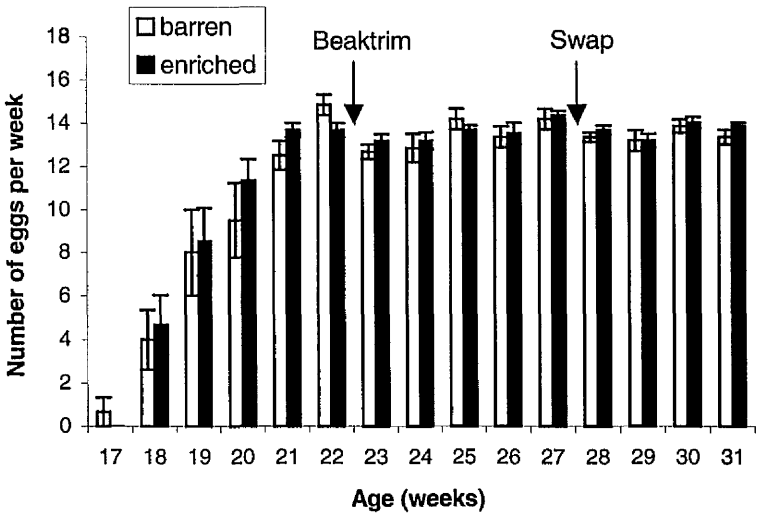


Figure 3.34: Total number of eggs collected each week. Legend shows which environment birds were living in at time of production (Graph taken from Lord, 2001).

3.14: DISCUSSION

Partial beak amputation, which is often performed on commercially reared poultry in order to control feather pecking and cannibalism, has been reported to produce a number of significant alterations to bird behaviour (Gentle *et al.*, 1990). Within this experiment birds significantly increased the amount of time spent drinking and decreased maintenance behaviour, changes which can be accounted for by the birds adjustment to the plastic 'bit' placed between their mandibles (Lord, 2001). It is also possible that drinking itself is soothing to the newly trimmed beak.

A rise in plasma corticosterone is associated with stress in laying hens (Beuving and Vonder, 1978) and, within the current work, faecal glucocorticoid levels, measured on the day of the beak and claw trim, were found to be elevated above all pre-stress days (Lord, 2001; Figure 3.33).

Following the swap in environments there was a significant interaction between day and environment for both substrate pecking and scratching. This was mainly due to a significant increase in these behaviours in the 'barren to enriched birds' who were now in a position to utilise the diversity of inclusions within their new environment. This result supports the

findings of Chamove and Anderson (1989) who stated that successful enrichment will increase an animal's behavioural repertoire.

After the swap treatment the faecal glucocorticoid results revealed a significant interaction between day and treatment with the birds from the 'Barren to Enriched' treatment demonstrating a significantly elevated faecal glucocorticoid level on the day following the swap. Jones *et al.* (1987a) and Boissy (1995) stated that any novelty can elicit a fear response, it is perhaps not surprising therefore that these birds reacted more fearfully to the more complex environment, clearly supporting the views of Rushen (1986) and Broom (1988) who stated that corticosterone elevation can also occur following non-aversive stimuli. As previously discussed, these birds also significantly increased their activity levels following the move and this may account for the increased faecal glucocorticoid levels reported by Lord (2001).

The environment type in which the birds were housed (barren or enriched) did not have any effect on egg production, however a significant effect of day was detected (Figure 3.34). During the week following the beak-trim, egg production was considerably reduced although such a decline was not detected following the swap, possibly as this result is overlying the recovery in productivity seen in the weeks following the beak /claw trim event. These results corroborate the findings of Wolford *et al.* (1983) who reported that feeding corticosterone resulted in a decrease in egg production within 4 – 8 days. Hemsworth *et al.* (1993) commented that high levels of fear are associated with reduced egg production suggesting that the beak and claw trim procedure used in this experiment elicited a fear response while the swap treatment did not.

Many stressors including handling, adrenaline injection, change in environment and exclusion from nest-sites have been reported to result in an increase in the proportion of eggs displaying superficial abnormalities (Hughes *et al.*, 1986; Watt, 1989). Within this study no significant changes in external eggshell quality were detected following the beak and claw trimming procedure (Figure 3.17). Following the swap treatment however there was an increase in the proportion of abnormal shells (Figure 3.28) but this change was only at the $P<0.1$ level probably due to the weak non-parametric statistical test used to analyse this data set. Watt (1989) observed an increase in eggshell abnormalities during the four days following an environmental stress event. The results reported within the current study, which were more rigorously analysed, follow the same pattern and demonstrate a similar level of change. There

was no effect of treatment type on the proportion of abnormal shells produced which is surprising considering that many authors have reported that nest-site deprivation results in egg retention and delayed laying (Duncan, 1970; Van Middelkoop, 1971; Kite, 1986). In the light of these views it might have been anticipated that there would be an increase in eggs exhibiting extraneous calcification in the 'enriched to barren' treatment birds.

With respect to the physical eggshell quality parameters measured during the period encompassing the beak and claw trim procedure, non-destructive deformation, total shell thickness and effective shell thickness all revealed significant changes with day. Non-destructive deformation increased significantly between days 5 and 6 (pre-stress) and day 9 (48 hours post-stress) before decreasing again by day 12 (Figure 3.19). Total shell thickness and effective thickness appeared to decrease between the pre-stress days (1-7) and day 9 (48 hours post-stress) but this difference was not significant. There was however a significant increase in these measures between day 9 and day 12 following the stress (Figures 3.20, 3.21). Shell thickness is influenced by the time the egg spends in the shell gland pouch and therefore it is possible that the thinner shells reported in this study were the result of premature oviposition of these eggs. A change in the proportion of specific matrix proteins, responsible for controlling the calcification process, may also exert an affect on the thickness of the eggshell. A significant effect of environment was also found with the eggs from the barren pens displaying greater non-destructive deformation values (Figure 3.22) and effectively thinner shells (Figure 3.24) throughout the study period.

During the swap procedure the only physical measure to change significantly with respect to day was non-destructive deformation but this change could not be associated with any day in particular (Figure 3.29). The birds from the 'barren to enriched' treatment revealed a reduction in total shell thickness following the swap but this was only at the $P<0.1$ level. As previously discussed this reduction in shell quality was most likely due to a short-term fear reaction by the birds in response to the increased complexity of the new environment. It is likely that the change in shell thickness was not as marked as that observed Experiment 1 as the birds were moved in pairs and so did not experience the social stress of being removed from con-specifics.

These results suggest that the swap procedure was not as disruptive to the birds as the beak-trim event. However they confirm that the deformation measure can provide a non-destructive

measure of eggshell thickness, but as stiffness is also influenced by the curvature of the shell (Bain, 1990) there will never be complete agreement between these two measures.

The birds initially housed in the barren pens consistently laid poorer quality shells with respect to non-destructive deformation, total shell thickness and effective thickness (Figures 3.30, 3.31, 3.32). Following the move to the enriched environment this situation did not change i.e. these birds persisted in laying inferior quality shells despite the fact that they had been afforded a more complex and theoretically 'welfare friendly' environment. Again this supports the theory that environmental enrichment will not, in the short-term, have any desirable affect on eggshell quality parameters due to the increased fear response in the animals concerned.

Neither the acute nor the chronic stress event encouraged the structural changes in the mammillary layer of the shell described by many authors (Solomon *et al.,* 1987; Watt, 1989; Brackpool, 1995). This is perhaps surprising given the observed changes in faecal glucocorticoid concentration reported by Lord (2001) and the hypothesised association between the latter measure and fear levels (Solomon *et al.,* 1987). The only common factor in all these experiments however is that the work used laying hybrids and strain, age and prior experience will all have a role to play in the means by which fear is elicited. Although it is tempting to assume, from the absence of structural defects, that the stressors applied in this study were of a minor nature, by taking only one parameter, in this case the structure of the mammillary layer, into account an erroneous impression would have been derived.

The results discussed above suggest that the beak and claw trim event did elicit a short-term acute stress response within the birds, with effects tending to return to pre-stress levels within 72 hours following the treatment. The fact that birds from both environment types reacted in a similar way to this short-term stress demonstrates that the experience of increased environmental novelty within the home pen did not result in reduced fearfulness. This result is in disagreement with many authors who suggest that environmental enrichment will reduce underlying fearfulness and not simply stimulus-specific fears (Broom, 1969; Jones, 1982; Gvaryahu *et al.*, 1989; Jones *et al.*, 1991).

The swap procedure in comparison did not appear to elicit the chronic stress response intended, with the 'barren to enriched' birds only showing a short-term faecal glucocorticoid response, probably due to the increased novelty present within the new environment. The apparent lack of response in the 'enriched to barren' treatment birds suggests that the removal

of nest-boxes and litter facilities were not in themselves stress inducers contradicting the views held that these facilities fulfil behavioural 'needs' for the hen (Meijsser and Hughes, 1989, Vetergaard, 1981, 1982, Baxter, 1994). However the noticeable increase in superficial shell defects should not be ignored as this is evidence of egg retention which could equally be a response to the lack of a suitable nest-site.

The simpler protocol adopted in this experiment has facilitated a more critical analysis of the results of the experiment 1. Therefore when designing an experiment to address the effect of any stress factor, it is essential to minimise additional variables such as separation from con-specifics. It would appear therefore that the protocol described in experiment 1 inadvertently resulted in an increased level of stress than initially intended.

Furthermore it is clear from the results of experiments 1 and 2 that both temporal and spatial changes in eggshell quality can occur as a result of a stress event, and that these must also be taken into consideration when using eggshell quality as a non-invasive measure of welfare. In chapters 4 and 5 eggshell quality is further defined and applied to studies within the commercial environment.

3.15: CONCLUSIONS

Significant differences in behaviour were recorded following the beak-trim procedure and these changes were not affected by the pen type from which the bird originated. Following the swap event the birds experiencing the 'barren to enriched' treatment demonstrated an increase in behavioural repertoire. This result is consistent with the preference for the enriched environment detected within the first experiment.

Faecal glucocorticoid levels were elevated on the day following the beak-trim procedure. Following the swap event only the 'barren to enriched' birds demonstrated increased faecal glucocorticoid levels.

With regard to physical eggshell quality, non-destructive deformation increased approximately 48 hours following the beak trim event and then returned to pre-treatment levels 3 days later. As this is a measure of shell stiffness its increase is indicative of a reduction in the latter and is thus suggestive of a weaker shell. No change in eggshell quality was detected during the swap treatment indicating that this chronic stressor was not of sufficient strength to elicit a response in terms of eggshell quality.

Neither the chronic nor the acute stress event encouraged structural diversity within the mammillary layer.

CHAPTER 4

THE EFFECT OF ENRICHMENT DURING THE REARING PHASE ON SUBSEQUENT SHELL QUALITY IN BROILER BREEDERS

4.1: INTRODUCTION

Genetic selection over the last 30 years, by primary breeding companies, has resulted in the development of a genotype which is completely different from that which was farmed in the 1960's. For broiler production this selection process has concentrated on improving feed conversion, growth rate and body conformation (FAWC, 1998).

Broiler breeder birds are normally reared from day old to 18 weeks of age on deep litter rearing farms. Following this, 'point of lay' pullets and the appropriate number of cockerels are transferred to laying farms.

During the rearing phase broiler breeders are fed a restricted quantity of food to limit their body weight at the onset of lay (Hocking *et al.*, 1993). Controlling the weight of hens prevents a rapid growth rate and deposition of fat, both of which influence longevity and egg-laying performance (FAWC, 1998). Feed restriction during rearing however can result in birds becoming aggressive due to competition at the feeders (Craig, 1992) and the resulting frustration can cause dominant birds to become highly abusive of subordinates (Rosales, 1994). Beak trimming can prevent pecking and pulling of feathers but if done carelessly causes pain and results in a reduction in feed intake and weight gain (Rosales, 1994). Injurious pecking is a significant problem within the broiler industry, and there is a continual search for ways to minimise feather pecking and cannibalism without using beak-trimming (FAWC, 1998).

When birds are transported they display physiological changes consistent with stress (Freeman, 1984; Duncan, 1989) and so the reduction of fear is an important goal in the improvement of their welfare (Nicol, 1992). Some authors have reported that environment enrichment reduced the birds' reaction to novel stimuli experienced in fear-inducing situations (Jones, 1982; Nicol, 1992).

Early environmental enrichment involves increasing the complexity and stimulus value of the home surroundings of the young bird usually by the introduction of novel stimuli (Jones, 1982; Jones, 1996b). It has been suggested that successful enrichment will increase an animal's behavioural repertoire, reduce the occurrence of abnormal and undesirable behaviours and allow the animal to cope with challenges in a more normal fashion (Chamove and Anderson, 1989). There is also substantial evidence that environmental enrichment reduces underlying fearfulness and not simply stimulus-specific fears (Broom, 1969; Jones, 1982; Gvaryahu *et al.*, 1989). In addition environmental enrichment has been shown to result in improved growth and feed conversion in broilers and layers (Thompson, 1976; Jones *et al.*, 1980; Jones and Hughes, 1981; Gross and Seigel, 1982; Gvaryahu *et al.*, 1989). FAWC (1998) suggested that perches in the rearing house may provide a form of enrichment to allow the birds to perform their natural perching behaviour. They went further to suggest that adding perches may aid the bird's adaptation from litter to raised perforated floors when they move to the laying house. In an earlier communication Jones (1996b) suggested that it is important to find out if environmental enrichment can be applied economically at any stage of development.

To date there has been very little work reported on the effect of early environmental enrichment on productivity and indeed on eggshell quality measures in general within broiler breeder flocks. Significant losses occur in breeding flocks due to thin shells and other abnormalities which can compromise hatchability (Brake, 1987; Roque and Soares, 1994). A number of findings suggest that eggshell quality and hatchability are reduced in fearful birds (Jones, 1996b). This result leads to the hypothesis that any factor that reduces fear may result in improved production traits.

4.2: AIMS

Poor shell quality is not the prerogative of the layer bird. Thus the aim of this trial was to look for any advantages, in terms of egg production or eggshell quality (ESQ), in providing broiler breeder chicks with a pecking and perching enrichment during the rearing period between 0 and 18 weeks of age. In addition to effects on ESQ and production, bird behaviour was monitored during the rearing period as part of another Ph.D. programme (King, 2001). This study also provided the opportunity to examine the effects of flock age on resulting eggshell quality within broiler breeders using a number of methods originally developed for use on layer eggs.

4.3: MATERIALS AND METHODS

4.3.1: REARING PERIOD

From 0 - 18 weeks of age, 16388 Ross 508 Broiler Breeder pullets were reared under standard husbandry conditions within three identical commercial rearing houses. Each of the houses was split into two with half the birds experiencing an environmental enrichment (enriched treatment) whilst the other half were subject to standard rearing conditions (control treatment). The enrichment consisted of bales of plastic-wrapped wood-shavings placed randomly across the house floor (Plate 4.1). These bales facilitated the development of perching and pecking behaviours.

4.3.2: BEHAVIOURAL OBSERVATIONS DURING THE REARING PERIOD

The following behavioural observations were carried out by King (2001). In order to eliminate any observer effect, eight video cameras were set up within each house, four matching cameras above each pen. One camera in one pen recorded the birds for 10 minutes and then switched off and another continued for 10 minutes. 10 minute focal bird observations were made from these videos, with a bird being chosen at random from its position on the television screen. When the birds were 18 weeks old instantaneous scan samples were used to record the behavioural time budgeting in each pen. The time of day was controlled for in all these observations.

4.3.3: MOVEMENT TO LAYING FARM

At 19 weeks of age the birds were transported, separately in terms of treatment type, to a commercial laying farm. Upon arrival the birds were transferred into two commercial laying houses – House A and House B. Each house was split into two separate pens by wire mesh and each half house was filled with either enriched birds or control birds. There was no enrichment provided within the laying houses.

Plate 4.1: Enriched birds using the bales of plastic-wrapped wood-shavings for perching and pecking behaviours.

4.3.4: EGG COLLECTION

Eggs were collected at four periods throughout lay: Beginning of lay (25 weeks old), peak production (31 weeks old), mid lay (45 weeks old) and end of lay (57 weeks old). At each collection period, 30 eggs in total were collected from each house, 15 from each half of the house (treatment type). The eggs were collected at random from nest-boxes throughout each house by the farm staff. Eggs which would normally be classed as seconds were disregarded and an alternative egg chosen. The house and pen number were written on the blunt pole of each egg. The eggs were then placed on egg trays and boxed for courier transport to Glasgow.

4.3.5: PRODUCTION AND HATCHERY DATA

Throughout the trial, the production data, including total number of eggs produced, number of floor eggs and number of seconds (cracked, dirty and substandard eggs which do not go to the hatchery) were recorded separately for each treatment group on a weekly basis. Hatchery data, including number of eggs set, infertiles, culls and numbers of chicks produced, were also recorded for each treatment group separately.

4.3.6: EGGSHELL QUALITY ASSESSMENT

4.3.6.1: Basic Physical Measurements

Fresh Weight: On arrival at the laboratory the eggs were weighed and measured to an accuracy of 10mg.

Shape Index

The length and breadth of intact eggs were measured with hand callipers to the nearest mm and these values used to calculate a Shape Index for each egg (Bain, 1990):

SI = Length of Egg (mm) / Breadth of Egg (mm)

4.3.6.2: Other Physical Measurements

Non-destructive deformation

This was calculated as described in section 3.10.4.2.

The eggs were then randomly re-coded allowing the following measurements to be assessed blind with regard to pen and house.

Eggshell Breaking Strength and Stiffness

Quasi-static compression tests were carried out on a J.J. Lloyd screw driven testing machine fitted with a 100N load cell. Compression speed was standardised at 0.5cm / minute and deformation was measured throughout the test using a transducer coupled to an XY chart recorder. Each egg was compressed at the equator and maximum force (F) and resulting deformation (d) at fracture were used to calculate stiffness (F/d).

Shell Thickness Profiles

Sections of shell were prepared and measured as detailed in section 3.3.5.2.

4.3.6.3: Assessment of the Material Properties of the Eggshell

The material properties of the eggshell allow direct comparisons to be made between samples by taking into account differences in shape, curvature and thickness (Bain, 1990).

Elastic Modulus (E Shell): This is a measure of the elastic flexibility of a material. In engineering terms this value is expressed as Newton's per mm^2 and is calculated by: E = Stress / Strain. Bain (1990) studied this relationship in terms of shell geometry and derived a formula, which takes into account shell curvature, to calculate the elastic modulus for any given shell (E Shell).

$$\text{E. Shell} = C.\ \frac{F R}{dt^2}$$

where $C = C\text{sphere} \times A$

in this expression $C\text{sphere} = \dfrac{0.408 + 3.026t^2}{b}$

and $A = \dfrac{-0.666+(1.866SI)-(0.907SI^2)+(0.153SI^3)}{0.444}$

E. Shell = elastic modulus

t = effective thickness

C = compliance (dependent on shape)

R = radius of curvature (egg breadth / 2)

F = Force

d = Deformation

b = egg breadth

SI = Shape Index

***Fracture Toughness (Kc)*:** The ultimate strength of a material is determined, not by its stiffness or elastic modulus, but by its ability to resist fracture. Ultimate failure of a material is the result of growth of pre-existing flaws or cracks (Gordon, 1974). The greater the pre-existing flaws the earlier a given specimen will fail. Bain (1990) established a method for calculating the Kc value for eggshells. This can be derived by the formula:

$$Kc = Knd\ (F / t^{3/2})$$

where $$Knd = 0.777(2.388+2.934\ \sigma)^{1/2}$$

and $$\sigma = \frac{\text{critical crack length}}{R}$$

The critical crack length is assumed to equal 6mm which is the point at which crack growth becomes unstable (Bain, 1990)

4.3.6.4: Ultrastructural Assessment

Mammillary Layer Ultrastructure

Sections of shell were prepared for mammillary analysis as detailed in section 3.3.6.1. Mammillary layer ultrastructure was only analysed in shells from three time periods (Peak, Middle and End) as other measurements had detected no treatment differences at 25 weeks of age (beginning of lay). Once all samples were prepared and mounted, for all age periods, they were again randomly re-coded. This allowed samples from all three time periods, to be analysed blind with regard to age, pen and house.

The broiler breeder eggs in this trial were all scored using the modified scoring system devised by Darnell-Middleton (1999) specifically for use on eggs set for hatching (Table 4.1). The differences between this and the table egg system exist in the scores for fusion and cuffing. In the table eggs early fusion and cuffing can be considered highly beneficial as they increase shell strength. In the hatching eggs there is a trade off between the need for shell strength and the requirement for adequate gas exchange and water loss, as well as the requirement for the chick to break out at the end of incubation. For these reasons the scores have been adjusted accordingly.

CONFLUENCE	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
CAPS	GOOD 1	AVERAGE 3	BELOW AVG. 5	POOR 7	
FUSION	EARLY 10	MAINLY EARLY 8	50:50 3	MAINLY LATE 4	LATE 7
ALIGNMENT	RANDOM 1	MOD. ORGAN. 3	FISSURED 5		
TYPE B	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
DEPRESSION	NONE 1	LIMITED 2	MODERATE 4	DETRIMENTAL 6	
EROSION	NONE 1	LIMITED 3	MODERATE 5	DETRIMENTAL 8	
ARAGONITE	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
TYPE A	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
RHOMBOHEDRAL CALCITE	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
CUFFING	NONE /ISOL. 1	ISOL./MOD. 3	MOD. EXT. 7		
CHANGED MEMBRANE	NONE /ISOL. 1	ISOL./MOD. 4	MOD. EXT. 7		

Table 4.1: Ultrastructural weighted scoring system modified for hatching eggs by Darnell-Middleton (1999).

Cuticle Ultrastructure

Sections of shell were prepared for cuticular analysis as detailed in section 3.3.6.2.

4.3.7: STATISTICAL ANALYSIS

4.3.7.1: Physical and Material Data

Treatment, House and Age Effects:

All physical and material data were analysed using a three-way ANOVA as all data were normally distributed and there were no missing values. The model used was:

*Response = Treatment + House + Age + Treatment*Age*

All p-values were two-tailed. An interaction term between treatment and age (treatment *age) was included to check for any significant differences in patterns seen between each treatment type with age. Preliminary analyses using two-way ANOVA's revealed no significant house interaction and so this was not included in the model. For a significant factor or interaction, Tukey's pair-wise comparisons were used to identify which factor levels differed most.

Adjusted critical *P*-values were used to counter the problem of multiple comparisons between the treatment levels and the inherent increased likelihood of a Type 1 error.

4.3.7.2: Ultrastructural Data

The ultrastructural data, based on a weighted scoring system, were highly non-parametric and could not be transformed to fulfill the assumptions of a parametric test.

Treatment Effect within each age group:

The data were analysed by means of Kruskal-Wallis analyses within each age group separately to look for any treatment effects.

Age Effect within each treatment group:

The data were analysed by means of Kruskal-Wallis analyses within each treatment group separately to look for any age effects. Kruskal Wallis tests were also used to conduct pair-wise comparisons when analyses revealed significant results.

4.3.7.3: Production and Hatchery Data

The production and hatchery results, which were recorded on a treatment basis, were analysed by means of a two way ANOVA, with the model:

Response = Treatment + Week

No interaction term was added as there were too many possible week combinations to consider. Similarly no pair-wise comparisons were run for any significant week differences again as there were too many possible comparisons.

All statistical analyses were carried out using Minitab release 12.1 (© Minitab Inc., 1998).

4.4: RESULTS FROM LAYING FARM

4.4.1: PHYSICAL AND MATERIAL DATA

Treatment, House and Age Effects

When all the physical and material data were analysed, for all ages together, significant effects were found for age, treatment and age / treatment interactions (Table 4.2). These results are discussed in detail below. No significant effects were found between houses and so house was dropped from the model.

	Treatment	House	Age	Treatment /Age Interaction
Egg Weight	F = 0.59 *P*= 0.443 df = 1	F = 0.33 *P*= 0.564 df = 1	**F = 166.62** ***P* < 0.001** **df = 3**	
Shape Index	F = 0.06 *P* = 0.809 df = 1	F = 0.03 *P* = 0.859 df = 1	**F = 6.09** ***P* = 0.001** **df = 3**	
Mean Deform.	**F = 4.80** ***P* = 0.030** **df = 1**	F = 1.20 *P* = 0.275 df = 1	F = 0.07 *P* = 0.975 df = 3	**F = 3.70** ***P* = 0.012** **df = 3**
Total Thickness	**F = 4.71** ***P* = 0.031** **df = 1**	F = 0.86 *P* = 0.354 df = 1	**F = 3.50** ***P* = 0.016** **df = 3**	
Mamm. Thickness	F = 1.55 *P* = 0.215 df = 1	F = 0.06 *P* = 0.806 df = 1	**F = 9.37** ***P* < 0.001** **df = 3**	
Effect. Thickness	*F = 3.04* *P = 0.083* *df = 1*	F = 0.75 *P* = 0.387 df = 1	**F = 8.25** ***P* < 0.001** **df = 3**	
Break. Strength	F = 1.25 *P* = 0.265 df = 1	F = 1.37 *P* = 0.242 df = 1	**F = 7.79** ***P* < 0.001** **df = 3**	**F = 4.77** ***P* = 0.003** **df = 3**
Stiffness	F = 1.96 *P* = 0.163 df = 1	F = 0.42 *P* = 0.518 df = 1	**F = 5.01** ***P* = 0.002** **df = 3**	**F = 5.65** ***P* = 0.001** **df = 3**
Kc	F = 0.09 *P* = 0.764 df = 1	F = 0.30 *P* = 0.582 df = 1	**F = 34.98** ***P* < 0.001** **df = 3**	
E Shell	F = 0.23 *P* = 0.635 df = 1	F = 0.18 *P* = 0.669 df = 1	**F = 15.53** ***P* < 0.001** **df = 3**	

Table 4.2: Test statistics and P-values for treatment, house and age effects on physical and material data.

4.4.1.1: Treatment Age Interactions

Treatment-age interactions were detected within the physical data for non-destructive deformation (F=3.70, df=3, *P*=0.012), breaking strength (F=4.77, df=3, *P*=0.003) and stiffness (F=5.65, df=3, *P*=0.001; Table 4.2).

Non-destructive deformation: The interaction effect was found to occur between week 31 and 45 (Figure 4.1). Further analysis however revealed no significant differences between any of the ages or treatments (Table 4.3) suggesting that differences, in this non-destructive measure of stiffness, were marginal.

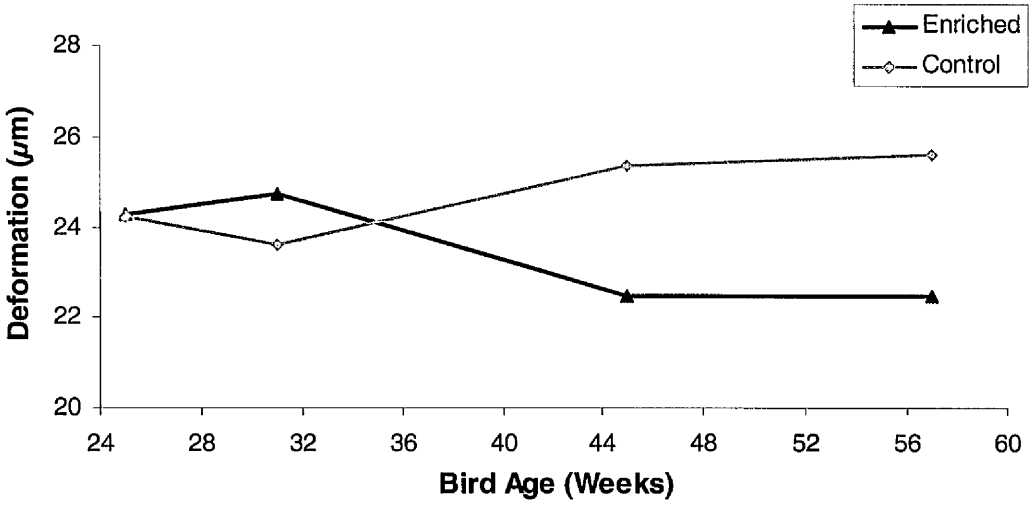


Figure 4.1: Pooled means (S.E. bars not shown) for non-destructive deformation with regard to treatment type.

		Enriched				*Control*			
		25	**31**	**45**	**57**	**25**	**31**	**45**	**57**
Enriched	**25**								
	31	0.999							
	45	0.734	0.458						
	57	0.728	0.451	1.000					
Control	**25**	1.000							
	31		0.970			0.999			
	45			0.161		0.972	0.758		
	57				*0.090*	0.918	0.609	1.000	

Table 4.3: Adjusted P-values for Tukey's paired comparisons of non-destructive deformation across all treatments and ages.

Breaking Strength: The control birds showed a steady decrease in this measure while the enriched birds showed a more changeable response throughout (Figure 4.2). Further analysis revealed a significant increase in breaking strength in eggs from the enriched birds between weeks 31 and 45 (Table 4.4). There was however no significant differences in breaking strength between treatment types at each age.

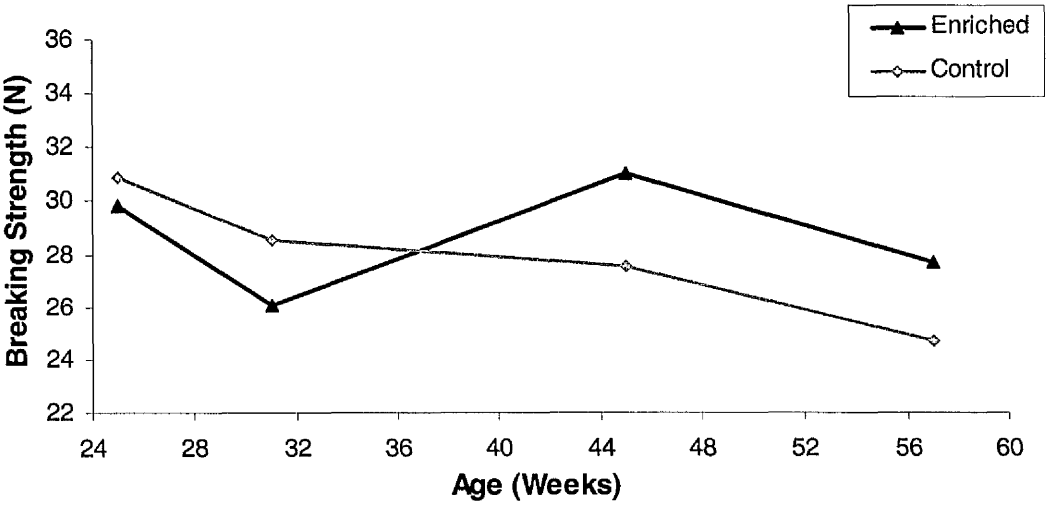


Figure 4.2: Pooled means (S.E. bars not shown) for breaking strength with regard to treatment type.

		Enriched				*Control*			
		25	**31**	**45**	**57**	**25**	**31**	**45**	**57**
Enriched	**25**								
	31	0.102							
	45	0.990	**0.008**						
	57	0.757	0.930	0.227					
Control	**25**	0.994							
	31		0.614			0.647			
	45			0.168		0.197	0.995		
	57				0.333	**0.000**	*0.089*	0.420	

Table 4.4: Adjusted P-values for Tukey's paired comparisons of breaking strength across all treatments and ages.

Stiffness: Again the interaction term appeared to occur between week 31 and 45. It is interesting to note that once more the control birds demonstrated a steady decrease in this measure whereas the enriched birds displayed a more variable response (Figure 4.3). Further analysis revealed significant differences in eggs from the enriched birds between weeks 31 and 45 (Table 4.5). This parallels the changes previously observed in breaking strength but in this case the changes resulted in significant differences between groups at this time.

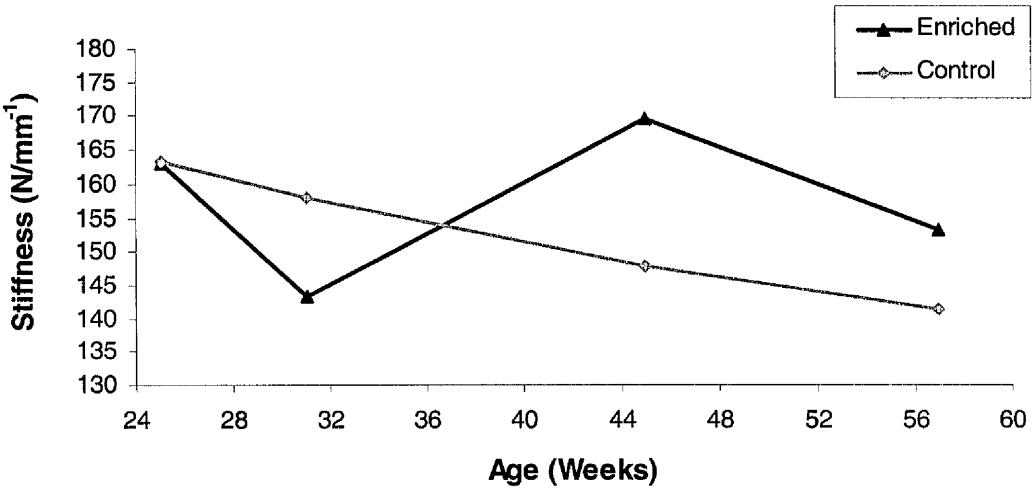


Figure 4.3: Pooled means (S.E. bars not shown) for stiffness with regard to treatment type.

		Enriched				*Control*			
		25	**31**	**45**	**57**	**25**	**31**	**45**	**57**
Enriched	**25**								
	31	0.056							
	45	0.978	**0.002**						
	57	0.793	0.811	0.202					
Control	**25**	1.000							
	31		0.339			0.991			
	45			**0.026**		0.273	0.799		
	57				0.628	**0.021**	0.191	0.973	

Table 4.5: Adjusted P-values for Tukey's paired comparisons of stiffness across all treatments and ages.

4.4.1.2: Treatment Effect

A significant treatment effect was found for total thickness (F=4.79, df=1, *P*=0.030, Table 4.2). Figure 4.4 demonstrates that eggs from the enriched birds had significantly thicker shells. There was a matching difference in effective thickness between treatments but this was only at the P<0.1 level (Figure 4.5).

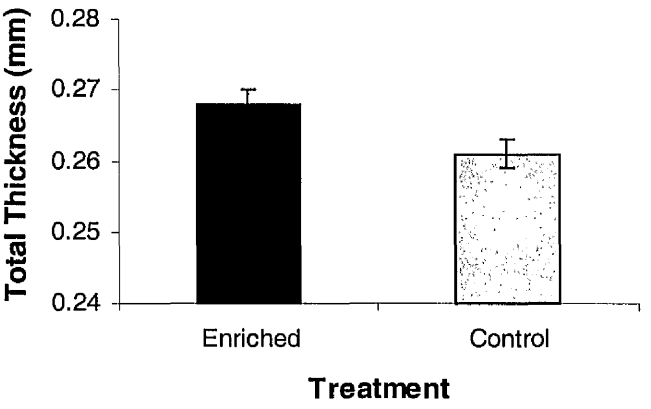


Figure 4.4: Pooled means (± S.E.) for total thickness with regard to treatment type.

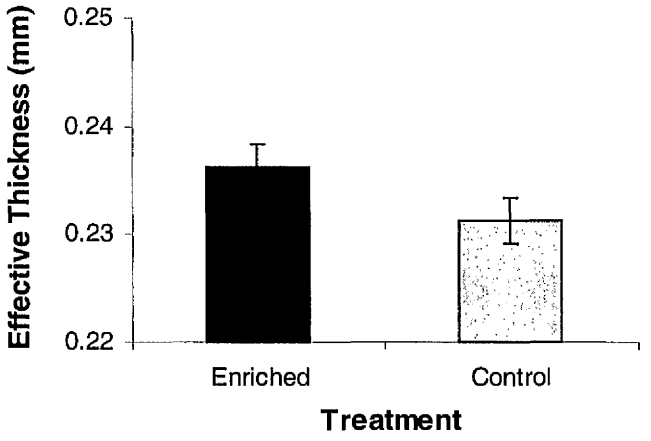


Figure 4.5: Pooled means (± S.E.) for effective thickness with regard to treatment type.

4.4.1.3: Age Effect

A significant age effect was found for egg weight (F=166.62, df=3, $P<0.001$), shape index (F=6.09, df=3, $P=0.001$), total thickness (F=3.50, df=3, $P=0.016$), mammillary thickness (F=9.37, df=3, $P<0.001$), effective thickness (F=8.25, df=3, $P<0.001$), Kc (F=34.98, df=3, $P<0.001$) and E Shell (F=15.53, df=3, $P<0.001$, Table 4.2).

Fresh Egg Weight: This measure increased significantly between week 25 and mid lay (week 45) before remaining constant through to the end of lay (week 57) (Figure 4.6). Further analysis revealed significant differences in weight between all weeks ($P<0.001$) apart from week 45 and week 57 (*Appendix 5 Table A5.1*).

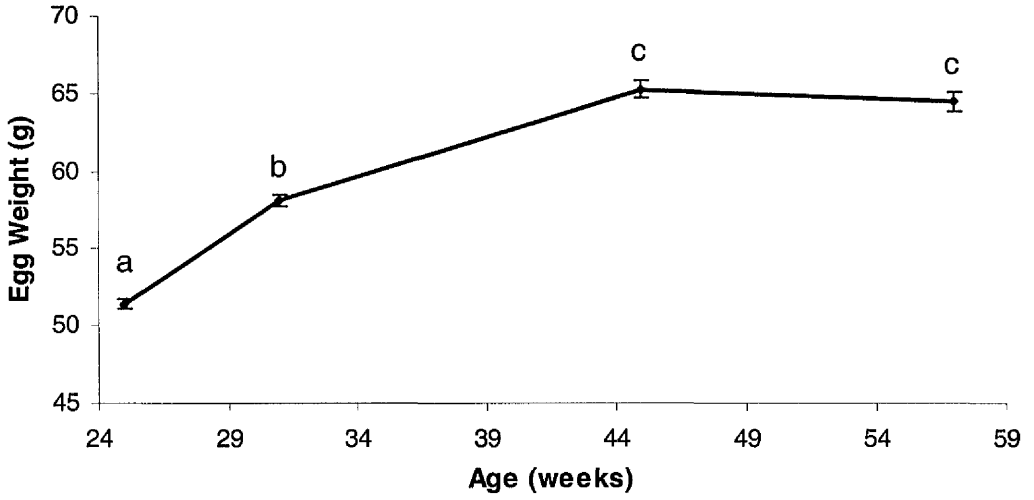


Figure 4.6: Pooled means (± S.E.) for fresh egg weight for all birds throughout lay. Data points sharing letters are not significantly different (P>0.05).

Shape Index: This measure increased significantly between week 31 and week 45 (*P*=0.019) indicating that the eggs became more elongate. Shape index then remained constant through to the end of lay (Figure 4.7; *Appendix 5 Table A5.2*).

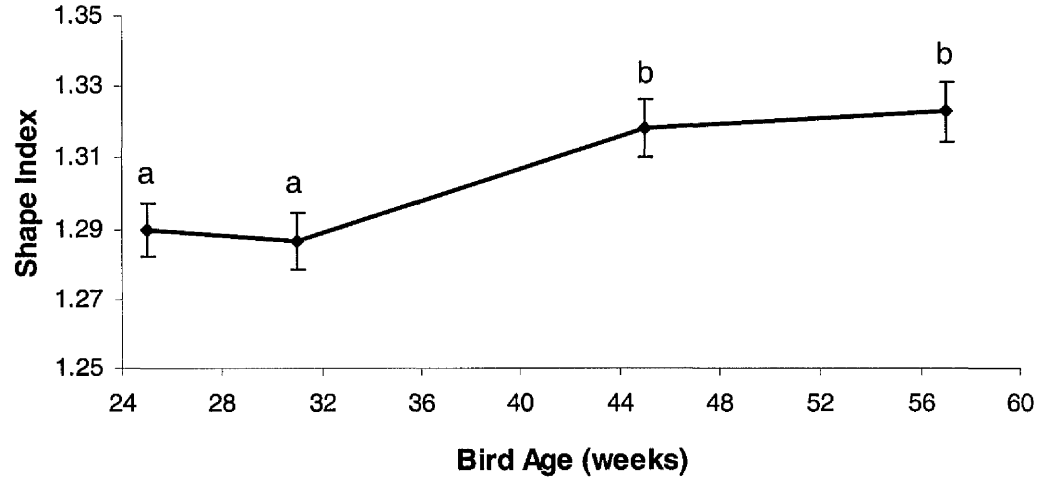


Figure 4.7: Pooled means (± S.E.) for shape index for all birds throughout lay. Data points sharing letters are not significantly different (P>0.05).

Total Thickness: This measure increased significantly from the beginning of lay (week 25) through to mid lay (week 45) (*P*=0.008; Figure 4.8; *Appendix 5 Table A5.3*) before decreasing slightly, although not significantly, at the end of lay.

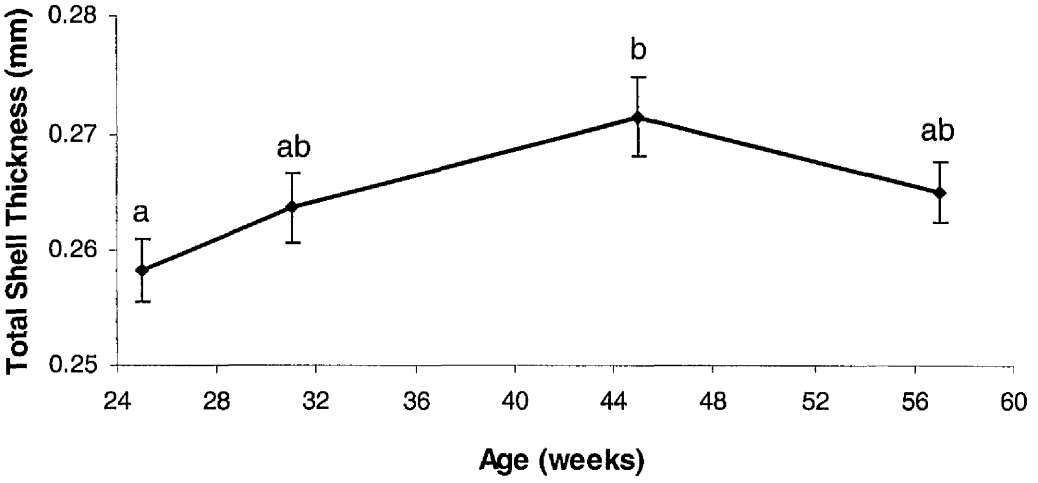


Figure 4.8: Pooled means (± S.E.) for total thickness for all birds throughout lay. Data points sharing letters are not significantly different (P>0.05).

Mammillary Thickness: This measure decreased significantly between week 25 and 31 (P=0.012) and then remained constant throughout the rest of lay (Figure 4.9). Further analysis revealed significant differences between week 25 and all other ages ($P \leq 0.012$; *Appendix 5 Table A5.4*).

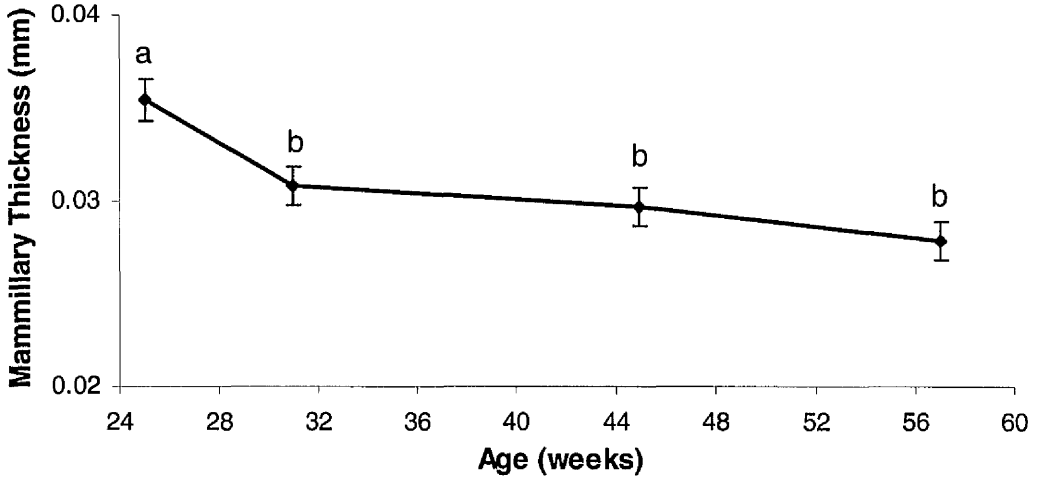


Figure 4.9: Pooled means (± S.E.) for mammillary thickness for all birds throughout lay. Data points sharing letters are not significantly different (P>0.05).

Effective Thickness: This measure increased significantly between week 25 and mid lay (week 45) (P<0.001) before decreasing slightly but not significantly at the end of lay (Figure 4.10; *Appendix 5 Table A5.5*).

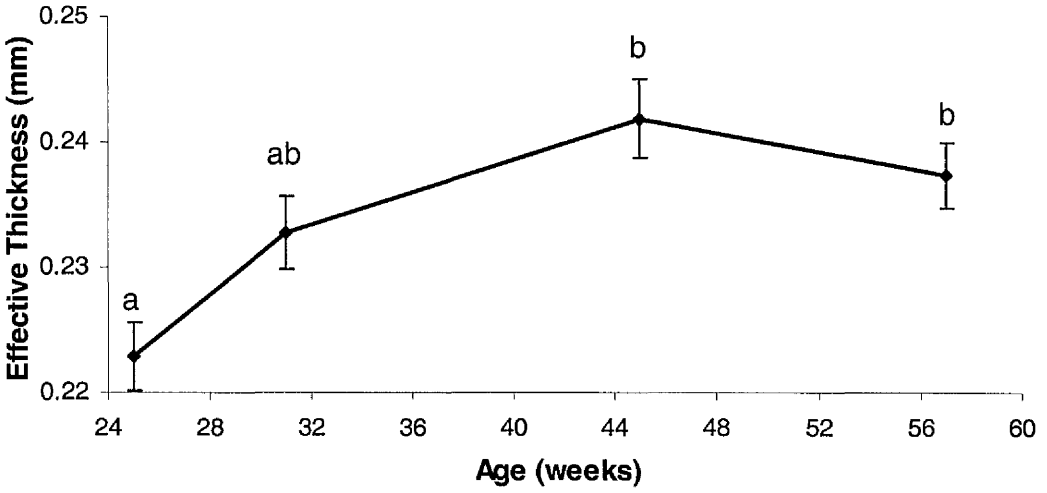


Figure 4.10: Pooled means (± S.E.) for effective thickness for all birds throughout lay. Data points sharing letters are not significantly different (P>0.05).

Fracture Toughness (Kc): This measure decreased significantly between weeks 25 and 31 ($P<0.001$) and again between weeks 45 and 57 ($P=0.037$; Figure 4.11). Further analysis revealed significant differences in Kc between week 25 and all other weeks ($P<0.001$; *Appendix 5 Table A5.6*) suggesting that the shells contained an increased number of defects at the beginning of lay.

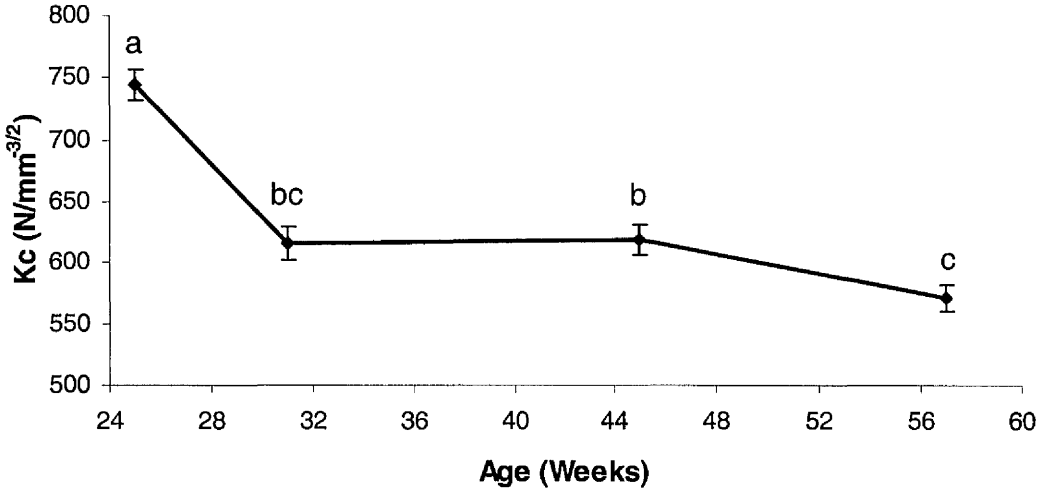


Figure 4.11: Pooled means (± S.E.) for fracture toughness for all birds throughout lay. Data points sharing letters are not significantly different (P>0.05).

E Shell: This measure decreased between weeks 25 and 31 and then remained consistent until the end of lay (Figure 4.12). Further analysis revealed significant differences in E shell between week 25 and all other weeks ($P<0.001$; *Appendix 5 Table A5.7*) suggesting that the elasticity of the shell material was significantly different than that observed at other times during the laying year.

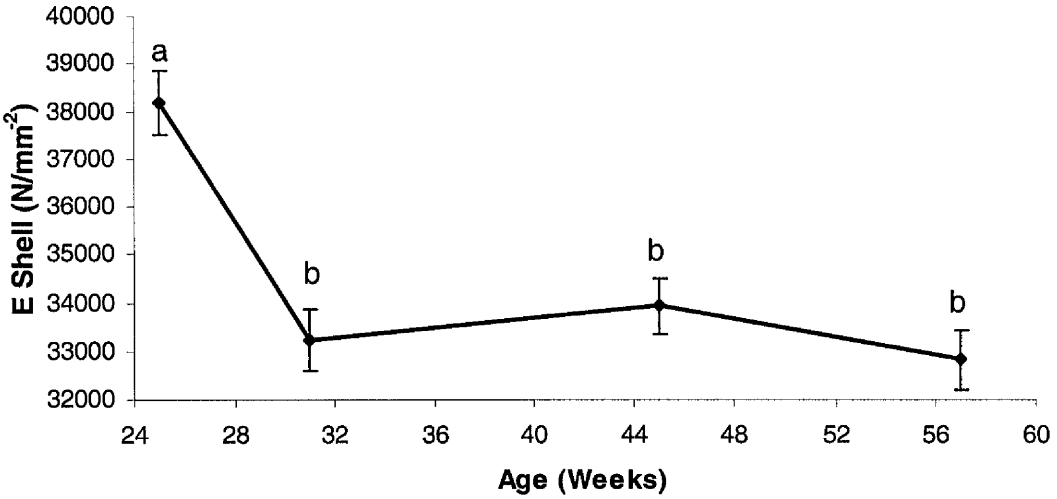


Figure 4.12: Pooled means (± S.E.) for E Shell for all birds throughout lay. Data points sharing letters are not significantly different (P>0.05).

4.4.2: ULTRASTRUCTURAL DATA

4.4.2.1: Treatment Effect within each age group

Analysis revealed a significant difference between treatment types in cuffing score at 57 weeks (H=4.93, df=1, P=0.26, Table 4.6). This was the only significant difference detected.

	31 Weeks	45 Weeks	57 Weeks
Confluence	H=0.03 df=1 *P*=0.866	H=2.41 df=1 *P*=0.120	H=0.09 df=1 *P*=0.767
Caps	*H=3.54* *df=1* *P=0.060*	H=0.00 df=1 *P*=0.985	H=0.41 df=1 *P*=0.523
Fusion	H=0.38 df=1 *P*=0.539	H=0.82 df=1 *P*=0.366	H=0.03 df=1 *P*=0.870
Alignment	H=0.43 df=1 *P*=0.512	H=0.14 df=1 *P*=0.711	H=0.95 df=1 *P*=0.329
Type B	H=0.23 df=1 *P*=0.633	H=0.73 df=1 *P*=0.393	H=0.00 df=1 *P*=0.973
Depression	H=0.00 df=1 *P*=1.00	*H=3.30* *df=1* *P=0.069*	H=2.32 df=1 *P* =0.128
Erosion	H=0.07 df=1 *P*=0.792	H=1.05 df=1 *P*=0.305	H=0.00 df=1 *P*=0.973
Aragonite	H=0.00 df=1 *P*=1.00	H=1.00 df=1 *P*=0.317	H=0.35 df=1 *P*=0.557
Type A	H=0.34 df=1 *P*=0.560	H=1.36 df=1 *P* =0.244	H=0.54 df=1 *P*=0.464
Rhombohedral calcite	H=1.00 df=1 *P*=0.317	H=0.35 df=1 p=0.557	H=0.00 df=1 *P*=1.00
Cuffing	H=0.17 df=1 *P*=0.680	H=1.09 df=1 *P*=0.296	**H=4.93** **df=1** ***P*=0.026**
Total Score	H=0.38 df=1 *P*=0.538	H=0.02 df=1 *P*=0.882	H=0.90 df=1 *P*=0.342
Cuticle	H=0.06 df=1 *P*=0.804	H=0.04 df=1 *P*=0.849	H=0.07 df=1 *P*=0.797

Table 4.6: P-values and Kruskal-Wallis test statistics (H) for differences in treatments for data from each age separately.

Cuffing Score: There was a significant difference in this score between treatment types at 57 weeks. At 31 and 45 weeks the eggs from both treatment types had cuffing scores which were not significantly different. By 57 weeks of age the enriched birds had a significantly higher mean cuffing score (Figure 4.13).

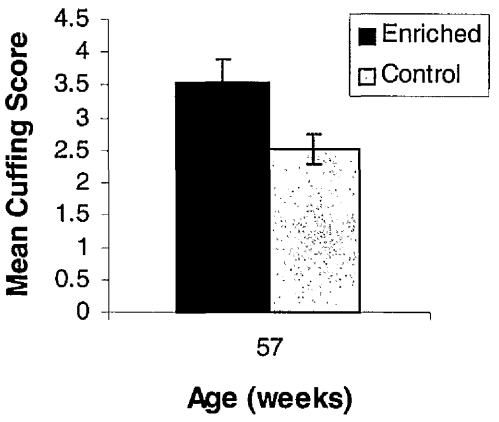


Figure 4.13: Pooled means (± S.E.) for mean cuffing score for each treatment at 57 weeks of age.

4.4.2.2: Age Effect within each treatment group

Analysis revealed that the only significant difference between ages occurred with regard to the cuffing score in the enriched data (H=8.53, df=2, *P*=0.014, Table 4.7).

	Enriched	Control
Confluence	H=3.48 df=2 *P*=0.175	H=1.26 df=2 *P*=0.534
Caps	H=0.54 df=2 *P*=0.763	H=2.71 df=2 *P*=0.258
Fusion	H=0.30 df=2 *P*=0.861	H=0.21 df=2 *P*=0.902
Alignment	H=0.38 df=2 *P*=826	*H=4.69* *df=2* *P=0.096*
Type B	H=0.31 df=2 *P*=0.858	H=0.84 df=2 *P*=0.658
Depression	H=3.49 df=2 *P*=0.175	H=2.26 df=2 *P*=0.323
Erosion	*H=5.13* *df=2* *P=0.077*	H=1.51 df=2 *P*=0.469
Aragonite	H=0.00 df=2 *P*=1.00	H=2.00 df=2 *P*=0.368
Type A	H=2.58 df=2 *P*=0.275	H=0.86 df=2 *P*=0.652
Rhombohedral calcite	H=2.05 df=2 *P*=0.360	H=2.00 df=2 *P*=0.368
Cuffing	**H=8.53** **df=2** ***P*=0.014**	H=3.75 df=2 *P*=0.153
Changed Membrane	H=0.00 df=2 *P*=1.00	H=0.00 df=2 *P*=1.00
Total Score	H=3.23 df=2 *P*=0.199	H=0.65 df=2 *P*=0.723
Cuticle	H=1.29 df=2 *P*=0.525	H=0.21 df=2 *P*=0.900

Table 4.7: P-values and Kruskal-Wallis test statistics (H) for differences in age in data from both treatments.

Cuffing Score: There were significant differences between ages for this score in the enriched data and further analysis revealed significant differences between the end of lay (57 weeks) and both peak production (*P*=0.034) and mid lay (*P*=0.008; Figure 4.14; *Appendix 5 Table A5.8*).

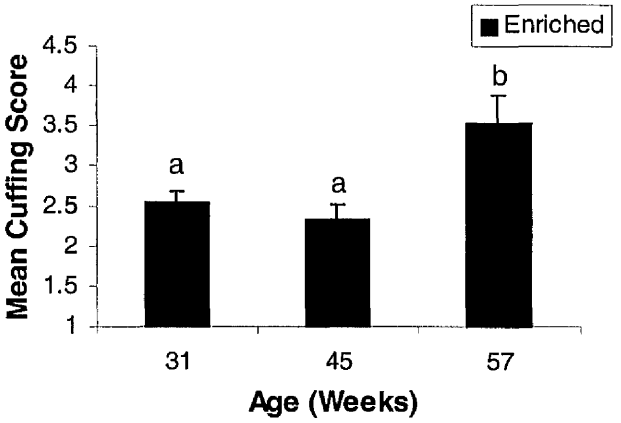


Figure 4.14: Pooled means (± S.E.) for mean cuffing score for enriched birds at each age. Columns sharing letters are not significantly different (P>0.05).

4.4.3: PRODUCTION AND HATCHERY DATA

4.4.3.1: Production Data

No significant treatment differences were found for any of the production variables (Table 4.8). Significant age effects were found for all measures.

	Treatment	Age
Number of Eggs	F=1.16 *P*=0.289	**F=46.69** ***P*<0.001**
Number of Seconds	F= 2.73 *P*=0.110	**F=74.48** ***P*<0.001**
Number of Floor Eggs	F=2.75 *P*=0.100	**F=46.6** ***P*<0.001**

Table 4.8: P-values and paired t-test statistics (F) for differences in hatchery data between treatments.

Figure 4.15 shows the percentage egg production throughout the laying year. The percentage of seconds produced was relatively high during the first 4 weeks of lay and then dropped to below 5% until the end of lay (after 52 weeks old) before rising slightly again (Figure 4.16). Figure 4.17 shows the mean number of floor eggs laid throughout lay. This shows that there was quite a high percent of floor eggs for the first two weeks of lay and then there was a reduction to below 4% which remained throughout the rest of lay.

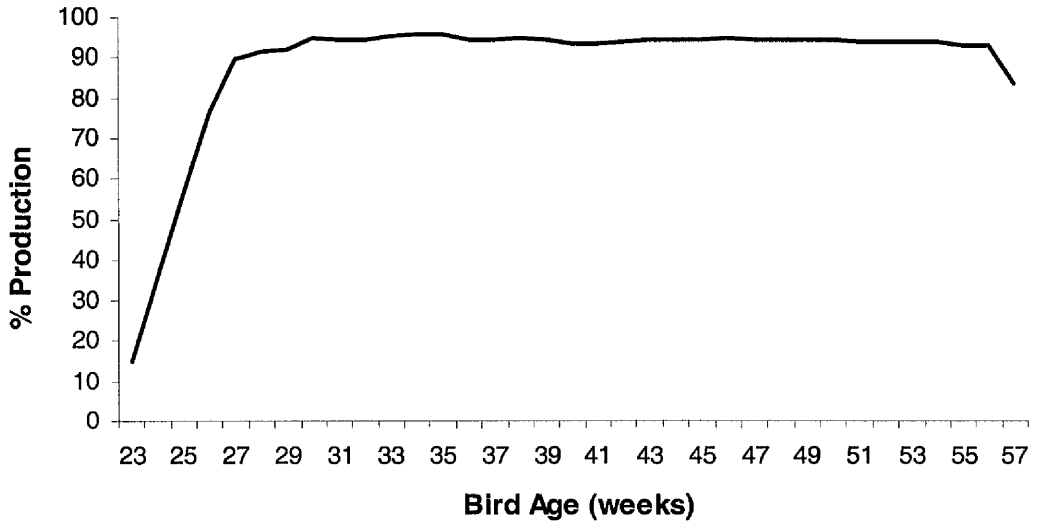


Figure 4.15: Percentage production throughout lay (S.E. bars not shown).

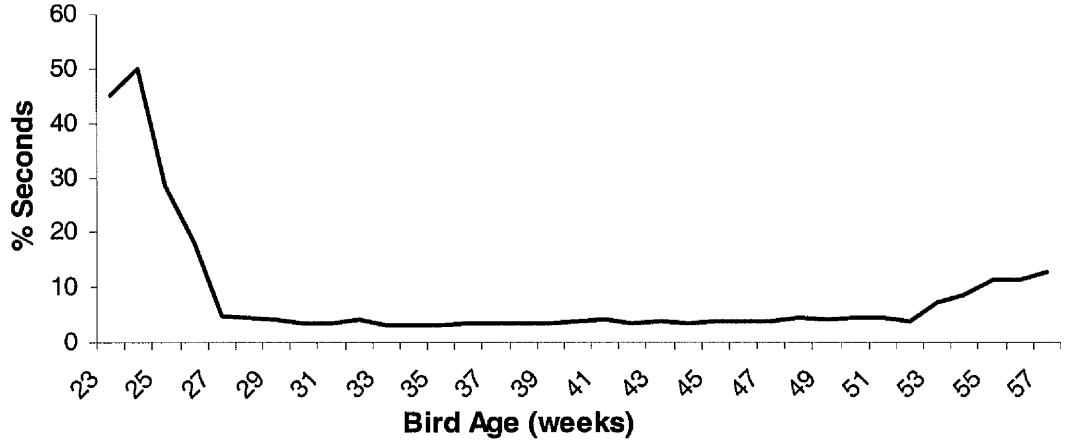


Figure 4.16: Percentage seconds produced by birds throughout lay (S.E. bars not shown).

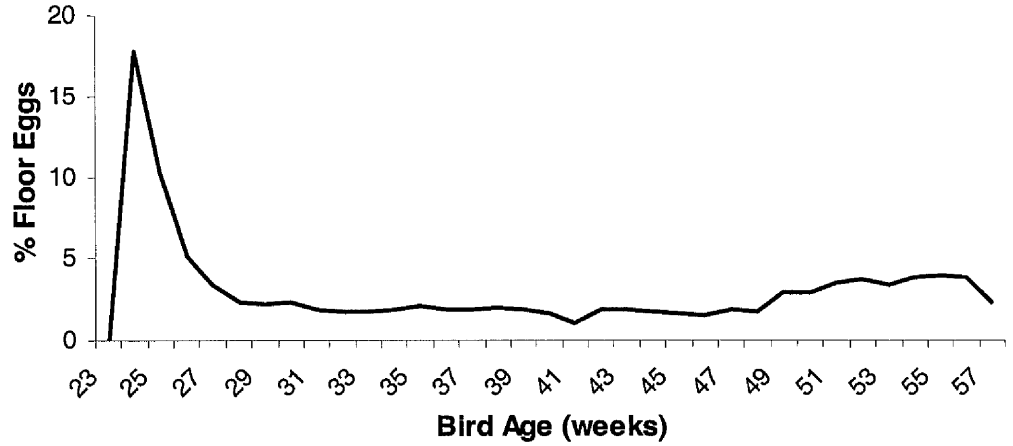


Figure 4.17: Percentage floor eggs throughout lay (S.E. bars not shown).

4.4.3.2: Hatchery Data

Analysis revealed no significant differences between treatment groups for any of the variables recorded in the hatchery data (Table 4.9). Significant age effects were again found for all measures and followed the patterns expected with a curvilinear increase in percentage hatch of fertiles throughout lay and a curvilinear increase in percentage hatch of set until mid lay followed by a decrease into the end of lay.

	Treatment	Age
Eggs Set	F=0.04 *P*=0.838	**F=44.41** ***P*<0.001**
Infertiles	F=0.49 p=0.490	**F=28.74** ***P* <0.001**
Culls	F=0.80 *P*=0.379	**F=15.14** ***P* <0.001**
Chicks Produced	F=0.08 *P*=0.779	**F=56.06** ***P*<0.001**
Hatch of Set %	F=1.01 *P*=0.325	**F=178.17** ***P*<0.001**
Hatch of Fertile %	F=0.29 *P*=0.593	**F=11.83** ***P*<0.001**

Table 4.9: P-values and paired t-test statistics (F) for differences in hatchery data between treatments.

4.4.4: Behavioural Observations during the Rearing Period

Figures 4.18 and 4.19 show the time budgets of hens from each treatment. Enriched birds spent significantly less time drinking. The drinking bouts were of similar length for both treatment types but the control birds had increased visits to the drinker. Enriched birds spent significantly less time standing, not engaged in any activity and more time pecking objects in the pen, primarily the bales. The proportion of time spent air-pecking was significantly reduced in enriched birds. Focal bird sampling revealed that the enriched birds had significantly fewer head-peck interactions (F=12.5, *P*=0.003) and that this effect was pronounced when birds were located at the drinker (F=15.04, *P*=0.001). These data are taken from King (2001).

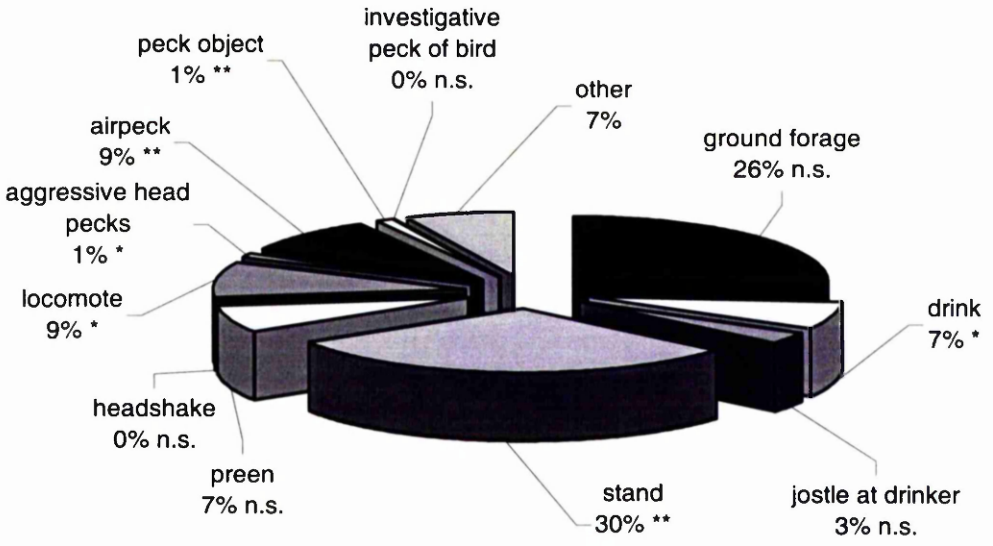


Figure 4.18: Time budget of hens reared in standard breeder production system.

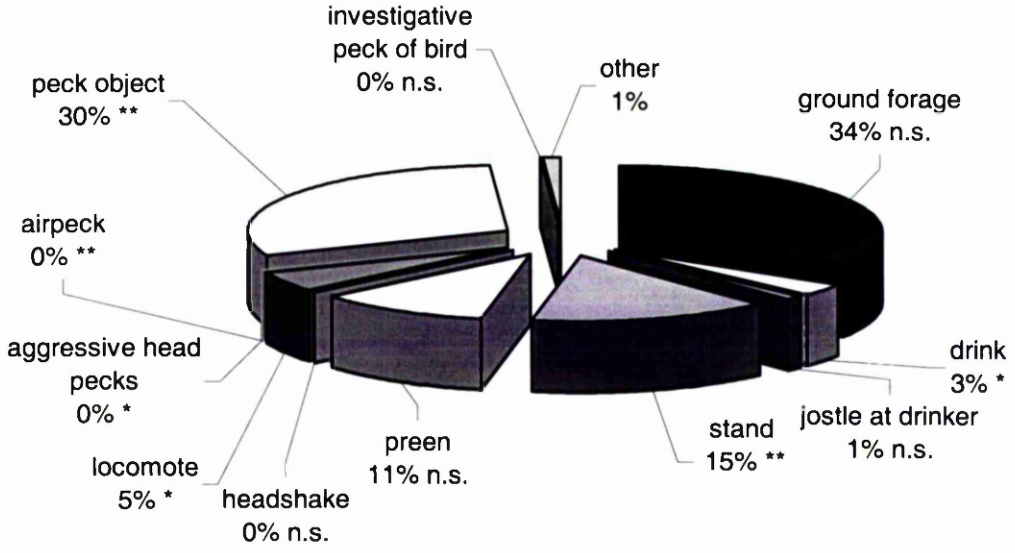


Figure 4.19: Time budget of hens reared in enriched conditions.

* $p<0.05$ ** $p<0.005$

Both figures are taken from King (2001).

4.5: DISCUSSION

Much has been written concerning the beneficial effects of environmental enrichment on behaviour (Broom, 1969; Jones, 1982, Gvaryahu *et al.*, 1989), on bone mass and strength (Hughes and Appleby, 1989) and on fear reduction (Reed *et al.*, 1993). The latter authors reported that when an enrichment was applied over the first 5 weeks of life then its effects persisted into adulthood. Norgaard-Nielsen *et al.* (1993) found that providing layers with an enrichment of peat or sand significantly reduced their tendency to feather peck later in life. In a series of communications Jones (1982, 1989a, 1996b) provided evidence to suggest that environmental enrichment could reduce general or underlying fearfulness and not simply stimulus-specific fears. In the light of this it is interesting to note that both the enriched and control groups within the current study produced shells of comparable quality at the onset of lay following movement into standard laying accommodation. It can only be assumed that the stressors experienced during this time far outweighed any benefits received from the enrichment.

Jones (1996b) also reported that environmental enrichment reduced mortality, aggressiveness and cannibalism and improved bird performance. The results of the behavioural aspect of this work (King, 2001) concluded that the provision of litter bales as an enrichment altered time-budgets and resource use during the rearing phase (Figures 4.18, 4.19). Thus enriched birds demonstrated a reduction in visits to the drinker and spent significantly less time air-pecking or standing, and instead appeared to spend more time pecking at the bales. There was also an overall reduction in the incidence of aggressive and potentially injurious head-pecking within the enriched birds. Hocking et al. (2001) reported similar behavioural changes in broilers fed ad libitum and thus it is suggested that the provision of litter bales in this trial reduced the frustration experienced by the birds. In so far as litter bales are safe and affordable, they achieve the aims of the criteria set out by Jones (1997) who in addition maintained that to be successful, such enrichment devices must also be accessible to all members of the flock and retain the bird's interest.

In general terms, egg production followed the standard pattern with its characteristic decline at the end of lay prior to the point of culling (Figure 4.15). The earlier work of Appleby *et al.* (1983) and Brake (1987) reported a reduction in the incidence of floor laying in chickens reared with perches, however the present work does not support these findings. Within the

current study the bales provided within the rearing phase were at a lower height than the perches subsequently available in front of the nest-boxes in the laying farm. Appleby *et al.* (1983) and Brake (1987) however provided perches in the rearing phase which were of comparable heights and configuration to those found in the laying phase and it is therefore very likely that the experienced birds in these trials would have made the transition to using these perches more readily than the birds within the current experiment. It is also worth noting that the percentage of floor eggs found within the present trial were low (mainly < 4%) compared to the figures found by both Appleby *et al.* (1983) and Brake (1987). The number of floor eggs laid did however diminish with increasing bird age in both groups (Figure 4.17), an observation which is in agreement with the results presented by Brake (1987).

In this investigation significant interactions between treatment and age were found for non-destructive deformation, breaking strength and stiffness, indicating that the egg produced by the enriched birds at mid to end of lay were stronger (Figures 4.1, 4.2, 4.3). As the enriched birds aged so the incidence of cuffing also significantly increased (Figure 4.14). Thus during palisade layer formation extra deposits of calcium carbonate insert between the columns filling in the spaces which naturally occur during the growth process. The net result is to increase the effective thickness of the shell although this feature can also be responsible for blocking gas exchange pores. The enriched birds in this study demonstrated a trend for increased effective thickness throughout the laying year (Figure 4.5). These eggs also displayed a significantly increased total shell thickness throughout lay (Figure 4.4). If these eggs had been destined for the table market then the increased thickness would have rendered them less liable to breakage during routine handling (Bain, 1990). In hatchery eggs, however, a compromise has to be achieved in terms of satisfying gas exchange and the ease with which the chick can emerge. It was gratifying to observe that even with their increased thickness the eggs from enriched birds displayed similar hatchability results.

A significant age effect was found for all other physical and material data, thus egg weight changed significantly with age and showed a standard increase towards mid lay followed by a plateau until the end of the laying cycle (Figure 4.6). This curvilinear increase in egg weight is in agreement with the findings of French and Tullett (1991). Associated with the change in egg weight, the eggs became more elongated (Figure 4.7) but this was not accompanied by a decrease in shell thickness (Figure 4.8). In layers, the age related decline in total shell thickness can be quite marked and has been related to the fact that the bird is attempting to encase a larger egg mass, at the end of lay, with the same amount of shell material (Roland,

1979; Kaminska and Skraba, 1992). The lack of significance in the present results may reflect the fact that these birds were culled at 57 weeks i.e. ten to twelve weeks earlier than their layer counterparts as illustrated by Bain (1990). Total shell thickness comprises two aspects, viz mammillary layer thickness and effective thickness, the results reported in this chapter illustrate that their individual contribution to total thickness varied during the laying year (Ruiz and Lunam, 2000).

The measures of fracture toughness and elastic modulus (E shell), both of which generally decline with increasing bird age in layers (Bain, 1990; Roberts *et al.*, 1997) remained fairly constant following peak production (Figures 4.11, 4.12). Given the consistency observed with regard to shell thickness at the end of the experimental period this is not surprising and corroborates the notion that age at cull is responsible for the lack of change observed.

Between 24 and 31 weeks of age significant changes were observed in all birds, regardless of treatment, for most of the physical and material measures of shell quality. This is a time of great physiological stress and such variations are to be anticipated (Solomon, pers. comm.). In the absence of any treatment effect at 24 weeks of age it was decided not to undertake an ultrastructural investigation of the mammillary layer.

Many authors have reported on the changes in shell ultrastructure which occur as the bird ages (Bain, 1990; Nascimento, 1992; Brackpool, 1995). Thus, layer birds normally culled at 72 weeks of age consistently display a variety of intra-shell defects e.g. rhombohedral calcite, aragonite and erosion. Broiler breeders however lay fewer eggs and this fact together with the earlier cull time would reduce the incidence of morphological variation at the mammillary layer (Fraser, pers. comm.) a feature also borne out in this study.

In the context of the observed improvements in eggshell quality as seen in the enriched birds, it is feasible that access to a perching enrichment early in life may be sufficient to allow birds to develop an improved skeletal system in comparison to birds reared on the floor. Work by Martin *et al.* (1987, cited by Darnell-Middleton, 1999) revealed that the skeletal system of reproductive birds stores 89% of body calcium, and eggshell formation relies on the mobilisation of this mineral. Medullary bone is a specific type of trabecular bone which develops, under the influence of sex hormones, in the medullary cavity of long bones during the egg laying cycle (de Bernard *et al.*, 1980). Birds which are calcium deficient utilise medullary bone as a calcium source before beginning to break down cortical bone (Bloom *et*

al., 1958) and during times of calcium depletion the eggshell produced becomes progressively thinner until soft-shelled eggs are laid or the laying cycle is interrupted (Common, 1938; Deobald *et al.*, 1936).

Hopping up and down on perches has been shown to stress leg bones and hence stimulate bone mass and bone strength and it is suggested that perches may have the effect of increasing both dynamic and static load (Lanyon and Rubin, 1984; Hughes *et al.*, 1993; Hughes and Appleby, 1989).

Tullett (1987) reported that older birds have a decreased ability to absorb calcium from the intestine and to mobilise skeletal calcium (Tullett, 1987). Therefore it could be hypothesised that anything which increases the amount of available mineral within the bones of laying birds, early in lay, may be sufficient to maintain eggshell quality, later into lay, when calcium metabolism and / or matrix protein production begin to reduce in efficiency.

4.6: CONCLUSIONS

King (2001) concluded that providing the bird with bales, during the rearing period, as an enrichment altered time-budgeting and resource use and significantly reduced the incidence of aggressive head pecking.

Significant advantages, in terms of eggshell quality, of providing the birds with a bale enrichment were detected at the middle and end of lay periods. These improvements included increased breaking strength and stiffness measurements and demonstrated that these birds were producing a stronger shell at this time. There was no advantage however, in terms of egg production or number of seconds produced, in the provision of this enrichment. The hatchery data also revealed no differences between groups proving that the increased shell thickness and breaking strength, observed in the enriched birds, did not compromise hatchability in any way.

In this study the age of the broiler breeder flock was proven to affect the resulting eggshell quality, although the dramatic reductions at the end of lay, observed in layers, were not recorded.

Given that the provision of litter bales, during the rearing period, is a safe and affordable option for farm managers, then the results of this study suggest that this enrichment is worth applying in broiler rearing systems.

CHAPTER 5

EGGSHELL QUALITY IN A MULTI-TIER SYSTEM

5.1: INTRODUCTION

In the UK 78% of all eggs produced for human consumption are produced within layer cage systems (MAFF, 1999 cited by BEIS, 2001). The 1986 report by FAWC suggested that the current layer cage system does not provide an adequate environment for laying hens or meet their basic needs viz the small space allocated imposes a restriction on movement and the barren environment inhibits the performance of the hen's natural behaviour (Baxter, 1994). In contradistinction layer cages are acknowledged to provide a uniform environment for the birds, in terms of temperature, lighting and humidity, and reduce the incidence of disease and cannibalistic tendencies (Dun *et al.*, 1991; Baxter, 1994).

Jones (1985a) has reported variation in levels of fear in birds housed within multi-tier cage systems and demonstrated that the birds on the top tier reacted more fearfully during a variety of behavioural tests. It was suggested that these differences were the result of variation in illumination and temperature between the tiers or may have been due to the restricted visual field experienced by these birds in terms of familiarity to humans and cleaning tools. Hemsworth and Barnett (1989) also observed that birds on the top tier had a lower hen day production, lower egg mass output, higher corticosteroid response to humans and demonstrated greater avoidance to the approach of a human being. Sparks (1991) observed that eggs laid in the top tier of a system displayed a reduced covering of cuticle, consistent with premature oviposition.

Environmental temperature has also been shown to influence eggshell quality with higher temperatures resulting in paler eggs with thinner eggshells (Peterson, 1965; Emery *et al.*, 1984; Brackpool, 1995).

5.2: AIMS

The cage environment is perceived, by the general public, as being "stressful" and so in response to increased consideration for animal welfare the current cage system will cease to exist from 2012 (EU Directive 1999/74/EC). Within the U.K., 3-tier housing is the norm,

although multi-tier systems, making use of the full vertical height within the house, do exist. This trial, carried out under commercial conditions, considered the stability of a multi-tier system with regard to temperature and light intensity. Several authors have reported differences in the behaviour of birds within each tier (Jones, 1985a; Hemsworth and Barnett, 1989) and this investigation was intended to establish if there was any difference in the quality of eggs being laid by birds on different tiers of the production system and, if so, to establish which conditions or tier level promoted the best egg and eggshell quality traits.

5.3: MATERIALS AND METHODS

5.3.1: ANIMALS AND HOUSING

24815 ISA Brown layers were transferred to a commercial five tier laying house at 17 weeks of age. The house consisted of 4 double-side banks, each with 124 cages x 5 tiers on each side (1240 cages per bank). The dimensions of each cage were $50cm^3$ and housed 5 birds. Each cage was supplied with 2 nipple drinkers and a feed trough. The birds were fed *ad libitum* via an automatic feeder and eggs were collected via an automated conveyor belt at the front of the cages. The five tiers were labelled A through to E with A corresponding to the top tier and E the bottom. Hanging Tungsten lights with shades were present along each aisle between the banks of cages. A standard light pattern of 15 hours per day was employed.

5.3.2: FARM DATA

5.3.2.1: Environmental Conditions

Temperature:

Five thermometers were suspended from the roof in the centre of the house (Figure 5.1), one hanging at the level of each tier (A – E). The maximum, minimum and average daily temperature was recorded throughout the study period.

Lighting Levels:

At three periods during lay (corresponding to each egg collection period) lux measurements were taken at the level of each tier (A – E) at fifteen points throughout the laying house, as detailed in figure 5.1. Measurements were taken from both within the cage and over the feed trough thus providing two values for each sample point. The lux meter used was a hand held TES 1330 digital lux meter .

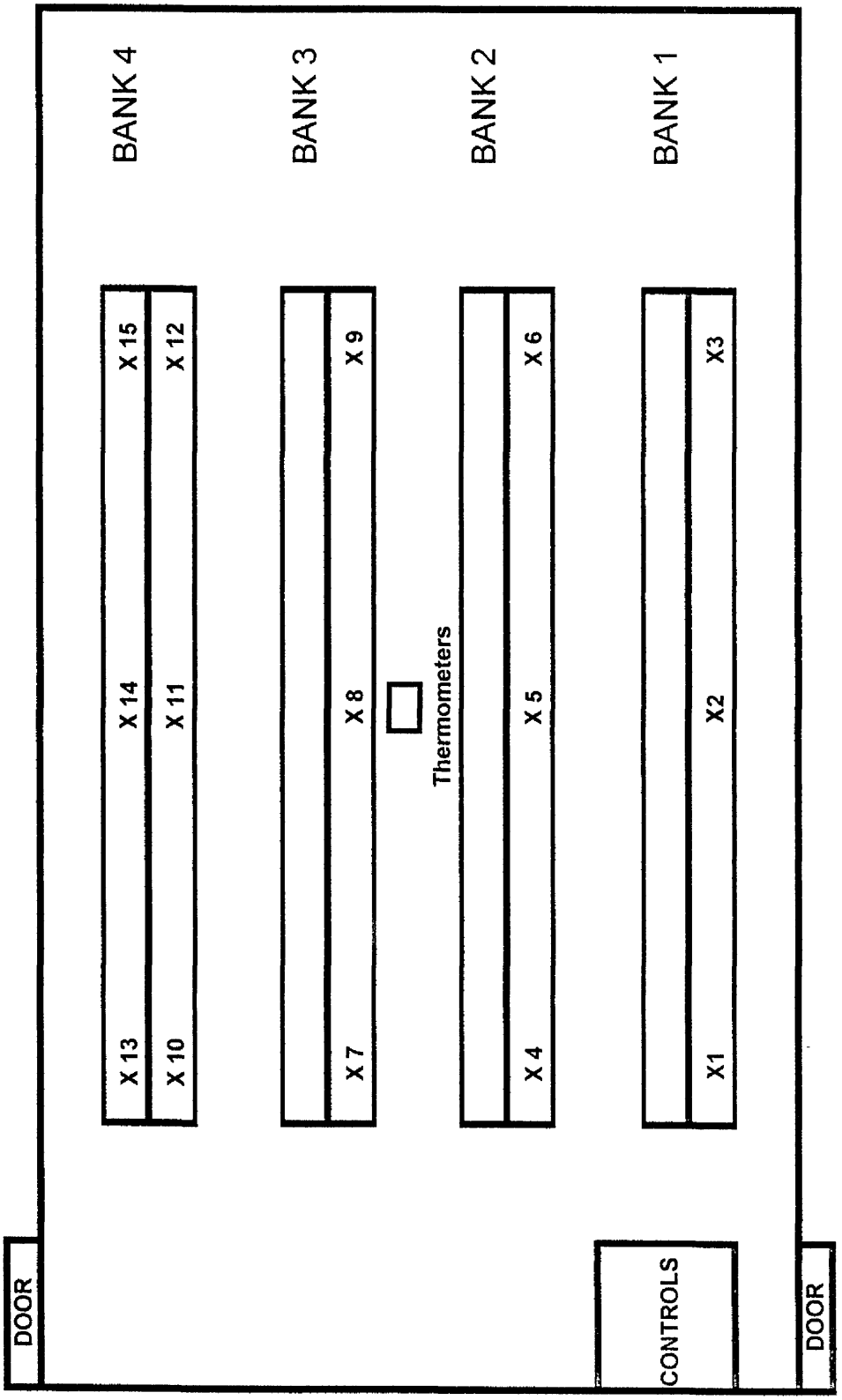


Figure 5.1: Plan of laying house. X 1 - X 15 - Points in house where lux meter readings were taken.

Relative Humidity:

The minimum, maximum and average value was recorded by an electrical monitor, placed in the control area of the house, on every day throughout the trial.

5.3.2.2: Animal Data

Mortality (Cull) Data:

The number of hens which were culled or dead was recorded on an individual tier basis every day throughout the trial.

5.3.2.3: Production Data

Eggs were collected from the house each day via an automatic conveyor belt system.

Total Egg Production:

This was recorded daily on a whole house basis throughout lay.

Number of Seconds:

The total number of eggs classed as seconds including cracks were recorded on a whole house basis throughout lay. This measure was expressed as the percentage seconds of all eggs laid for each day.

Number of Cracked Eggs:

The number of eggs which were cracked or broken was recorded daily on a whole house basis throughout the study period. This measure was expressed as the percentage cracks of all eggs laid for each day.

5.3.3: EGG COLLECTION

Egg samples were collected at three periods throughout lay viz beginning of lay (26 weeks old), mid lay (46 weeks old) and end of lay (71 weeks old). At each period a sample of 60 eggs were collected from each of three tiers (tiers A, C and E). These eggs were selected at random from the conveyor belt throughout the day's collection in order to get a representative sample from throughout the house. For identification purposes a letter, corresponding to the tier from which it was collected, was written on the blunt pole of each egg in pencil. The eggs were then placed on cardboard flats, boxed and transported to Glasgow for further analysis.

5.3.4: EGGSHELL QUALITY ASSESSMENT

The 60 eggs from each tier were split into two groups of 30, one for eggshell quality analyses (Sections 5.3.4.1 through to 5.3.4.4) and the other for internal analyses (Section 5.3.4.5). Both the eggshell analyses and internal analyses were subsequently carried out blind in terms of tier.

5.3.4.1: Basic Physical Measurements

Fresh Weight and Shape Index: These were measured as described within section 4.3.6.1.

5.3.4.2: Other Physical Measurements

Eggshell Breaking Strength and Stiffness

Quasi-static compression tests were carried out to determine the egg's breaking strength and stiffness as described in section 4.3.6.2.

Shell Thickness Profiles

Sections of shell were prepared and measured as detailed in section 3.3.5.2.

5.3.4.3: Assessment of the Material Properties of the Eggshell

The elastic modulus (E Shell) and Fracture Toughness (Kc) were determined as described in section 4.3.6.3.

5.3.4.4: Mammillary Layer Ultrastructure

Sections of shell were prepared and analysed as detailed in section 3.3.5.3. and using the scoring system detailed in Table 3.2.

5.3.4.5: Internal Quality

The internal quality of each egg was evaluated in break-out tests using an EQM Egg Quality Dedicated Microprocessor (Version 3.1, Release F, Technical Services and Suppliers, York, 1993) with the full range of peripheral instruments. Eggs were first weighed in grams and then the contents were emptied out onto a glass surface plate, taking care not to break the yolk. The albumen height was then measured in mm using a tripod micrometer as described by Buckley and Reid (1971). Using the weight of the egg calculated previously, this value was automatically converted into Haugh Units (HU) as described by Buckley *et al.* (1981). Following this the yolk colour was measured by the EQR reflectometer and the values

converted to a Roche value following the Roche Colour Fan edition (1965) described by Vuilleumier (1969). Finally the number of meat spots and blood spots present were counted.

5.3.5: STATISTICAL ANALYSIS

5.3.5.1: Environmental Conditions

The farm data including weekly temperature, light intensity and mortality which was recorded on a tier basis were analysed by means of a two way ANOVA with the model:

Response = Tier + Week

No interaction term was added as there were too many possible interactions present with each week. When a tier factor was found to be significant a GLM was run using Tukey's pair-wise comparisons to find the source of any significant differences. Adjusted critical *P*-values were used to counter the problem of multiple comparisons between the treatment levels and the inherent increased likelihood of a Type 1 error. No pair-wise comparisons were run for any significant week differences as there were too many possible comparisons. The humidity data were analysed by means of a one-way ANOVA as this measurement was not recorded on an individual tier basis.

5.3.5.2: Productivity Data

The productivity data including total production, number of seconds and number of cracked eggs were not recorded on a tier basis and so were analysed by means of a one-way ANOVA with the model: *Response = Week*

No pair-wise comparisons were run for any significant week differences as again there were too many possible comparisons.

5.3.5.3: Egg Physical and Material Data

Tier and Age Effects:

All physical and material data were analysed using a two-way ANOVA as the data were normally distributed and there were no missing values. The model used was:

*Response = Tier + Age + Tier*Age*

All *P*-values were two-tailed. An interaction term between Tier and Age (Tier*Age) was included to check for any significant differences in patterns seen between each tier with age. When a factor was found to be significant a GLM was run using Tukey's pair-wise

comparisons to find the source of any significant differences. Adjusted critical *P*-values were used to counter the problem of multiple comparisons between the treatment levels and the inherent increased likelihood of a Type 1 error.

5.3.5.4: Eggshell Ultrastructural Data

Tier Effect – within each age:

The ultrastructural data which were based on the scoring system (described in table 2.2) was not normal in distribution and therefore highly non-parametric. The data associated with each collection period were analysed by means of Kruskal-Wallis tests to look for any tier effects. Kruskal Wallis tests were also used to conduct pair-wise comparisons when analyses revealed significant results.

Age Effect – within each tier:

The data associated with each tier were analysed by using the Kruskal-Wallis test in order to look for any age effects. Kruskal Wallis tests were also used to conduct pair-wise comparisons when analyses revealed significant results.

5.3.5.5: Egg Internal Quality Data

Tier and Age Effects:

Most of the data were analysed by means of a two-way ANOVA as described for the physical and material data using the model: $Response = Tier + Age + Tier*Age$

When a factor was found to be significant a GLM was run using Tukey's pair-wise comparisons to find the source of any significant differences.

The data relating to blood spots and meat spots however were not normally distributed and therefore were analysed by means of Kruskal-Wallis tests for each tier and then each age separately, as with the ultrastructural data. Again Kruskal Wallis tests were also used to conduct pair-wise comparisons when analyses revealed significant results.

All statistical analyses were carried out using Minitab release 12.1 (© Minitab Inc., 1998).

5.4: RESULTS

5.4.1: ENVIRONMENTAL CONDITIONS

Significant tier effects were found for temperature, mortality and light intensity (inside and outside cages). Significant age effects were found for temperature and mortality levels (Table 5.1).

	Tier	Age
Temperature	**F=280.04** **df=4** ***P*<0.001**	**F=9.70** **df=47** ***P*<0.001**
Mortality	**F=11.56** **df=4** ***P*<0.001**	**F=2.44** **df=49** ***P*<0.001**
Light Intensity (Outside Cages)	**F=56.78** **df=4** ***P*<0.001**	F=2.52 df=2 *P*=0.083
Light Intensity (Inside Cages)	**F=36.66** **df=4** ***P*<0.001**	F=2.30 df=2 *P*=0.103
Humidity		**F= 6.61** **df=46** ***P*<0.001**

Table 5.1: Effect of tier and week on farm data.

Temperature:

The temperature within the poultry house fluctuated significantly throughout the laying year (F=9.70, df=47, *P*<0.001; Figure, 5.2).

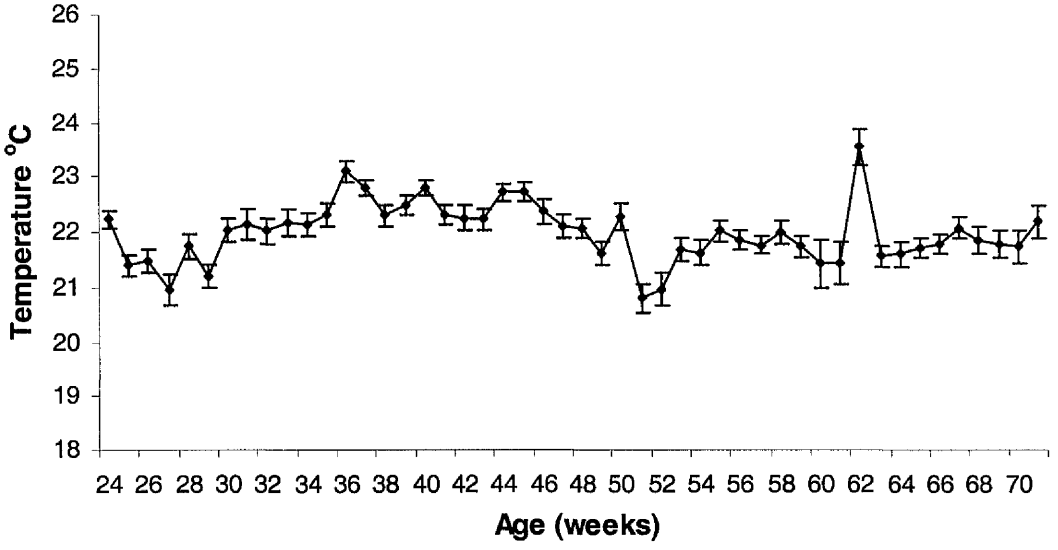


Figure 5.2: Pooled means (± S.E.) for temperature throughout lay.

There was also a tier effect, with regard to average temperature, with significant differences found between the upper three tiers (A, B and C) and lower two tiers (D and E) ($P<0.001$; Figure 5.3; *Appendix 6 Table A6.1*).

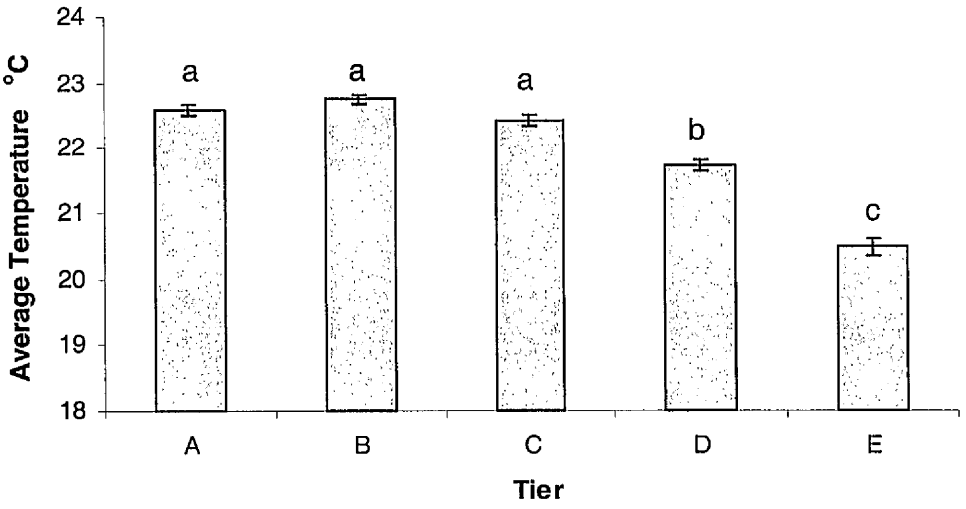


Figure 5.3: Means (±S.E.) for temperature at the level of each tier throughout lay. Columns sharing the same letter are not significantly different (P>0.05).

Mortality:

The weekly mortality levels within the poultry house fluctuated throughout the laying year (F=2.44, df=49, $P<0.001$; Figure 5.4).

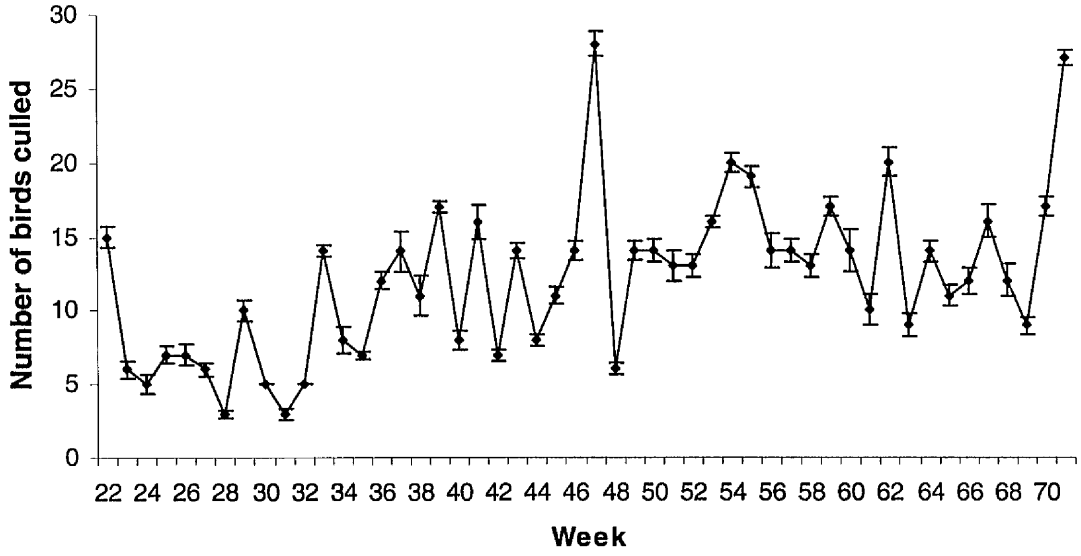


Figure 5.4: Pooled means (± S.E.) for mortality levels from each tier throughout lay.

There was also a significant tier effect occurring, with tiers A and B having significantly higher numbers of birds culled ($P \leq 0.005$; Figure 5.5; *Appendix 6 Table A6.2*).

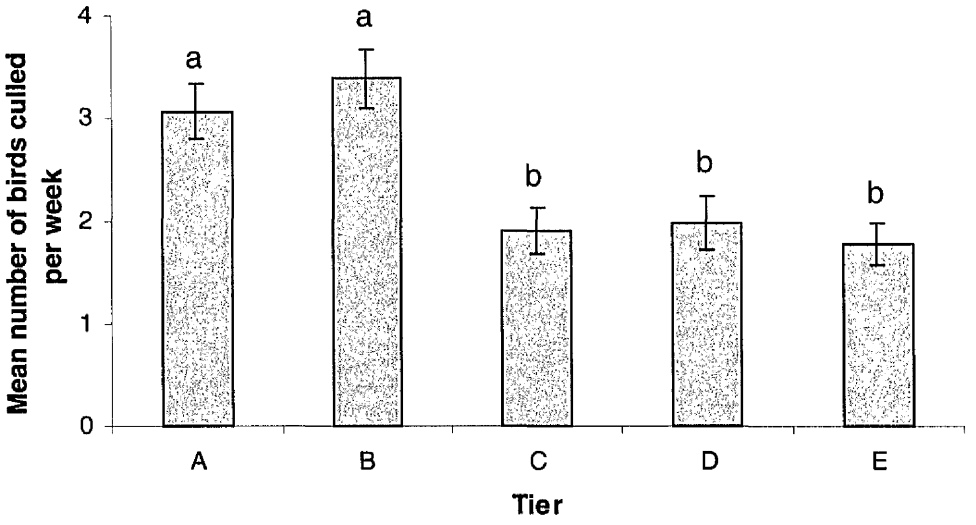


Figure 5.5: Means (±S.E.) for mortality levels from each tier throughout lay. Columns sharing the same letter are not significantly different (P>0.05).

Light Intensity (Lux):

There was a significant tier effect on light intensity both within and outwith the cages. The upper two tiers (A and B) had significantly higher light levels within the cages than the lower three tiers ($P \leq 0.001$). The light intensity within the cages of tier B was also significantly higher than that in tier A ($P<0.001$; Figure 5.6; *Appendix 6 Table A6.3*).

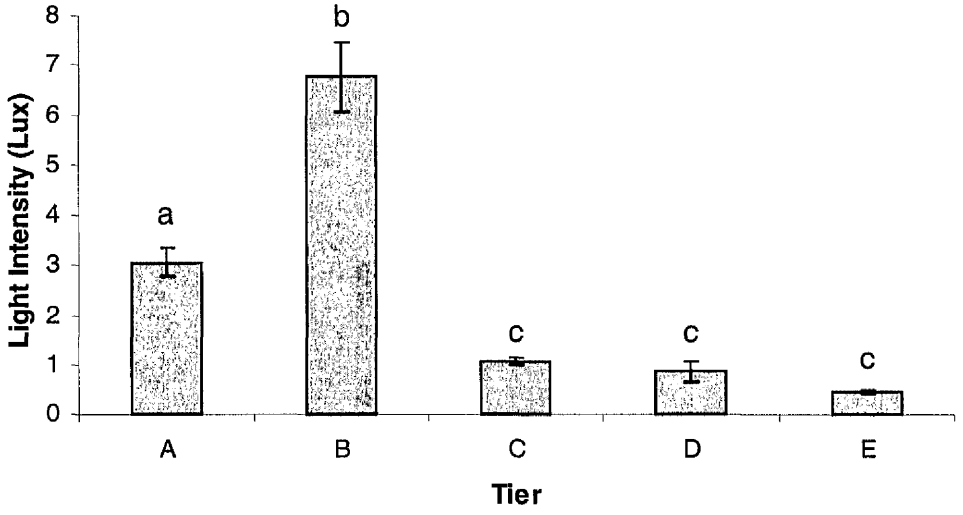


Figure 5.6: Means (±S.E.) for light intensity within the cages from each tier. Columns sharing the same letter are not significantly different (P>0.05).

Figure 5.7 shows the differences in light intensity levels outwith the cages at each tier level. Tiers B and C demonstrated significantly increased lighting levels compared to all other tiers ($P<0.001$). The light intensity outside Tier A was significantly lower than the levels found outside tiers B and C ($P<0.001$) and significantly higher than the level found outside tier E ($P=0.007$; *Appendix 6 Table A6.4*).

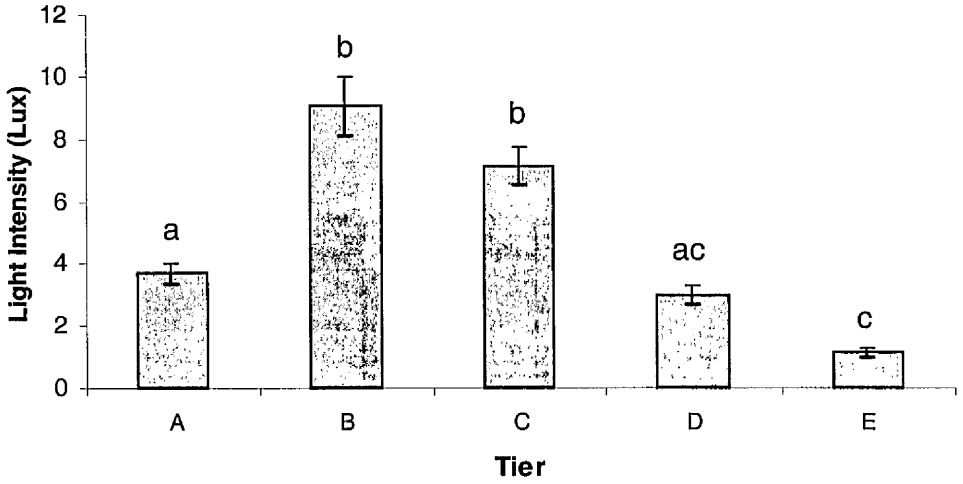


Figure 5.7: Means (±S.E.) for lighting levels outside cages from each tier throughout lay. Columns sharing the same letter are not significantly different (P>0.05).

Humidity:

The weekly humidity levels within the poultry house fluctuated significantly throughout the laying year (F=6.61, df=46, $P<0.001$; Figure 5.8). No information relating to tier differences was available.

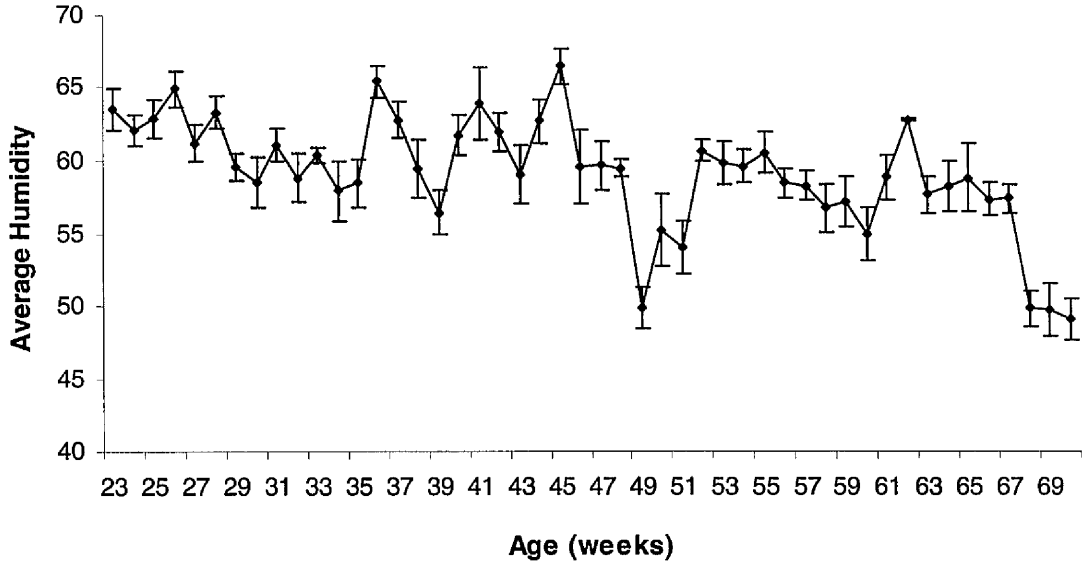


Figure 5.8: Pooled means (±S.E.) for humidity throughout lay.

5.4.2: PRODUCTION DATA

Significant age effects were found for the number of seconds and number of cracked eggs produced (Table 5.2).

	Age
Productivity	F=0.71 df=54 *P*=0.935
Percentage Seconds	**F=27.38**** **df=51** ***P*<0.001**
Percentage of Cracked Eggs	**F=22.97**** **df=51** ***P*<0.001**

Table 5.2: Effect of age on production data

Productivity:

The total egg production was fairly consistent throughout lay and followed a standard production curve (Figure 5.9).

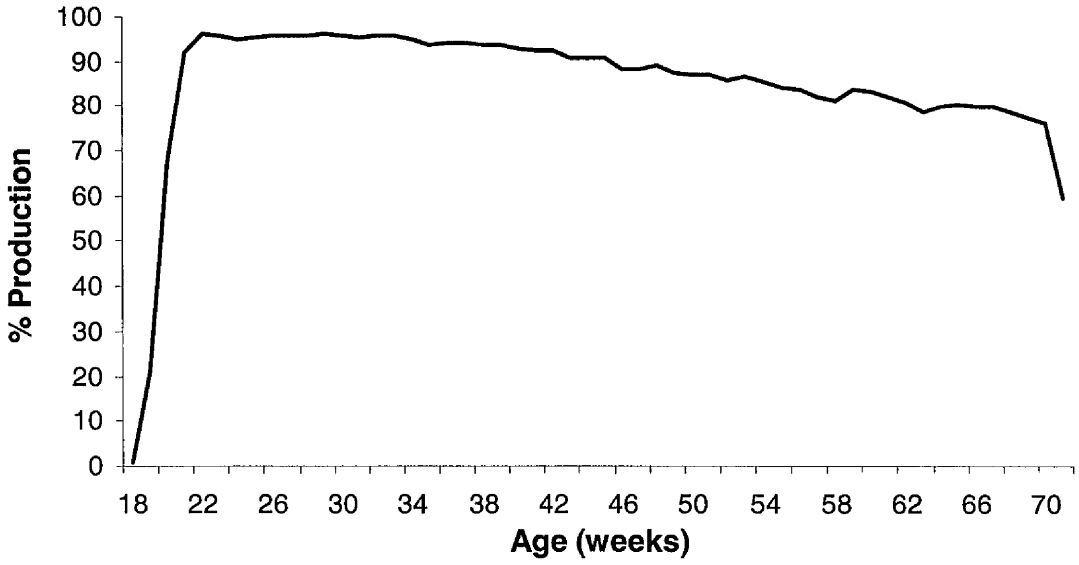


Figure 5.9: Percentage egg production throughout lay (S.E. bars not shown).

Number of Seconds:

This measure was fairly consistent throughout the laying year although there was a peak at the beginning of lay and a slight increase towards the end (F=27.38, df=51, *P*<0.001; Figure 5.10).

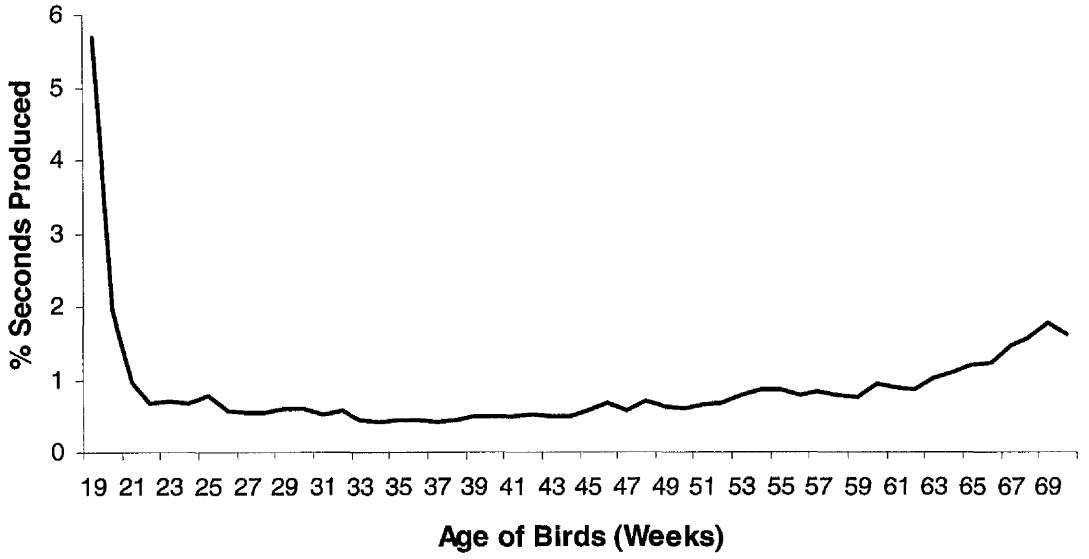


Figure 5.10: Percentage seconds produced throughout lay (S.E. bars not shown).

Number of Cracked Eggs:

The percentage of seconds due to cracked eggs is shown in Figure 5.11. Between 20 and 25 weeks there was an increased number of large eggs accounting for a greater percentage of the seconds. The average percentage of seconds due to cracked eggs was fairly constant throughout lay.

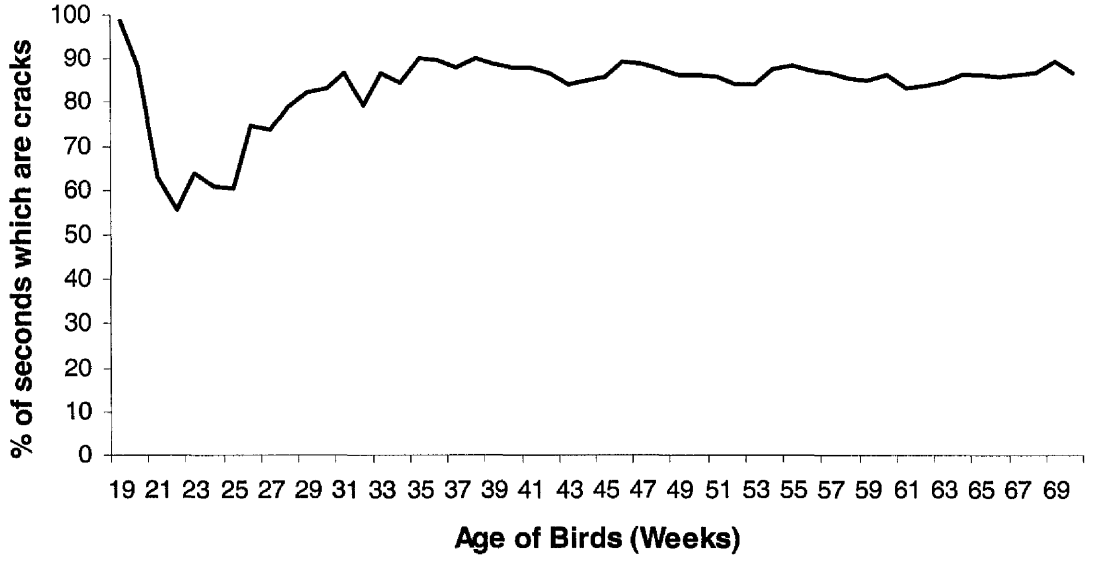


Figure 5.11: Percentage of seconds which are due to cracked eggs throughout lay (S.E. bars not shown).

5.4.3: PHYSICAL AND MATERIAL EGGSHELL QUALITY

5.4.3.1: Tier and Age Effects:

No tier effect was found with regard to any of the physical and material measurements (Table 5.3). Significant age effects were found for egg weight, shape index, shell total thickness, mammillary thickness (T.Mamm), effective thickness, breaking strength, stiffness, fracture toughness (Kc) and elastic modulus (E Shell) (Table 5.3).

	Tier	Age
Weight	F=1.69 df=2 *P*=0.186	**F=16.60**** **df=2** ***P*<0.001**
Shape Index	F=0.56 df=2 *P*=0.570	**F=94.33**** **df=2** ***P*<0.001**
Total Thickness	F=0.25 df=2 *P*=0.778	**F=4.74*** **df=2** ***P*=0.010**
Mammillary Thickness	F=0.05 df=2 *P*=0.952	**F=4.58*** **df=2** ***P*=0.011**
Effective Thickness	F=0.21 df=2 *P*=0.809	**F=9.00**** **df=2** ***P*<0.001**
Breaking Strength	F=1.45 df=2 *P*=0.237	**F=46.10**** **df=2** ***P*<0.001**
Stiffness	F=0.09 df=2 *P*=0.917	**F=6.49**** **df=2** ***P*=0.001**
Kc	F=1.51 df=2 *P*=0.223	**F=60.21**** **df=2** ***P*<0.001**
E Shell	F=0.48 df=2 *P*=0.622	**F=3.91*** **df=2** ***P*=0.021**

Table 5.3: Effect of tier and age on physical and material measurements of eggshell quality.

Fresh Egg Weight:

This measure increased throughout the laying period (Figure 5.12) and further analysis revealed significant increases in weight between beginning of lay and mid lay (*P*=0.005) and between mid lay and end of lay (*P*=0.024; *Appendix 6 Table A6.5*).

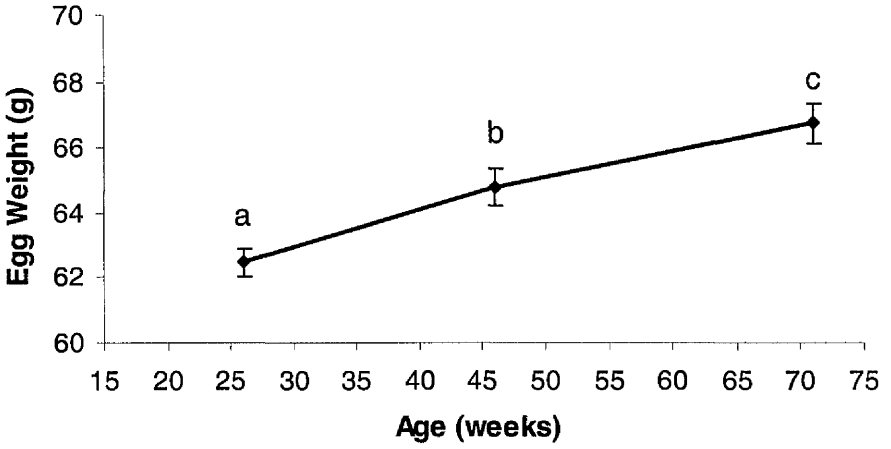


Figure 5.12: Pooled means (± S.E.) for fresh weight for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

Shape Index:

This measure increased throughout the laying period suggesting that eggs were becoming more elongated (Figure 5.13) and further analysis revealed significant differences in shape index between all collection periods ($P<0.001$; *Appendix 6 Table A6.6*).

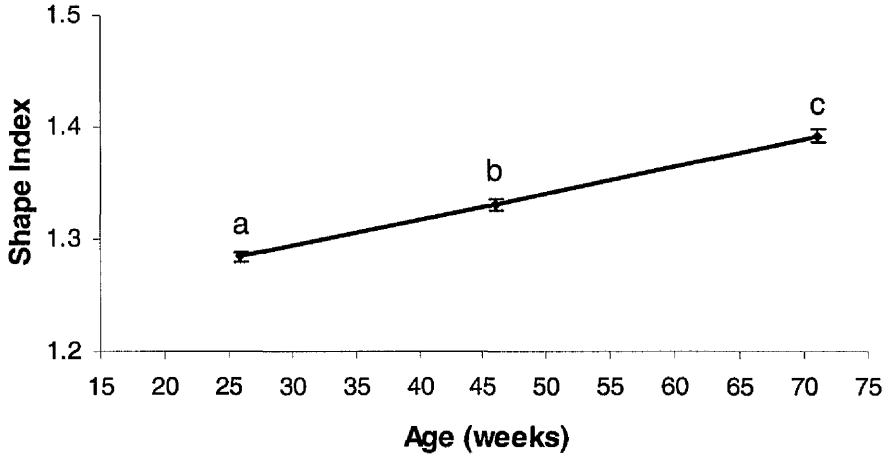


Figure 5.13: Pooled means (± S.E.) for shape index for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

Shell Total Thickness:

This measure increased significantly between beginning of lay (25 weeks) and mid lay (46 weeks) ($P=0.016$) and then remained constant through to the end of lay (71 weeks) (Figure 5.14; *Appendix 6 Table A6.7*).

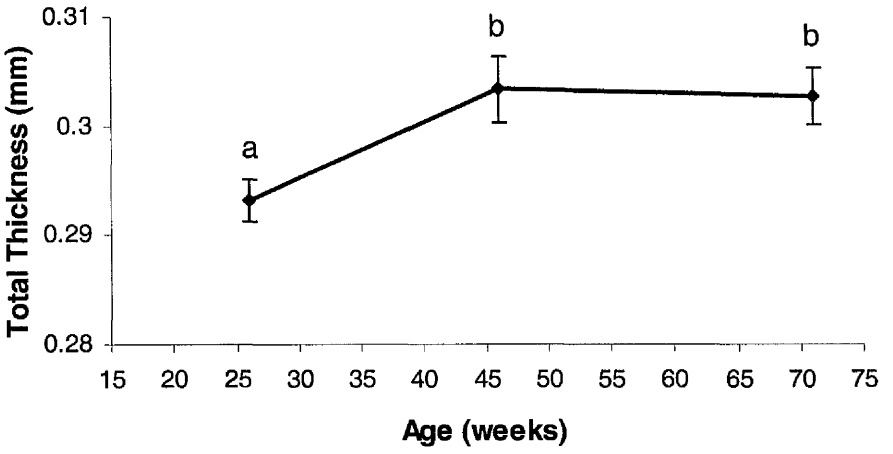


Figure 5.14: Pooled means (± S.E.) for shell total thickness for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

Mammillary Thickness:

This measure decreased steadily throughout lay (Figure 5.15) and further analysis revealed significant differences between week 26 and week 71 (*P*=0.010; *Appendix 6 Table A6.8*).

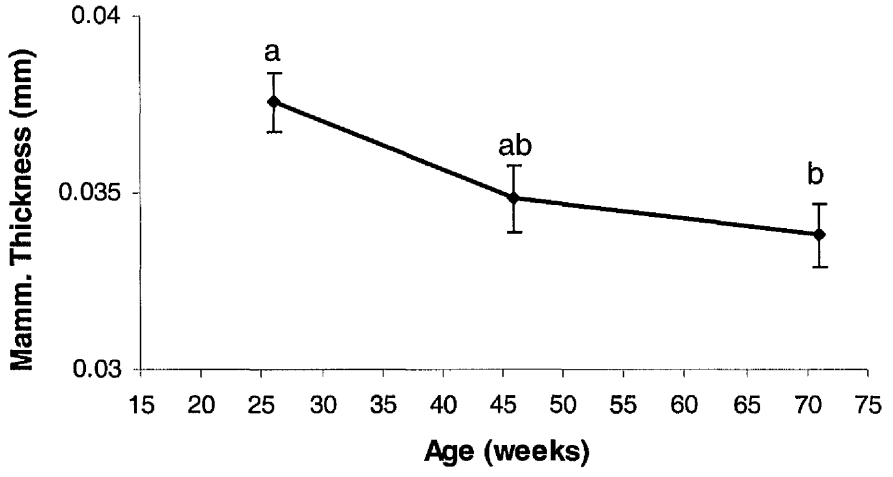


Figure 5.15: Pooled means (± S.E.) for shell mammillary thickness for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

Effective Thickness:

This measure followed a similar pattern to the total shell thickness. The effective thickness increased significantly between beginning of lay and mid lay (*P*=0.001), and then remained relatively constant through to the end of lay (Figure 5.16; *Appendix 6 Table A6.9*).

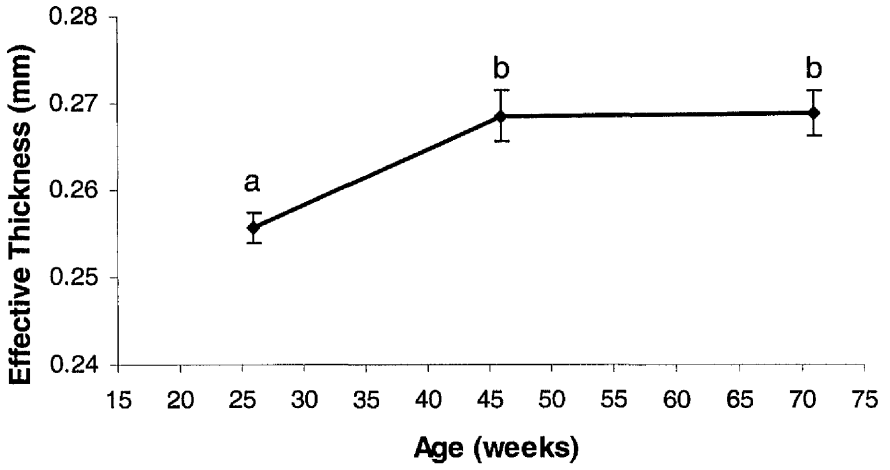


Figure 5.16: Pooled means (± S.E.) for shell effective thickness for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

Breaking Strength:

This measure decreased throughout lay (Figure 5.17) and further analysis revealed significant differences in breaking strength between eggs from all ages ($P<0.001$; *Appendix 6 Table A6.10*).

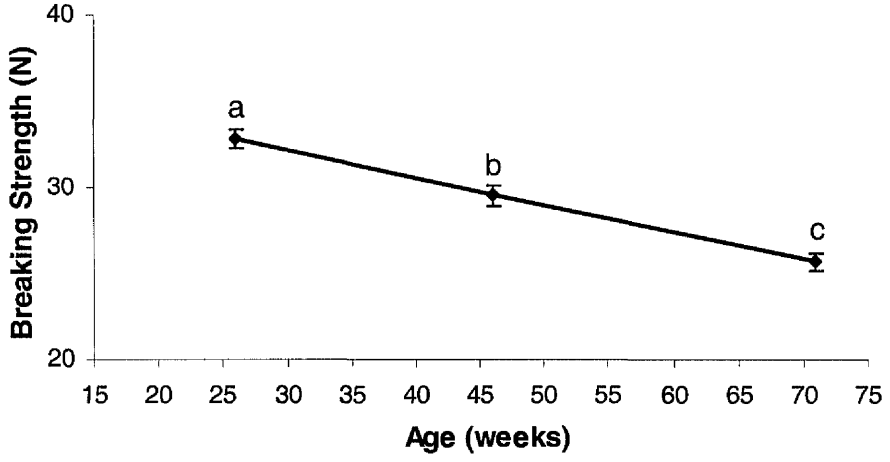


Figure 5.17: Pooled means (± S.E.) for breaking strength for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

Stiffness:

This measure increased significantly between beginning of lay and mid lay ($P=0.004$) and then decreased significantly between mid and end of lay ($P=0.003$; Figure 5.18; *Appendix 6 Table A6.11*).

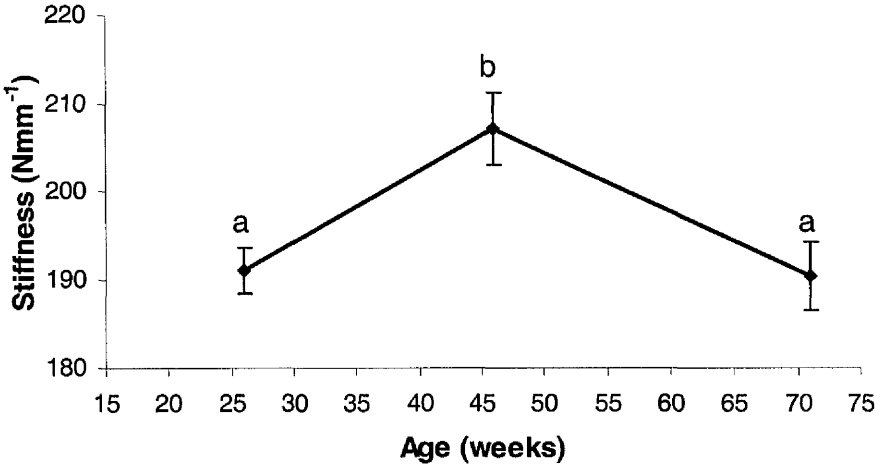


Figure 5.18: Pooled means (± S.E.) for stiffness for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

Fracture Toughness (Kc):

This measure decreased throughout lay (Figure 5.19) and further analysis revealed significant differences in Kc between eggs from all ages ($P<0.001$; *Appendix 6 Table A6.12*).

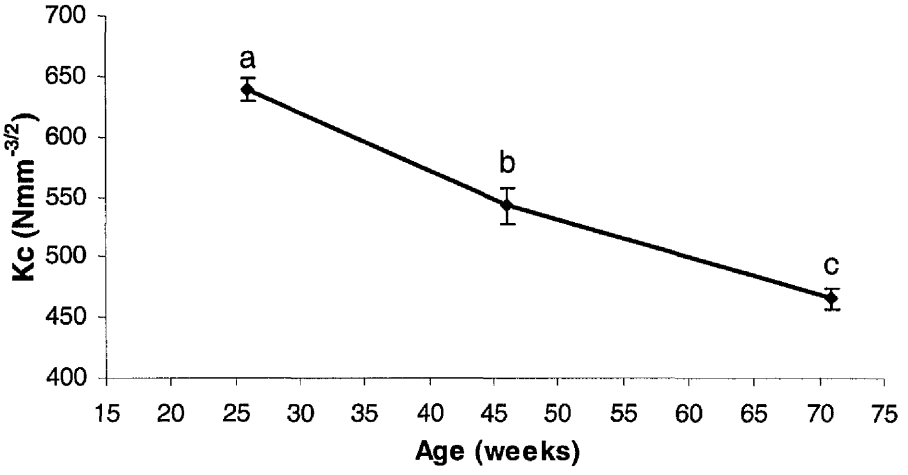


Figure 5.19: Pooled means (± S.E.) for Kc for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

E Shell:

This measure was constant between beginning of lay and mid lay and then decreased significantly between the middle and end of lay ($P=0.001$; Figure 5.20; *Appendix 6 Table A6.13*).

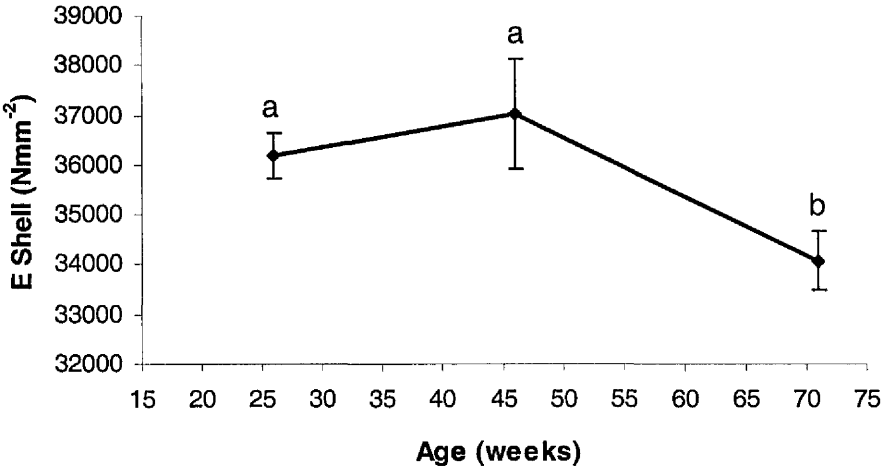


Figure 5.20: Pooled means (± S.E.) for E Shell for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

5.4.4: MAMMILLARY LAYER ULTRASTRUCTURE

5.4.4.1: Tier Effect – within each age

No tier effect was found for total score at any age. With regard to individual ultrastructural scores, significant tier effects were found in eggs at beginning of lay (26 weeks) at which time significant differences with respect to the incidence of erosion were found. At mid lay (46 weeks) significant differences were found for cuffing. In eggs from the end of lay (71 weeks) significant differences were found for type B scores (Table 5.4).

	26 Weeks	**46 Weeks**	**71 Weeks**
Total Score	H=0.48 df=2 *P*=0.786	H=2.72 df=2 *P*=0.256	H=4.27 df=2 *P*=0.118
Confluence	H=1.76 df=2 *P*=0.415	H=1.68 df=2 *P*=0.432	H=1.51 df=2 *P*=0.471
Caps	H=0.82 df=2 *P*=0.663	H=1.67 df=2 *P*=0.433	H=1.10 df=2 *P*=0.576
Fusion	H=3.36 df=2 *P*=0.186	H=1.65 df=2 *P*=0.439	*H=5.60* *df=2* *P=0.061*
Alignment	H=0.59 df=2 *P*=0.746	H=2.01 df=2 *P*=0.366	H=0.49 df=2 *P*=0.784
Type B	H=2.20 df=2 *P*=0.333	H=0.80 df=2 *P*=0.669	**H=10.91** **df=2** ***P*=0.004**
Depression	H=2.07 df=2 *P*=0.355	H=1.82 df=2 *P*=0.403	H=1.65 df=2 *P*=0.439
Erosion	**H=8.75** **df=2** ***P*=0.031**	H=2.14 df=2 *P*=0.342	H=3.13 df=2 *P*=0.210
Aragonite	H=1.01 df=2 *P*=0.603	H=1.01 df=2 *P*=0.603	H=0.30 df=2 *P*=0.860
Type A	H=0.38 df=2 *P*=0.827	H=0.53 DF=2 *P*=0.767	H=1.02 df=2 P=0.601
Rhombohedral Calcite	H=1.06 df=2 *P*=0.589	H=2.00 df=2 *P*=0.368	H=0.00 df=2 *P*=1.00
Cuffing	H=0.12 df=2 *P*=0.942	**H=6.08** **df=2** ***P*=0.048**	H=1.53 df=2 *P*=0.464
Changed Membrane	H=0.22 df=2 *P*=0.895	H=2.00 df=2 *P*=0.368	H=1.33 df=2 *P*=0.515

Table 5.4: P-values and Kruskal-Wallis test statistics (H) for differences in tier for data from each age separately.

Beginning of Lay (26 weeks):

A significant tier effect was found for erosion scores in eggs from the beginning of lay period. Further analysis revealed that erosion scores in eggs from tier A were significantly higher than those from both Tier C (*P*=0.014) and Tier E (*P*=0.028; Figure 5.21; *Appendix 6 Table A6.14*).

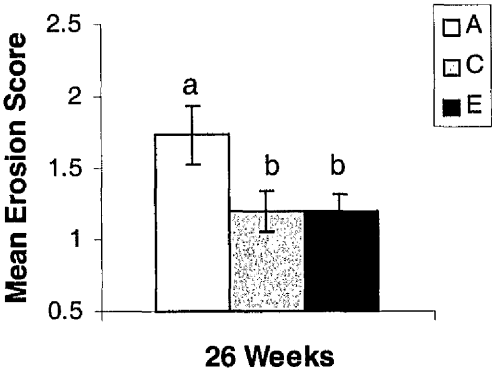


Figure 5.21: Means (±S.E.) for erosion score in eggs at 26 weeks for each tier. Columns sharing the same letter are not significantly different (P>0.05).

Mid Lay (46 weeks):

A significant tier effect was found for cuffing scores in eggs at mid lay and further analysis revealed significant differences between eggs from Tier C and those from Tier E (P=0.012; Figure 5.22; *Appendix 6 Table A6.15*).

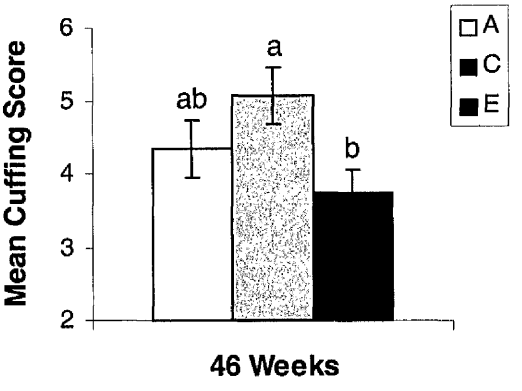


Figure 5.22: Means (±S.E.) for cuffing score in eggs at 46 weeks for each tier. Columns sharing the same letter are not significantly different (P>0.05).

End of Lay (71 weeks):

A significant tier effect was found for type B scores in eggs from the end of lay and further analysis revealed significant differences between eggs from Tier C and those from Tier E (P=0.001; Figure 5.23; *Appendix 6 Table A6.16*).

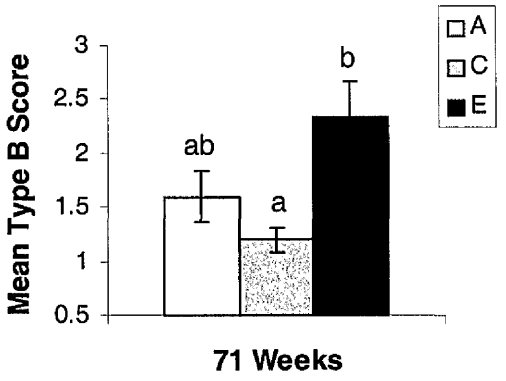


Figure 5.23: Means (±S.E.) for type B score in eggs at 71 weeks for each tier. Columns sharing the same letter are not significantly different (P>0.05).

5.4.4.2: Age Effect – within each tier

A significant age effect with regard to total score was only apparent in tier E. With respect to individual structural characteristics significant age effects were found in eggs from tier A for the presence of rhombohedral calcite and changed membrane scores. In eggs from tier C significant differences were found for depression, rhombohedral calcite and changed membrane scores. In eggs from tier E significant differences were found for type B's and erosion (Table 5.5).

	Tier A	Tier C	Tier E
Total Score	*H=4.70* *df=2* *P=0.095*	H=0.86 df=2 *P*=0.650	**H=8.02** **df=2** ***P*=0.018**
Confluence	H=2.66 df=2 *P*=0.264	H=4.36 df=2 *P*=0.113	H=1.79 df=2 *P*=0.409
Caps	H=1.45 df=2 *P*=0.484	H=1.72 df=2 *P*=0.424	H=3.11 df=2 *P*=0.211
Fusion	H=0.26 df=2 *P*=0.879	H=3.97 df=2 *P*=0.137	*H=4.82* *df=2* *P=0.090*
Alignment	H=0.27 df=2 *P*=0.875	*H=5.02* *df=2* *P=0.081*	H=0.28 df=2 *P*=0.868
Type B	H=3.25 df=2 *P*=0.197	H=0.23 df=2 *P*=0.892	**H=17.22** **df=2** ***P*<0.001**
Depression	*H=4.95* *df=2* *P=0.084*	**H=7.38** **df=2** ***P*=0.025**	H=2.09 df=2 *P*=0.352
Erosion	H=4.60 df=2 *P*=0.100	H=1.09 df=2 *P*=0.581	**H=9.63** **df=2** ***P*=0.008**
Aragonite	H=0.54 df=2 *P*=0.765	H=4.05 df=2 *P*=0.132	H=1.79 df=2 P=0.409
Type A	H=0.19 df=2 *P*=0.909	H=1.12 df=2 *P*=0.570	H=1.53 df=2 *P*=0.465
Rhombohedral Calcite	**H=6.14** **df=2** ***P*=0.046**	H=1.01 df=2 *P*=0.603	H=4.11 df=2 *P*=0.128
Cuffing	H=1.95 df=2 *P*=0.377	H=3.35 df=2 *P*=0.187	H=1.28 df=2 *P*=0.528
Changed Membrane	**H=6.59** **df=2** ***P*=0.037**	**H=10.71** **df=2** ***P*=0.005**	H=4.07 df=2 *P*=0.131

Table 5.5: P-values and Kruskal-Wallis test statistics (H) for age differences in ultrastructural data from each tier separately.

Tier A:

A significant age effect was found with regard to rhombohedral calcite scores in eggs from tier A. It appeared that the scores were higher at the beginning of lay (26 weeks), but further analysis revealed no significant differences between any of the age groups (Figure 5.24).

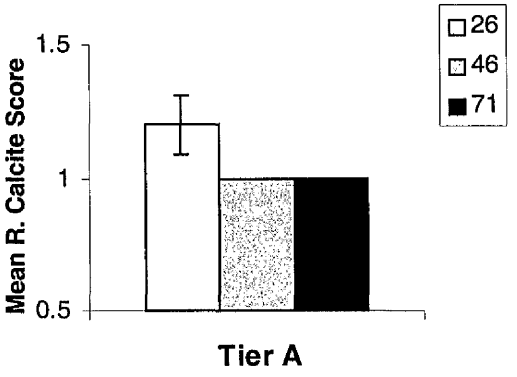


Figure 5.24: Means (±S.E.) for rhombohedral calcite score in eggs from tier A at each age.

A significant age effect was also found with regard to changed membrane scores in eggs from tier A. Further analysis revealed that the eggs from the mid lay period (46 weeks) had significantly lower changed membrane scores than eggs from the end of lay (71 weeks) (*P*=0.010; Figure 5.25; *Appendix 6 Table A6.17*).

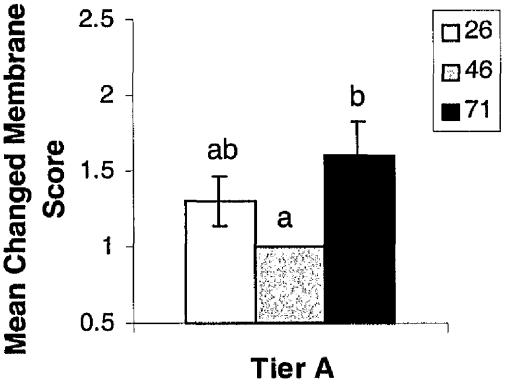


Figure 5.25: Means (±S.E.) for changed membrane score in eggs from tiers A at each age. Columns sharing the same letter are not significantly different (P>0.05).

Tier C:

A significant age effect was found for depression score in eggs from tier C. Further analysis revealed that depression scores in eggs from mid lay (46 weeks) were significantly lower than those from both beginning of lay (*P*=0.019) and end of lay periods (*P*=0.011; Figure 5.26; *Appendix 6 Table A6.18*).

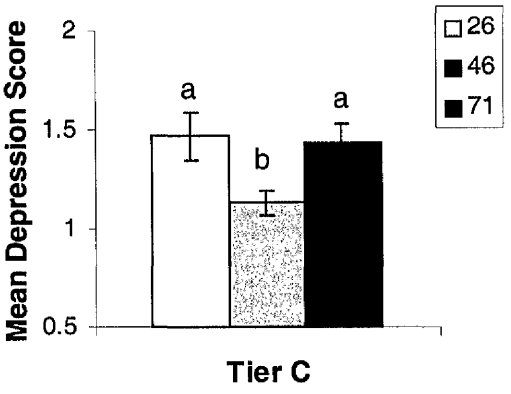


Figure 5.26: Means (±S.E.) for depression score in eggs from tier C at each age. Columns sharing the same letter are not significantly different (P>0.05).

A significant age effect was also found for changed membrane scores in eggs from tier C. Further analysis revealed that changed membrane scores in eggs from mid lay (46 weeks) were also significantly lower than those from both beginning of lay (P=0.040) and end of lay periods (P=0.001; Figure 5.27; *Appendix 6 Table A6.19*).

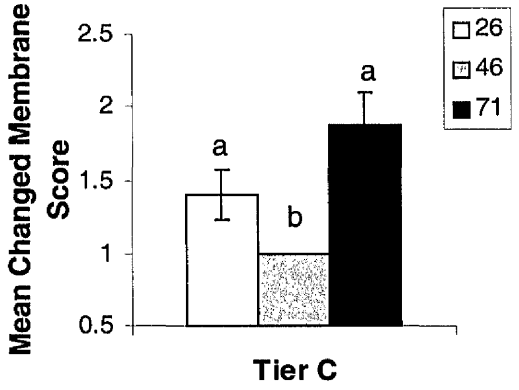


Figure 5.27: Means (±S.E.) for changed membrane score in eggs from tiers C at each age. Columns sharing the same letter are not significantly different (P>0.05).

Tier E:

A significant age effect was found for the total score in eggs from tier E. Further analysis revealed that the total score scores in eggs from the end of lay (71 weeks) were significantly higher than those from both beginning of lay (P=0.015) and mid lay periods (P=0.015; Figure 5.28; *Appendix 6 Table A6.20*).

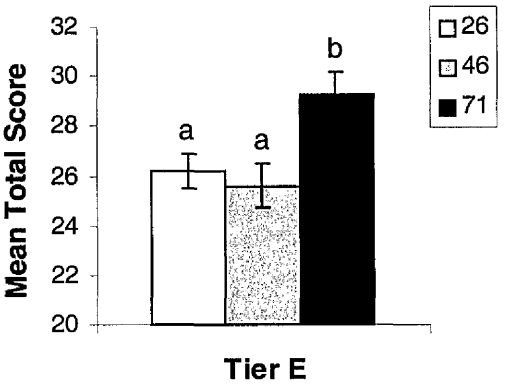


Figure 5.28: Means (±S.E.) for total score in eggs from tier E at each age. Columns sharing the same letter are not significantly different (P>0.05).

A significant age effect was also found for type B score in eggs from tier E. Further analysis revealed that the type B scores in eggs from the end of lay (71 weeks) were also significantly higher than those from both beginning of lay ($P<0.001$) and mid lay periods ($P=0.011$; Figure 5.29; *Appendix 6 Table A6.21*).

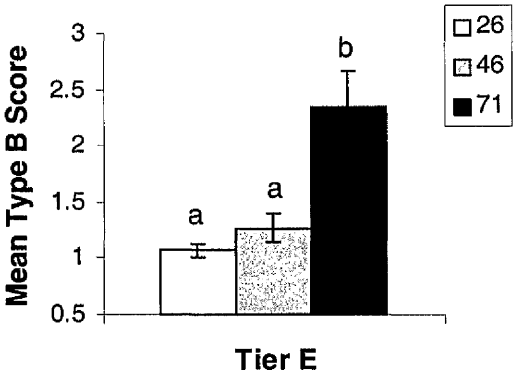


Figure 5.29: Means (±S.E.) for type B score in eggs from tier E at each age. Columns sharing the same letter are not significantly different (P>0.05).

A significant age effect was also found for erosion scores in eggs from tier E. Further analysis revealed that the eggs from the mid lay period (46 weeks) had significantly lower erosion scores than eggs from the end of lay (71 weeks) ($P=0.005$; Figure 5.30; *Appendix 6 Table A6.22*).

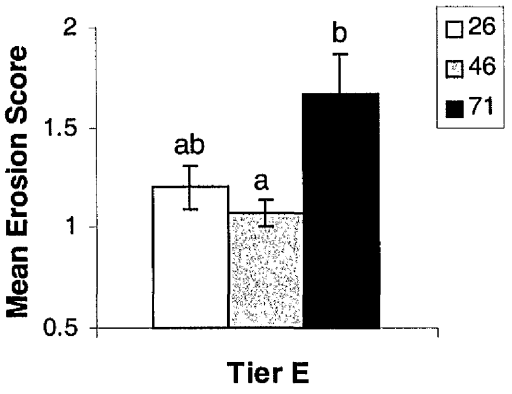


Figure 5.30: Means (±S.E.) for erosion score in eggs from tier E at each age. Columns sharing the same letter are not significantly different (P>0.05).

5.4.5: INTERNAL QUALITY DATA

5.4.5.1: Albumen and Yolk Data

Tier and Age Effects

Significant tier effects were only found for yolk colour (Table 5.6). Significant age effects were found for Haugh units and yolk colour.

	Tier	Age
Haugh Units	F=0.19 df=2 *P*=0.829	**F=71.07** **df=2** ***P*<0.000**
Yolk Colour	**F=4.60** **df=2** ***P*=0.011**	**F=20.36** **df=2** ***P*<0.000**

Table 5.6: Effect of tier and age on internal egg quality.

Tier Effect

There was a significant effect of tier on egg yolk colour and further analysis revealed significant differences between eggs from tier A and those from tier E (*P*=0.007; Figure 5.31; *Appendix 6 Table A6.23*).

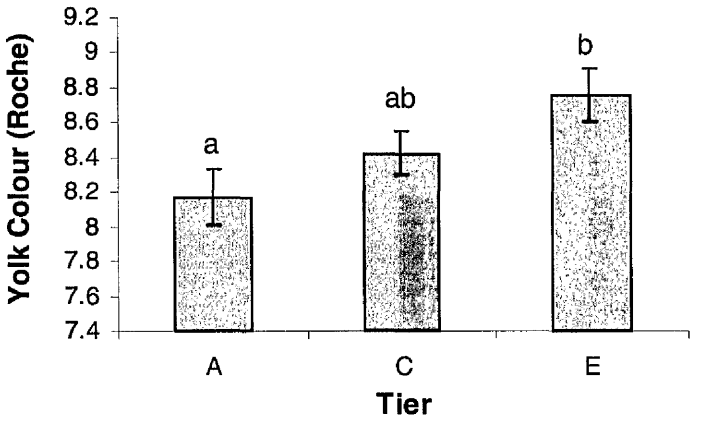


Figure 5.31: Means (±S.E.) for yolk colour for all eggs from each tier. Columns sharing the same letter are not significantly different (P>0.05).

Age Effect

The mean haugh units decreased throughout the laying period and further analysis revealed significant differences between all age groups (*P*<0.001; Figure 5.32; *Appendix 6 Table A6.24*).

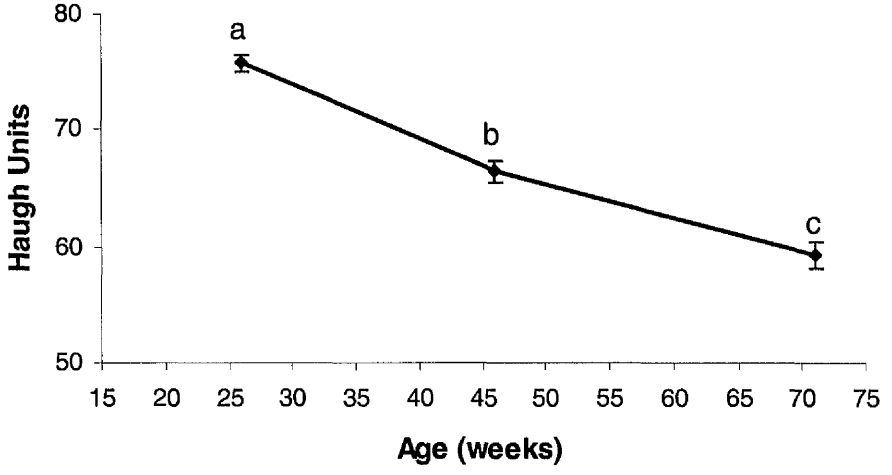


Figure 5.32: Pooled means (± S.E.) for haugh units for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

A significant age effect was also found with respect to yolk colour and further analysis revealed that Roche values at both the beginning and end of lay periods were significantly higher than those found at mid lay (*P*<0.001; Figure 5.33; *Appendix 6 Table A6.25*).

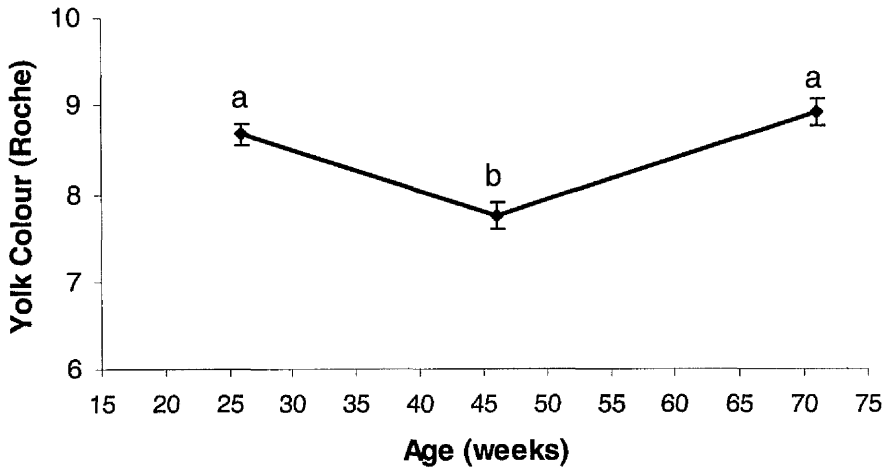


Figure 5.33: Pooled means (± S.E.) for yolk colour for all eggs throughout lay. Data points sharing the same letter are not significantly different (P>0.05).

5.4.5.2: Blood and Meat Spot Counts

Effect of tier within each age:

When the data were analysed for each age separately no significant tier effects were found for either measure (Table 5.7).

	26 weeks	46 weeks	71 weeks
Blood Spots	H=1.01 df=2 *P*=0.603	H=0.00 df=2 *P*=0.999	H=1.24 df=2 *P*=0.539
Meat Spots	H=0.60 df=2 *P*=0.741	H=1.88 df=2 *P*=0.390	H=4.01 df=2 *P*=0.135

Table 5.7: Effect of tier on blood and meat spot occurrence at each age.

Effect of age within each tier:

When the data were analysed for each tier separately significant age effects were found for meat spots in tiers C and E and blood spots in tier E (Table 5.8).

	A	C	E
Blood Spots	H=1.79 df=2 *P*=0.408	*H=5.55* *df=2* *P=0.062*	**H=8.26*** **df=2** ***P*=0.016**
Meat Spots	H=4.07 df=2 *P*=0.131	**H=6.67*** **df=2** ***P*=0.036**	**H=9.26**** **df=2** ***P*=0.010**

Table 5.8: Effect of age on blood and meat spot occurrence in eggs from each tier.

Blood Spots:

The mean number of blood spots remained low in all tiers throughout (< 0.4). Nevertheless the number of blood spots found in the eggs from tier E increased significantly between the beginning of lay (26 weeks) and end of lay (71 weeks) (*P*=0.005; Figure 5.34; *Appendix 6 Table A6.26*).

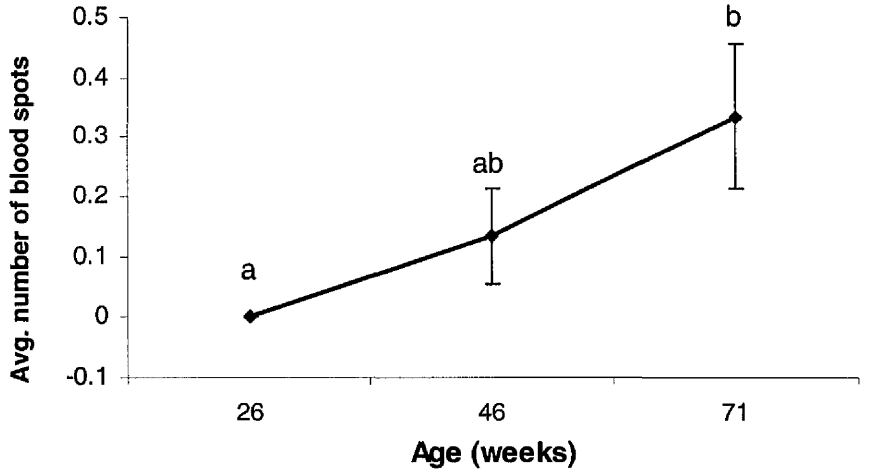


Figure 5.34: Average number (± S.E.) of blood spots in eggs from tier E at each age. Data points sharing the same letter are not significantly different (P>0.05).

Meat Spots:

A significant age effect on the number of meat spots was found in eggs from tier C and tier E (Table 5.8). Figure 5.35 shows that the number of meat spots in eggs, from both of these tiers, increased with bird age. Further analysis of data from tier C revealed significant differences between the beginning of lay (week 26) and the end of lay (week 71) (*P*=0.010; *Appendix 6 Table A6.27*). Analysis of data from tier E revealed a significant increase in meats spots at the end of lay period (71 weeks) compared to the beginning of lay (*P*=0.004) and mid lay periods (*P*=0.040; *Appendix 6 Table A6.28*).

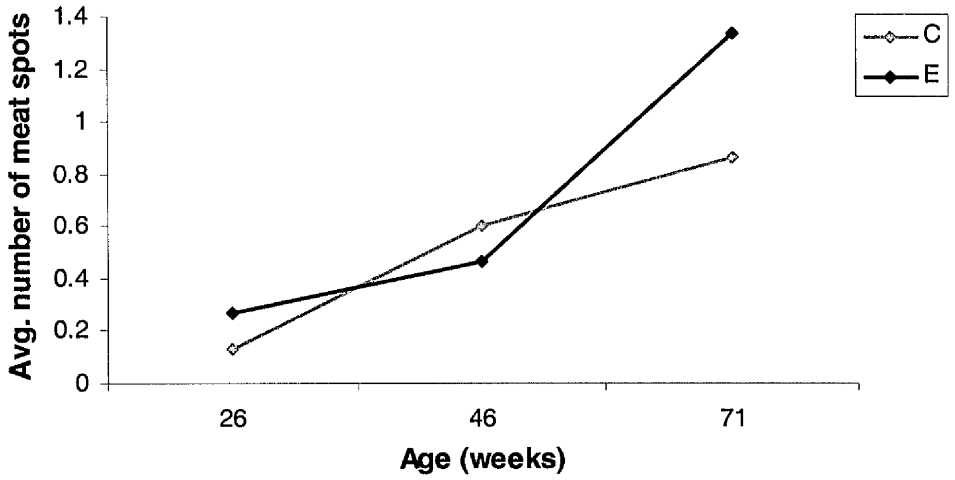


Figure 5.35: Average number of meat spots (S.E. bars not shown) in eggs from tier C and E at each age.

5.5: DISCUSSION

In a house of the size utilised in this study it was anticipated that certain environmental factors would fluctuate during the laying year (Figures 5.2, 5.8). Thus temperature and humidity levels changed significantly within the layer shed. The top tiers consistently displayed higher temperatures (Figure 5.3), an observation previously recorded by Jones (1985a). This having been said the actual range in temperature was as little as 3°C and the levels only fluctuated within 20–24 °C. According to Say (1987; cited by Brackpool, 1995) and Cobb (1991) such variation is unlikely to induce any form of thermal stress.

In a multi-tier system, uniformity of lighting levels is difficult to achieve as illustrated herein, viz the top tiers (A and B) were significantly brighter inside while tiers B and C were brighter outside (Figures 5.6, 5.7). The maximum lighting levels were found in tier B rather than tier A and this is explained by the presence of the shades on the house lights at the level of tier A. The results of Jones (1985a) (from work on a three tier system) corroborate the pattern observed.

Mortality levels fluctuated significantly throughout the year within the entire house (Figure 5.4) and also between tiers, with tiers A and B displaying higher recordings (Figure 5.5). Of the environmental factors measured, or at best noted, viz lighting, temperature, humidity,

ammonia levels and ventilation, only the increased lighting in the higher tiers can be cited as having a potential role to play in this pattern of mortality. An increase in light intensity encourages increased bird activity (Kull, 1948; Hughes and Black, 1974) and so establishes a cascade of behavioural changes leading to feather pecking (Hughes and Duncan, 1972) which according to El-lethey (2000) is suggestive of increased levels of stress. The earlier work of Jones (1984a cited by Jones 1985a) however refutes these hypotheses. In a similar experimental set-up (3 tier system) this author recorded equivalent levels of fear response from birds in the middle and bottom tiers irrespective of the substantial differences in light and temperature. In the current trial, it was reported by the stockmen that the birds in the top tier (A) were more 'flighty' which is in accordance with the findings of Jones (1985a) who demonstrated increased fear responses in birds from the top tier. It is the current author's opinion that the birds in the top tier in this system would have an increased level of activity due to the increased lighting levels and that this in turn may have led to aggressive interactions resulting in increased mortality on this tier.

The production data generated a standard productivity curve with a slight depression in productivity towards the end of lay (Figure 5.9). This decline has been reported by many authors (Hemsworth and Barnett, 1989; Lillpers and Wilhelmson, 1993). The percentage of second-class eggs was high at the beginning of lay but then remained under 2% throughout the rest of lay (Figure 5.10). This result is less than the values found by Hughes *et al.* (1985) who reported that the production of cracked eggs increased from 1% at the beginning of lay to nearer 10% at the end. It is likely that the observed discrepancy in the results found within this study were due to differences in system, stocking densities, cage design and strain of bird. The percentage of seconds which were due to cracked shells remained high throughout lay (Figure 5.11).

No tier effect was found with regard to any of the physical and material measurements of eggshell quality which is in contrast to the findings of Hurnik *et al.* (1974) who reported that eggs laid on the top tier were heavier. Significant age effects however were found for all physical and material measurements. Fresh egg weight increased throughout lay (Figure 5.12) and this is in accordance with the findings of many other authors (Cunningham *et al.,* 1960, Roland 1979; Nys, 1986; O'Sullivan *et al.,* 1991; Shalev and Pasternak, 1993; Brackpool, 1995; Roberts *et al.,* 1997). Shape index also increased throughout lay. Thus the eggs became less spherical and more elongate (Figure 5.13). Total shell thickness and effective thickness increased between the beginning and middle of lay and then remained stable (Figures 5.14,

5.16), findings which are in agreement with Kaminska and Skraba (1993). The latter authors reported an increase in shell weight, indicative of an increase in shell thickness, at mid lay (41 weeks) which was maintained until the end of lay (62 weeks). In the current study breaking strength and fracture toughness both decreased significantly throughout lay (Figures 5.17, 5.19) indicating that shell quality was nevertheless deteriorating. E shell showed a significant decrease between the middle and end of lay signifying that the shells were also becoming more elastic (Figure 5.20).

There was no consistency with regard to the morphology of the specific shell faults at the level of the mammillary layer within the house, although each tier displayed a greater incidence of one form or another between the middle and end of lay periods, results which are in agreement with the findings of Bain (1990), Nascimento (1992) and Brackpool (1995). It is perhaps pertinent to note however that in both tiers A and C i.e. tiers with observed higher temperature and higher lighting, the changed membrane phenomena increased at the end of lay (Figures 5.25, 5.27). This variation accompanies a change in the pH of the oviduct and signifies a trend towards increased acidity (Watt, 1989). The cumulative effects of environmental stress and age might be implicated in its occurrence. The total score is representative of the overall structural integrity of the mammillary layer and many authors have reported an increase in this score with increasing bird age, suggestive of a deterioration in mammillary quality (Bain, 1990; Nascimento, 1992; Darnell-Middleton, 1999). Within this study a significant increase in total score was found only in eggs from tier E (Figure 5.28) although the eggs from tier A did demonstrate a trend for increased total score at the end of lay (Table 5.5). In contrast total score in tier C was not affected by bird age, suggestive of a maintenance of mammillary quality at the end of lay in these birds. This may be a consequence of the less extreme environmental conditions found at this level with respect to lighting and temperature.

Albumen quality deteriorates with increasing bird age and with storage time, an observation recorded over many years and confirmed within this investigation (Figure 5.32) (Romanoff and Romanoff, 1949; Roberts *et al.*, 1997). No tier effects were observed with regard to this measure. This investigation did however highlight tier effects with regard to yolk colour with an increased intensity of colour being detected in eggs from tier E i.e. the bottom tier (Figure 5.31). Although this difference was found to be significant, the intensity of colour in all yolks was within the acceptable range and differences would not have been detected visually. Emery *et al.* (1984) reported a reduction in food consumption associated with increased environmental temperature and at this stage it is hypothesised that the differences noted may

be the result of the variation in temperature and lighting having an effect on bird activity patterns perhaps resulting in altered feed intake i.e. birds from the top tier being more active and spending less time feeding. It is also possible that the increased temperature itself may be responsible for a reduced appetite in the birds again resulting in reduced feed intake (Hughes, pers. comm.). Wakeman (pers. comm.) has noted differences in yolk colour as a result of reduced food consumption. At this time no further conclusions can be made.

Age effects on internal quality were also detected with respect to the inclusion of blood and meat spots (Figures 5.34, 5.35). Solomon (2000) classified the latter into three distinct groups i.e. tissue, blood and tissue and calcified deposits. The results reported in this chapter were generated prior to the establishment of this classification system and so no comment can be made as to the nature of the inclusions found. Nevertheless the number of blood and meat spots were significantly increased, with increasing bird age, in tier E i.e. the tier with poorer eggshell quality, where ammonia levels, although not recorded, were noted to be elevated. Thus environmental factors may be related to the appearance of these inclusions.

5.6: CONCLUSIONS

The cage environment within this multi-tier system varied in relation to tier level and time of year. Temperature and lighting levels were greater in the upper tiers (A to C) and temperature and humidity levels varied significantly throughout the laying year within the entire house.

Mortality levels were greatest on the top two tiers (A and B) and the birds from tier A were reported to be more active. It is possible that the increased lighting and activity on these upper levels is responsible for the increased mortality due to the increased likelihood of feather pecking.

There was no significant difference in the physical and material eggshell quality of eggs laid on each tier although all parameters did vary significantly with increasing bird age. Between mid and end of lay periods the characteristic deterioration in breaking strength, stiffness, fracture toughness (Kc) and E Shell values was demonstrated.

Ultrastructural analysis revealed that eggs from each tier displayed an increase in at least one form of mammillary layer fault with increasing bird age. However an increase in total structural score, i.e. poorer shell quality, was only significant in tier E at the end of lay. This bottom tier also exhibited an increase in blood and meat spots at this time which may be indicative of oviduct breakdown.

With regard to internal egg quality, yolk colour was also more intense in the eggs from the bottom tier (E). No tier effect was observed for albumen quality although it did deteriorate with increasing bird age.

CHAPTER 6

THE APPLICATION OF RESONANCE FREQUENCY TESTING TO MONITOR EGGSHELL QUALITY TRAITS

6.1: INTRODUCTION

Downgrading due to damaged shells accounts for approximately 6 – 8 % of all the eggs laid in commercial production. In monetary terms this gives rise to in excess of £19 million deficit within the UK alone (BEIS, pers. comm.). Obviously this loss is a significant problem within the poultry industry and therefore maintaining eggshell quality is of prime importance.

Eggshell breakage depends both on the strength of the eggshell and the magnitude of the mechanical load applied. Variables associated with eggshell strength are biological in nature (Hamilton, 1982) and involve the material and structural properties of the different layers which comprise the eggshell. The material properties depend on the type and the association between the mineral and the organic components of the shell. The structural properties depend on eggshell thickness, the size and shape of the egg and the distribution of shell over the egg surface. Most methods used for evaluating the mechanical properties of eggshells cannot quantify these aspects separately because of their complex relationships. For example shell curvature, and its brittle nature make it difficult to measure material properties directly (Bain, 1990).

The mechanical stiffness and strength of the whole eggshell are traditionally measured by quasi-static compression (Hunton, 1987). Bain (1991) showed that the mechanical stiffness depends on the thickness of the shell, the curvature of the eggshell, the breadth of the egg and the elastic modulus of the composite formed by the various materials in each layer of the shell. The participation of each material and structural variable in the mechanical stiffness behaviour of the eggshell has been calculated by Bain (1991) in a computer model for the mechanical analysis of structures. However it has been suggested that the quasi-static compression test may bear little relation to the type of stress likely to be encountered in the field and a number of other mechanical methods have been devised to measure the aforementioned properties (Voisey and Hunt, 1974; Hunton, 1987). Since each event which leads to shell fracture in the field is unique to that particular occasion, the view has been taken that experimental

convenience can play a part in deciding which predictive measure to use on any occasion (Hunton, 1987). In a review paper, Voisey and Hunt (1974) reported vibration tests as a possible alternative method to measure the mechanical properties of biological products.

Vibration tests have been developed, within engineering sciences, to allow the assessment of the elastic properties of homogenous materials. Resonance frequency (RF) testing is such a technique, used for the assessment of elastic properties of metals, alloys, ceramics and composites (Lemmens, 1990). For objects with homogenous material properties and simple geometry, analytical relationships are derived that describe the relationship between the RF and its material properties (Blevins, 1993, cited by Coucke *et al.*, 1999). It has also been shown that relationships can be determined between these dynamic values and the compressive strength, porosity, density and crystallisation form of the test specimen (Allison, 1987; Lemmens, 1990; Roebben *et al.,* 1997 cited by Coucke *et al.*, 1999).

In the case of the chicken egg, the interpretation of the vibration response is very complex as the egg is non-homogenous (Voisey and Hunt, 1974), consisting of several layers of material including yolk, albumen, the air chamber and the shell, itself comprising several distinct layers penetrated by gaseous exchange pores (Johnson, 2000). Eggs also have a complex geometry which varies between eggs. Coucke *et al.* (1999) nevertheless devised a model based on a simple engineering concept, to convert the RF value of an egg into a measure of its dynamic stiffness ($K._{DYNAMIC}$). These authors also established relationships between the dynamic stiffness value and some commonly measured eggshell quality parameters but not breaking strength. Nevertheless they concluded that the resonance frequency test is potentially useful as a non-destructive predictor of other, traditionally destructive, means of analysing ESQ. It has also been suggested by De Ketelaere *et al.* (In Press) that this measure may be useful for genetic selection as in practice dynamic loads rather than static loads cause eggshell breakage. Since these publications modifications to the original formulae have been derived but as yet remain untested (Coucke, pers. comm.).

The requirements for good eggshell quality differ between layer flocks and broiler breeder flocks. Eggs laid by broiler breeders are quite different from those laid for the table egg market due to the different demands placed by each sector. Eggs from the table industry are required to be relatively standard in size with an appealing appearance and must be robust enough to withstand packaging, transportation and handling. Aesthetic qualities have little role to play with respect to the broiler breeder egg in so far as they are required to support

embryonic growth and they must be strong enough to withstand the handling and transportation from farm to hatchery.

6.2: AIMS

The previous chapters have described the relationships between certain aspects of bird behaviour and faecal glucocorticoid levels with reference to a variety of shell quality parameters. This chapter addresses the need to consolidate the diversity of measurements used to assess shell quality.

The aim of this pilot investigation was to determine whether the non-destructive resonance frequency test (RFT) including the modifications as detailed by Coucke (pers. comm.) could be used to predict other eggshell quality measures accurately. Within this trial the RFT was used on eggs collected throughout the laying year from one broiler breeder flock and one layer flock. Many authors have reported deterioration in other eggshell quality parameters with increasing bird age (Bain, 1990; Fraser, 1996) and this trial also included the effect of bird age on RFT values.

6.3: MATERIALS AND METHODS

6.3.1: EGGS COLLECTED FOR CORRELATION ANALYSES

270 eggs were collected from a range of layer and broiler breeder flocks. Any cracked or damaged eggs were discarded.

The following measures of eggshell quality were carried out on these eggs. Fresh egg weight, shape index, shell thickness, T.Mamm., T.Effective (section 3.3.5.2), eggshell breaking strength and stiffness (section 4.3.6.2.) and the material properties of the eggshell (section 4.3.6.3).

6.3.2: EGGS COLLECTED FOR ASSESSING AGE EFFECTS ON RF VALUES

6.3.2.1: Layer Eggs

The eggs were collected at random from ISA Brown layers housed in a commercial layer house under standard husbandry conditions. 270 eggs were collected and assessed from three

periods during lay (90 eggs from each period). The first collection occurred at 26 weeks (Beginning of Lay), the second at 46 weeks (Mid Lay) and the third at 71 weeks (End of Lay).

6.3.2.2: Broiler Breeder Eggs

Eggs were collected from Ross 508 hens housed within a standard broiler breeder house. 240 eggs were collected and assessed from four periods during lay (60 eggs from each period). The first collection took place when the birds were 25 weeks old (beginning of lay), the second when the birds were 31 weeks old (Peak Production), the third at 45 weeks (Mid Lay) and the final collection at 57 weeks of age (End of Lay).

6.3.3: Resonance Frequency Analysis

6.3.3.1: Resonance Frequency

Resonance frequency (sometimes called Natural Frequency) is the frequency at which a given structure will vibrate if acted upon by an external energy source. A good analogy is striking a bell to cause it to ring; the tone (a measure of the resonant frequency) is always the same. Striking it harder or softer increases or decreases the volume of the sound (a measure of the energy imparted and the amplitude of vibration) but the tone always remains the same. Resonant frequencies depend upon geometry, thickness, elastic properties and density of the structure. Damping describes the rate of the exponential decay of the vibration amplitude with time.

6.3.3.2: Resonance Frequency Test (RFT)

The resonance frequency test simply involves tapping an egg at the equator thus causing excitation. The impulse of energy imparted to the egg causes it to vibrate. The energy impulse initiates random vibration waves to run around the surface of the egg. These waves are quickly damped out at non-resonant frequencies and the remaining energy causes the egg structure to vibrate at its resonant frequency (analogous to ringing a bell). The natural damping of the egg structure itself however quickly damps out this vibration.

In this trial the tapping energy was applied with a lightweight hammer, made from a plastic strip and a ball bearing (Plate 6.1). Each egg was tapped three times on the equator of the egg. The equator of the egg was chosen as the site for the application of the test because it is the standard site for most eggshell quality analyses. The vibration response of the egg was recorded by a small microphone placed under the egg which recorded the frequency opposite

the point of excitation (Plate 6.2). The acoustic output was recorded and amplified before being analysed by a Dynamic Signal Analyser (HP 36555, Hewlett Packard, USA). The latter provides an output of the average measured resonant frequency (of the three taps). The Matlab computer program (Version 5.1, 1984 – 1997, The Math Works Inc.) was used to display the output from the dynamic signal analyser in terms of the average resonance frequency and damping levels, and then the model developed by Coucke *et al.* (1999) was used to convert this value along with egg weight to produce a value for $K_{DYNAMIC}$.

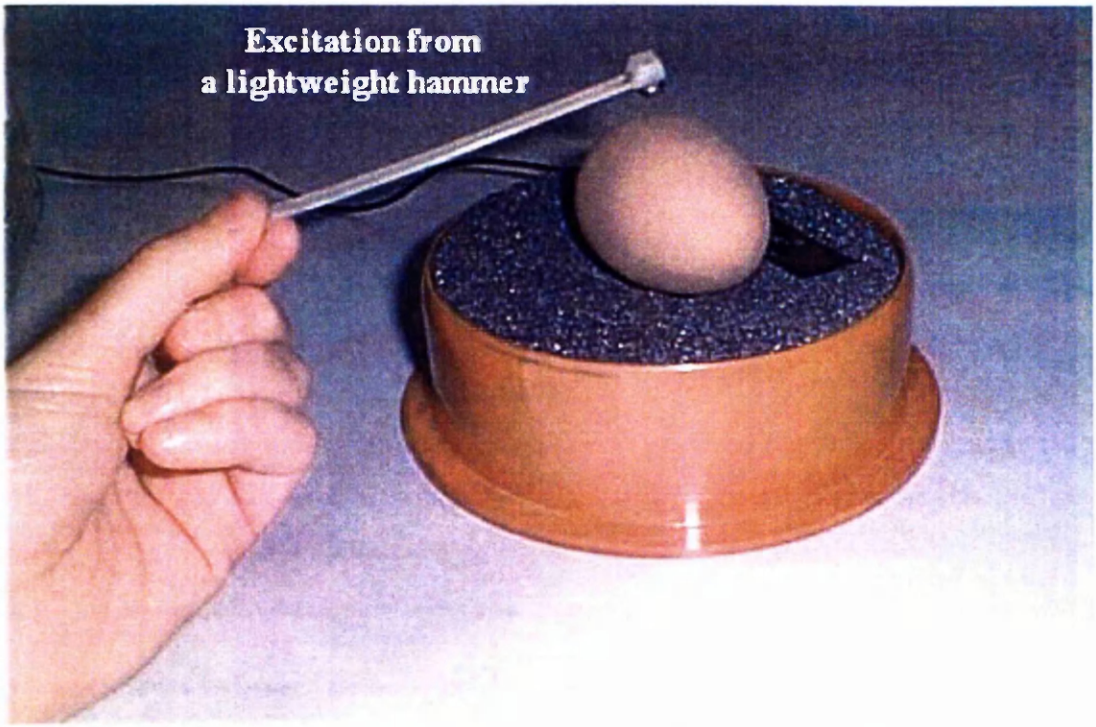


Plate 6.1: Excitation of the egg with a light-weight hammer.

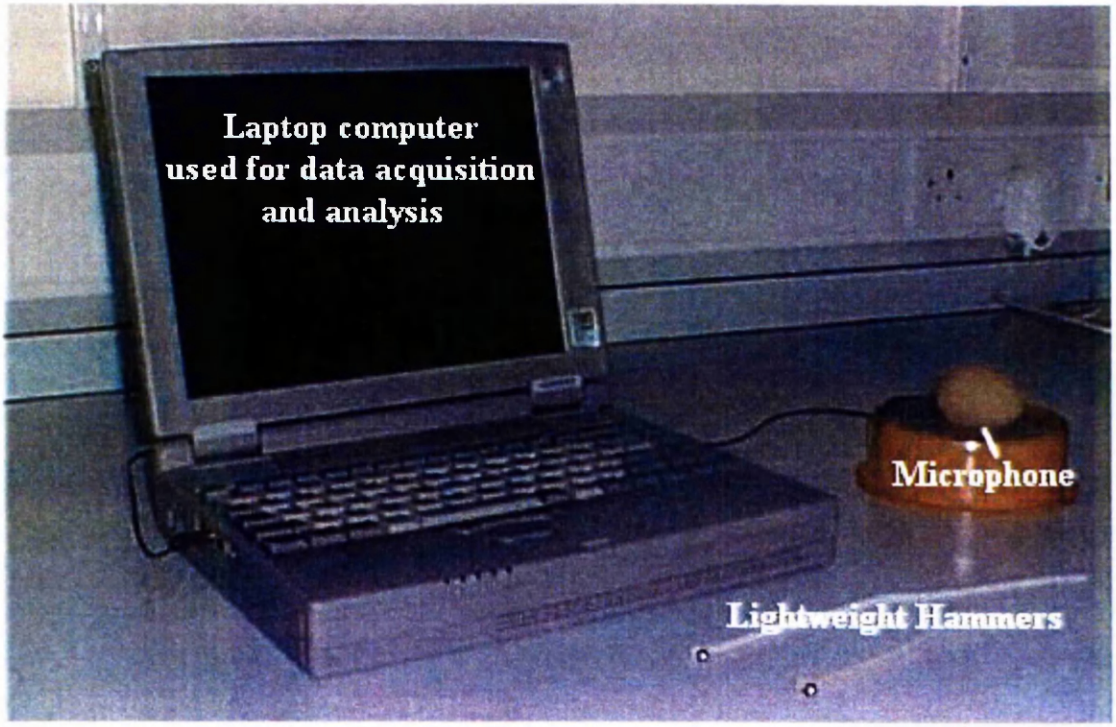


Plate 6.2: Experimental set-up for the measurement of the resonance frequency response of an egg.

Figure 6.1 illustrates a typical time and corresponding frequency signal demonstrating that the vibration response is damped out in under 30 ms. The frequency spectrum of the acoustic signal contains several peaks (natural frequencies) for each egg (Figure 6.2). The RF of the dominant peak is the one used in further calculations.

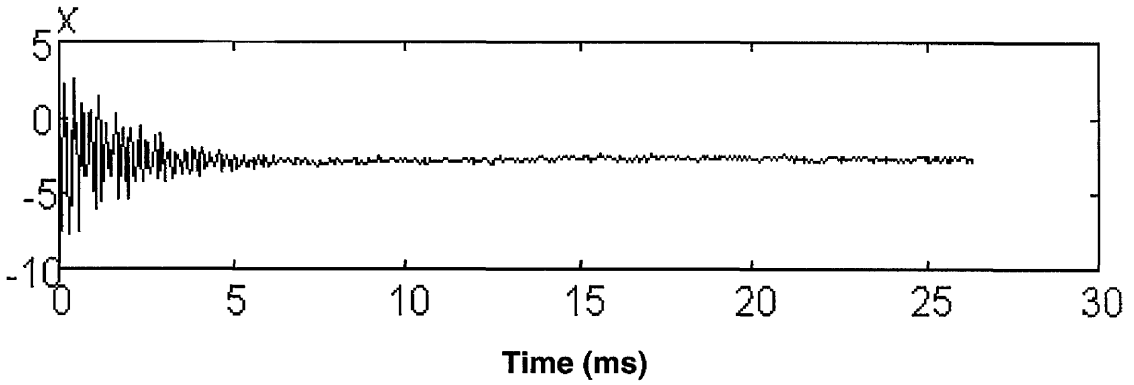


Figure 6.1: Graph showing the damping response of an egg following excitation.

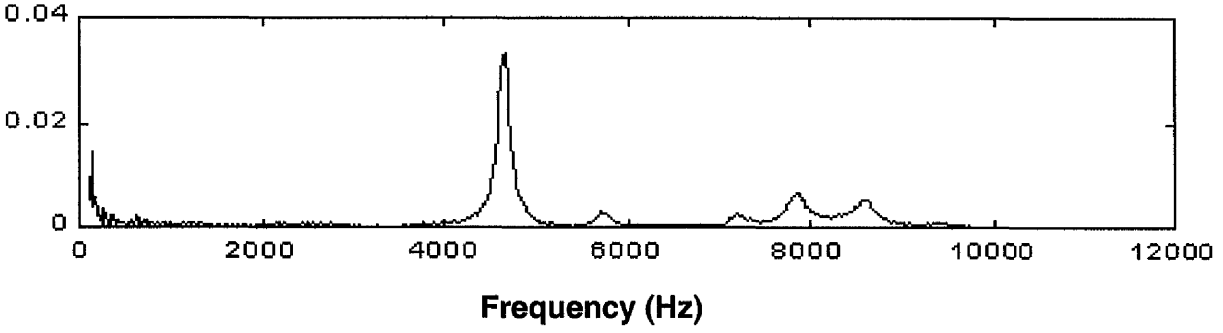


Figure 6.2: Graph showing the frequency signal of an intact egg.

As previously mentioned the geometry and density of the egg will influence RF values. For this reason Coucke *et al.* (1999) included a compensation for egg mass. The model developed by these authors is based on the assumption that the dynamic egg behaviour can be modeled as a linear undamped mass-spring system. Voisey and Hunt (1974) used this mass-spring model for the purpose of modeling the elastic behaviour of the eggshell in a quasi-static compression test.

The model developed by Coucke for determination of the $K_{DYNAMIC}$ value is described in detail in Coucke (1998) and Coucke *et al.* (1999).

The model used was:

$$K._{DYNAMIC} = (2.\pi.\ RF)^2.m$$

Where: m = mass

RF = resonance frequency of the egg

Coucke (pers. comm.) later described a modification to this original formula which takes the average damping into consideration, viz

$$K._{DYNAMIC}\ (correction) = 68.7\ .\ K._{DYNAMIC} + 12.44 \times Average\ Damping.\ K._{DYNAMIC}$$

This corrected model allows the egg's response to the excitation to be considered as a 'Mass-Spring-Damper' system (De Ketelaere, pers. comm.).

In this thesis both $K._{DYNAMIC}$ and $K._{DYNAMIC}$ (correction) have been computed. The average damping is also considered as an independent measure.

6.3.4: STATISTICAL METHOD

6.3.4.1: Relationship between measures

Pearson's correlations were used to establish if there were any relationships between damping, $K._{DYNAMIC}$ and $K._{DYNAMIC}$ (correction) with the other measured eggshell quality traits. Scatter-plots were created for correlations of $R>0.5$ as this corresponds to the predictor value accounting for approximately 25% of the response value. Regression lines and R^2 values were then fitted to these scatter-plots.

6.3.4.2: Age Effect

One-way ANOVA's were used on each data set to look for any changes in the damping, $K._{DYNAMIC}$ and $K._{DYNAMIC}$ (correction) values with increasing bird age. When a result was found to be significant a GLM was run using Tukey's pair-wise comparisons to find the source of significant differences. Adjusted critical *P*-values were used to counter the problem of multiple comparisons between the treatment levels and the inherent increased likelihood of a Type 1 error.

All statistical analyses were carried out using Minitab release 12.1 (© Minitab Inc., 1998).

6.4: RESULTS

6.4.1: RELATIONSHIP BETWEEN RFT RESULTS AND OTHER MEASURES OF ESQ

The relationships which were found between the average damping, K. $_{DYNAMIC}$ and K. $_{DYNAMIC}$ (correlation) values with all other physical and material measures of eggshell quality are shown in Table 6.1 (layer eggs) and Table 6.2 (broiler breeder eggs). It should be noted that the strong correlations amongst the physical and material variables are almost certainly a result of them being related by definition (or related through intermediate variables by definition) and so are not of interest in the context of this chapter.

6.4.1.1: Average Damping in Layer Eggs

In the layer eggs the average damping measure was found to be significantly correlated with fresh weight (R=-0.302, $P<0.001$), shape index (R=-0.611, $P<0.001$), mammillary thickness (T. Mamm) (R=-0.180, $P=0.004$), breaking strength (R=-0.503, $P<0.001$) and fracture toughness (Kc) (R=-0.478, $P<0.001$; Table 6.1). With the exception of shape index and breaking strength these correlations are all relatively weak (R<0.5).

	DAMPING	K. DYN.	K. DYNA (CORR)	WEIGHT	SHAPE INDEX	TOTAL THICK.	MAMM. THICK.	EFFECTIVE THICK.	BREAKING STRENGTH	STIFFN.	KC	E SHELL
DAMPING	1.000											
K. DYNAMIC	**-0.615****	1.000										
K. DYNAMIC (CORR)	0.297**	**0.559****	1.000									
WEIGHT	-0.302**	0.179**	-0.102	1.000								
SHAPE INDEX	**-0.611****	0.225**	-0.377**	0.186**	1.000							
TOTAL THICK.	0.051	0.357**	0.468**	0.098	0.102	1.000						
MAMM THICK.	0.180**	-0.102	0.075	0.011	-0.085	0.245**	1.000					
EFFECT. THICK.	-0.017	0.405**	0.450**	0.098	0.138*	**0.932****	-0.122*	1.000				
B STRENGTH	**0.503****	-0.077	0.432**	-0.232**	-0.491**	0.274**	0.092	0.245**	1.000			
STIFFNESS	0.108	0.308**	0.478**	-0.204**	-0.088	0.556**	0.077	**0.539****	**0.534****	1.000		
KC	0.478**	-0.325	0.125*	-0.320**	**-0.514****	-0.297**	0.144*	-0.359**	**0.806****	0.185**	1.000	
E SHELL	0.036	-0.047	-0.008	-0.155*	-0.118	-0.337**	0.208**	-0.423**	0.260**	**0.500****	**0.506****	1.000

Table 6.1 : Pearson's correlation coefficients between all physical and material measures of ESQ in layer eggs.

Those for which correlation >±0.5 are shown in bold.

Those for which p<0.01 are shown with **

Those for which p<0.05 are shown with *

	Damping	K.Dynamic	K.Dynamic (Corr)	Weight	Shape Index	Total Thick.	Mamm. Thick.	Effect. Thick.	Break. Stren.	Stiff.	Kc	E Shell
Damping	1.000											
K.Dynamic	**-0.639****	1.000										
K.Dynamic (Corr)	0.058	**0.721****	1.000									
Weight	-0.343**	0.238**	0.000	1.000								
Shape Index	-0.268**	-0.128	-0.388**	0.249**	1.000							
Total Thick.	0.016	0.284**	0.400**	0.171**	0.215**	1.000						
Mamm. Thick.	0.156*	-0.082	0.027	-0.231**	-0.141*	0.198**	1.000					
Effect. Thick.	-.044	0.321**	0.396**	0.259**	0.270**	**0.930****	-0.175**	1.000				
B. Strength	0.182**	0.159*	0.368**	-0.148*	-0.099	**0.556****	0.082	**0.529****	1.000			
Stiffness	0.165*	0.193**	0.403**	-0.169	0.010	**0.651****	0.146*	**0.601****	**0.752****	1.000		
Kc	0.261**	-0.111	0.071	-0.444**	-0.304**	-0.157*	0.238**	-0.246**	**0.681****	0.350**	1.000	
E Shell	0.145*	-0.135*	-0.062	-0.292**	-0.201**	-0.359**	0.338**	-0.486**	0.166*	0.369**	**0.607****	1.000

Table 6.2 : Pearson's correlation coefficients between all physical and material measures of ESQ in broiler breeder eggs.

Those for which correlation $\geq \pm 0.5$ are shown in bold.

Those for which $p<0.01$ are shown with **

Those for which $p<0.05$ are shown with *

Average Damping and Shape Index:

Average damping demonstrates a significant but negative correlation with shape index (Figure 6.3) therefore the higher the damping measurement the lower the shape index value (R=-0.611, $P<0.001$).

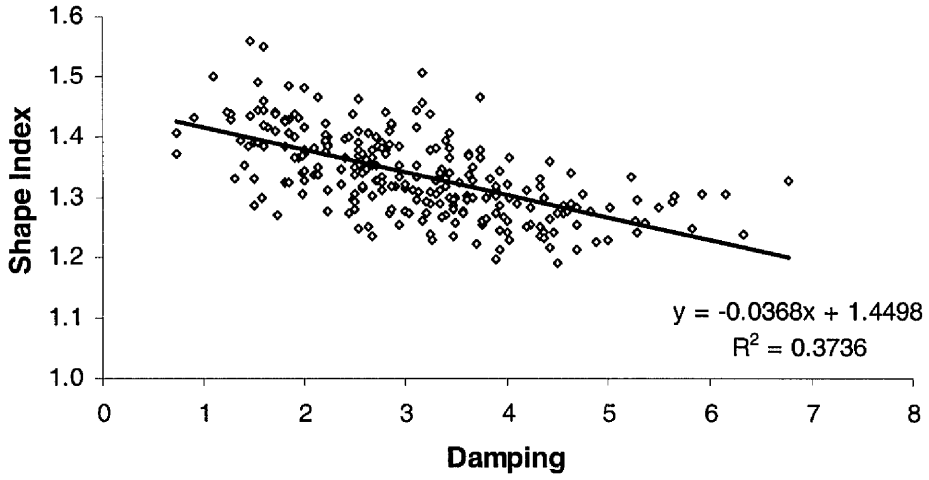


Figure 6.3: Regression between average damping measures and egg shape index in layer eggs (displaying equation and R^2 value).

Average Damping and Breaking Strength:

Average damping demonstrates a significant positive correlation with breaking strength (Figure 6.4) thus the higher the damping measurement the higher the breaking strength (R=-0.503, $P<0.001$).

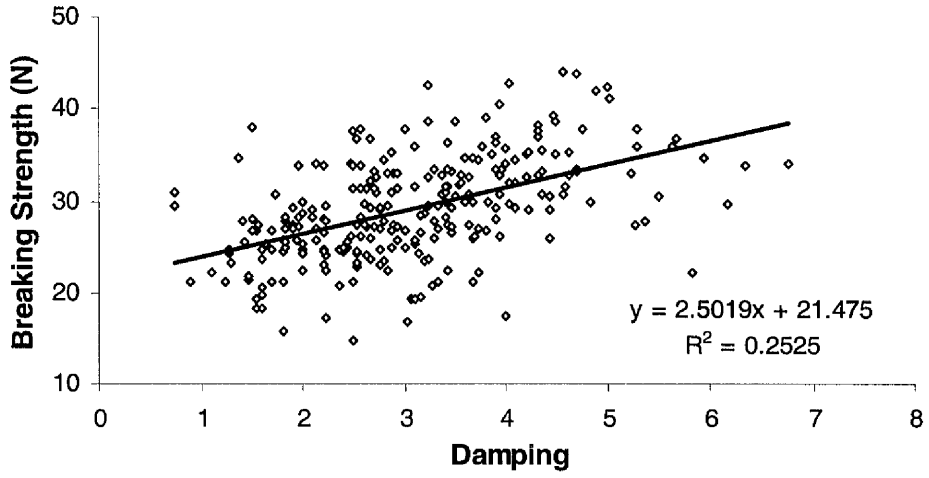


Figure 6.4: Regression between average damping measures and breaking strength in layer eggs (displaying equation and R^2 value).

6.4.1.2: Average Damping in Broiler Breeder Eggs

In the broiler breeder eggs the average damping measure was found to be significantly correlated with fresh weight (R=-0.343, $P<0.001$), shape index (R=-0.268, $P<0.001$), T.Mamm (R=0.156, $P=0.019$), breaking strength (R=0.182, $P=0.06$), stiffness (R=0.165, $P=0.013$), Kc (R=0.261, $P<0.001$) and elastic modulus (E shell) (R=0.145, $P=0.029$; Table 6.2). These correlations however were all very weak.

6.4.1.3: K. $_{DYNAMIC}$ in Layer Eggs

In the layer eggs the average K.$_{DYNAMIC}$ measurement was found to be significantly correlated with fresh weight (R=0.179, $P=0.004$), shape index (R=0.225, $P<0.001$), total thickness (R=0.357, $P<0.001$), T. Effective (R=0.405, $P<0.001$), stiffness (R=0.308, $P<0.001$), and Kc (R=-0.325, $P<0.001$; Table 6.1). These correlations were again relatively weak (R>0.5).

6.4.1.4: K. $_{DYNAMIC}$ in Broiler Breeder Eggs

In the broiler breeder eggs the average K. $_{DYNAMIC}$ measure was found to be significantly correlated with fresh weight (R=0.238, $P<0.001$), total thickness (R=0.284, $P<0.001$), effective thickness (T. Effective) (R=0.321, $P<0.001$), breaking strength (R=0.159, $P<0.001$), stiffness (R=0.193, $P<0.004$) and E shell (R=0.135, $P=0.043$; Table 6.2). Again these correlations were all quite weak (R<0.5).

6.4.1.5: K. $_{DYNAMIC}$ (correction) in Layer Eggs

In the layer eggs the K. $_{DYNAMIC}$ (correction) measure was found to be significantly correlated with shape index (R=-0.377, $P<0.001$), total thickness (R=0.468, $P<0.001$), T. Effective (R=0.450, $P<0.001$), breaking strength (R=0.432, $P<0.001$), stiffness (R=0.478, $P<0.001$) and Kc (R=0.125, $P=0.045$; Table 6.1). These correlations were again weak (R<0.5) but all were greater than the correlations found with K. $_{DYNAMIC}$ with the exception of that for Kc.

6.4.1.6: K. $_{DYNAMIC}$ (correction) in Broiler Breeder Eggs

In the broiler breeder eggs the K. $_{DYNAMIC}$ (correction) measure was found to be significantly correlated with shape index (R=-0.388, $P<0.001$), total thickness (R=0.400, $P<0.001$), T. Effective (R=0.396, $P<0.001$), breaking strength (R=-0.368, $P<0.001$) and stiffness (R=0.403, $P<0.001$; Table 6.2). These correlations are again relatively weak (R<0.5) but are still stronger than the correlations found with K.$_{DYNAMIC}$.

6.4.2: EFFECT OF AGE

In the layer eggs, effects with increasing age were found for Average Damping, K. DYNAMIC and K. DYNAMIC (correction). In the broiler breeder eggs significant effects with increasing age were found for Average Damping, and K. DYNAMIC only (Table 6.3).

	Average Damping	K. Dynamic	K. DYNAMIC (Correction)
Layers	**F=92.02** **df=2** ***P*<0.001**	**F=35.79** **df= 2** ***P*<0.001**	**F=7.34** **df= 2** ***P*=0.001**
Broiler Breeders	**F=18.38** **df=3** ***P*<0.001**	**F=16.69** **df= 3** ***P*<0.001**	F=1.75 df= 3 *P*=0.158

Table 6.3: Test statistics and P-values for age effects on resonance frequency data in both layer and broiler breeder eggs.

6.4.2.1: Average Damping

In the layer eggs this measure decreased steadily with age (Figure 6.5 A) and a post-hoc test revealed that there were significant differences between the average damping values at all ages ($P<0.001$; *Appendix 7 Table A7.1*). In the broiler breeder eggs average damping values decreased significantly between peak production and mid lay ($P<0.001$; Figure 6.5 B; *Appendix 7 Table A7.2*). Broiler breeder eggs in general displayed lower damping values than those found in layer eggs.

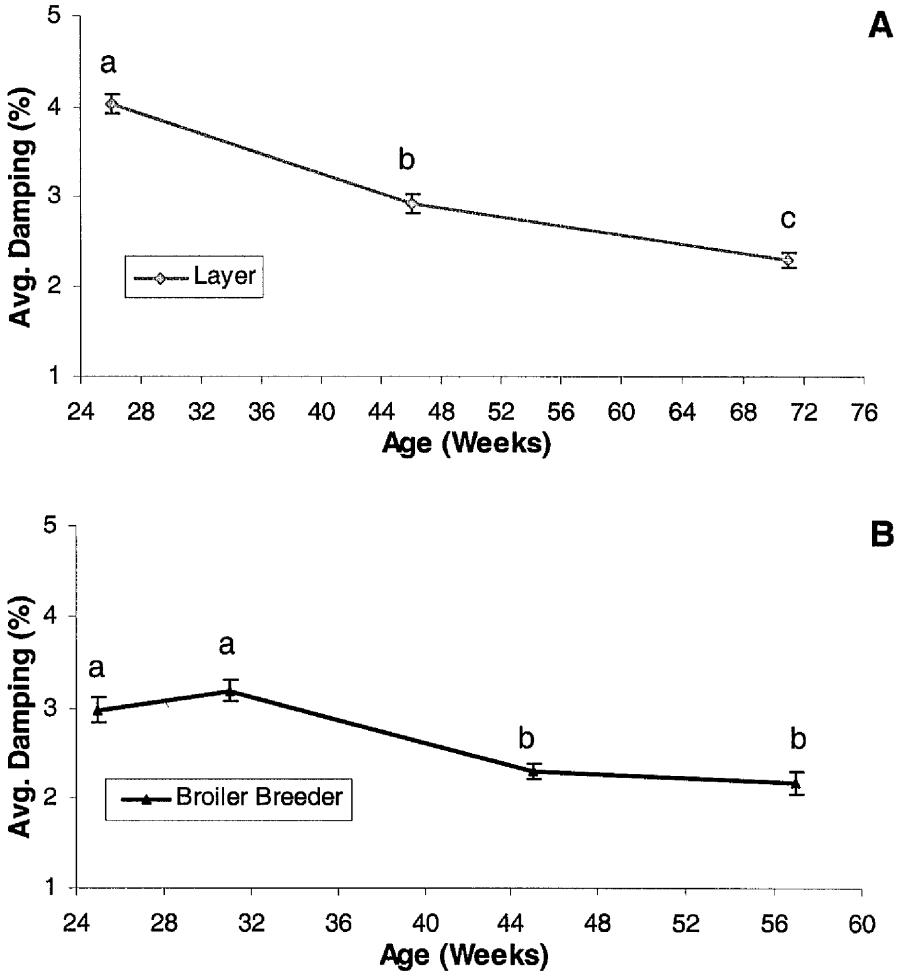


Figure 6.5: Average damping values (± S.E.) throughout lay in (A) layer and (B) broiler breeder eggs. Data points sharing the same letter are not significantly different (P>0.5).

6.4.2.2: K. DYNAMIC

In the layer eggs this measure significantly increased between beginning of lay and mid lay ($P<0.001$; Figure 6.6 A; *Appendix 7 Table A7.3*). In the broiler breeder eggs K. DYNAMIC values increased significantly between peak production and mid lay ($P<0.001$; Figure 6.6 B; *Appendix 7 Table A7.4*). The eggs from both types of bird therefore show a similar pattern. The range of values with increasing bird age was also similar.

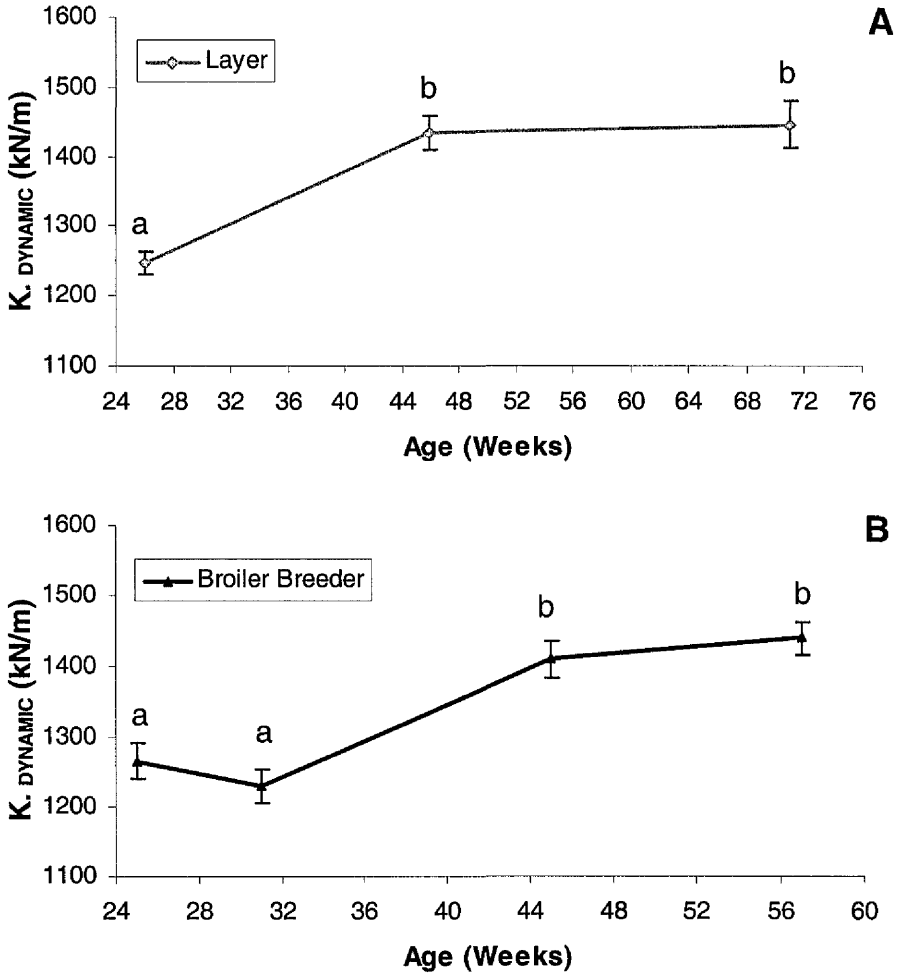


Figure 6.6: K. $_{Dynamic}$ values (± S.E.) throughout lay in (A) layer and (B) broiler breeder eggs. Data points sharing the same letter are not significantly different (P>0.05).

6.4.2.3: K. DYNAMIC (correction)

In the layer eggs this measure decreased significantly between mid and end of lay ($P<0.001$; Figure 6.7 A; *Appendix 7 Table A7.5*). In the broiler breeder eggs no significant differences in K.DYNAMIC (correction) values were found between different ages (Figure 6.7 B). In general the layer eggs displayed higher K. DYNAMIC (correction) values.

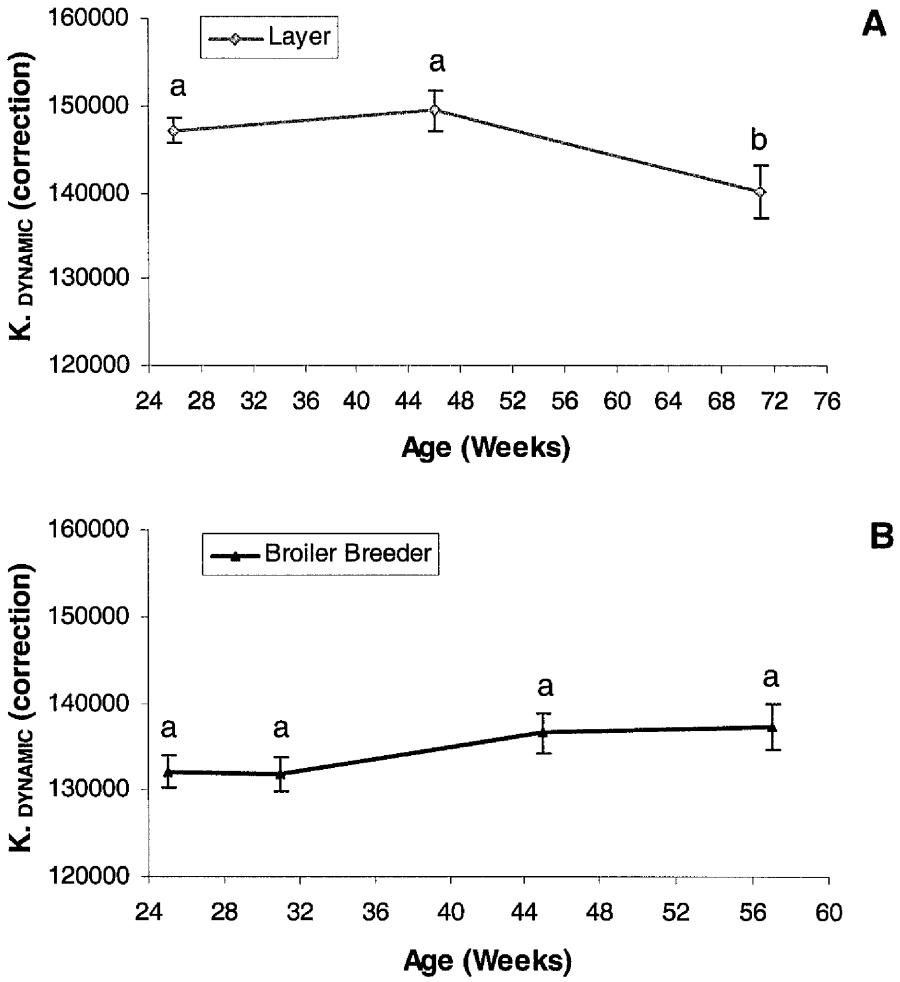


Figure 6.7: Average K. $_{DYNAMIC}$ (correction) values (± S.E.) throughout lay in (A) layer and (B) broiler breeder eggs. Data points sharing the same letter are not significantly different (P>0.05).

6.5: DISCUSSION

In developing the resonance frequency test (RFT) Coucke (1998) clearly demonstrated that this type of analysis lended itself most readily to the detection of cracked and broken shells. The author also highlighted the fact that intact eggs demonstrated varying ranges of K.$_{DYNAMIC}$ and Damping values. Following this Coucke *et al.* (1999) reported highly significant correlations between K. $_{DYNAMIC}$ values and shell stiffness, thickness and shape index. Within the current study the relationships between the measurements obtained from the RFT and eggshell quality parameters were investigated further.

In general the correlations established within this study, for layer and broiler eggs, are not as strong as those demonstrated by Coucke *et al.* (1999) and there are a number of factors which must be taken into consideration when comparing these studies. Coucke *et al.* (1999) used a non-destructive test to derive a static stiffness value whilst a destructive fracture test was used within this study. The compression speed of the quasi-static test used by Coucke was also considerably slower (1mm per minute) than that used within this study (5mm per minute). Voisey and Hunt (1974) demonstrated the difference in results obtained by using different speeds of compression and suggested that the optimum compression speed is 200mm per minute, which is considerably faster than either of the above. These authors also went on to state that the problem with using such high compression rates is the requirement for high-speed recording apparatus. Finally, Coucke *et al.* (1999) measured shell thickness using a screw gauge micrometer, with rounded tips, a method which does not allow the computation of the shell's effective thickness, a measure which has been shown to be of prime importance with regard to shell strength Bain (1990).

Within the context of the current work there are some noteworthy relationships between the values from the RFT and other eggshell quality parameters. Thus, with reference to the layer eggs, the correlation between damping and shape index (Figure 6.3) and damping and breaking strength (Figure 6.4) are credible. The results suggest that by measuring the damping value, eggs which characteristically display lower breaking strength could be identified with some accuracy without actually carrying out destructive tests. Interestingly these eggs are also more elongate, a feature consistent with eggs from the end of lay and indicative of poor eggshell quality. The results presented in figure 6.5(A) support this hypothesis viz damping decreases with increasing bird age in layer eggs.

Within the broiler breeder eggs the above relationships were less prominent perhaps reflecting the reduced variation in shape index and other eggshell quality parameters in general. Thus the damping value is probably most useful in detecting extremes in eggshell quality.

$K._{DYNAMIC}$ and $K._{DYNAMIC}$ (correction) values did not relate strongly to any of the eggshell quality parameters measured in layer or broiler breeder eggs. Compared to the $K._{DYNAMIC}$ values, however the $K._{DYNAMIC}$ (correction) measures demonstrate improved correlations. The modification involved in calculating $K._{DYNAMIC}$ (correction) incorporates the damping value and this probably explains the improved correlations particularly in the case of the layer eggs. Recent work by De Ketelarere and De Baerdemaeker (2000) suggests the inclusion of both the

damping value (discussed within this chapter) and the shape index to create a multivariate model for K. $_{DYNAMIC}$ which is closer to a 'Mass-Spring-Damper' system and which may improve the correlations between this and static stiffness values. Furthermore these authors have now automated the equipment for on-line crack detection and it is possible that measurements thus obtained on intact eggs will reveal improved correlations with other eggshell quality parameters. In this context the RFT measurement may facilitate the diversion of potentially weaker eggs within the commercial environment, although no indirect non-destructive test will ever replace direct methodology.

The current study also provided the opportunity to monitor age related changes in RFT measurements in layer and broiler breeder flocks which is of particular interest given that eggshell quality is known to deteriorate with increasing bird age (Bain, 1990; Fraser, 1996).

The damping measurement demonstrated a decrease with the age of the bird in both the layer and broiler breeder strains (Figure 6.5), confirming that eggshells become more brittle in nature as the bird ages. Given that the avian eggshell is a composite bioceramic formed by an interaction between organic and inorganic materials (Arias and Fernandez, 1995), then a change in the damping characteristic is likely to reflect a change in the composition of these organic and inorganic factions. Panheleux *et al.* (1999) found that the total amount of protein in the soluble matrix did not change with age but that the concentrations of specific proteins were greater in older hens. Fraser *et al.* (1998) reported an increase in percentage of shell matrix and matrix vesicles present at the end of lay and speculated that the decline in shell quality associated with the end of lay is related to a change in matrix quality. The current study also revealed that breeder eggs are consistently more brittle than the layer eggs throughout the laying year viz lower damping values suggesting that the matrix quality differs between these two eggs types.

The K. $_{DYNAMIC}$ values were similar in both layer and breeder eggs, generally increasing with age until mid lay where they plateau (Figure 6.6). This increase in K. $_{DYNAMIC}$ suggests that both types of eggs increased in stiffness at this time which is probably a reflection of the fact that egg shape increased most between the beginning of lay and mid lay in both layer and breeder flocks. The damping values in broiler breeder eggs were consistently lower than those observed in layer eggs. Given that K. $_{DYNAMIC}$ (correction) is empirically derived from K. $_{DYNAMIC}$ together with the damping value, it is not surprising that this measure was lower in the former (Figure 6.7).

6.6: CONCLUSIONS

The eggs produced by the layer birds demonstrated significant correlations between damping and shape index and damping and breaking strength. It is concluded that a decreased damping value is to be anticipated in elongate eggs with a lower breaking strength. Within the broiler breeder eggs these relationships were less evident. $K._{DYNAMIC}$ and $K._{DYNAMIC}$ (correction) values did not relate strongly to any of the eggshell quality parameters measured in layer or broiler breeder eggs. However the $K._{DYNAMIC}$ (correction) measure demonstrated improved correlations, with all aspects of shell quality, when compared to the original $K._{DYNAMIC}$ measure. The correlations established within this study were weaker than those found by Coucke *et al.* (1999) but this can be explained by differences in the techniques used to measure the eggshell quality parameters.

With respect to age, damping decreased significantly with increasing bird age in both the layer and broiler breeder eggs and was lower in the broiler breeder eggs throughout suggesting differences in the organic matrix within these eggs. The $K._{DYNAMIC}$ values were similar in both layer and breeder strains, generally increasing with age until mid lay at which time they plateau. $K._{DYNAMIC}$ (correction) values did not change with age in either the layer or broiler breeder eggs and were generally lower in the broiler breeder eggs throughout.

Given that the resonance frequency test is quick, affordable and non-invasive it could be applied within a commercial situation to detect weak as well as cracked eggshells. It is also possible that improvements in the formulae for deriving $K._{DYNAMIC}$ may allow this technique to be of use for a more detailed assessment of egg quality in the future.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

This thesis has addressed a number of issues relating to bird welfare and its assessment and the main conclusion derived from the various 'in-house' and 'field' experiments is that no one measure will suffice. If a series of tests are applied to the same situation then a variety of answers will emerge, not all correlating to the same extent.

When in 1986 Hughes *et al.* demonstrated that stress could cause egg retention and hence 'splashing' of the cuticular surface of the shell, there was general enthusiasm for the use of the shell as a non-invasive measure of welfare. Such splashed eggs are commonplace within the commercial sector but are generally removed from the production line prior to packing. However, within the housing systems currently in vogue, the identification of individual stress factors is virtually impossible. The enthusiasm for the appearance of the shell as an indicator of stress was however tempered by the work of Watt (1989) which demonstrated the relationship between the position of the egg in the oviduct, at the time of the stress event, and the nature of the faults induced. This author's critical evaluation of oviduct breakdown and repair and the associated intra and extra shell defects, served to highlight the fact that even when repair of the oviduct was effected, a return to a state of complete normality was never achieved. It is now clear from the work contained in this thesis that if the egg is being used as a welfare predictor then every aspect of its architecture must be considered.

Selection for high egg production has put the commercial hybrid under considerable stress and, the work contained within chapter 2 highlighted that, even at the onset of lay these birds produced a greater number of visually poor quality shells compared with the traditional breeds. Acute stress events triggered a different response in individual breeds. As to the question 'do poor shell formers continue to lay poor shells?', it would appear from this work that this is indeed the case.

The use of a range of shell quality parameters is both time consuming and laborious, but irrespective of these constraints has provided a more comprehensive data set. Without such extensive evaluation the shell thinning phenomena observed in the in-house trial, described in chapter 3, designed to assess the bird's reaction to novelty would have gone undetected.

Likewise the timing of this event would have remained obscure. Taken in isolation, the elevated faecal glucocorticoid levels observed by Lord (2001) signaled a response to the stressor within 24 hours. The effect of this change in stress hormone level was however not observed in terms of product quality until two days later. Within the commercial environment this observation has serious implications viz a stress event occurring on Monday might not influence production until Wednesday by which time the nature of the stressor will have been forgotten, if indeed it had been noted.

The layer house provides the bird with a number of diverse stress events which will vary on a daily basis. There is no doubt that acclimatisation to the environment will occur but whether such acclimatisation is uniform or not is debatable. In broad terms the bird will experience both chronic and acute stress within its lifetime e.g. within a multi-tier system the birds on the lower tier, as shown in chapter 5, lived in a darker, colder and ammonia enriched environment and, in response to these conditions, produced structurally inferior shells at the end of lay. In terms of acute stress one need look no further than the random replacement of an escaped hen into its non-home territory, or the process of beak trimming, normally carried out on younger birds. Once again, a time delay was recorded between the process of beak and claw trimming and changes in shell quality. The shell thinning, reported in this work, also produced significant changes in the stiffness of the shell as measured by non-destructive deformation.

The nest-box is generally accepted as being a basic requirement (Duncan 1978b; Duncan and Kite, 1987, Baxter, 1994) but its use is likely to be a learned behavioural pattern as demonstrated by the fact that, when deprived of a suitable nest-site, birds retained their eggs beyond the anticipated time of oviposition and thereafter laid a characteristic splashed or dusted product (Duncan, 1970).

Faecal glucocorticoid levels, reported in chapter 3, indicated, once again, a more immediate response to the acute event but, in terms of chronic stress, it was only when the birds were moved from the barren pen into an enriched enclosure that these hormone levels were elevated.

The dictionary definitions of the terms 'chronic' and 'acute' are quite specific. In practice the distinction between the two terms is less pronounced, particularly when the time span of the experimental phase is measured in days. Under such conditions adverse environmental situations have no time to become chronic and might be better defined as minor acute stress

events. The environmental conditions experienced by the birds housed in the multi-tier system can be considered chronic. In this particular situation it is likely that the birds become adapted to the specific conditions of each tier and this was reflected, to some extent, by the similarity in eggshell quality found in the eggs from each of the tiers. The non-invasive measures of welfare tested in this thesis related more easily to major acute events.

Stress, of course, is not the prerogative of the commercial layer, neither are the variations in shell quality observed within this sector of the industry. Breeder birds with their lower egg output do have an advantage and several authors, including Fraser (pers. comm.), have reported on the stability of the shell, in terms of its structure, at the end of lay. The rearing phase for broiler breeders is critical. During this time food is restricted to control body weight and lighting is restricted to delay the development of the reproductive tract. Many authors have advocated increasing novelty in the rearing environment in order to reduce fearfulness and allow animals to cope with the challenges they will face thereafter (Broom, 1969; Jones, 1982; Chamove and Anderson, 1989).

Transporting birds from the rearing sheds to the production unit is a stressful event and can be described as an acute stressor (Broom, 1990). The process is therefore carried out prior to the onset of lay in order to permit acclimatisation to the new environment. At the beginning of lay analysis of the shells, described within chapter 4, revealed no advantage, in terms of the previously enriched experience. In this novel environment all birds reacted similarly and product quality reflected the immature status of the egg forming system at this time. Thereafter those birds which had access, in their earlier life, to litter bales, upon which they perched, demonstrated, from mid lay onwards, improved eggshell quality parameters. Once again a time delay in response was observed but, in this instance, of weeks rather than days. Bone and shell formation are closely related and the maintenance of skeletal structure and function are pre-requisites for egg formation in so far as medullary bone acts as a labile source of calcium on a cyclical basis. As the provision of bales facilitated bird perching and the use of their legs can only have increased dynamic loading, then there is circumstantial evidence to support the hypothesis of a correlation between improved skeletal condition and shell quality.

The confined conditions of the laying house are perceived by many as unacceptable in terms of welfare. The debate is set to continue for several years since no environment can provide a uniform stress-free existence for birds reared at the current stocking density required to meet consumer demand.

The multi-tier system considered in chapter 5 made maximum use of the vertical space within the shed and as such provided evidence of the variation in environmental conditions which birds experience, occasionally to the detriment of product quality. This having been said the system investigated was of the highest order with regard to plant materials and husbandry. Any deficiencies were inherent in the design, but these effects were minimised by the existence of an automated control system. This is not the case in every situation as illustrated by the results of Hemsworth and Barnett (1989) and Sparks (1991).

Ultrastructural changes in the shells of layer birds manifest themselves particularly at the end of lay when there has been a corresponding change in certain physical parameters of the egg e.g. size, weight etc. Such ultrastructural changes have also been observed in response to acute stress situations (Watt, 1989; Solomon *et al.* 1987) and to specific farm conditions such as over-flight of aircraft which can cause egg retention or premature oviposition (Solomon, pers. comm.). The experiments conducted in the course of this thesis did not explore these extremes and for this reason alone may have failed to demonstrate the sub-structural shell changes which pre-dispose these eggs to easy breakage. Nevertheless in several of the studies, e.g. multi-tier experiment described in chapter 5 and to a lesser extent the work reported in chapter 3, trends for changes in shell ultrastructure were documented but in both instances the experiments were ended prior to the birds reaching 72 weeks of age.

The results reported herein demonstrate correlations between the timing of stress events, the nature of the stressor, the role of environmental enrichment and a variety of shell quality parameters. It takes on average 2 days to complete a profile of shell structure for one egg and assess its physical and material properties. This timing is acceptable within the confines of the laboratory but does not sit easily within the rapidly moving industrial sector. For this reason many attempts have been made to devise instrumentation which will provide an accurate and rapid assessment of quality. Currently on-line detectors will remove all eggs with cracks, but what of those eggs pre-disposed to cracking because of the presence of inherent structural defects? Resonance frequency testing is a promising tool, it is both quick and cost-effective but what exactly is it measuring? The egg is a prolate spheroid with a fluid content which changes in its consistency according to storage conditions. Resonance technology is detecting subtle changes in shell construction and the consistency of the contents. The work described in chapter 6 has developed the interpretation of data a little further with respect to the correlation between extremes in quality and both damping and $K._{DYNAMIC}$ values. The way is now open to further develop the concept and to take into account the multi-layered nature of the shell, it's

composition as a bioceramic and the interaction between it's solid and fluid components. When this is achieved future investigators will have a reliable tool that can be applied to the eggs of any species to provide an accurate assessment of their viability with regard to their role as embryonic chambers and by using this simple non-destructive method enable more rapid correlations to be made with the other measures of bird welfare.

With respect to future work within this field, there are many areas which could be developed. Firstly the labeling method for marking eggs from specific birds should be further investigated with a view to tracing each egg back to an individual hen. In the case of the multi-strain experiment, this would have allowed a sequence of eggs from one individual bird, rather than from the pen as a whole, to be identified at the onset of lay.

The competition for calcium for bone and shell formation will always favour the reproductive effort (Darnell-Middleton, 1999). In terms of the broiler enrichment trial, it would be interesting to carry out bone breaking strength and bone volume analysis on the leg bones from culled broilers. This would permit a comparison to be made between bones from those birds which were enriched, and appeared to lay superior eggshell quality at the end of lay, and the control birds reared on the floor.

The multi-tier system analysis was carried out in the absence of any measures of welfare, other than shell quality. Given the results relating to tier differences it would be advantageous to assess whether these effects are accompanied by differences in bird behaviour or basal plasma corticosterone levels.

Finally one of the most important findings within this thesis was the fact that changes, in terms of eggshell quality, following a stress tended to affect the physical shell parameters such as thickness and breaking strength. These shell characteristics are the direct result of the interaction between the organic (protein matrix) and inorganic (calcite) fractions of the shell. Where calcium is not the limiting factor, then protein production must be cited as the causative agent. Watt (1989) demonstrated a breakdown in the oviduct following stress. Such disruption to this vital organ will no doubt influence the formation and secretion of the matrix proteins. Future studies should concentrate on the isolation and identification of these organic components.

Appendix 1

CORTICOSTERONE ASSAY PROCEDURE

A1.1: EXTRACTION PROCEDURE

1. Rinse flat bottomed glass tubes in methanol
2. Add 200µls of plasma to each tube
3. In fume cupboard – add 2mls of dichloromethane to each tube
4. Vortex for 2mins on multi-tube vortexer
5. Remove 1ml of dichloromethane layer (bottom layer) and add to methanol washed pyrex tubes
6. Evaporate to dryness using water bath at 45°C in fume cupboard.
7. Add 100µls of assay buffer to each tube

A1. 2: ASSAY PROCEDURE

Radio immunoassay kit used was the Gamma-B ^{125}I Corticosterone (Cambridge Diagnostics Kit, Biogenesis Ltd., Bournemouth) The capped reagent vials and bottles were brought to room temperature prior to use and returned to 4°C immediately after to minimize deterioration.

A serial dilution of the standard was prepared as follows :

1. Label 9 LP4's A to I
2. To a different LP4 add 100µls of top standard (1000ng/ml) to 0.9mls of assay buffer. This is stock standard at 100ng/ml and can be stored in the freezer
3. Add 0.25mls of this stock standard to tube I and add 0.75mls of assay buffer. Vortex. (25ng/ml)
4. Add 0.5mls of assay buffer to each tube from A to H
5. Add 0.5mls of to top standard from tube I to tube H. Vortex. (12.5ng/ml)
6. Add 0.5mls of to top standard from tube H to tube G. Vortex. (6.25ng/ml)
7. Add 0.5mls of to top standard from tube G to tube F. Vortex. (3.12ng/ml)
8. Add 0.5mls of to top standard from tube F to tube E. Vortex. (1.56ng/ml)
9. Add 0.5mls of to top standard from tube E to tube D. Vortex. (0.78ng/ml)
10. Add 0.5mls of to top standard from tube D to tube C. Vortex. (0.39ng/ml)
11. Add 0.5mls of to top standard from tube C to tube B. Vortex. (0.195ng/ml)
12. Leave tube A with only assay buffer.

13. Label glass pyrex tubes as follows : Total Counts (TC) tube 1 and 2; Non-specific binding (NSB) tube 3 and 4; Maximum binding (Bo) tubes 5 and 6; standards (in duplicate) A,B,C,D,E,F,G,H and I tubes 7 to 22; quality control (QC) in tubes 23 and 24. Then place samples in tubes from 25 onwards.

A1.3: ASSAY

14. Add 200µls of assay buffer to the NSB tubes (3 and 4).
14. Add 100µls of assay buffer to the Bo tubes (5 and 6).
15. Add 100µls of standards A to I in duplicate tubes (7 to 22).
16. Qc and sample tubes already have 100µls of assay buffer added to them.
17. Add 100µls of I_{125} corticosterone to all tubes. Shake rack gently.
18. Add 100µls of corticosterone anti-serum to all tubes except TC and NSB tubes.
19. Vortex tubes and incubate for 16-24 hours at 4°C.
20. Add 100µls of second antibody to all tubes except TC.
21. Vortex tubes and incubate at room temperature for 1 hour.
22. Add 1ml of ice cold saline to all tubes except TC
23. Centrifuge at 1500-1600g for 15 minutes at 5oC on the DAMON centrifuge.
24. Immediately aspirate supernatants from all tubes except TC, taking care not to dislodge the pellet.
25. Count assay on gamma counter for 60 seconds per tube.

Appendix 2

ULTRASTRUCTURE OF THE MAMMILLARY LAYER AND CUTICLE OF THE EGGSHELL

Plate A2.1: ***Ultrastructural variations (indicated by arrows) present within the mammillary layer of the hen's eggshell: A – Confluence; B – Alignment; C – Good quality caps; D – Poor quality caps.***

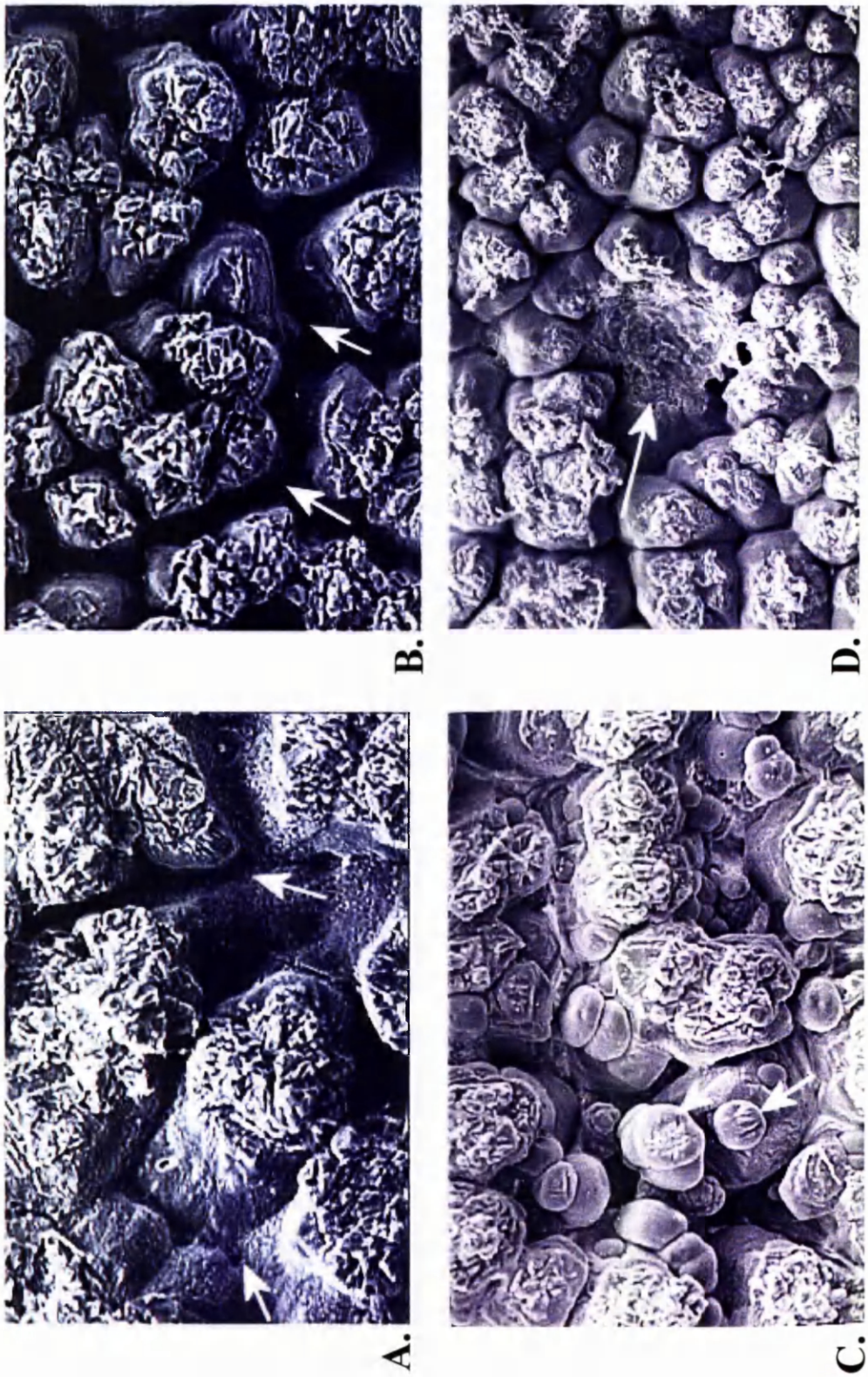


Plate A2.2: ***Ultrastructural variations (indicated by arrows) present within the mammillary layer of the hen's eggshell: A – Early fusion; B – Late fusion; C – Type B bodies; D – Depression.***

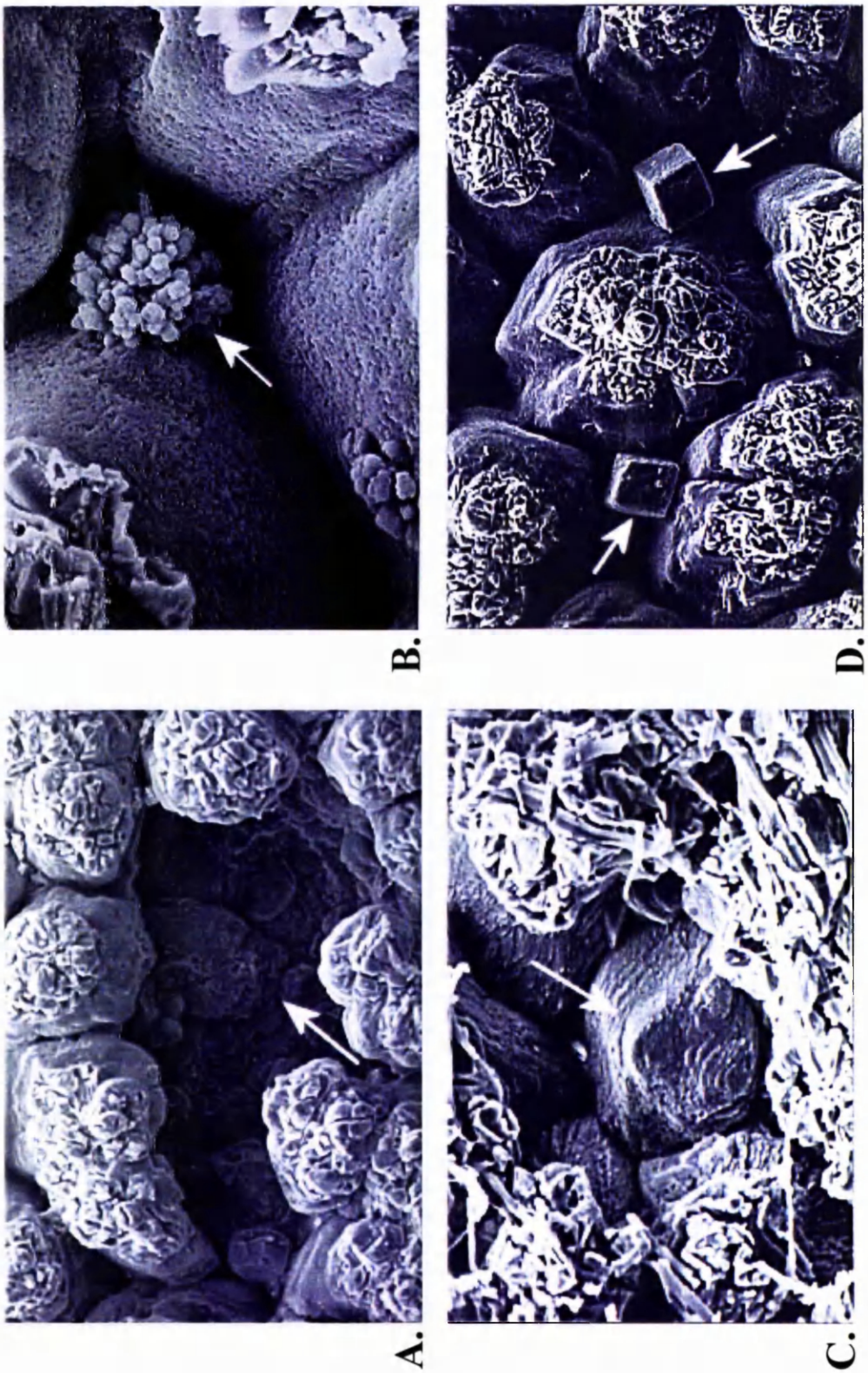


Plate A2.3: ***Ultrastructural variations (indicated by arrows) present within the mammillary layer of the hen's eggshell: A – Erosion; B – Aragonite; C – Type A body; D – Rhombohedral calcite.***

A.

B.

Plate A2.4: ***Ultrastructural variations (indicated by arrows) present within the mammillary layer of the hen's eggshell: A – Cuffing; B – Changed membrane on an aragonitic cap.***

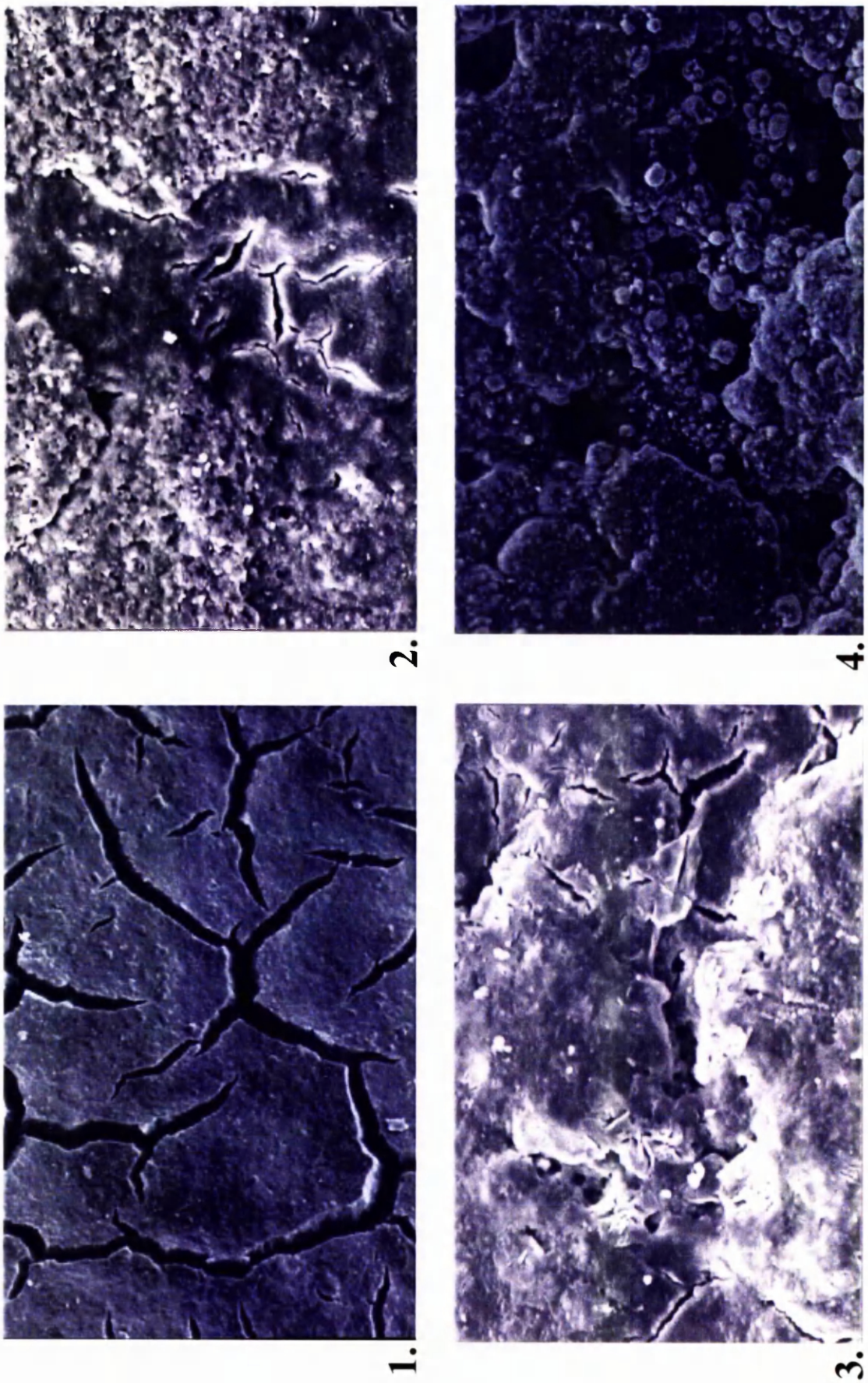


Plate A2.5: Examples of cuticle covering for each score: 1- Good covering; 2 - Patchy covering; 3 - Poor covering; 4 - Cuticle-less.

Appendix 3

POST HOC PAIRED COMPARISON TABLES FOR CHAPTER 3

A3.1: EXPERIMENT 1

	Day					
	0 - Control	**1**	**2**	**3**	**4**	**5**
0						
1	1.000					
2	0.4118	1.000				
3	**0.0001**	**0.015**	0.1735			
4	1.000	1.000	1.000	**0.008**		
5	1.000	1.000	1.000	**0.001**	1.000	

Table A3.1: Adjusted P-values for Bonferroni paired comparisons of total thickness for each day.

	Day					
	0 - Control	**1**	**2**	**3**	**4**	**5**
0						
1	1.000					
2	0.479	*0.085*				
3	0.179	**0.031**	1.000			
4	1.000	1.000	1.000	1.000		
5	1.000	1.000	1.000	0.529	1.000	

Table A3.2: Adjusted P-values for Bonferroni paired comparisons of mammillary thickness for each day.

	Day					
	Control - 0	**1**	**2**	**3**	**4**	**5**
0						
1	1.000					
2	1.000	1.000				
3	**0.014**	0.865	0.397			
4	1.000	1.000	1.000	0.103		
5	1.000	1.000	1.000	**0.044**	1.000	

Table A3.3: Adjusted P-values for Bonferroni paired comparisons of effective thickness for each day.

	Day					
	0 - Control	**1**	**2**	**3**	**4**	**5**
0						
1	1.000					
2	0.855	1.000				
3	**0.002**	0.107	0.366			
4	1.000	1.000	1.000	*0.079*		
5	1.000	1.000	1.000	0.1180	1.000	

Table A3.4: Adjusted P-values for Bonferroni paired comparisons of total shell thickness for each day for enriched data only.

	Day					
	0 - Control	**1**	**2**	**3**	**4**	**5**
0						
1	0.529					
2	**0.014**	0.917				
3	*0.080*	0.529	0.294			
4	0.441	0.600	*0.080*	0.183		
5	0.294	0.208	**0.042**	*0.059*	0.353	

Table A3.5: Adjusted P-values for Wilcoxon test on differences in total score for each day for enriched data only.

	Day					
	0 - Control	**1**	**2**	**3**	**4**	**5**
0						
1	*0.059*					
2	0.141	0.787				
3	0.726	0.584	**0.036**			
4	0.800	0.371	0.151	1.000		
5	0.554	0.181	0.108	1.000	0.893	

Table A3.6: Adjusted P-values for Wilcoxon test on differences in cap score for each day for enriched data only.

	Day					
	0 - Control	**1**	**2**	**3**	**4**	**5**
0						
1	**0.036**					
2	0.294	*0.059*				
3	**0.030**	**0.036**	**0.022**			
4	0.944	0.116	0.447	0.181		
5	**0.014**	*0.059*	**0.014**	**0.014**	**0.043**	

Table A3.7: Adjusted P-values for Wilcoxon test on differences in cuticle score for each day for enriched data only.

	Day					
	0 - Control	**1**	**2**	**3**	**4**	**5**
0						
1	0.624					
2	**0.014**	**0.014**				
3	1.000	0.141	**0.042**			
4	**0.014**	**0.014**	0.141	**0.014**		
5	**0.044**	**0.030**	0.183	**0.014**	0.529	

Table A3.8: Adjusted P-values for Wilcoxon test on differences in cuticle score for each day for barren data only.

A3.2: EXPERIMENT 2

	Day													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1														
2	1.000													
3	1.000	1.000												
4	1.000	1.000	1.000											
5	1.000	1.000	1.000	1.000										
6	1.000	1.000	1.000	1.000	1.000									
7	1.000	1.000	1.000	1.000	1.000	1.000								
8	1.000	1.000	1.000	1.000	1.000	1.000	1.000							
9	0.206	**0.016**	*0.060*	*0.071*	**0.003**	**0.017**	*0.095*	1.000						
10	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000					
11	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000				
12	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.190	**0.001**	1.000	1.000			
13	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
14	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	**0.018**	1.000	1.000	1.000	1.000	

Table A3.9: Adjusted P-values for Bonferroni paired comparisons of non-destructive deformation for each day

	Day													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1														
2	1.000													
3	1.000	1.000												
4	1.000	1.000	1.000											
5	1.000	1.000	1.000	1.000										
6	1.000	1.000	1.000	1.000	1.000									
7	1.000	1.000	1.000	1.000	1.000	1.000								
8	1.000	1.000	1.000	1.000	1.000	1.000	1.000							
9	1.000	0.231	0.477	0.418	1.000	1.000	1.000	1.000						
10	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000					
11	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000				
12	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	**0.050**	1.000	0.705			
13	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
14	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	

Table A3.10: Adjusted P-values for Bonferroni paired comparisons of total shell thickness for each day.

	Day													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1														
2	1.000													
3	1.000	1.000												
4	1.000	1.000	1.000											
5	1.000	1.000	1.000	1.000										
6	1.000	1.000	1.000	1.000	1.000									
7	1.000	1.000	1.000	1.000	1.000	1.000								
8	1.000	1.000	1.000	1.000	1.000	1.000	1.000							
9	1.000	0.603	0.314	1.000	1.000	0.291	0.804	1.000						
10	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000					
11	1.000	1.000	0.652	1.000	1.000	0.609	1.000	1.000	1.000	1.000				
12	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	**0.028**	1.000	*0.076*			
13	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
14	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.892	1.000	1.000	1.000	1.000	

Table A3.11: Adjusted P-values for Bonferroni paired comparisons of effective thickness for each day

	Day													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1														
2	1.000													
3	1.000	1.000												
4	1.000	1.000	1.000											
5	1.000	1.000	1.000	1.000										
6	1.000	1.000	1.000	1.000	1.000									
7	1.000	1.000	1.000	1.000	1.000	1.000								
8	1.000	1.000	1.000	1.000	1.000	1.000	1.000							
9	1.000	0.369	1.000	0.361	0.133	0.171	**0.042**	1.000						
10	1.000	1.000	1.000	1.000	1.000	1.000	0.391	1.000	1.000					
11	1.000	1.000	1.000	1.000	0.772	0.957	0.291	1.000	1.000	1.000				
12	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.283	1.000	1.000			
13	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
14	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.536	1.000	1.000	1.000	1.000	

Table A3.12 : Adjusted P-values for Bonferroni paired comparisons of non-destructive deformation for enriched data only

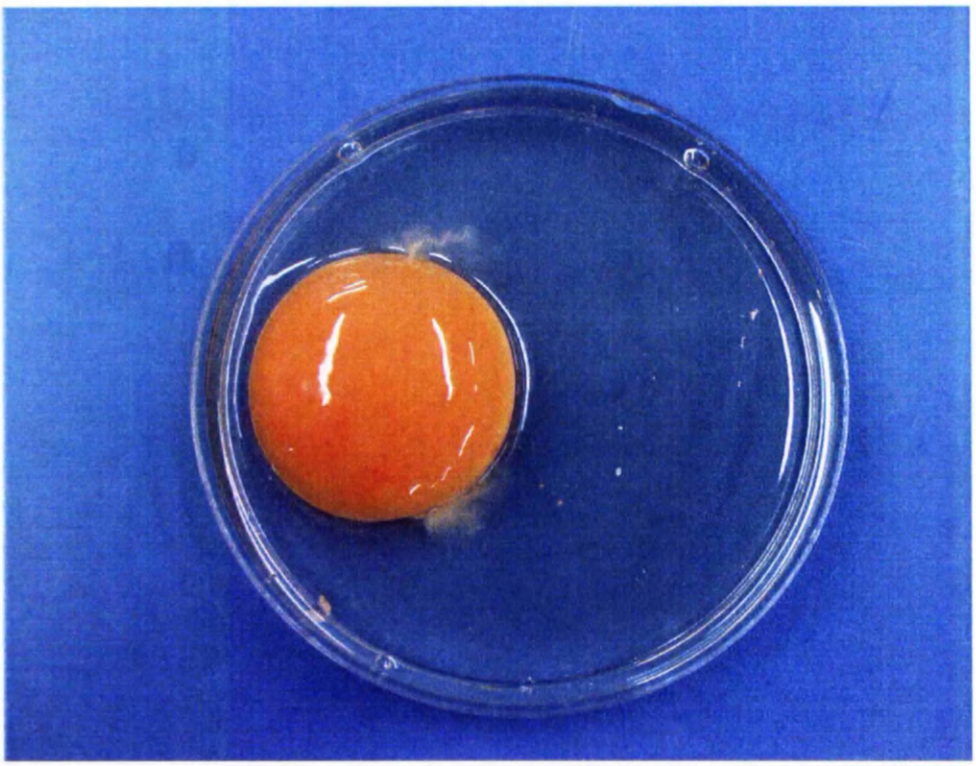
Appendix 4

PILOT STUDY INTO THE ADMINISTRATION OF COLOURED DYE WITHIN GELATINE CAPSULES TO FACILITATE THE IDENTIFICATION OF EGGS FROM INDIVIDUAL BIRDS

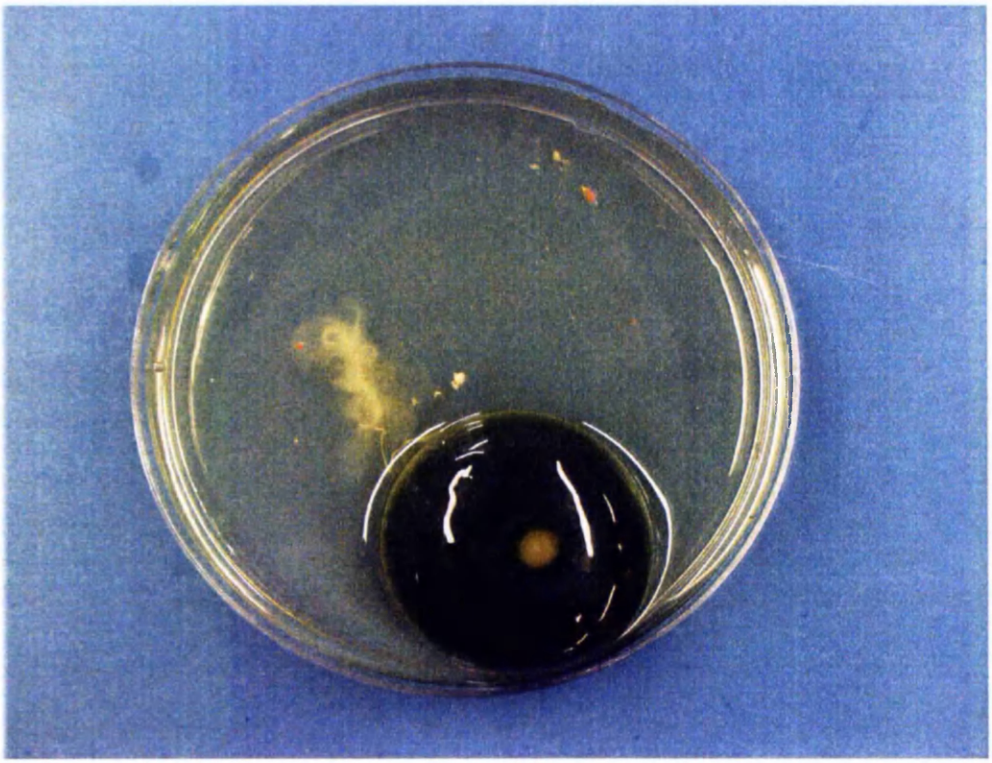
Within the current study coloured dye was added to gelatine capsules and fed to the birds as part of a stress protocol. The aim of this pilot investigation was to gauge how easily eggs could be identified by this colour and to note for how many days the colour existed. Only two colours of dye were used within this trial, Red and Black.

Following the administration of these capsules, no effect on yolk colour was noted on the egg laid on the subsequent morning (Day 1), but on the morning of day 2 intensive colour was noted in the egg yolks (Plate A4.1 A and B). The colour was also obvious on day 3 (Plate A4.2 A and B) but by day four the yolk had to be burst to allow detection of the red dye within (Plate A4.3 A). On day 4 the black dye was still obvious (Plate A4.3 B) and continued to be so until day 7.

This type of marker is particularly useful for stress studies as the capsule can by administered as part of the protocol. More studies are required to demonstrate which colours will be individually identifiable within the egg yolks and how long these colours last. Within the studies presented within this thesis a technique such as this would have been useful and would have allowed the eggs from one bird within a pen to be singled out and analysed.

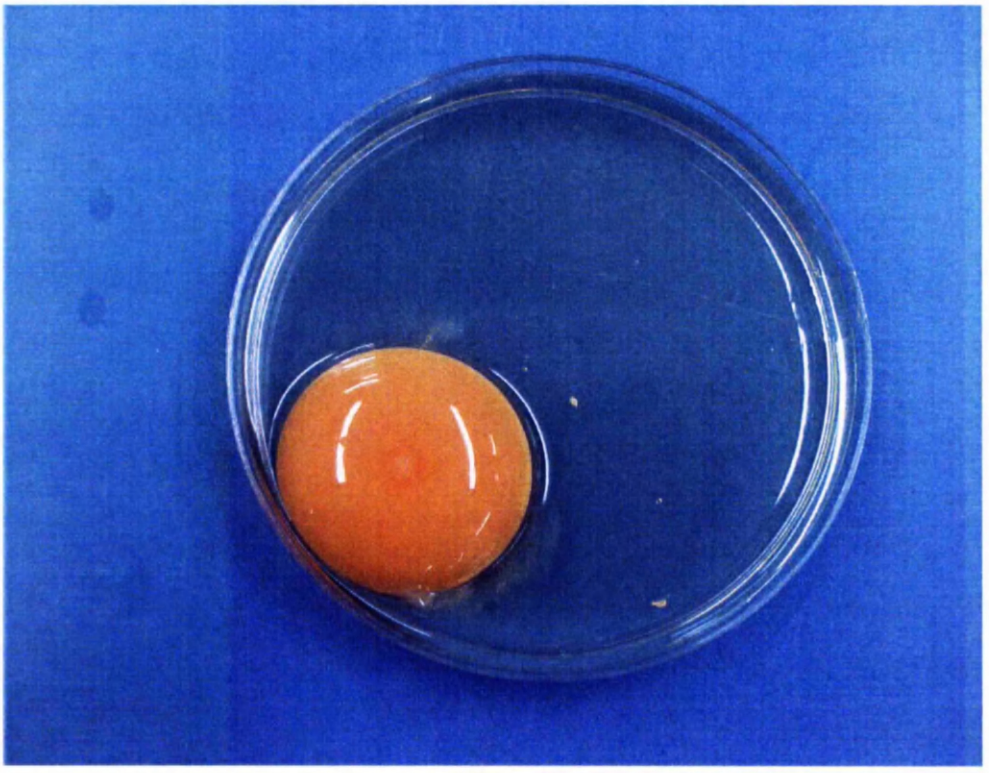


A.

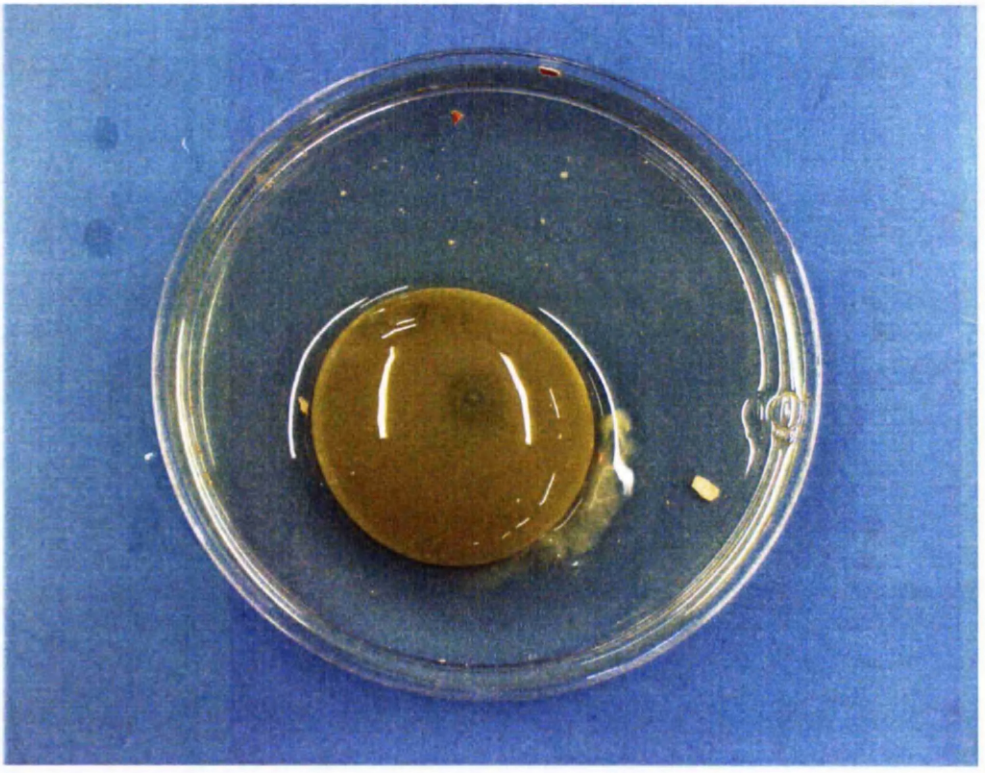


B.

Plate A4.1: Colour intensity of the yolk on day two following oral administration of (A) Red dye and (B) black dye within gelatine capsules.

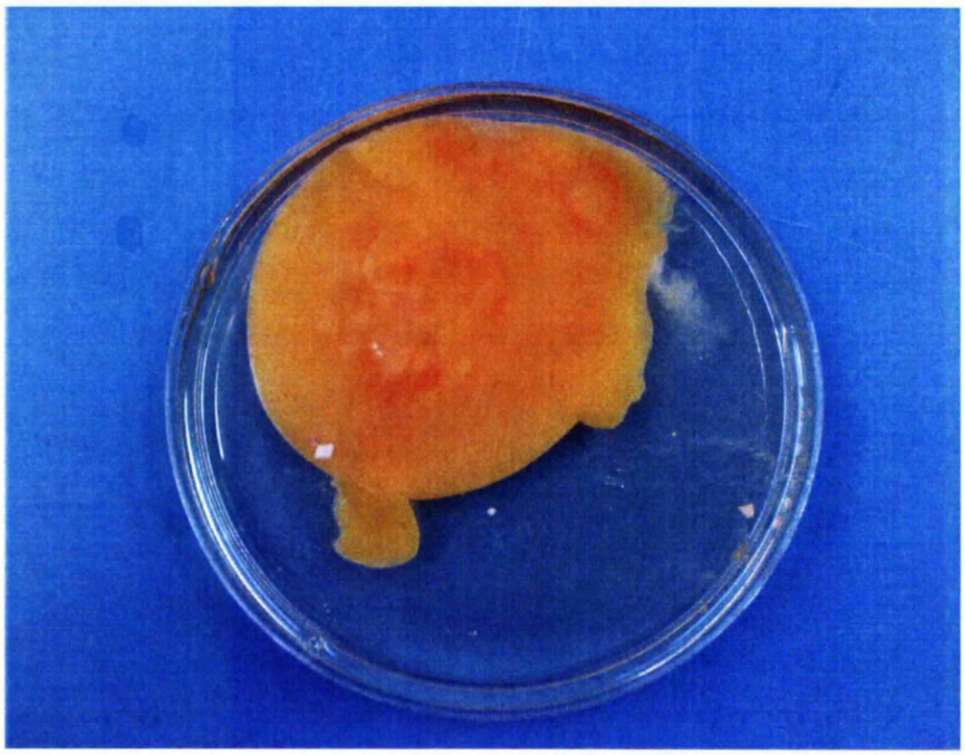


A.

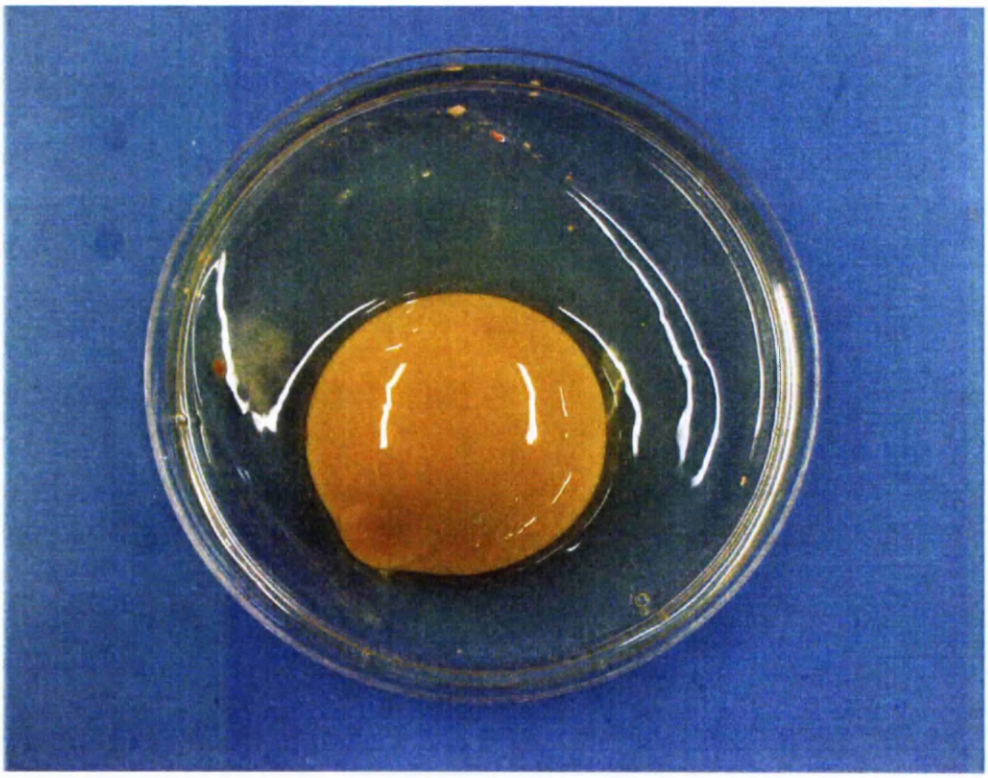


B.

Plate A4.2: Colour intensity of the yolk on day three following oral administration of (A) Red dye and (B) black dye within gelatine capsules.



A.



B.

Plate A4.3: Colour intensity of the yolk on day four following oral administration of (A) Red dye and (B) black dye within gelatine capsules.

Appendix 5

POST HOC PAIRED COMPARISON TABLES FOR CHAPTER 4

	Age (weeks)			
	25	31	45	57
25				
31	**0.000**			
45	**0.000**	**0.000**		
57	**0.000**	**0.000**	0.731	

Table A5.1: Adjusted P-values for Tukey's paired comparisons of fresh egg weight across all ages.

	Age (weeks)			
	25	31	45	57
25				
31	0.991			
45	**0.043**	**0.019**		
57	**0.013**	**0.005**	0.976	

Table A5.2: Adjusted P-values for Tukey's paired comparisons of shape index across all ages.

	Age (weeks)			
	25	31	45	57
25				
31	0.545			
45	**0.008**	0.235		
57	0.344	0.987	0.408	

Table A5.3: Adjusted P-values for Tukey's paired comparisons of total thickness across all ages.

	Age (weeks)			
	25	31	45	57
25				
31	**0.012**			
45	**0.001**	0.876		
57	**0.000**	0.189	0.593	

Table A5.4: Adjusted P-values for Tukey's paired comparisons of mammillary thickness across all ages.

	Age (weeks)			
	25	31	45	57
25				
31	*0.065*			
45	**0.000**	0.111		
57	**0.002**	0.664	0.680	

Table A5.5: Adjusted P-values for Tukey's paired comparisons of effective thickness across all ages.

	Age (weeks)			
	25	31	45	57
25				
31	**0.000**			
45	**0.000**	0.999		
57	**0.000**	*0.052*	**0.037**	

Table A5.6: Adjusted P-values for Tukey's paired comparisons of Kc across all ages.

	Age (weeks)			
	25	31	45	57
25				
31	**0.000**			
45	**0.000**	0.863		
57	**0.000**	0.964	0.591	

Table A5.7: Adjusted P-values for Tukey paired comparisons of E Shell across all ages.

	Age (weeks)		
	31	45	57
31			
45	0.399		
57	**0.034**	**0.008**	

Table A5.8: P-values for Kruskal Wallis comparisons of cuffing score in enriched birds across all ages.

Appendix 6

POST HOC PAIRED COMPARISON TABLES FOR CHAPTER 5

AGE	A	B	C	D	E
A					
B	0.276				
C	0.111	**0.000**			
D	**0.000**	**0.000**	**0.000**		
E	**0.000**	**0.000**	**0.000**	**0.000**	

Table A6.1: Adjusted P-values for Tukey's paired comparisons of temperature between tiers.

AGE	A	B	C	D	E
A					
B	0.838				
C	**0.002**	**0.000**			
D	**0.005**	**0.000**	0.999		
E	**0.000**	**0.000**	0.995	0.967	

Table A6.2: Adjusted P-values for Tukey's paired comparisons of mortality between tiers.

TIER	A	B	C	D	E
A					
B	**0.000**				
C	**0.001**	**0.000**			
D	**0.000**	**0.000**	0.997		
E	**0.000**	**0.000**	0.728	0.903	

Table A6.3: Adjusted P-values for Tukey's paired comparisons of light intensity within the cages between tiers.

TIER	A	B	C	D	E
A					
B	**0.000**				
C	**0.000**	0.079			
D	0.874	**0.000**	**0.000**		
E	**0.007**	**0.000**	**0.000**	0.110	

Table A6.4: Adjusted P-values for Tukey's paired comparisons of light intensity outside the cages between tiers.

AGE	26	46	71
26			
46	**0.005**		
71	**0.000**	**0.024**	

Table A6.5: Adjusted P-values for Tukey's paired comparisons of fresh egg weight across all ages.

AGE	26	46	71
26			
46	**0.000**		
71	**0.000**	**0.000**	

Table A6.6: Adjusted P-values for Tukey's paired comparisons of shape index across all ages.

AGE	26	46	71
26			
46	**0.016**		
71	**0.027**	0.982	

Table A6.7: Adjusted P-values for Tukey's paired comparisons of shell total thickness across all ages.

AGE	26	46	71
26			
46	*0.079*		
71	**0.010**	0.729	

Table A6.8: Adjusted P-values for Tukey's paired comparisons of mammillary thickness across all ages.

AGE	26	46	71
26			
46	**0.001**		
71	**0.001**	0.994	

Table A6.9: Adjusted P-values for Tukey's paired comparisons of effective thickness across all ages.

AGE	26	46	71
26			
46	**0.000**		
71	**0.000**	**0.000**	

Table A6.10: Adjusted P-values for Tukey's paired comparisons of breaking strength across all ages.

AGE	26	46	71
26			
46	**0.004**		
71	0.992	**0.003**	

Table A6.11: Adjusted P-values for Tukey's paired comparisons of stiffness across all ages.

AGE	26	46	71
26			
46	**0.000**		
71	**0.000**	**0.000**	

Table A6.12: Adjusted P-values for Tukey's paired comparisons of Kc across all ages.

AGE	26	46	71
26			
46	0.732		
71	0.123	**0.018**	

Table A6.13: Adjusted P-values for Tukey's paired comparisons of E Shell across all ages.

TIER	A	C	E
A			
C	**0.014**		
E	**0.028**	0.677	

Table A6.14: P-values for Kruskal Wallis comparisons of erosion score in eggs from 26 weeks for each tier.

TIER	A	C	E
A			
C	0.184		
E	0.278	**0.012**	

Table A6.15: P-values for Kruskal Wallis comparisons of cuffing score in eggs from 46 weeks for each tier.

TIER	A	C	E
A			
C	0.160		
E	*0.056*	**0.001**	

Table A6.16: P-values for Kruskal Wallis comparisons of type B score in eggs from 71 weeks for each tier.

AGE	26	46	71
26			
46	*0.078*		
71	0.282	**0.010**	

Table A6.17: P-values for Kruskal Wallis comparisons of changed membrane score in eggs from tier A at each age.

AGE	26	46	71
26			
46	**0.019**		
71	0.884	**0.011**	

Table A6.18: P-values for Kruskal Wallis comparisons of depression score in eggs from tier C at each age.

AGE	26	46	71
26			
46	**0.040**		
71	0.131	**0.001**	

Table A6.19: P-values for Kruskal Wallis comparisons of changed membrane score in eggs from tier C at each age.

AGE	26	46	71
26			
46	0.648		
71	**0.015**	**0.015**	

Table A6.20: P-values for Kruskal Wallis comparisons of total score in eggs from tier E at each age.

AGE	26	46	71
26			
46	0.094		
71	**0.000**	**0.011**	

Table A6.21: P-values for Kruskal Wallis comparisons of type B score in eggs from tier E at each age.

AGE	26	46	71
26			
46	0.289		
71	*0.051*	**0.005**	

Table A6.22: P-values for Kruskal Wallis comparisons of erosion score in eggs from tier E at each age.

AGE	A	C	E
A			
C	0.388		
E	**0.007**	0.201	

Table A6.23: Adjusted P-values for Tukey's paired comparisons of yolk colour between tiers.

AGE	26	46	71
26			
46	**0.000**		
71	**0.000**	**0.000**	

Table A6.24: Adjusted P-values for Tukey's paired comparisons of haugh units across all ages.

AGE	26	46	71
26			
46	**0.000**		
71	0.421	**0.000**	

Table A6.25: Adjusted P-values for Tukey's paired comparisons of yolk colour across all ages.

AGE	26	46	71
26			
46	*0.078*		
71	**0.005**	0.164	

Table A6.26: P-values for Kruskal Wallis comparisons of number of blood spots in eggs from tier E across all ages.

AGE	26	46	71
26			
46	*0.095*		
71	**0.010**	0.329	

Table A6.27: P-values for Kruskal Wallis comparisons of number of meat spots in eggs from tier C across all ages.

AGE	26	46	71
26			
46	0.355		
71	**0.004**	**0.040**	

Table A6.28: P-values for Kruskal Wallis comparisons of number of meat spots in eggs from tier E across all ages.

Appendix 7

POST HOC PAIRED COMPARISON TABLES FOR CHAPTER 6

AGE (WEEKS)			
	26	**46**	**71**
26			
46	**0.000**		
71	**0.000**	**0.000**	

Table A7.1: Adjusted P-values for Tukey's paired comparisons of average damping for each age in layer eggs.

AGE (WEEKS)				
	25	**31**	**45**	**57**
25				
31	0.574			
45	**0.000**	**0.000**		
57	**0.000**	**0.000**	0.865	

Table A7.2: Adjusted P-values for Tukey's paired comparisons of average damping for each age in broiler breeder eggs.

AGE (WEEKS)			
	26	**46**	**71**
26			
46	**0.000**		
71	**0.000**	0.904	

Table A7.3: Adjusted P-values for Tukey's paired comparisons of K. Dynamic for each age in layer eggs.

AGE (WEEKS)				
	25	**31**	**45**	**57**
25				
31	0.729			
45	**0.000**	**0.000**		
57	**0.000**	**0.000**	0.897	

Table A7.4: Adjusted P-values for Tukey's paired comparisons of K. Dynamic for each age in broiler breeder eggs.

AGE (WEEKS)			
	26	**46**	**71**
26			
46	0.635		
71	**0.014**	**0.000**	

Table A7.5: Adjusted P-values for Tukey's paired comparisons of K. Dynamic for each age in layer eggs.

REFERENCES

AKBAR, M.K., GAVORA, J.S., FRIARS, G.W. & GOWE, R.S. (1983) Composition of eggs by commercial size categories: effects of genetic group, age and diet. *Poultry Science*, **62**: 925-933.

ALLISON, R.J. (1987) Non-destructive determination of Young's modulus and its relationship with compressive strength, porosity and density in deformation of sediments and sedimentary rocks, in: JONES, M.E. & PRESTON, R.M.F. (Eds) *Geological Society Special Publication,* No 29, pp. 63-69.

ALLS, A.A., COVER, M.S., BENTON, W.J. & KRAUSS, W.C. (1964) Treatment of Hatching Eggs for Disease Prevention - Factors affecting Permeability and a Visual Detection of Drug Absorption. *Avian Diseases*, **8**: 245-246.

ANDERSON, G.B. & CARTER, T.C. (1972) The hen's egg: hell cracking in eggs dropped on cage floors coated with plastic or zinc. *British Poultry Science*, **13**: 115.

ANDERSON, G.B., CARTER, T.C. & JONES, R.M. (1969) Some factors affecting downgrading in eggs, especially damage in transit. *British Poultry Science*, **10**: 45.

ANTHONY, N.B., KATANBAF, M.N. & SIEGEL, B. (1988) Responses to social disruption in two lines of White Leghorn chickens. *Applied Animal Behaviour Science*, **21**: 243-250.

APPLEBY, M.C., MCRAE, H.E. & DUNCAN, I.J.H. (1983) Nesting and floor laying by domestic hens: effects of individual variation in perching behaviour. *Behav. Anal. Lett.*, **3**: 345-352.

ARAD, Z. & MARDER, J. (1982) Differences in eggshell quality among the Sinai Bedouin fowl, the commercial White leghorn and their crossbreds. British *Poultry Science*, **23**: 107.

ARIAS, J.L. & FERNANDEZ, M.S. (1995) Role of extracellular matrix on eggshell formation. *Proceedings of the 6th European Symposium on Quality of Egg and Egg Products*, pp. 89-93.

ARIAS, J.L., FINK, D.J., XIAO, S.Q., HEUER, A.H. & CAPLAN, A.I. (1993) Biomineralization and eggshells: cell-mediated acellular compartments of mineralized extracellular matrix. *Int. Rev. Cytol.*, **145**: 217-250.

BAIN, M.M. (1990) Eggshell Strength: A Mechanical-Ultrastructural Evaluation, *Ph. D. Thesis*, University of Glasgow.

BAIN, M.M. (1991) A reinterpretation of eggshell strength, in: SOLOMON, S.E. (Ed) *Egg and Eggshell Quality*, pp. 131-142 (Mason Publishing, Iowa State University Press).

BALM, P.H. (1999) *Stress Physiology in Animals* (Sheffield Academic Press, USA).

BARNETT, J.L. & HEMSWORTH, P.H. (1990) The validity of physiological and behavioural measures of animal welfare. *Applied Animal Behaviour Science*, 25: 177-187.

BARNETT, J.L., HEMSWORTH, P.H. & NEWMAN, P.H. (1992) Fear of humans and its relationship with productivity in laying hens at commercial farms. *British Poultry Science*, **33**: 699-710.

BAXTER, M.R. (1989) Philosophical problems underlying the concept of welfare, in: FAURE, M. & MILLS, A.D. (Eds) *Proceedings of the 3rd European Symposium on Poultry Welfare,* Tours, pp. 59-66.

BAXTER, M.R. (1994) The welfare problems of laying hens in battery cages. *Veterinary Record*, **134**: 614-619.

BAXTER, J.D. & HARRIS, A.W. (1975) Mechanisms of glucocorticoid action: general features with reference to steroid-mediated immunosuppression. *Transplantation Proceed*ings, **7**: 55-65.

BECK, J.R. (1991) Understanding and minimizing stress in broilers and broiler breeders. *Zootecnica International*, **14 (3)**: 30-38.

BEIS (2001) Egg Market Data [online]. Available from: http://www.britegg.co.uk/beis/beis_frm.htm (December 2001).

BELYAVIN, C.G. (1986) Egg Quality, Important but not understood. *Poultry-Misset*, June: 22-25.

BELYAVIN, C.G., SOLOMON, S.E. & BAIN, M.M. (1991) Eggshell Structure as an Indicator of Shell Quality, in: Oosterwoud, A. & DeVries, A.W. (Eds.), *Proceedings of the 4th European Symposium on the Quality of Eggs and Egg Products*, Vol. II (Beekbergen, Holland), pp. 9-17.

BEUVING, G. & VONDER, G.M.A. (1977) Daily rhythm of corticosterone in laying hens and the influence of egg laying. *J. Reproductive. Fertility*, **51**: 169-173.

BEUVING, G. & VONDER, G.M.A. (1978) Effect of stressing factors on corticosteroid levels in the plasma of laying hens. *General and Comparative Endocrinology,* **35:** 153–159.

BEUVING; G. & VONDER, G.M.A. (1986) Comparison of the adrenal sensitivity to ACTH of laying hens with immobilization and plasma baseline levels of corticosterone. *General and Comparative Endocrinology*, **62 (2)**: 353-358.

BLEVINS, R.D. (1993) Formulas for natural frequency and mode shapes, (Krieger Publishing Company, Florida, USA).

BLOCKHUIS, H.J. (1994) *Revue Scientifique et Technique de L. Office International Des Epizooties*, **13 (1)**: 67-78.

BLOOM, M.A., DOMM, L.V., NALBANDOV, A.V. & BLOOM, W. (1958) Medullary bone of laying chickens. *American Journal of Anatomy*, **102**: 411-453.

BOISSEY, A. (1995) Fear and Fearfulness in animals. *Quarterly Review of Biology*, **70**: 165-191.

BRACKPOOL, C.E. (1995) Eggshell Structure as an Indicator of Eggshell Quality in Laying Hens. *Ph. D. Thesis,* University of New England.

BRACKPOOL, C.E., ROBERTS, J.R., HECKER, J. & HUGHES, R. (1993) How heat stress affects eggshell ultrastructure, in: *Proceedings of the Australian Poultry and Feed Convention*, Gold Coast.

BRAKE, J. (1987) Influence of presence of perches during rearing on incidence of floor laying in broiler breeders. *Poultry Science*, **66**: 1587-1589.

BRAKE, J., BAKER, M, MORGAN, G.W. & THAXTON, P. (1982) Physiological changes in caged layers during a forced moult. 4. Leukocyte and packed cell volume. *Poultry Science*, **61**: 790-795.

BRAMBELL, F.W.R. (1965) Chairman. *Report of the technical committee to enquire into the welfare of animals kept under intensive livestock husbandry systems*. Cmnd 2836. (H.M.S.O., London).

BROADHURST, P.L. (1960) Abnormal Animal Behaviour, in: EYSENCK, H.J. (Ed) *Handbook of Abnormal Psychology*, pp. 726-763 (Pitman, London).

BROOM, D.M. (1969) Effects of visual complexity during rearing on chicks' reactions to environmental change. *Animal Behaviour*, **17**: 773–780.

BROOM, D.M. (1986) Indicators of Poor Welfare. *British Veterinary Journal*, **142**: 524.

BROOM, D.M. (1988) Stress, Welfare and the State of Equilibrium, in : Wegner, R,-M. (Ed) *Proceedings of the 2nd European Symposium on Poultry Welfare*, (Celle), pp. 72–81.

BROOM, D.M. (1990) Effects of handling and transport on laying hens. *World's Poultry Science Journal*, **46**: 48-50.

BROOM, D.M. & JOHNSON, K.G. (1993) *Stress and Animal Welfare* (Chapman and Hall, London).

BROOM, D.M., KNIGHT, P.G. & STANSFIELD, S.G. (1986) Hen behaviour and hypothalamic-pituitary-adrenal response to handling and transport. *Applied Animal Behavioural Science*, **16**: 19.

BUCKLEY, D.G., AMOUR, G. & FAIRFULL, R.W. (1981) An improved electronic gauge for measuring egg albumen height. *Poultry Science*, **60**: 777-780.

BUCKLEY, D.G. & REID, W.S. (1971) A digital egg albumen height measurement gauges. *Poultry Science*, **50**: 1326-1330.

BUCKNER, G.D., MARTIN, J.H., PIERCE, W.C. & PETER, A.M. (1922) Calcium in egg-shell formation. *Journal of Biological Chemistry*, **51**: 51-54.

BUSS, E.G. (1982) Genetic differences in avian egg shell formation. *Poultry Science*, **61**: 2048.

CANNON, W.B. (1929) *Bodily changes in pain, fear and rape: An account of recent researches into the function of emotional excitement*, 2nd edition (Appleton, New York).

CARMICHAEL, N.L., WALKER, A.W. & HUGHES, B.O. (1999) Laying hens in large flocks in a perchery system: influence of stocking density on location, use of resources and behaviour. *British Poultry Science*, **40 (2):** 165-176.

CARPENTER, E. (1980) *Animals and Ethics* (Watkins, London).

CARTER, T.C. (1971) The hens egg: shell cracking at oviposition in battery cages and its inheritance. *British Poultry Science*, **12**: 259.

CHAMOVE, A.S. (1989) Environmental enrichment: A review. *Animal Technology*, **40**: 155-178.

CHAMOVE, A.S. & ANDERSON, J.R. (1989) Examining environmental enrichment, in: SEGAL, E. (Ed.) *Housing Care and Psychological Well-Being of Primates,* pp. 183-202 (Noyes Publications, Philadelphia).

CHANCELLOR, L. & GLICK, B. (1960) Effect of temperature as a stressor on white blood cells, adrenals and bursa of fabricus of chicks. *American Journal of Physiology*, **198**: 1346-1348.

CHOUDARY, M.R. & CRAIG, J.V. (1972) Effects of early flock assembly on agonistic behaviour and egg production in chickens. *Poultry Science*, **51**: 1928-1937.

COBB, R. (1991) Managing chickens in the tropics. *Poultry International*. **April**: 24-28.

COLLIAS, N.E. & COLLIAS, E.C. (1967) A field study of the Red Jungle Fowl in North-Central India. *Condor,* **69**: 360-386.

COMMON, R.H. (1938) Observations on the mineral metabolism of pullets. *Journal of Agricultural Science,* **28**: 347-366.

COOK, A.S. & BALCH, D.A. (1970) Studies of membrane, mammillary cores and cuticle of the hens egg shell. *British Poultry Science*, **11**: 345-352.

COUCKE, P. (1998) Assessment of some physical quality parameters of eggs based on vibration analysis, *Ph. D. Thesis*, Faculty of Agriculture and Applied Biological Sciences, Katholieke Universiteit Leuven, Belgium.

COUCKE, P., DEWIL, E. & DECUYPERE, E. (1999) Measuring the mechanical stiffness of an eggshell using resonance frequency analysis. *British Poultry Science*, **40**: 227-232.

CRAIG, J.V. (1981) Domestication, in: CRAIG, J.V. (Ed.) Domestic Animal Behaviour: Causes and Implications for Animal Care and Management, pp. 21-31 (Prentice-Hall, New Jersey).

CRAIG, J.V.(1992) Measuring social behaviour in poultry. *Poultry Science*, 71: 650-657.

CRAIG, J.V. & CRAIG, J.A. (1985) Corticosteroid levels in white leghorn hens as affected by handling, laying-house environment and genetic stock. *Poultry Science*, **64**: 809-816.

CRANSTOUN, S. (1992) The influence of eggshell washing on bacterial transfer across the shell wall. *M. Sc. Thesis*, University of Glasgow.

CUNNINGHAM, F.E., COTTERILL, O.J. & FUNK, E.M. (1960) The effect of season and age of bird. *Poultry Science*, **39**: 289-299.

DARNELL-MIDDLETON, S.L. (1999) Relationship between eggshell quality and bone biology in the laying hen, *Ph. D. Thesis*, University of Glasgow.

DAVISON, T.F., ROWELL, J.G. AND REA, J (1983) Effects of dietary corticosterone on peripheral blood lymphocyte and granulocyte populations in immature domestic fowl. *Research in Veterinary Science*, **34**: 236-239.

DAWKINS, M.S. (1976) Towards an objective method of assessing welfare in domestic fowl. *Applied Animal Ethology*, **2**: 245-254.

DAWKINS, M.S. (1977) Do hens suffer in battery cages ? Environmental preferences and welfare. *Animal Behaviour*, **25**: 1034-1046.

DAWKINS, M.S. (1980) *Animal Suffering The Science of Animal Welfare* (Chapman and Hall, London).

DAWKINS, M.S. (1981) Priorities in the cage size and flooring preferences of domestic hens. *British Poultry Science*, **22**: 255-263.

DAWKINS, M.S. (1983a) Cage size and flooring preferences in litter-reared and cage-reared hens. *British Poultry Science*, **24**: 177-182.

DAWKINS, M.S. (1983b) The current status of preference tests in the assessment of animal welfare, in: BAXTER, S.H., BAXTER, M.R. & MACCORMACK, J.A.D. (Eds), *Farm Animal Housing and Welfare* (Martinus Nijhoff Publishers, The Hague, The Netherlands), pp. 20-26.

DAWKINS, M.S. (1988) Behavioural deprivation: a central problem in animal welfare. *Applied Animal Behaviour Science,* **20**: 209-225.

DAWKINS, M.S. (1989) Time budgets of Red Junglefowl as a baseline for the assessment of welfare in domestic fowl. *Applied Animal Behaviour Science*, **24**: 77-80.

DE BERNARD, B., STAGNI, N., CAMEROTTO, R., VITTUR, F., ZANETTI, M & ZAMBONIN ZALLONE, A. (1980) Influence of calcium depletion on medullary bone of laying hens. *Calcified Tissue International*, **32**: 221-228.

DE KETELAERE, B. & DE BAERDEMAEKER, J. (2000) Part II: Using high resolution resonant frequency and damping estimators for the non-destructive, on-line determination of the eggshell strength. *Proceedings of the 1st international workshop on noise and vibration in agriculture and biological engineering*, pp.149-154.

DE KETELAERE, B., GOVAERTS, T., COUCKE, P., DEWIL, E., VISSCHER, J. & DECUYPERE, E. (In Press) Measuring the eggshell strength of 6 different genetic strains of laying hens : Techniques and comparisons.

DEOBALD, H.J., LEASE, E.J., HART, E.B. & HALPIN, J.G. (1936) Studies on the calcium metabolism of laying hens. *Poultry Science*, **15**: 179-185.

DOUGHERTY, T.F., (1952) Effect of hormone on lymphatic tissue. *Physiological Reviews*, **32**: 379-403.

DUN, P., HUGHES, B.O., HOWIE, T.J., MITCHIE, W. & WRIGHT, D. (1991) Cages for Laying Hens. *Farm Building Progress*, **106**: 25-27.

DUNCAN, I.J.H. (1970) Frustration in the fowl, in: FREEMAN, B.M. & GORDON, R.F. (Eds). Aspects of Poultry Behaviour, p15 (British Poultry Science Association, Edinburgh).

DUNCAN, I.J.H. (1978a) An overall assessment of poultry welfare, in: SORENSEN, L.Y. (Ed), *Proceedings of the First Danish Seminar on Poultry Welfare*, Copenhagen, pp.79 (World Poultry Science Association).

DUNCAN, I.J.H. (1978b) The interpretation of preference tests in animal behaviour. *Applied Animal Ethology*, **4**: 197-200.

DUNCAN, I.J.H.(1980)Animal behaviour as a guide to welfare. *Feedstuffs*, **52**: 29-39.

DUNCAN I.J.H. (1981) Animal rights - Animal welfare : A scientist's assessment. *Poultry Science*, **60**: 489 - 499.

DUNCAN, I.J.H. (1985) How do fearful birds respond ? in : Wegner, R,-M. (Ed) *Proceedings of the 2nd European Symposium on Poultry Welfare*, Celle, pp. 96-106.

DUNCAN, I.J.H. (1989) The assessment of welfare during the handling and transport of broilers, in: FAURE, M. & MILLS, A.D. (Eds) *Proceedings of the 3rd European Symposium on Poultry Welfare,* Tours, pp. 93-107.

DUNCAN, I.J.H., APPLEBY, M.C. & HUGHES, B.O. (1992) Effect of perches in laying cages on welfare and production of hens. *British poultry Science*, **33**: 25-35.

DUNCAN, I.J.H. & DAWKINS, M.S. (1983) The problem of assessing 'well-being' and 'suffering' in farm animals, in: SMIDT, D. (Ed) *Indicators Relevant to Farm Animal Welfare*, pp. 13-24. (Martrius Nijhoff Publishers, The Hague, The Netherlands),

DUNCAN, I.J.H. & FILSHIE, J.H. (1979) The use of radio-telemetry devices to measure temperature and heart rate in the domestic fowl, in: AMLANER, C. AND MACDONALD, D.W.A. (Eds) *A Handbook on Biotelemetry and radio Tracking*, pp. 579-588. (Pergamon, Oxford).

DUNCAN, I.J.H. & KITE, V.G. (1987) Some investigations into motivation in the domestic fowl. *Applied Animal Behaviour Science*, 18: 387-388.

DUNCAN, I.J.H. & PETHERICK, J.C. (1991) The implications of cognitive processes for animal welfare. *Journal of Animal Science*, **69 (12)**: 5017-5022.

DUNCAN, I.J.H. & WOOD-GUSH, D.G.M. (1971) Frustration and aggression in the domestic fowl. *Animal Behaviour*, **19**: 500-504.

DUNCAN, I.J.H. & WOOD-GUSH, D.G.M. (1972) Thwarting of Feeding behaviour in the domestic fowl,. *Animal Behaviour*, **20**: 444-451.

EGGLETON, L.Z. & ROSS, W.J. (1971) Observed eggshell damage in a mechanical gathering system. *Poultry Science*, **50**: 1008.

EL-LETHEY, H., AERNI, V., JUNGI, T.W. & WECHSLER, B. (2000) Stress and feather pecking in laying hens in relation to housing conditions. *British Poultry Science*, **41**: 22-28.

EMERY, D.A., VOHRA P., ERNST, R.A. & MORRISON, S.R. (1984) The effects of cyclic and constant ambient temperature on feed consumption, egg production, egg weight and shell thickness in hens. *Poultry Science*, **63(10)**: 2027-2035.

ENGSTROM, B. & SCHALLER, G. (1993) Experimental studies of the health of laying hens in relation to housing systems, in: SAVOURY, C.J. & HUGHES, B.O. (Eds.), *Proceedings of the 4th European Symposium on Poultry Welfare,* pp. 87 (Potters Bar, UFAW).

E.U. DIRECTIVE 1999/74/EC of July 19th 1999 Laying down minimum standards for the protection of laying hens.

FAWC (1998) The Farm Animal Welfare Council's Report on the Welfare of Broiler Breeders.

FAWC (2001) The Five Freedoms [online]. Available from: http://www.fawc.org.uk/fawc-Welfare.htm (December 2001).

FOX, M.W. (1968) *Abnormal Behaviour in Animals* (Saunders, Philadelphia).

FRASER, A.C. (1996) Environmental and physiological factors influencing the formation of the eggshell of the domestic fowl, *Ph. D. Thesis*, University of Glasgow.

FRASER, A.C., BAIN, M.M. & SOLOMON, S.E. (1998) Organic matrix morphology and distribution in the palisade layer of eggshells sampled at selected periods during lay. *British Poultry Science,* **39**: 225-228.

FRASER, A.F. (1968) Behaviour disorders in domestic animals, in: FOX, M.W. (Ed), *Abnormal Behaviour in Animals*, pp.179-187 (Saunders, Philadelphia).

FREEMAN, B.M. (1984) Transportation in Poultry. *World's Poultry Science Journal*, **40**: 19-30.

FREEMAN, B.M. (1985) Stress and the Domestic Fowl: Physiological Fact or Fantasy? *World's Poultry Science Journal*, **41**: 45–51.

FRENCH, N.A. & TULLETT, S.G. (1991) Variation in the eggs of poultry species, in: TULLETT, S.G. (Ed) *Poultry Science Symposium 22, Avian Incubation*, pp. 59-77 (Butterworth-Heinemann Ltd.).

GALLUP, G.G. (1979) Tonic immobility as a measure of fear in the domestic fowl. *Animal Behaviour*, **27**: 316-317.

GAUTRON, J., HINCKE, M.T., MANN, K., PANHÉLEUX, M., BAIN, M., MCKEE, M.D., SOLOMON, S.E. & NYS, Y. (2001) Ovocalyxin-32, a novel chicken eggshell matrix protein. *Journal of Biological Chemistry*, **276 (42)**: 39243-39252.

GENTLE, M.J., WADDINGTON, D., HUNTER, L.N. & JONES, R.B. (1990) Behavioural evidence for persistent pain following partial beak amputation in chickens. *Applied Animal Behavioural Science,* **27**: 149-157.

GROSS, W.B. & COLMANO, G. (1970) The effect of social stress on infectious diseases. *Poultry Science*, **43**: 1390.

GROSS, W.B. & SIEGEL, P.B. (1982) Socialization as a factor in resistance to infection, feed efficiency and response to antigens in chickens. *American Journal of Veterinary Research*, **43**: 2010-2012.

GROSS, W.B. & SIEGEL, H.S. (1983) Evaluation of the heterophil : lymphocyte ratio as a measure of stress in chickens. *Avian Diseases*, **27**: 972–979.

GROSS, W.B. & SIEGEL, P.B. (1985) Selective breeding of chickens for corticosterone response. *Poultry Science*, **64**: 2230-2233.

GROSS, W.B., SIEGEL, P.B. & DUBOSE, R.B. (1980) Some effects of feeding corticosterone to chickens. *Poultry Science*, **59**: 516-522.

GVARYAHU, G., ARARAT, E., ASAF, E., LEV, M., ROBINZON, B., WELLER, J.I. & ROBINZON, B. (1994) An enrichment object that reduces aggressiveness and mortality in caged laying hens. *Physiology and Behaviour*, **55**: 313-316.

GVARYAHU, G., CUNNINGHAM, D.L. & VAN TIENHOVEN, A. (1989) Filial Imprinting, environmental enrichment, and music application effects on behaviour and performance of meat strain chicks. *Poultry Science*, **68**: 211 – 217.

HALL, A.L. (2001) The effect of stocking density on the welfare and behaviour of broiler chickens reared commercially. *Animal Welfare - Potters Bar,* **10 (1)**: 23-40.

HAMILTON, R.M.G. (1982) Methods and factors that affect the measurement of egg shell quality. *Poultry Science*, **61**: 2022-2039.

HARRISON, R. (1964) *Animal Machines* (Stuart, London).

HEMSWORTH, P.H. & BARNETT, J.L. (1989) Relationships between fear of humans, productivity and cage position of laying hens. *British Poultry Science*, **30**: 505–518.

HEMSWORTH, P.H., BARNETT, J.L. & COLEMAN, G.J. (1993) The human animal relationship in agriculture and the consequences for the animal. *Animal Welfare*, **2**: 33-51.

HINCKE, M.T., BERNARD, A.-M., LEE, E.R., TSANG, C.P.W. & NARBAITZ, R. (1992) Soluble protein constituents of the domestic fowl's eggshell. *British Poultry Science*, 33: 505-516.

HOCKING, P.M., CHANNING, C.E., ROBERTSON, G.W., EDMOND, A. & R.B. JONES (In Press) Between breed genetic variation for behavioural traits in domestic fowl.

HOCKING, P.M., MAXWELL, M.H. & MITCHELL, M.A. (1993) Welfare assessment of broiler breeder and layer females subjected to food restriction and limited access to water during rearing. *British Poultry Science*, **34**: 443-458.

HOCKING, P.M., MAXWELL, M.H., ROBERTSON, G.W. & MITCHELL, M.A. (2001) Welfare assessment of modified rearing programmes for broiler breeders. *British Poultry Science*, **42**: 424-432.

HOGAN, J.A. (1965) An experimental study of conflict and fear: an analysis of behaviour of young chicks towards a mealworm: Part 1 . The behaviour of chicks which do not eat the mealworm. *Behaviour*, **25-26**: 45-95.

HUGHES, B.O. (1973) Animal Welfare and the intensive housing of domestic fowl. *Veterinary Record*, 93: 658-662.

HUGHES, B.O. (1976) Behaviour as an index of welfare, in: *Proceedings of the 5th European Poultry Conference*, Vol. II, Malta (WPSA) pp. 1005-1018.

HUGHES, B.O. & APPLEBY, M.C. (1989) Increase in bone strength of spent laying hens housed in modified cages with perches. *Veterinary Record*, **124**: 483-484.

HUGHES, B.O. & BLACK, A.J. (1973) The preference of domestic hens for different types of battery cage floor. *Poultry Science*, **14**: 615 – 619.

HUGHES, B.O. & BLACK, A.J. (1974) The effect of environmental factors on activity, selected behaviour patterns and "fear" of fowls in cages and pens. *British Poultry Science*, **15**: 375-380.

HUGHES, B.O. & BLACK, A.J. (1976)The influence of handling on egg production, egg shell quality and avoidance behaviour of hens. *British Poultry Science*.**17**: 135–144.

HUGHES, B.O., DUN, P. & MCCORQUODALE, C.C. (1985) Shell strength of eggs from medium-bodied hybrid hens housed in cages or on range in outside pens. *British Poultry Science*, **26**: 129-136.

HUGHES, B.O. & DUNCAN, I.J.H. (1972) The influence of strain and environmental factors upon feather pecking and cannibalism in fowls. *British Poultry Science*, 13: 525-547.

HUGHES, B.O. & GILBERT, A.B. (1984) Induction of egg shell abnormalities in domestic fowls by injection of adrenaline. *IRCS Medical Science*, **12**: 969-970.

HUGHES, B.O., GILBERT, A.B. & BROWN, M.F. (1986) Categorisation and causes of abnormal eggshells : Relationship with stress. *British Poultry Science,* **27**: 325 – 337.

HUGHES, B.O., WILSON, S., APPLEBY, M.C. & SMITH, S.F. (1993) Comparison of bone volume an strength as measures of skeletal integrity in caged laying hens with access to perches. *Research in Veterinary Science*, **54**: 202-206.

HUNTON, P. (1987) Laboratory evaluations of egg quality, in: WELLS, R.G. & BELYAVIN, C.G. (Eds.), *Poultry Science Symposium 20, Egg Quality - Current Problems and Recent Advances*, pp. 87-102 (London and Boston, Butterworths).

HUNTON, P. (1995) Understanding the architecture of the egg shell. *World's Poultry Science Journal*, **51(2)**: 141-147.

HURNICK J.E., PIGGINS, D.J., REINHART, B.S. & SUMMERS, J.D. (1974) The effect of visual pattern complexity of feeders on food consumption in laying hens. *British Poultry Science*, **15**: 97-105.

HURWITZ, S. (1987) The effect of disease on egg quality, in: WELLS, R.G. & BELYAVIN, C.G. (Eds) *Poultry Science Symposium 20, Egg Quality - Current Problems and Recent Advances*, pp. 235-254 (London and Boston, Butterworths).

JAY, M., TANDRON, E. & RAMOS, M. (1992) Effect on duration and conditions of storage on egg quality of crossbred E1 x E23 heavy hens. [Spanish]. *Revista de Salud Animal*, **14**: 7-11.

JOHNSON, A.L. (2000) Chapter 22: Reproduction in the Female, in: WHITTOW, G.C. (Ed) *Sturkie's Avian Physiology*, pp. 569-596 (Academic Press, London).

JOHNSON, R.A. (1963) Habitat preference and behaviour of breeding junglefowl in central Western Thailand. *Wilson Bull.*, **75**: 270-272.

JONES, D.R. (1996a) Effects of genetic selection on the production parameters, heterophil:lymphocyte ratios, and plasma concentrations of corticosterone, 3,5,3'-Triiodothyronine, and Thyroxine in single comb white Leghorn hens. *M. Sc. Thesis*, North Carolina State University.

JONES, R.B. (1982) Effects of early environmental enrichment upon open-field behaviour and timidity in the domestic chick. *Developmental Psychobiology*, **15**: 105–111.

JONES, R.B. (1985a) Fearfulness of hens caged individually or in groups in different tiers of a battery and the effects of translocation between tiers. *British Poultry Science*, **26**: 399-408.

JONES, R.B. (1985b) Fearfulness and adaptability in the domestic fowl. *IRCS Medical Science*, **13**: 797-800.

JONES, R.B. (1986) The tonic immobility reaction of the domestic fowl : A review. *World's Poultry Science Journal*, **42**: 82-96.

JONES, R.B.(1987a) Social and environmental aspects of fear in the domestic fowl, in: ZAYAN, R. & DUNCAN, I.J.H (Eds) *Cognitive Aspects of Social Behaviour in Domestic Fowl*, pp. 82-149 (Elsevier, Amsterdam).

JONES, R.B. (1987b) The assessment of fear in the domestic fowl, in: ZAYAN, R. & DUNCAN, I.J.H (Eds) *Cognitive Aspects of Social Behaviour in Domestic Fowl*, pp. 40-81 (Elsevier, Amsterdam).

JONES, R.B. (1987c) Assessment of fear in adult laying hens: Correlational analysis of methods and measures. *British Poultry Science*, **28**: 319-326.

JONES, R.B. (1989a) Development and alleviation of fear in poultry, in: FAURE, M. & MILLS, A.D. (Eds) *Proceedings of the 3rd European Symposium on Poultry Welfare,* Tours, pp.123–136.

JONES, R.B. (1996b) Fear and adaptability in poultry: Insights, implications and imperatives. *World's Poultry Science Journal*, **52**: 131–174.

JONES, R.B. (1997) Fear and Distress, in: APPLEBY, M.C. & HUGHES, B.O. (Eds) *Animal Welfare*, pp. 75–87 (CAB International).

JONES, R.B., BEUVING, G. & BLOKHUIS, H.J.(1988) Tonic immobility and heterophil / lymphocyte responses of the domestic fowl to corticosterone infusion. *Physiology and Behaviour*, **42**: 249-253.

JONES, R.B., CARMICHAEL, N.L. & RAYNER, E. (2000) Pecking preferences and predispositions in domestic chicks: implications for the development of environmental enrichment devices. *Applied Animal Behaviour Science*, **69 (4)**: 317-324.

JONES, R.B., DUNCAN, I.J.H. & HUGHES, B.O. (1981) The assessment of fear in domestic hens exposed to a looming human stimulus. *Behavioural Processes*, **6**: 121-133.

JONES, R.B., HARVEY, S., HUGHES, B.O. & CHADWICK, A. (1980) Growth and the plasma concentration of growth hormone and prolactin in chicks: effects of ' environmental enrichment', sex and strain. *British Poultry Science*, **21**: 457-462.

JONES, R.B. & HUGHES, B.O. (1981) Effect of regular handling on growth in male and female chicks of broiler and layer strains. *British Poultry Science*. **22**: 461-465.

JONES, R.B. & HUGHES, B.O. (1986) Fearfulness and abnormalities in the chickens eggshell - Is there a link ? *Proceedings of the International Symposium on Applied Ethology in Farm Animals*, Balatonfured, Hungary, pp. 171 – 175.

JONES, R.B., MILLS, A.D. & FAURE, J.M. (1991) Genetic and experimental manipulation of fear-related behaviour in Japanese Quail chicks (*Coturnix corturnix japonica*). *Journal of Comparative Psychology*, **105**: 15-24.

KAMINSKA, B.F. & SKRABA, B. (1992) Changes in egg shell quality during the first year of laying in hens. *Journal of Animal and Feed. Science*, **1**: 51-58.

KAMINSKA, B.F & SKRABA, B. (1993) Correlation between some parameters of estimating egg shell quality and breaking strength in five periods of laying cycle in hens. *Proceedings of the 11th European symposium on the quality of eggs and egg products*, Tours, pp. 206-210.

KEER-KEER, S., HUGHES, B.O., HOCKING, P.M. & JONES, R.B. (1996) Behavioural comparison of layer and broiler fowl: measuring fear responses. *Applied Animal Behaviour Science*, **49**: 321 - 333 .

KING, L. (2001) Environmental enrichment for broiler breeders, *Ph. D. Thesis*, University of Oxford.

KITE, V.G. (1986) Does a Hen Require a Nest. *Archiv Fur Geflugelkunde*, **50(3)**:117-118.

KOELKEBECK, K.W., AMOSS JR, M.S. & CAIN, J.R. (1987) Production, physiological and behavioural responses of laying hens in different management environments. *Poultry Science*, **66**: 397 - 407.

KRETCHMER, K.R. & FOX, M.I. (1975) Effects of domestication on animal behaviour. *Veterinary Record*, **96**: 102-108.

KRUIJT, J.P. (1964) Ontogeny of social behaviour in Burmese Red Junglefowl (*Gallus gallus spadiceus Bonnaterre*). *Behaviour Suppl.*, **12**: 1-201.

KULL, K, -E, (1948) The prevention and treatment of cannibalism and feather eating in fowls. *Proceedings of the 8th World Poultry Congress*, Copenhagen, pp. 124-125.

LAGADIC, H., FAURE, J.M., MILLS, A.D. & WILLIAMS, J.B. (1990) Effects of blood sampling on plasma concentrations of corticosterone and glucose in laying hens caged in groups. *British Poultry Science*, **31**: 823-829.

LANYON, L. & RUBIN, C.T. (1984) Static versus dynamic loads as an influence on bone remodelling. *Journal of Biomechanics*, **17**: 897-905.

LEMMENS, J.W. (1990) Impulse Excitation: A technique for dynamic modulus measurement, in: WOLFENDEN, A. (Ed) *Dynamic Elastic Modulus Measurements in Materials*, pp. 90-99 (Philadelphia, American Society for Testing of Materials).

LILLPERS, K. & WILHELMSON, M. (1993) Age-dependant changes in oviposition pattern and egg production traits in the domestic fowl. *Poultry Science*, **72**: 2005-2011.

LITTIN, K.E. & COCKREM, J.E. (2001) Individual variation in corticosterone secretion in laying hens. *British Poultry Science*, **42**: 536-546.

LORD, A. (2001) Faecal Glucocorticoid as an Indicator of Welfare in Chickens. *Ph. D. Thesis*, University of Oxford.

MANKOVICH, N.J. & BANKS, E.M. (1982) An analysis of social orientation and the use of space in a flock of domestic fowl. *Applied Animal Ethology*, **9**:177-193.

MARTIN, T.G., RAISZ, L.G. & RODAN, G. (1987) *Clinical Endocrinology of Calcium Metabolism,* (Macel Dekker, New York).

MAXWELL, M.H. (1993) Avian blood leukocyte response to stress. *World's Poultry Science Journal*, **49**: 34–43.

MAXWELL, M.H. & ROBERTSON, G.W. (1995) The avian basophilic leukocyte : A review. *World's Poultry Science*, **51**: 307-325.

MAXWELL, M.H., ROBERTSON, G.W., MITCHELL, M.A. & CARLISLE, A.J. (1992) The fine structure of broiler chicken blood cells, with particular reference to basophils after severe heat stress. *Comparative Haematology International,* **2**: 90–200.

MAXWELL, M.H., ROBERTSON, G.W., SPENCE, S. & MCCORQUADALE. C.C. (1990) Comparisons of haematological values in restricted and ad libitum fed domestic fowls: White blood cells and thrombocytes. *British Poultry Science*, **31**: 399-405.

MEIJSSER, F.M. & HUGHES, B.O. (1989) Comparative analysis of pre-laying behaviour in battery cages and in three alternative systems, *British Poultry Science*, **30**: 747-760.

MENCH, J.A. (1992) The welfare of poultry in modern production systems. *Poultry Science Reviews*, **4**: 107-128.

MILLS, A.D., MARCHE, M. & FAURE, J.M. (1987) Extraneous Egg shell calcification as a measure of stress in poultry. *British Poultry Science*, **28**: 177 - 181 .

MILLS, A.D., NYS, Y., GAUTRON, J. & ZAWADSKI, J. (1991) Whitening of brown shelled eggs: individual variation and relationships with age. *British Poultry Science*, **32**: 117-129.

MILLS, A.D. & WOOD-GUSH, D.G.M. (1985) Pre-laying behaviour in battery cages. *British Poultry Science*, **26**: 247-252.

MITCHELL, M.A., MACLEOD, M.G. & RAZA, A. (1986) The effects of ACTH and dexamethasone upon plasma thyroid-hormone levels and heat production in the domestic-fowl. *Comparative Biochemistry and Physiology A*, **85 (2)**: 207-215.

NASCIMENTO, DO. V. P. (1992) The ease of translocation of *S. enteritidis* through the eggshell wall: An immunocytochemical / ultrastructural study, *Ph. D. Thesis*, University of Glasgow.

NICOL, C.J. (1992) Effects of environmental enrichment and gentle handling on behaviour and fear responses of transported broilers. *Applied Animal Behavioural Science*, **33**: 367-380.

NICOL, C.J., GREGORY, N.G., KNOWLES, T.G. & PARKMAN, I.D. (1999) Differential effects of increased stocking density, mediated by increased flock size, on feather pecking and aggression in laying hens. *Applied Animal Behaviour Science*, **65 (2):** 137-152.

NOLES, R.K. & TINDELL, D. (1967) Observations on the inter-relationships of egg quality traits and their association with season, age and strain of bird. *Poultry Science*, **46**: 943-952.

NORDSTROM, J.O. (1973) Duration of egg formation in chickens during heat stress. *Poultry Science,* **51**: 1687-1690.

NORGAARD-NIELSEN, G., VESTERGAARD, K. & SIMONSEN, H.B. (1993) Effect of rearing experience and stimulus enrichment on feather damage in laying hens. *Applied Animal Behaviour Science*, **38**: 345–352.

NYS, Y. (1986) Relationship between age, shell quality and individual rate and duration of shell formation in domestic hens. *British Poultry Science*, **27**: 253–259.

NYS, Y., HINCKE, M.T., ARIAS, J.L., GARCIA-RUIZ, J.M. & SOLOMON, S.E. (1999) Avian Eggshell Mineralization. *Poultry and Avian Biology Reviews* ,**10**:143-166.

O'SULLIVAN, N.P., DUNNINGTON, E.A. & SIEGEL, P.B. (1991) Relationship among age of dam, egg components, embryo lipid transfer, and hatchability of broiler breeder eggs. *Poultry Science*, **70**: 2180-2185.

OUART M.D. & ADAMS A.W. (1982) Effects of cage design and bird density on layers. 1. Productivity, feathering and nervousness. *Poultry Science*, **61**: 1606-1613.

OVERFIELD, N.D. (1995) Egg quality assessment techniques at laboratory and field level. in: *Proceedings of the 6th European Symposium on the Quality of Egg and Egg Products*, pp.11-19.

PANHÉLEUX, M., NYS, Y., WILLIAMS, J. & GAUTRON, J. (1999) Quantification by ELISA of eggshell matrix proteins laid by young and old hens. *Proceedings of the 8th European Symposium on Quality of Eggs and Egg Products*, Bologna, Italy, pp.37-42.

PARSONS, A.H. (1982) Structure of the Eggshell. *Poultry Science*, 61: 2013-2021.

PATTERSON, H.D. & THOMPSON, R. (1971) Recovery of inter-block information when block sizes are unequal. *Biometrika*, **58**: 345-554.

PETERSON, C.F. (1965) Factors influencing eggshell quality. *World's Poultry Science Journal*, **21**: 110–138.

REED, H.J., WILKINS, L.J., AUSTIN, S.D. & GREGORY, N.G. (1993) The effect of environmental enrichment during rearing on fear reactions and depopulation trauma in adult caged hens. *Applied Animal Behaviour Science*, **36**: 39–46.

REID, J. (1983) The use of the plasma chemistry unit as an aid to the scanning electron microscope study of avian eggshell structure. *British Poultry Science*, **24**: 233-235.

ROBERTS, J.R. & BRACKPOOL, C.E. (1994) The ultrastructure of avian egg shells. *Poultry Science Review*, **5**: 245–272.

ROBERTS, J.R., LEARY, A.M., BALL, W. & NOLAN, J.V. (1997) Egg and egg shell quality in five strains of laying hen and the effect of hen age. *Proceedings of the Australian Poultry Science Symposium*, pp.126-128.

ROBERTSON, E.S., APPLEBY, M.C., HOGARTH, G.S. & HUGHES, B.O. (1989) Modified cages for laying hens: a pilot trial. *Research and Development in Agriculture*, **6**: 107-114.

ROEBBEN, G., BOLLEN, B., BREBELS, A., VAN HUMBEECK, J. & VAN DER BIEST, O. (1997) Impulse excitation apparatus to measure resonance frequencies, elastic moduli and internal friction at room and high temperature. *Review of Scientific Instruments*, **16**: 4511-4515.

ROLAND, D.A. SR. (1979) Factors influencing shell quality of aging birds. *Poultry Science*, **58**: 774–777.

ROMANOFF, A.L. & ROMANOFF, A.J. (1949) *The Avian Egg* (Chapman and Hall, London).

ROQUE, L. & SOARES, M.C. (1994) Effects of eggshell quality and broiler breeder age on hatchability. *Poultry Science*, **73 (12)**:1838-1845.

ROSALES, A.G. (1994) Managing stress in broiler breeders : a review. *Journal of Applied Poultry Research*, **3**: 199-207.

ROSE, S.P. (1997) Chapter 4 : Female Reproduction, in: *Principles of Poultry Science*, pp. 41-69 (Cab International, London).

RUIZ, J. & LUNAM, C.A. (2000) Ultrastructural analysis of the eggshell: contribution of the individual calcified layers and the cuticle to hatchability and egg viability in broiler breeders. *British Poultry Science*, **41**: 584-592.

RUSHEN, J. (1986) Some problems with the physiological concept of 'stress'. *Australian Veterinary Journal*, **63**: 359-360.

SAADOUN, A., SIMON, J.R. & LECLERQ, B. (1987) Effect of exogenous corticosterone in genetically fat and lean chickens. *British Poultry Science*, **28**: 519-528.

SAHIN, A. & FORBES, J.M. (1999) Effects of corticosterone on diet selection for protein in relation to egg production by laying hens. *British Poultry Science*, **40**: S54-S55.

SANOTRA, G.S., LAWSON, L.G. & VESTERGAARD, K.S. (2001) Influence of stocking density on tonic immobility, lameness, and tibial dyschondroplasia in broilers. *Journal of Applied Animal Welfare Science*, **4 (1)**: 71-87.

SAPOLSKY, R. (1987) Stress, social status and reproductive physiology in free-living baboons, in: CREWS, D (Ed.), Psychobiology of reproductive behaviour: An evolutionary perspective (Prentice Hall, Englewood Cliffs, New Jersey), pp. 291-322.

SAY, R.R. (1987) *Manual of Poultry Production in the Tropics (Translated)* (CAB International, U.K.).

SCHALLER, G.B. & EMLEN, J.T. (1962) The ontogeny of avoidance behaviour in some precocial birds. *Animal Behaviour*, **10**: 370-381.

SCHÜTZ, K.E., FORKMAN, B. & JENSEN, P. (2001) Domestication effects on foraging strategy, social behaviour and different fear responses: a comparison between the red Jungle fowl (*Gallus gallus*) and a modern layer strain. *Applied Animal Behavioural Science*, **74**: 1-14.

SELYE, H. (1932) The general adaptation syndrome and the diseases of adaptation. *Journal of Clinical Endocrinology*, **6**:117-152.

SELYE, H. (1950) *The physiology and pathology of exposure to stress* (Acta, Montreal).

SELYE, H. (1973) The evolution of the stress concept. *The American Scientist*, **61**: 692–699.

SHALEV, B.A. & PASTERNAK, H. (1993) Increment of egg weight with hen age in various commercial avian species. *British Poultry Science*, 34: 915.

SHERWIN, C.M. & NICOL, C.J. (1992) Behaviour and production of laying hens in three prototypes of cages incorporating nests. *Applied Animal Behaviour Science*, **35**: 41-54.

SIEGEL, P.B. (1971) Adrenals, stress and the environment. *World's Poultry Science Journal*, **27**: 327-349.

SIEGEL, H.S. (1985) Immunological responses as indicators of stress. *World's Poultry Science Journal,* **41**: 36-44.

SIEGEL, H.S. (1995) Stress, strains and resistance. *British Poultry Science*, **36**: 3-23.

SIMKISS, K. (1968) The structure and formation of the shell and shell membranes, in: CARTER, T.C. (Ed.) *Egg Quality* (Edinburgh, Oliver and Boyd).

SIMONS, P.C.M. (1971) Ultrastructure of hens eggshell and its physiological interpretation, Centre for Agricultural Publishing and Documentation, (Wageningen).

SOLOMON, S.E. (1973a) Studies on the reproductive tract of the domestic fowl, *Ph. D. Thesis*, University of Glasgow.

SOLOMON, S.E. (1973b) The ultrastructural localisation of acid phosphatase in the avian shell gland. *British Poultry Science*, **14**: 241–243.

SOLOMON, S.E. (1975) Studies on the Isthmus region of the domestic fowl. *British Poultry Science*, **16**: 255–258.

SOLOMON, S.E. (1991) *Egg and Eggshell Quality* (Mason Publishing, Iowa State University Press).

SOLOMON, S.E. (2000) The Oviduct in Chaos. *Proceedings of the 21st Worlds Poultry Congress*, Montreal, Canada.

SOLOMON, S.E., HUGHES, B.O. & GILBERT, A.B. (1987) Effect of a single injection of adrenalin on shell ultrastructure in a series of eggs from domestic hens. *British Poultry Science*, **28**: 585-588.

SPACKMAN, D. (1987)The effect of disease on egg quality, in: WELLS, R.G. & BELYAVIN, C.G. (Eds) *Poultry Science Symposium 20, Egg Quality - Current Problems and Recent Advances*, pp. 255-282 (London and Boston, Butterworths).

SPARKS, N.H.C. (1991) Cuticle distribution on table eggs. *International Hatchery Practice,* **5(8)**: 22.

STRUWE, F.J., GLEAVES, E.A. & DOUGLAS, J.H. (1992) Stress measurement on beak-trimmed and untrimmed pullets. *Poultry Science*, **71 (7):** 1154-1162.

TERLOUW, E.M.C., SCHOUTEN, W.G.P. & LADEWIG, J. (1997) Physiology, in: APPLEBY, M.C. & HUGHES, B.O. (Eds), Animal Welfare, pp. 143–158 (CAB International).

THOMPSON, C.I. (1976) Growth in the Hubbard broiler: increased size following early handling. *Developmental Psychobiology*, **9**: 459-464.

TULLETT, S.G. (1987) Egg Shell Formation and Quality, in: WELLS, R.G. & BELYAVIN, C.G. (Eds) *Poultry Science Symposium 20, Egg Quality - Current Problems and Recent Advances*, pp. 123-146 (London and Boston, Butterworths).

TYLER, C. (1961) Studies on egg shells. XVI. Variations in shell thickness over different parts of the same shell. *Journal of the Science of Food and Agriculture*, **12**: 459–470.

TYLER, C. & GEAKE, F.H. (1965) Egg shell thickness patterns as shown by individual domestic hens. *Poultry Science*, **6**: 235.

VAN LIERE, D.W., KOOJMAN, J. & WIEPKEMA, P. (1989) Conflict dustbathing behaviour on wood shavings? IN: FAURE, M. & MILLS, A.D., *Third European Symposium on Poultry Welfare*, pp. 265-268.

VAN MIDDELKOOP, J.H. (1971) Shell abnormalities due to the presence of 2 eggs in the shell gland. *Archiv Fur Geflugelkunde*, **35**: 122–127.

VAN MIDDELKOOP, J.H. (1972) Hatching problems of hard-shelled eggs with additional shell deposition produced by broiler mothers. *Archiv Fur Geflugelkunde*, **36**: 63-70.

VESTERGAARD, K. (1981) The well-being of the caged hen - an evaluation based on behaviour of fowls. *Tierhaltung*, **12**: 145-165.

VESTERGAARD, K. (1982) The significance of dustbathing for the wellbeing of the domestic hen. *Tierhaltung*, **13**: 109-118.

VESTERGAARD, K. (1989) Environmental influences on the development of behaviour and their relation to welfare, in: FAURE, M. & MILLS, A.D. (Eds) *Proceedings of the 3rd European Symposium on Poultry Welfare,* Tours, pp.109-121.

VOISEY, P.W. & HUNT, J.R. (1974) Measurement of Eggshell Strength. *Journal of Texture Studies*, **5**: 135-182.

VUILLEUMIER, J.P. (1969) The 'Roche Yolk Colour Fan'. An instrument for measuring yolk colour. *Poultry Science*, **48**: 767-779.

WASSER, S. K., BEVIS, K., KING, G. & HANSON, E. (1997) Non-invasive physiological measures of disturbance in the northern spotted owl.*Conservation Biology*, **11**: 1019-1022.

WASSER, S.K., HUNT, K.E., BROWN, J.L., COOPER, K., CROCKETT, C.M., BECHERT, U. MILLSPAUGH, J.J., LARSON, S. & MONFORT, S. (2000) A generalised fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *General and Comparative Endocrinology*, **120**: 260-265.

WASSER, S.K., MONFORT, S.L., SOUTHERS, J. & WILDT, D.E. (1994) Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*) faeces. *Journal of Reproduction and Fertility*, **101**: 213-220.

WATT, J.M. (1985) An ultrastructural evaluation of eggshell quality in the domestic fowl. *M.Sc. Thesis*, University of Glasgow.

WATT, J.M. (1989) The effect of stress on the Reproductive tract of the domestic fowl. *Ph. D. Thesis*, University of Glasgow.

WHITTEN, P.L., BROCKMAN, D.K. & STAVISKY, R.C. (1998) Recent advances in non-invasive techniques to monitor hormone - behaviour interactions. *Yearbook of Physical Anthropology*, **41**: 1-23.

WINGFIELD, J.C., VLECK, C.M. & MOORE, M.C. (1992) Seasonal changes of the Adreno-cortical response to stress in birds of the Sonoran desert. *Journal of Experimental Biology*, **264**: 419-428.

WOLFORD, J.H., GROSS, W.B., DUNNINGTON, E.A. & SIEGEL, P.B. (1983) Reproductive response of laying hens to corticosterone feeding. *Poultry Science*, **62**: 1525.

WOLFORD, J.H. & TANAKA, K. (1970) Factors influencing egg shell quality - a review. *World's Poultry Science Journal*: **26**, 763-780.

WOOD-GUSH, D.G.M. & GILBERT, A.B. (1964) The control of nesting behaviour of the domestic hen II, *Animal Behaviour*, **12**: 451-453.

WOOD-GUSH, D.G.M. & GILBERT, A.B. (1973) Some hormones involved in the nesting behaviour of hens, *Animal Behaviour*, **21**: 98-103.

ZIMMERMAN, R.A., SNETSINGER, D.C., GREENE, D.E. & MARR, J.E. (1972) Effect of environmental stress on layer performance and egg characteristics. *Poultry Science*, 51: 1889.

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