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# A non-invasive test of stress in laying hens

**A report for the Rural Industries Research and Development Corporation**

by J.A. Downing and W.L. Bryden

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# Foreword

The adoption of intensive production systems for poultry production has resulted in remarkable increases in productive efficiency during the last thirty years. These changes have arisen from intensified genetic selection, as well as improvements in nutrition, management and disease treatment and prevention. Although all these factors have had a positive impact on animal welfare, intensive confinement systems also impose costs on birds.

Of increasing concern to the egg industry is the growing public perception that laying birds exist in a state of chronic stress for the duration of their productive life. In view of the public concern of the welfare issues associated with egg production it is important to try to identify or define what is a contented bird exposed to minimum stress.

In this project, a novel and practical, non-invasive means of monitoring the stress status of hens by measuring stress hormone concentrations in eggs was developed. In a series of studies the relationship between circulating concentrations of stress hormones, and their sequestering into egg albumen was determined under normal conditions and experimentally induced stress.

This two year project was funded by industry revenue from the Egg Program which is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 700 research publications, forms part of our Egg R & D program,which aims to initiate, support and manage R & D to meet the requirements of a profitable and responsible Australian egg industry.

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**Peter Core**
Managing Director
Rural Industries Research and Development Corporation

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# Executive Summary

The egg industry faces continued criticism of the ethics of husbandry practices used in egg production especially the welfare of hens kept in cages. Assessment of hen welfare is difficult because it encompasses many factors. Measurements based on health, production, behaviour and physiology have been made. These measurements are based on some level of change and a major difficulty is how much change constitutes a risk to the hen's welfare?

Physiological changes can be a sensitive measure of welfare as these relate to changes in the stress-axis. Stress describes the way an organism responds to a stressful stimuli in an endeavor to maintain homeostasis. Stressors are events perceived as a real or anticipated threat which elicit responses and these depend on the severity and duration of the stimuli. Many of the responses to noxious stimuli highlight the importance of the adrenal gland in regulating physiological changes. As a general adaptive response corticosterone increases gluconeogenesis and blood glucose, catabolism of muscle tissue, increases fatness and depresses immunity. Short–term responses to stress result in catecholamine release from the adrenal gland.

There are inherent difficulties with the interpretation of circulating hormone concentrations because of intrinsic patterns and changes occurring in response to sampling. Non-invasive techniques of measuring levels of stress hormones would reduce these problems. Hormone levels in the egg could provide a non-invasive method of measuring stress levels in hens and prove helpful in identifying conditions responsible for poor welfare. The gradual accumulation of albumen over six hours during egg formation potentially provides an accurate reflection of circulating hormone levels over this time. The overall objective of the project was to develop procedures for measuring stress hormones in egg albumen and then assess whether albumen levels of these hormones reflect stress experienced by laying hens.

As part of the project a comprehensive literature review was completed entitled 'Stress, hen husbandry and welfare' (see Appendix 1). Assays to determine corticosterone and catecholamines (adrenaline and noradrenaline) in albumen samples have been developed and validated. The corticosterone assay is a competitive protein binding radioimmunoassay using a specific antiserum raised against corticosterone. In essence it is a one step extraction procedure followed by an overnight incubation with antiserum and radio-labelled corticosterone and charcoal separation of free label. The catecholamine assay is more complicated requiring lengthy extraction procedures and then determination of catecholamines by HPLC separation and electrochemical detection.

Once the assays were developed the next objective was to determine the relationship between plasma corticosterone and catecholamine levels and albumen levels. In these studies attempts were made to modify plasma hormone levels by infusing or injecting the hormones subcutaneously and measuring the levels in egg albumen. As detailed in the full report it was not possible to determine the relationship between plasma and albumen hormone levels in the injection studies.

The final objective of the project was to assess what effect known stressors have on albumen stress-hormone levels. In the short-term, increasing housing temperature from $18^0$ C to $32^0$ C increased egg albumen corticosterone concentrations on some days. Long-term exposure to $30^0$ C increased albumen corticosterone concentration compared to hens held at $18^0$ C. In both studies there were no effects on adrenaline levels. Handling in various forms is stressful to hens. When hens were handled there was an increase in egg albumen corticosterone levels especially during early episodes of handling. It is possible hens adjust to the handling procedures and differences in corticosterone levels abate. While handling is known to stimulate catecholamine release there were no differences in albumen catecholamine levels following handling. Moving hens from one cage to another increases egg albumen corticosterone concentration although the differences last for only a short time. Increasing cage density increased albumen corticosterone levels but again it appears hens adjust to this

change in about 7 days as no differences in albumen corticosterone were seen after this time. Changing cages or increasing the density had no effect on adrenaline levels in albumen.

During the course of the studies detailed in this report:

i) A literature review of stress in hens was prepared.
ii) Assays to measure corticosterone and catecholamines in egg albumen were established.
iii) The relationships between corticosterone and catecholamine in plasma and egg albumen were evaluated.
iv) In hens the effects of heat, handling and cage density on egg albumen corticosterone and catecholamine concentrations were evaluated.

From the limited number of bird studies conducted during the 2 years of the project it appears that the corticosterone but not catecholamine concentrations in egg albumen can provide a non-invasive measure of stress in hens.

# 1. Introduction

An understanding of the relationship between animal production characteristics and stress is important in issues of animal welfare and animals rights. This has been comprehensively reviewed by Downing and Bryden (1999) and the following relies predominantly on that paper. As the keeping of poultry progressed from small flocks with the products for home consumption, to large commercial enterprises, there were major improvements in production traits. Increasing stocking density could reduce costs and so hens were housed in single or multiple cages. As this occurred there was a real or perceived increase in behavioral problems associated with the housing of layers.

## 1.1. Hen welfare

Production systems should provide hens with:

freedom from hunger and thirst

thermal and physical comfort

freedom from pain and disease

freedom from fear and distress

sufficient space to exercise

The last of these five freedoms (see Downing and Bryden, 1999) is presently a very controversial tropic. Intensive farming practices often deprive animals' access to conditions that allow for the performance of normal behavior. It has been considered that such deprivation leads to stress and a decline in the animals well being.

An interest in welfare can stem from numerous factors and include economics, culture, philosophical attitudes, scientific, aesthetics, knowledge and religion (Craig and Swanson, 1994). Attitudes to animal welfare vary greatly. Kellert (1988) found that the attitude had a lot to do with the relationship that different professions had with animals. While there are many possible definitions according to the individual interest, perhaps an appropriate general definition suitable to all interests is that of Hurnik (1988). "Animal wellbeing (welfare) is a state or condition of physical and psychological harmony between the organism and its surroundings characterized by the absence of deprivation, aversive stimulation, over-stimulation or any other imposed condition which adversely affects health and productivity of the organism".

An assessment of welfare is difficult because it encompasses many factors that have input to the final state of the animal. It is a sum of all the factors that impinge on the animal. Most definitions of welfare have been based on physiological assessments and measurements based on health, production, behaviour and physiology (Mench and van Tienhoven, 1986; Broom, 1991). Assessments of welfare rely on some measure of change and change itself may not be a measure of stress as animal's behaviour and physiology changes to maintain homeostasis. Any parameter provides only prima face evidence that the animal's welfare is compromised. The real question becomes how much change signifies a risk to welfare?

## 1.2. Stress and stress hormones

Physiology and behaviour can be sensitive measures of welfare but with limitations. Physiological responses are related to changes in the stress-axis. The results are elevated heart rate, increased plasma corticosterone and catecholamine levels, adrenal hypertrophy and atrophy, immunosuppression, changes in growth and reproductive hormones and neurochemical changes (Freeman and Manning, 1976; Seigel, 1980). The difficulty with such measures is what is normal?

An increase in stress hormones could be beneficial or harmful depending on the duration and level of responses. One major difficulty is that often measurements are made as point samples and this can lead to misleading interpretations of data.

Stress as a term describes the way an organism responds to environmental stimuli it perceives as a threat, real or anticipated, to its survival or well being (Harvey *et al*., 1984). A simplistic attempt to categorize the stress response, has divided it into a reflex, 'alarm or emergency reaction" component, principally mediated by adrenomedullary activation, followed by a period of adaptation, accompanied by increased adrenal function, then by a stage of exhaustion which results in death if adaptation fails (Selye, 1950).

Stressors are events internal or external that elicit a response by the hen aimed at maintaining homeostasis. The ability to respond depends on the severity of the stress and the inherent ability of the hen to respond. Responses can be specific or non-specific (generalized). While exposure to a stress evokes a range of physiological responses many highlight the significance of the adrenal gland in these responses.

The adrenal gland provides an example of the co-operation existing between the endocrine, nervous and immune systems in the control of a variety of functions (Ganong, 1963; Siegel, 1985; Hendricks *et al.*, 1991). While there is no division of the avian adrenal gland into distinct regions as in mammals, it is still appropriate to consider the hormones synthesized and secreted from the adrenal gland as two groups. The cortical hormones, those secreted by the cortical tissue, and the medullary hormones, those secreted by the chromaffin cells.

The hormones of the cortical tissue are mainly steroids and are divided into the glucocorticoids and the mineralocorticoids. For mammal’s corticosterone, cortisol, cortisone and 11-dehydrocorticosterone are the principal glucocorticoids while 11-deoxycorticosterone, 17-hydroxy-11-deoxycorticosterone and aldosterone are the principal mineralocorticoids. The situation is less clear with hens where the main steroids are corticosterone, cortisol, cortisone and aldosterone with the principle glucocorticoid being corticosterone. The regulation of corticosterone release involves a sequence of events starting with the release of hypothalamic factors, then ACTH (Adrenocorticotrophic hormone) from the pituitary and eventually corticosterone from the adrenal gland.

The chromaffin cells, homologous to neural tissue, contain adrenaline and noradrenaline. Stimulation of the sympathetic nervous system results in release of catecholamines from the chromaffin cells. The response is apparently unrelated to the severity of the stress imposed (Lahiri, *et al*., 1982). These hormones are released in high concentration in a matter of seconds following perception of a noxious stimulus.

As part of the general adaptive response to stress, corticosterone increases gluconeogenesis and blood glucose, causes catabolism of muscle tissue and increases fatness and decreases immunological function. The last of these effects result in increased susceptibility to diseases and this is especially important in intensive housing systems. As part of the short-term ‘flight or fight’ response of hens to acute stress, the catecholamines are released from the adrenal gland. The essential function of the catecholamines is to mobilise energy reserves to assist the hen to evade the stressor.

## 1.3. Stress measurement

Of increasing concern to the egg industry is the growing public perception that the laying hen exists in a state of chronic stress for the duration of its productive life. At present there are no practical means of assessing stress in hens other than behavioral observations, which do not provide accurate determinations of well being. The consequences of deprivation or over-stimulation are often quite subtle and difficult to observe and quantify (Ewbank, 1988). Animal behaviouralists have made efforts to improve methods of assessing behavioural needs of hens and to determine how particular production systems interact to provide or deprive hens of these needs (Hughes and Duncan, 1988). However good these determinations are, they have limitations. What is required is an entirely objective, quantifiable indicator which unambiguously reflects a hens well being.

Not much is known about the physiological levels of the “stress” hormones in the hen. There are difficulties with the interpretation of circulating concentrations of hormones because of diurnal patterns and the rapid changes that occur in response to handling and blood sampling. For some species, hormone levels are assessed in milk and saliva. It is apparent that the secretory products of animals contain a wide range of growth factors and hormones which vary in concentration according to the physiological status of the animal (Prosser *et al*., 1991). Using non-invasive means of measuring levels of stress hormones has reduced these problems. Saliva contains cortisol among other hormones and the concentration is closely related to the degree of stress (Fell *et al*., 1985).

Could the egg provide a non-invasive means of measuring stress levels in hens? Many of the stresses to which a hen is subjected result in activation of the HPA-axis. Final consequences being changes in plasma and tissue levels of glucocorticoids and catecholamines, secreted by the adrenal gland (Harbutz and Lightman, 1992). Non-invasive measures could be helpful in identifying conditions responsible for poor welfare. Solomon (1991) has stated that shell quality is a very good indicator of a hen’s harmony with its environment. Misshapen eggs or ones with calcium carbonate deposits are probably evidence of disturbances to the hen. Microscopic observations of shell changes could be useful indicators of hen health. Diffusion of plasma constituents into egg white has received little attention but as the degradative metabolites of vitamin D are found in albumen (Fraser and Emtage, 1976), it is likely that other plasma solutes are also sequested into the albumen. The gradual accumulation of albumen over 6 hours during egg formation potentially provides a very accurate and integrated reflection of circulating hormones over this period. Determination of stress hormone levels in egg albumen could provide a non-invasive measure of acute and chronic stress in hens.

Management conditions influence plasma corticosterone levels (Edens *et al*., 1982; Mashlay *et al*., 1984; Koelkebeck and Cain, 1984; Gibson *et al*., 1986). Higher plasma corticosteroid concentrations have been reported for hens housed in floor pens compared to cages (Edens *et al*., 1982; Craig *et al*., 1985; Barnett *et al*., 1997a & b). Koelkebeck & Craig, (1984) found a similar pattern of elevated plasma corticosterone in floor-housed hens compared to either cage- or range-housed hens. Of three different housing systems, cages, strawyards and free-range, hens in cages had the highest circulating plasma corticosterone concentrations as reported by Gibson and colleagues (1986). These discrepancies highlight the need for further evaluation of the effects of housing-type on physiological measures of stress.

Alterations in floor space or 'personal space' can elevate plasma corticosterone (Mashaly *et al*., 1984; Compton *et al*., 1981). Corticosterone levels are elevated when the space allowance is below 400 cm2/bird (Craig *et al*., 1986). Housing density appears to strongly influence plasma corticosterone in different housing systems (Craig *et al*., 1986). The effects of space allowance can be influenced by temperature (Edens *et al*., 1982). When space allocation is adequate there appears to be no difference with individual or group housing (Koelebeck and Cain, 1984). Short term stressors such as heat (Beuving, 1980), food and water deprivation (Beuving, 1980) transport (Broom and Knowles, 1989) and fear (Beuving *et al*., 1989) give rise to elevated corticosterone levels.

## 1.4. Project objectives

The overall objective of the project was to develop assay procedures for measuring stress hormone levels in egg albumen and to then assess whether albumen levels of these hormones are measures of stress in laying hens. The specific objectives of the project were:

1) Establish egg hormone assays

2) Establish a data base and prepare a literature review of stress in laying hens

3) Establish the relationship between circulating blood and egg albumen concentrations of stress hormones.

4) Evaluate stress hormone concentrations in egg albumen when hens are exposed to known stressors.

# 2. Development of Egg Assays

The principle stress hormones of the avian adrenal gland are corticosterone and the catecholamines (adrenaline, noradrenaline and dopamine). Assays for these hormones were developed and characterized for determining levels in egg albumen.

## 2.1. Corticosterone assay

The corticosterone assay can be segregated into two components, the technique for the determination of the corticosterone concentration in the albumen extract and the actual procedure for extraction of corticosterone from egg albumen.

*(a) Determination of corticosterone concentration***:**

The procedure developed was a radioimmunoassay (RIA). It is a competitive binding assay where endogenous corticosterone in the albumen extract and added radio-labelled corticosterone compete for binding to a specific antiserum added to the assay at limiting concentration. The bound corticosterone and free corticosterone are separated using charcoal absorption. Unknown sample corticosterone levels are estimated by comparison to known standard levels.

(i) *Reagents:* Phosphate-buffered saline (PBS)

- 4.33 g Sodium dihydrogen phosphate
- 3.04 g Di-sodium Hydrogen Phosphate
- 9 g Sodium Chloride
- 1 g Gelatin
- 1 g Sodium Azide

all dissolved in 1 litre distilled water and the pH adjusted to 7.0

(ii) *Antiserum*: The antiserum was raised in rabbits against Corticosterone –21 Thyroglobulin and purchased from ICN Biomedicals.

(iii) *Label*: Radiolabelled Corticosterone - 2,4,6,7-$^{3}$H corticosterone was purchased from Amhersman Australia.

(iv) *Dextran-coated charcoal*

– 4 g charcoal

– 1 g dextran – T70 dissolved in 1 litre PBS.

(v) *Counting Scintillant* - Biodegradable Highsafe-3 purchased from Canberra Packhard

*(b)Assay development*

(i) *Antibody titre*: As a first step, a suitable antibody titre was established. A dilution that bound 40% of a fixed level of 2,4,6,7-$^{3}$H corticosterone (10,000 CPM) was chosen.

(ii) *Unextracted standard curve*: In the next step an unextracted standard curve was established. Corticosterone (Sigma Chemical Company) was dissolved in assay PBS-buffer to give concentrations ranging from 0.1 to 51.7 ng/ml and these were used in the assay.

(iii) *Assay procedure***:** To 12x75 mm glass tubes, 0.1 ml standard is added, 0.1 ml antiserum dilution and 0.1 ml corticosterone label. The contents are incubated overnight at $4^{0}$C. The following day 0.25 ml dextran-coated charcoal is added, vortexed and incubated for 15 min before centrifuging at 3000 rpm for 15 min. The supernatant is poured off into a scintillation vial and 3ml of counting scintillant added. Radioactivity was measured on a Wallace Beta counter. An example of a standard curve is given in Figure 1.

*(c) Extraction procedure*

To determine corticosterone levels, it needs to be removed from other constituents in the albumen. As a first step, a suitable solvent for extraction had to be assessed. Corticosterone is a steroid and has variable solubility in a range of organic solvents. Going on studies using similar RIA methods to measure other steroids, the following solvents were tested, neat hexane, dichlormethane, diethyl ether and combinations of hexane and diethyl ether. To assess extraction efficiency known amounts of both radiolabelled and unlabelled corticosterone were added to an albumen sample prepared from a collection of eggs and recovery rates determined. Dichloromethane was unsuccessful, as was neat hexane. Increasing the % of diethyl ether added to hexane increased the extraction efficiency. Diethyl ether alone gave an efficiency of around 90-92 % and was accepted as the solvent to use in the assay.

The procedure for extraction of corticosterone from egg albumen is as follows. A sample of 1 g (0.5 g albumen: 0.5 g water) is placed in a 20 ml glass scintillation vial and 12 ml of diethyl ether is added. The vial and contents are shaken for 10 min and then centrifuged at 1800 rpm for 10 min. The vial contents are frozen and the solvent fraction transferred to another vial. The solvent is dried down under nitrogen until approximately 2 ml solvent remained and this is transferred to a 12x75 mm glass assay tube and then dried completely. After drying, 0.1 ml of PBS buffer is added and left overnight. The next day the samples are treated as described for the standard curve. The extraction procedure initially entailed a double extraction step using 2 volumes of 8 ml but it was found that equal efficiency could be achieved using a single extraction step with 12 ml of solvent.

## 2.2. Catecholamine assay

As with the corticosterone assay there were two aspects to the procedure, extraction of the catecholamines from egg albumen and measurement of catecholamine levels in the extract using high performance liquid chromatography (HPLC) with electrochemical detection.

*(a) Extraction from albumen*

*(i) Reagents*

*Acidified n-butanol*: to 500 ml of n-butanol, 50 ml 1N sodium hydroxide was added and shaken for 2 min. The aqueous phase was decanted and then the butanol similarly treated with 50 ml of 1N hydrochloric acid. The butanol was then washed with three separate 50 ml volumes of Milli-Q water. The butanol was then saturated with sodium chloride and allowed to stand for 4 days when any aqueous phase was removed. To 500ml of the treated n-butanol was added 0.42 ml of

concentrated hydrochloric acid, 0.5g potassium bisulphite and 0.05g EDTA. The butanol was filtered and stored refrigerated until needed.

*Phosphate buffers*: 0.5M phosphate buffer was prepared by dissolving 35.5 g di-sodium hydrogen phosphate in 490 ml Milli-Q water and adjusting the pH to 6.5 with phosphoric acid and then adjusting the final volume to 500 ml with Milli-Q water. The 0.1M phosphate buffer was prepared by dissolving 7.1 g di-sodium hydrogen phosphate in 490 ml Milli-Q water and adjusting the pH to 2.5 with phosphoric acid and then adjusting the final volume to 500 ml with Milli-Q water.

After the extraction of albumen samples with acidified n-butanol, adrenaline is taken up into 0.1M phosphate buffer and noradrenaline and dopamine into 0.05 M phosphate buffer (Moudgal *et al*., 1992).

*(ii) Procedure*

A large effort was placed into developing the extraction procedure. Only limited details of the extraction procedure will be given. The procedure has been developed over the course of the project and there have been changes since the last project report. These changes were instigated by the observations made during the animal studies looking at catecholamine levels during stress. For each egg two 4 g samples of albumen were added to 20 ml scintillation vials. To each was added 10 ml of ice-cold acidified n-butanol and then shaken for 10 min. Each vial and contents were centrifuged at 1500 RPM for 15 min. The n-butanol fractions were removed to a 15 ml culture tube and to one, 3 ml of 0.5M Phosphate buffer (pH 6.5) was added and to the other, 3 ml of 0.1M phosphate buffer (pH 2.5). These are shaken for 15 min and then the n-butanol fraction were removed and the buffer fraction stored until assayed for catecholamines. By adding known amounts of catecholamines to egg albumen, the extraction efficiency was estimated to be 68-73%.

*(b) Extraction from plasma or phosphate buffer fractions*

To a 1.5 ml Eppendorf tube, 0.5 ml of plasma or albumen buffer-fraction was added followed by 0.025 ml of sodium metabisulphite (0.5 mg/ml), 0.025 ml of DHBA (the internal standard used to estimate the extraction efficiency) and 0.5 ml of Tris-buffer pH 8.6. The contents are vortexed and then approximately 5 mg of acid treated aluminum powder was added and the contents vortexed. After settling, the supernatant was removed and the aluminum washed 3 times with 1 ml of water. After the third washing the contents are centrifuged at 10,000 rpm for 5 min and then the supernatant removed. To the aluminum precipitate, 0.14 ml of 0.1M perchloric acid was added, the contents vortexed and then centrifuged. A 0.125 ml sample of the supernatant is removed and placed in a HPLC vial ready for injection onto the HPLC column.

*(c) The HPLC assay*

*(i) Reagents*

HPLC Buffer: this is 0.15M Phosphate made by adding

- 20.7 g sodium dihydrogen Phosphate
- 500 mg EDTA
- 40 ml Methanol
- 1 bottle waters Pic-8

to 1 litre with Milli-Q water and then filtered.

*(ii) Stock standards*

0.5M stock standard solutions of adrenaline, noradrenaline, dopamine and 3,4-dihydroxybenzylamine (DHBA) were made up in 0.2M HCl. These are stable for 12 months.

*(iii) Working standards*

5.0uM working solutions of adrenaline, noradrenaline, dopamine and 3,4- dihydroxybenzylamine (DHBA) were made up from (ii) in 0.1M perchloric acid.

*(iv) HPLC assay standards*

These are made from the working standards on the day samples were processed and run on the HPLC.

*(a) Internal standard*: 25ul of 3,4 dihydroxybenzylamine (DHBA) is made to 5 ml 0.1M perchloric acid.

*(b) Assay standards:* 25ul of adrenaline noradrenaline and dopamine are made to 5 ml of 0.1M perchloric acid. These were serially diluted too give standards of 25, 12.5 6.25, 3.12 and 1.56 ul in 5 ml. A 100 ul sample of each standard is added to a HPLC injection vial and to this is added 25ul of internal standard. A volume of 100ul is loaded onto the HPLC column.

*(v) HPLC conditions*

HPLC column: Waters C-18 bondapak

Flow rate: 1.0 ml/min of buffer

Potential: 600 mV

Current: 0.2 nA

Baseline: + 0.05 nA

Injection volume: 100ul

*(d) Determination of unknown concentrations*

The unknown concentrations are determined following integration of the areas for the plots of adrenaline, noradrenaline and dopamine and comparing these to the area for the standard plots. An adjustment is made for the extraction efficiency by comparing the area for the internal standard measured in the samples and the area of the internal standard plot from the standards. This gives the noradrenalin/DHBA, adrenaline/DHBA and dopamine/DHBA ratios and these are used to evaluate catecholamine concentrations in the sample. Adjustments were also made for the dilution factors involved during the preparations of the samples. Using the internal standard as a measure, extraction efficiency was 75-83% on best occasions but more often around 70%.

# 3. Experimental Studies

## 3.1. Establish relationship between circulating and egg concentrations of hormones

*3. 1. 1. Relationship between plasma corticosterone and egg albumen levels*

Two experiments investigating the relationship between blood corticosterone levels and those in egg albumen were completed. To establish if such a relationship exits, hens were infused with corticosterone at a constant rate using Alzet mini osmotic pumps (Alza corporation, USA). These pumps had been used previously for similar studies and have an accuracy of ± 5% of the prescribed flow rate. For the present studies, Model 2ML2 pumps with a flow rate of 5ul/h and patency over 14 days, were used.

(*a) Experiment 1*

In the first experiment hens 49 weeks of age, 5 per treatment were infused with 40, 30, 20, 10 or 0 ug/h of corticosterone (Sigma Chemical Company) dissolved in PEG-400 (Merck). These dose rates were chosen according to data published elsewhere showing that doses of 30ug/h using similar pumps affected egg production but 10 ug/h had no effect (Etches *et al*., 1984). Hens were bled on four occasions, days 1, 5, 8 and 13 after pump insertion. Time of sampling was recorded and only samples collected within 2 minutes were accepted for analysis. Data previously published indicates that samples taken within 2 min are free of the stress-induced increases in plasma corticosterone brought about by the bleeding procedure. Over the 14 days of treatment, food intakes and egg production were recorded. Eggs were collected on days 7, 8, 9, 11 and 13 after pump insertion. Following collection, eggs were weighed, cracked open and the albumen collected and weighed. Albumen samples were stored for later analysis. After the 14 day infusion hens were sacrificed and the ovaries observed. Significance of treatment effects was assessed by analysis of variance and Tukey's-Kramer multiple comparisons test.

It was found that doses of corticosterone greater than 10ug/h had a dramatic effect on egg production. Hens treated with doses of 40ug/h ceased egg production within 4 days of treatment, those receiving 30ug/h within 5 days and those treated with 20ug/h within 6 days. At the end of the infusion period all hens receiving doses above 10ug/h had no large yellow ovarian follicles on the ovary and a regressed oviduct.

The plasma corticosterone concentrations are shown in Figure 2. One day after pump insertion plasma levels were elevated by all corticosterone infusions. Levels for hens treated with 40 ug/h were significantly higher than all other treatments except the 30 ug/h dose. Infusion rates of 20 and 30 ug/h increased plasma levels significantly above those of control hens. Levels following treatment with 10 ug/h were not significantly different to those for hens given 20 or 0 ug/h. By day 5 plasma levels had declined in all treated groups and remained at these levels for the remainder of the trial. Plasma concentrations for hens treated with 40 ug/h were higher than for all other treatments on all days. Differences for all other groups were not significant from day 5 to day 13.

When designing this trial it was decided to allow blood corticosterone levels to stabilize before collecting eggs in the second week of the infusion. These decisions were regrettable as good egg numbers were only obtained for the 0 and 10 ug/h treatment groups during the second week. Only one hen in each of the 20 and 30 ug/h groups laid eggs during the second week. Egg albumen corticosterone concentrations are shown in Figure 3. There were no differences in levels for hens treated with 0 or 10 ug/h. This observation would be anticipated considering that there were no

differences in plasma corticosterone levels. A second experiment was planned because of the obvious problems with the dose rates used in this study.

*(b) Experiment 2*

Following the observations from experiment 1, a similar study was conducted using lower corticosterone doses and collecting eggs much earlier after the start of the treatments. Four hens per treatment had Alzet pumps inserted and were infused with corticosterone doses of 15, 10, 5, 2.5 or 0 ug/h for 14 days. The day of pump insertion was considered as day 0 of the study. Eggs were collected from day 2 to 13 and individual production was recorded. Hens were bled by venipuncture on 5 occasions (days 2, 6, 8, 10 and 13) with a 2 minute limit placed on the sampling time. Following collection, eggs were weighed, cracked and the albumen removed, weighed and stored until assayed. The samples of albumen and plasma were assayed for corticosterone. Treatment differences were assessed by a one-way analysis of variance and multiple comparisons between treatments were made using Tukey's–Kramer test.

Hens receiving 10, 5 and 2.5 ug/h had similar egg production to the control hens. Hens receiving 15ug/h had ceased egg production 5 days after the treatment starting and so, there is limited data available for this group. Plasma corticosterone concentrations are shown in Figure 4. While it would appear that the plasma levels for the 15 and 10 ug/h treatments are higher on days 2 and 6, these differences were not significant. This is probably due to the small sample number and the large variation at these sampling times. This is supported by the observations that levels for the 0, 2.5 and 5 ug/h treatments are similar and didn't change over the study period but were significantly lower than values for the 15 ug/h group on days 8, 10 and 13. Plasma levels for the 15 ug/h group remained constant over the period of the study.

The corticosterone concentration in egg albumen is given in Figure 5. Because of the small number of eggs, those collected for each treatment on days 2 and 3, 4-6, 7 and 8, 9 and 10 were analyzed together. On days 2 and 3 the levels in albumen for the controls were higher than for the 15 and 10 ug/h groups. There were no other significant differences for the rest of the study.

*(c) General considerations*

The infusion of corticosterone at rates above 10 ug/h had a major influence on egg production. Treatments of 15ug/h or greater ceased egg production within 4-6 days. The severity of the effect increased as the dose rate increased. From experiment 1 it seems clear that initially, the infusion rate of corticosterone influenced plasma levels but that levels declined to control values in all but those hens receiving the highest infusion rate (40 ug/h). Even for these hens there was a decrease in concentration after day 1. In experiment 2, doses below 15 ug/h had no significant effect on plasma levels.

From these studies it is difficult to establish that a relationship exists between plasma and egg albumen corticosterone concentration. As the studies were explorative there were flaws in the design. A major problem was that treatments giving high plasma levels of corticosterone resulted in poor egg production, while for treatments with ample eggs the plasma levels were not significantly different to controls. Egg collection should have started immediately in experiment 1 and this would have allowed collection of eggs for all treatments over the first two days when plasma levels were high. This may have allowed the relationship between plasma and egg levels to be determined.

It may be difficult to establish such a relationship for other reasons. If the pumps remain patent over the 14 days, which they are designed to do, the data indicate that mechanisms operate to reduce elevated plasma levels to physiological levels in the hen. This is probably brought about by negative feedback regulation acting to suppress endogenous corticosterone release. Corticosterone acts on the hypothalamic-pituitary-axis to inhibit endogenous corticosterone release and over rides

efforts to elevate plasma levels. With the information gathered from these studies it would be possible to design a study that would more fully achieve our initial aim.

*3. 1. 2. Relationship between catecholamine concentrations in plasma and egg albumen*

A difficulty in determining the relationship between plasma catecholamine levels and egg albumen levels exists because sampling procedures quickly raise blood catecholamine levels. When sampling for corticosterone there is a time lag between the stress-induced release of corticosterone and the actual rise in plasma corticosterone. This is not the case with the catecholamines were levels increase almost immediately. A simple approach to this problem was to inject hens subcutaneously with different doses of adrenaline and then establish what levels are in the albumen. This is the equivalent of a dose-response study**.**

*(a ) Experiment 1*

Five hens per treatment were given 0, 2.5, 5, 10 or 20 mg of adrenaline per day as a single injection. Adrenaline (Sigma Chemical Company) was dissolved in polyethyleneglycol-400 (Merck) to give the required dose in 1ml. Hens were injected at 0700-0800h daily for 4 days. Eggs were collected on days 2-5, weighed, cracked open and the albumen separated. From each egg, two sub-samples (approx. 1g) were taken and 1 g of Milli-Q water was added.

As with many explorative studies major problems were experienced. Adrenaline injection had disastrous effects on egg production. Hens treated with 2.5 and 5 mg/d of adrenaline had similar egg production to control hens. For hens receiving 10 or 20 mg/d only one hen laid an egg on the second or third day. No catecholamines were detected in the albumen samples. Later work indicated that 1 g sub-samples are too small to extract sufficient catecholamine to reach the detection limit of the assay. It was subsequently shown 4 g sub-samples are required to extract sufficient catecholamine to measure using the HPLC technique.

*(b) Experiment 2*

Five hens per treatment were given 0, 2.5, 5, 10 or 20 mg of adrenaline per day as a single injection. Adrenaline (Sigma Chemical Company) was dissolved in polyethyleneglycol-400 (Merck) to give the required dose in 1ml. Hens were injected at 0700-0800h daily for 2 days. Eggs were collected on days 2 and 3, weighed, cracked open and the albumen separated. From each egg, two sub-samples (approx. 4g) were taken and processed for catecholamine analysis.

As with the first study, adrenaline injection had dramatic effects on egg production. Only 3-4 eggs were collected from the hens treated with 10 or 20 mg adrenaline. Ten eggs were collected from the controls, 6 from hens treated with 5 mg and 5 eggs from those treated with 2.5 mg. Differences between treatments were assessed by analysis of variance and Tukey’s-Kramer multiple comparison test.

The albumen adrenaline and noradrenaline concentrations and total levels are shown in Figure 6. There were no significant differences in adrenaline levels and the only significant difference in noradrenaline was between the 2.5 and 5 mg treatment groups.

*(c) General considerations*

There were many problems associated with the two experiments described in this section. There were extreme effects of adrenaline on egg production with the high doses inhibiting production almost immediately. In experiment 2 the small number of eggs and the large variation between samples from the same treatment groups made it difficult to develop any strong conclusions. Dopamine was not detected in any of the samples processed.

## 3.2. Investigate effects of stressful situations on egg concentrations of hormones

The specific aims of these studies were to determine the concentrations of corticosterone and catecholamines in egg albumen collected from hens subjected to known stressors. The stressors used in these studies were heat, handling and cage density.

### *3.2.1. Effects of short duration exposure to high temperature.*

In this study, hens were subjected to heat stress over a short period. Two groups of 15 hens were moved to temperature controlled rooms and housed in individual cages. Hens were fed a commercial ration *ad libitium* and had free access to water at all times. The temperature in both rooms was set at $18^0$ C. After 7 days the temperature in one room was maintained at $18^0$ C and for the other, increased to $32^0$ C over 24 h. Hens were maintained at these temperatures for a further two weeks (weeks 1 and 2). Ten eggs from each group were collected at random on two occasions during weeks 1 and 2. The eggs were weighed and the albumen collected and weighed. A subsample of albumen was collected and stored for corticosterone analysis. Two samples of approximately 4g were also taken from 8 eggs for each treatment and processed for catecholamine analysis. Blood samples from all hens were collected by venipuncture at the end of each treatment week. Any differences between treatments were assessed by unpaired student's t-test.

The plasma corticosterone levels are shown in Figure 7. No significant differences in levels were observed. The corticosterone concentration and total albumen corticosterone levels are given in Figure 8. On days 4 and 9, corticosterone concentration and total levels were significantly elevated in the hens exposed to the higher temperature. Any differences on the other days were not significant. The adrenaline concentration and total adrenaline in albumen are given in Figure 9. On day 3 the levels were at the detection limit of the assay. On day 4 the levels in the hens at $18^0$ C were significantly higher than those for hens at $32^0$ C. There were no other significant differences.

### *3.2.2. Effects of long term exposure to high temperature for hens housed two/cage*

In this study hens were subjected to heat stress over a long period of egg production. This was part of a larger experiment investigating effects of diet composition on egg production under heat stress. The hens used in this study were those on the control diet in the larger experiment. Two groups of 20 hens, housed 2 per cage (30x40x45 cm) were used in the study. The groups were housed separately in temperature controlled rooms. The temperature of one room was set at $18^0$ C and the other at $30^0$ C. Hens were moved in to the rooms at 20 weeks of age and the treatment temperatures applied from 24 weeks of age. The hens were fed a diet based on commercial recommendations and prepared on the University site, Camden. Hens had free access to fresh water at all times. On two occasions each week during weeks 28, 29, 30, 31 and 32 after initial exposure to the treatment temperature, 10 eggs were collected from each group. The eggs were weighed, albumen collected and weighed and then sub-samples taken for corticosterone analysis. On one occasion for weeks 29-32 two sub-samples of approximately 4g of albumen were taken from 8 eggs for each treatment and processed for catecholamine analysis. At the end of week 32 a blood sample (2 ml) was collected from one hen in each cage. Differences between treatments on individual sampling days were assessed using unpaired student's t-test.

The mean corticosterone concentration and total corticosterone in egg albumen are shown in Figure 10. Mean corticosterone concentration in egg albumen samples collected from hens at $30^0$ C were never lower than those kept at $18^0$ C. Concentrations were significantly higher during all weeks of sampling although during some weeks this was the case on only one of the sampling days. Total albumen levels were significantly different during weeks 29, 30 and on one day during 32. In figure 11, the relationship between egg albumen corticosterone concentration and total corticosterone in the egg albumen is given. The relationship is highly significant and indicates that changes in albumen

concentration are real increases and not due to changes in albumen amount. Therefore it is likely that changes in concentration are due to increased corticosterone accumulation in the albumen.

There were no significant differences in adrenaline concentrations or total adrenaline levels when comparing the two heat treatments (Figure12). There were large variations in individual samples and this is highlighted by the large standard errors.

Plasma corticosterone concentration is given in Figure 13. The mean level for hens at $30^0$ C was significantly higher compared to those for hens at $18^0$ C. The higher egg levels in the heat treated hens is probably related to the higher plasma corticosterone levels found in these hens.

*3.2.3. Effects of handling*

In this study the effects of handling hens on egg albumen corticosterone and adrenaline concentrations were investigated. Two groups of 15 hens were housed individually in cages (30x40x45 cm) in a commercial type shed. Hens were fed a commercial diet and had free access to fresh water at all times. On treatment days hens from one group were removed from their pens and taken to a space out of sight of other hens. The hens were handled for one minute. During this time they were inverted 5 times, then moved side to side while being held aloft and eventually placed on a table until the one minute time period elapsed and then returned to their cage. This was repeated hourly for 6 hours starting at 0700 h on 3 consecutive days over two weeks with a one week rest period between the two treatment periods.

Eggs were collected from 10 hens from each treatment for three days, starting the day after the first handling and ending the day after the third day of handling. These collections were repeated during the second week of handling. Eggs were weighed, albumen collected and weighed and a sample removed for corticosterone analysis. On the first two collection days of each week two 4 g sub-samples were removed and processed for catecholamine analysis. A 2 ml blood sample was taken from all hens by venipuncture. Blood was collected 45 min after the final handling on the third day of the two treatment weeks. The blood was centrifuged, plasma harvested and stored until assayed for corticosterone. Any differences between treatments on individual collection days were assessed by unpaired student's t-test.

To determine what effect handling had on the profile of blood corticosterone levels a separate group of 36 hens were used. Thirty hens were handled in a similar manner to those used in the main experiment while 6 were left and not handled. Blood samples were collected from 6 different hens at 10, 20, 30, 40 and 60 min after being handled and from the 6 hens not handled. Blood was centrifuged, plasma harvested and stored until assayed for corticosterone.

Plasma corticosterone concentrations 45 min after the last handling episode on day 3 of each treatment week are given in Figure 14a. At 45 minutes after handling the corticosterone levels are elevated but not significantly above those for hens not-handled. The pattern of plasma corticosterone after handling is shown in Figure 14b. The pattern of corticosterone release shows that levels peaked at 10-20 min and approached those seen before handling at 60 min. From this pattern it is likely that levels at 45 min would not be significantly greater than pre-handling levels. This could be the reason that levels at 45 min in the main handling study were no significantly different for the treatments.

The corticosterone concentration and total in egg albumen are given in Figure 15. For handled hens the concentration in albumen was significantly higher on all days of week 1 and for the first day of week 2. The increased concentration was reflected in significantly higher total levels on the same days. There were not significant differences on the last two days of week 2.

The adrenaline concentration and total adrenaline in egg albumen are shown in figure 16. There were no significant effects of handling on concentration or total adrenaline.

*3.2.4. Cage density. The short-term effects of housing hens individually (single), in pairs (twin) or groups of five (multiple)*

In this experiment, hens, 56 weeks of age, in full egg production and housed in single pens were moved to new single cages, to cages housing two hens or to cages housing 5 hens. In this study 15 hens were housed in single cages (25x55x45 cm), 20 hens at 2/cage (25x55x45 cm) and 40 hens at 5/cage (50x55x60 cm). The hens had free access to a commercial diet and fresh water.

Eggs were collected randomly from 10 hens for each treatment 2, 4, 8, 10, 16 and 21 days after being moved to the cages. Eggs were weighed, albumen removed and weighed and a sample removed for corticosterone analysis. On the first two collection days of each week, two 4g sub-samples of albumen were removed from 8 eggs for each treatment and processed for catecholamine analysis. Blood samples (2 ml) from 10 hens of each treatment group were taken by venipuncture on day 5, 11 and 22 after being moved to the cages. The samples were taken within 2 min, blood centrifuged, plasma harvested and stored until assayed. The blood samples were taken on days where the stressed-induced rise in corticosterone due to sampling would not influence albumen levels on egg collection days. Differences between treatments were assessed by analysis of variance and individual differences by Tukey's-Kramer multiple comparison tests.

The plasma corticosterone levels are given in Figure 17. There were no significant differences in the levels. The egg albumen concentration and total egg corticosterone levels are given in Figure 18. On day 2 the concentration was significantly lower for hens housed individually but the difference was not apparent in total levels on the same day. On day 4 the concentration in all treatments were significantly different with highest levels in the twin group and lowest in the single group. These differences were also seen in the total corticosterone levels. On day 8, the concentration was lower in the single-housed group compared to the twin housed group and again there were similar differences in total levels. No other differences in corticosterone were observed.

The adrenaline concentration and total adrenaline levels are shown in Figure 19. There were no significant differences in adrenaline levels.

*3.2 5. Cage density. Effects of long-term housing of hens individually (single) or in groups of five (multiple)*

This experiment was part of a larger experiment were hens were housed in single cages (25x55x45 cm) or groups of five (50x55x60 cm). In the larger experiment the effects of diet and cage density on egg production were investigated. In the present study hens on the control diet were used. All other treatments were the same for each group. Twenty hens housed in single pens or forty housed in 8 cages of 5 were allocated to the experiment for egg collection. The hens were moved to the cages at the point of lay and remained for 50 weeks. As the hens were a part of a large study no invasive intervention was allowed and therefore no blood sampling was permitted.

Eggs were collected randomly from 10 hens for each treatment twice weekly during weeks 24, 27, 29, 32 and 34 weeks after being transferred to the cages. Eggs were weighed, albumen removed and weighed and a sample removed for corticosterone analysis. Two 4 g sub-samples were removed and processed for catecholamine analysis on one sampling day during weeks 24, 27, 32 and 34.

The corticosterone concentration and total corticosterone in egg albumen are shown in Figure 20. Concentrations were higher for the single caged hens on one sampling day (1.01 ± 0.05 Vs 0.84 ± 0.05 ng/ml) and on this same day total corticosterone was also significantly different. No other significant differences in concentration were noted. On four sampling days the total albumen corticosterone levels were significantly higher for hens housed in multiple cages. On these days there were no significant differences in total albumen weight but it tended to be higher in the multiple-caged group. This and the slightly higher concentration for this group on days where total corticosterone levels were significantly greater could explain the differences.

The adrenaline concentration and total adrenaline in egg albumen are given in Figure 21. Both the concentration and total adrenaline tended to be higher for hens housed as groups and differences approached significance at some sampling times. Individual variation was large in the multiple-caged group and this made interpretation of the data difficult.

# 4. Discussion and Implications

Responses to stress are a normal part of the survival strategies to changing environmental conditions. In the absence of such changes an animal is not capable of modifying its physiology to the needs of such changes. Normally the stress abates or the animal removes itself from the influence of the stress. Animal husbandry practices used in modern egg production are thought by some to impose stress on hens from which they have no escape.

Stress manifests itself in behavioural and physiological changes. The problem has always been what level of change is functionally beneficial and what level constitutes a chronic state to the animal. The difficulty has always been to objectively assess the effect of husbandry practices on hens. Stress imparts physiological responses from the HPA axis with consequent changes in stress hormone levels; corticosterone and catecholamines. Major difficulties are experienced in measuring these changes because sampling techniques are stressful.

The general purpose of this project has been to develop and assess the practicality of using a non-invasive means of measuring stress in hens. To this end, procedures to measure corticosterone and catecholamine levels in egg albumen have been developed. Egg albumen is laid down over approximately 6 h and so accumulation of substances in albumen would be a good indication of the physiological state over the period of albumen accumulation.

Development of the corticosterone assay was relatively easy. The assay is robust and easily adapted to processing large numbers of samples at low cost. A simple, one step extraction procedure and then an overnight RIA incubation result in good recoveries and replication.

Development of the catecholamine assay was tedious and many problems had to be overcome. The final procedure was developed over the course of the project. It was not until towards the end of the project that the technique was refined to a point where reliability could be expected with confidence. The difficulties experienced in development limited the time available to process samples. It was decided to process only adrenaline samples through the HPLC assay. In retrospect results indicate that assaying larger numbers of samples would have been of little extra value in assessing albumen adrenaline as a measure of stress in hens. A limited number of samples were processed for noradrenaline. No differences in treatments were found and so it was decided to put all efforts into processing the adrenaline samples.

The associations between either plasma corticosterone or adrenaline and levels in egg albumen were difficult to establish using data generated from the experimental work. Both corticosterone and adrenaline at high rates of administration have dramatic effects on ovarian function. Infusion rates not affecting egg production gave plasma levels not significantly different from untreated hens. This was true for some dose levels that inhibited egg production. The data on effects of adrenaline are limited and make it difficult to establish what relationship exists between plasma and albumen levels.

Why didn't high infusion rates transcend into high plasma levels? Blood corticosterone, like many hormones, is under regulation of negative feedback mechanisms where high plasma corticosterone acts at points in the HPA axis to inhibit endogenous release. The high infusion rates of corticosterone probably, initially, result in high plasma levels as indicated in Figure 2. High levels probably inhibit endogenous release so that plasma levels return to the normal physiological range. If abnormally high levels were maintained, it would be detrimental to the hen. Whatever the reason for the plasma levels failing to remain elevated, the experimental design failed to provide a conclusive determination of the relationship between plasma and albumen concentrations. For both the adrenaline and corticosterone studies it would have been better to have given the doses as a single injection to a large number of individual hens and collect eggs the following day knowing that in many cases production ceases after 1-2 days of treatment.

From Figure 11**,** it is clear that there is a significant relationship between albumen corticosterone concentration and total egg albumen corticosterone. This is important because it indicates that a measure of concentration by itself is a measure of differences between animals and not a consequence of changes in egg size or more particularly in egg albumen weight.

## 4.1. Effects of handling

Handling stresses hens and the magnitude of the plasma corticosterone response depends on the duration and type of handling (Kannan and Mench, 1996; Broom and Knowles, 1989). Upright and gentle handling is not as stressful as inverting a hen and being held by the legs (Kannan and Mench, 1996). In the present study, the combination of upright handling and inverting hens on their back caused an elevation in plasma corticosterone (Figure 14b). Maximum plasma corticosterone occurred by 10 min after handling and the increase was about 3 ng/ml above values for non-handled hens. Values at 45 min were approaching basal values and this would account for the lack of any difference in plasma levels seen in the main egg collection experiment (Figure 14a) where hens were bled 45 min after a handling episode. The fact that levels at 45 min were not different between handled and non-handled hens suggests that there was no carry over effect from each individual handling episode. This indicates that the handling procedure was moderately stressful because much more vigorous handling such as holding hens by their legs in an inverted position for 2 min results in plasma corticosterone significantly elevated (8 ng/ml) 3 h after the event (Kannan and Mench, 1996). Broom and Knowels (1989) found that for hens handled gently and upright, plasma corticosterone levels returned to pre-handled levels by 30 min.

In the first week of handling, egg albumen corticosterone levels were significantly elevated above levels for non-handled hens. This was also the case for the first day of the second week but not for later days. During the second week handled hens could have habituated to the procedure and found it less stressful. The levels in the non-handled hens were higher on the last two days during week 2 than in week 1. The hens were housed in commercial type housing and not under a controlled environment. Its possible that some other environmental stressor influenced plasma corticosterone levels.

Handling is a potent stimulator of catecholamine release (Beuving and Blokhius, 1997; Korte *et al*., 1997). While levels are elevated following handling they remain there for only a short period of 4-5 min and then return to pre-handling levels. While plasma catecholamines were not measured, blood levels would be elevated for only a short period, similar to the published patterns, and this is probably why no difference in egg albumen values was observed (Figure 16).

## 4.2. Effects of heat

High ambient temperature is a major stress of poultry in many parts of the world (El-Halawani *et al*., 1973; Geraert *et al*., 1996). In the present study the effects of heat on albumen levels of corticosterone and adrenaline were evaluated during both a period of short-term exposure (2 weeks) and over a much longer period. Exposure of hens to $18^0$ C or $32^0$ C had no effect on plasma corticosterone when samples were taken at the end of week 1 and 2 of exposure. These samples were taken mid-morning for the first week and mid-afternoon for week 2 and this may have contributed to the difference between the two weeks. Hens display a daily rhythm in corticosterone release (Beuving and Vonder, 1977), where levels are highest in the morning and decline during the afternoon until reaching a nadir at night. Any differences due to heat might be observed only at times when corticosterone levels are low during this daily rhythm. In turkeys subjected to $32^0$ C the plasma corticosterone levels were elevated but differences were only significant when compared to hens at $24^0$ C during the time when values were normally low during the daily rhythm. (El-Halawani, *et al*., 1973).

Heat stress in broilers increased plasma corticosterone by approximately 1 ng/ml (Deyhim and Teeter, 1991). If similar subtle changes occur in hens, processing large numbers of samples would be required to remove individual effects and detect significant differences. Small changes in plasma corticosterone might more readily be detected in egg albumen because it accumulates over 6 hours. Albumen corticosterone values were elevated in hens exposed to $32^0$ C on days 4 and 9 (Figure 8) after exposure and tended to be higher on day 3 but not day 10.

Little is known about the effects of heat on catecholamine levels in hens and in the present study short-term heat exposure had no effect on adrenaline levels in egg albumen. There were large variations in adrenaline levels but the levels were low and often at the detection limit of the assay.

Long-term exposure to heat results in differences in egg albumen corticosterone concentration (Figure 10). During weeks 28-32 after exposure to $30^0$ C, egg albumen corticosterone concentrations were higher at all times compared to hens exposed to $18^0$ C. On 7 of the 10 collection days values were significantly higher. Similar differences were not observed for adrenaline in albumen (Figure 12). Only one blood sampling was made, at the end of week 32, and at this sampling corticosterone levels were higher for hens at $30^0$ C, the difference being about 1.5 ng/ml. Its reasonable to suggest that plasma differences of 1-2 ng/ml in corticosterone can be detected as significant differences in egg albumen corticosterone.

Hens in the long-term study were housed 2 birds/pen. The increase in plasma corticosterone could be related to an interaction between heat and cage density. Cage density could have elevated plasma corticosterone in hens at $18^0$ C and the levels for hens at $32^0$ C could be magnified by effects of heat and any synergism. These possible intricacies especially in a commercial environment, of interactions between stressors, could be evaluated using this non-invasive model.

## 4.3. Effects of cage density

Many have proposed that housing hens in cages is stressful and in this study, the effect of cage density and the associated social stress on albumen levels of adrenaline and corticosterone was investigated. In one study it was reported that plasma corticosterone levels increase in hens in floor pens when the density increased but not for hens housed in cages (Koelkebeck, and Cain, 1984). In contrast, plasma corticosterone increased in hens housed in cages as number increased from 3 to 5 hens/cage (Mashaly, *et al*., 1984).

In this study, short-term and long-term effects were investigated. If there are effects of cage density on plasma corticosterone it was not evident 5 days after moving hens to cages (Figure 17). While it would have been useful to have plasma samples at earlier times following transfer to the cages this was not possible because samples can not be taken at times close to when egg collection takes place. This avoids any effect of sampling-induced rises in corticosterone influencing albumen levels.

The egg albumen corticosterone concentrations suggest initial plasma levels were probably higher in hens following their move to cages. Levels on days 2 and 4 of collection were higher in all groups compared to later sampling times. At early collection times, housing hens in groups of 2 or 5 significantly increased albumen corticosterone compared to hens housed individually. For the first few days, housing hens in pairs seemed to be more stressful than keeping them in groups of five. As with all previous studies in this project there was no effects on albumen adrenaline levels.

When hens are housed individually or in a group of 5 over a long period there was little effect on albumen corticosterone concentrations although levels tended to be higher for hens housed in groups. In this study there were differences in total corticosterone amounts in albumen. The tendency for concentration to be higher in group-housed hens coupled with a tendency for total albumen weights to be greater in this group probably accounts for the differences in total corticosterone. There were no

long-term effects on the corticosterone and adrenaline concentrations in albumen. These data suggest that following an initial period of adjustment, housing hens in groups may be no more stressful than housing hens individually.

# 5. Summary and Recommendations

The first significant contribution of the studies detailed in this project was the development of assay procedures for measuring corticosterone and catecholamines in egg albumen. The catecholamine assay is time consuming, expensive and involves two extraction steps. Extraction efficiency and assay sensitivity determine that large sample amounts need to be extracted. Albumen adrenaline appears not to be an indicator of stress in hens. None of the situations used in our studies and believed to impose stress on hens increased albumen adrenaline levels. While we analyzed limited numbers of samples, the data suggest that noradrenaline would not be a better measure.

Dantzer and Mormede (1983), citing work in mammals, argue that environmental stresses exert their effects on plasma corticosterone not by physical effects but rather through psychological effects. If this is the case, plasma corticosterone should be a good measure of well-being. We were not able to establish a relationship between plasma corticosterone and egg albumen levels. This failure was probably due to our experimental design rather than the non-existence of such a relationship.

From these studies, it appears that egg albumen corticosterone and total corticosterone concentrations reflect exposure of hens to stress. Plasma corticosterone levels are probably controlled within limits by feedback mechanisms. When hens are exposed to various stressors, corticosterone levels may not change greatly and therefore plasma changes might be difficult to detect especially when relying on single point blood samples. The problem is further complicated by the intrinsic diurnal pattern evident in hens. Subtle changes in plasma corticosterone may more readily be detected in albumen as it is accumulated over 6 h during egg formation. Small plasma changes can be magnified because of the accumulation time. This point is highlighted in the handling study. A single point blood sample at 45 min after handling indicated that there was little difference in plasma levels however, the profile over the hour following handling indicated that plasma levels are elevated for at least 30 min. The increase over this short period for 6 handling episodes resulted in significant elevation in albumen corticosterone concentration. This point is also reinforced by the short-term heat study. While it would require more rigorous examination, it is likely that albumen corticosterone could be a good assessment of stress in hens.

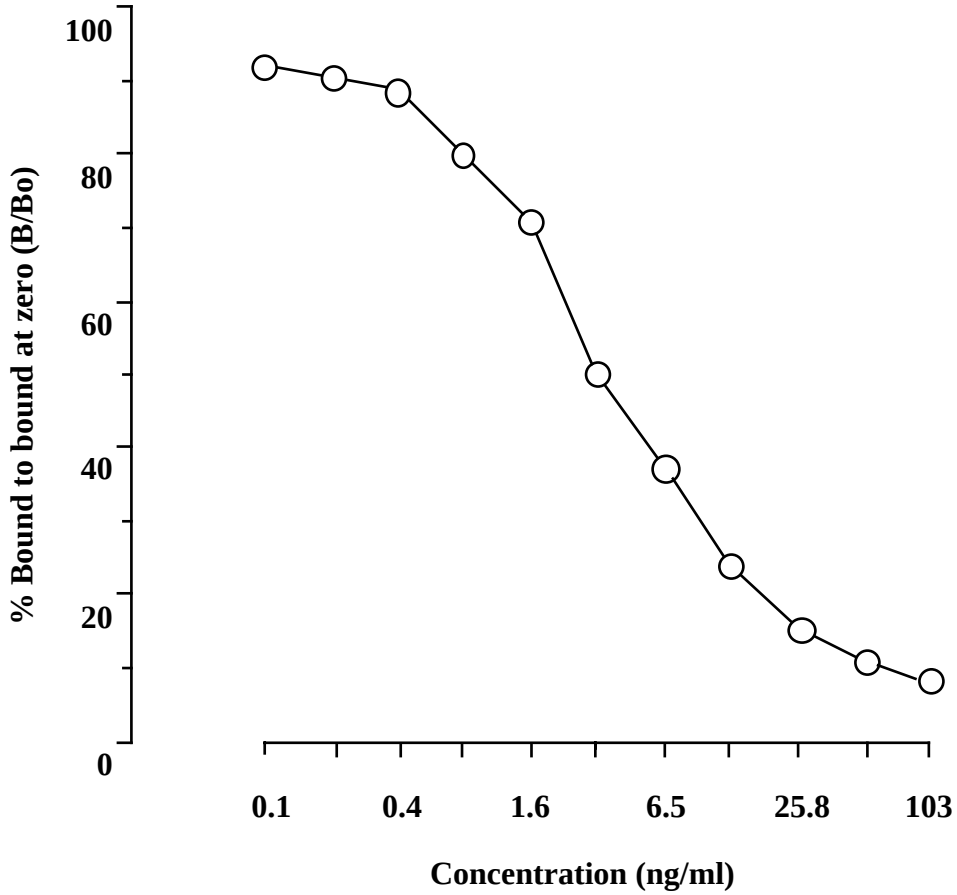
In summary the major findings arising from the project are:

1) Catecholamines are difficult to measure. No evidence was found to indicate that egg albumen levels of these hormones are increased by stressors, which influence corticosterone levels. It is concluded that adrenaline levels in albumen fail to provide a non-invasive measure of stress in hens.

2) Corticosterone levels in egg albumen increase when hens are exposed to stressors. Moreover, corticosterone is easily measured in albumen and sample processing is relatively inexpensive, both important aspects of a rapid assay method.

3) The results of the project indicate that a more extensive evaluation of albumen corticosterone concentrations as a measure of stress in hens is warranted and should provide a very useful tool in assessing the effects of husbandry and housing on hen welfare.

# 6. Figures




| | | |
|---|---|---|
| **Assay details:** | **reading range:** | **0.3-12.5 ng/ml**<br>**0.03-1.25 ng/tube** |
| | **sensitivity:** | **0.03 ng/tube** |
| | **zero binding** | **39.3% of total counts** |
| | **Counts added** | **10,000 cpm** |

**Figure 1:** The corticosterone standard curve.

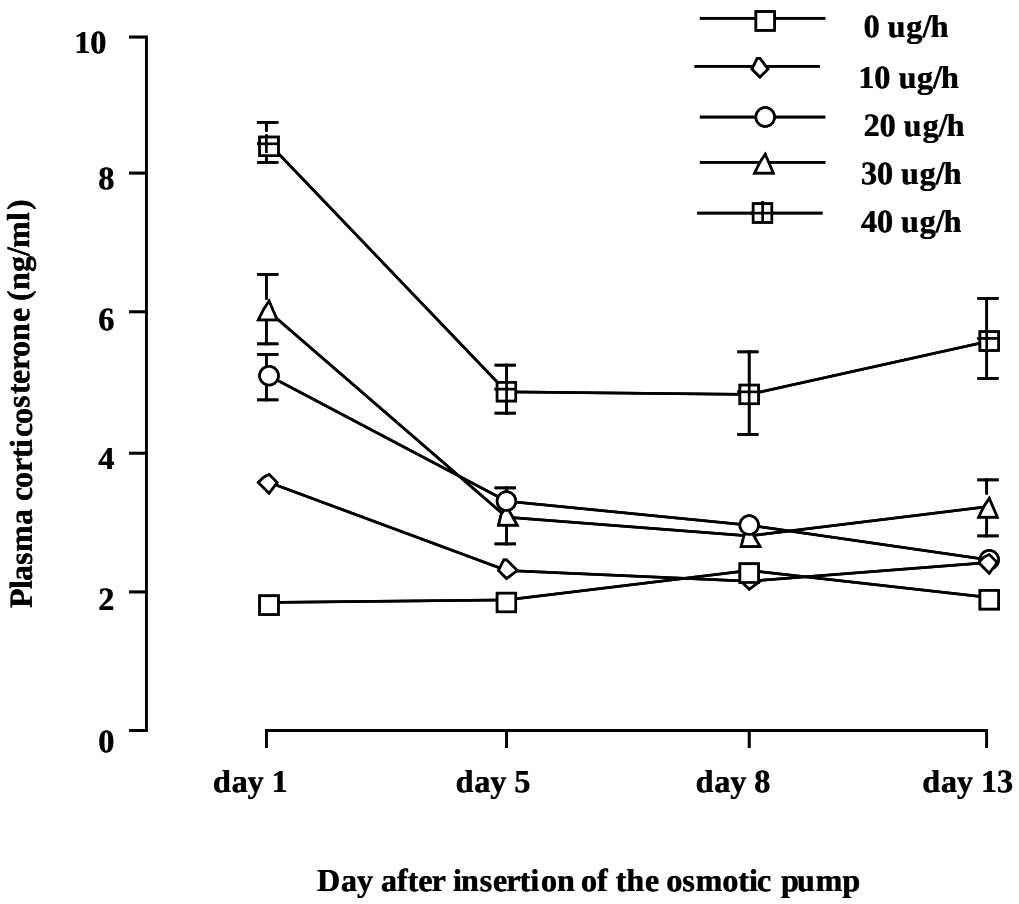



**Figure 2:** The mean (± SEM) plasma corticosterone concentration in hens infused with corticosterone for 14 days using subcutaneous osmotic pumps. Pumps were inserted on day 0 and blood samples were collected by venipuncture on days 1, 5, 8 and 13.

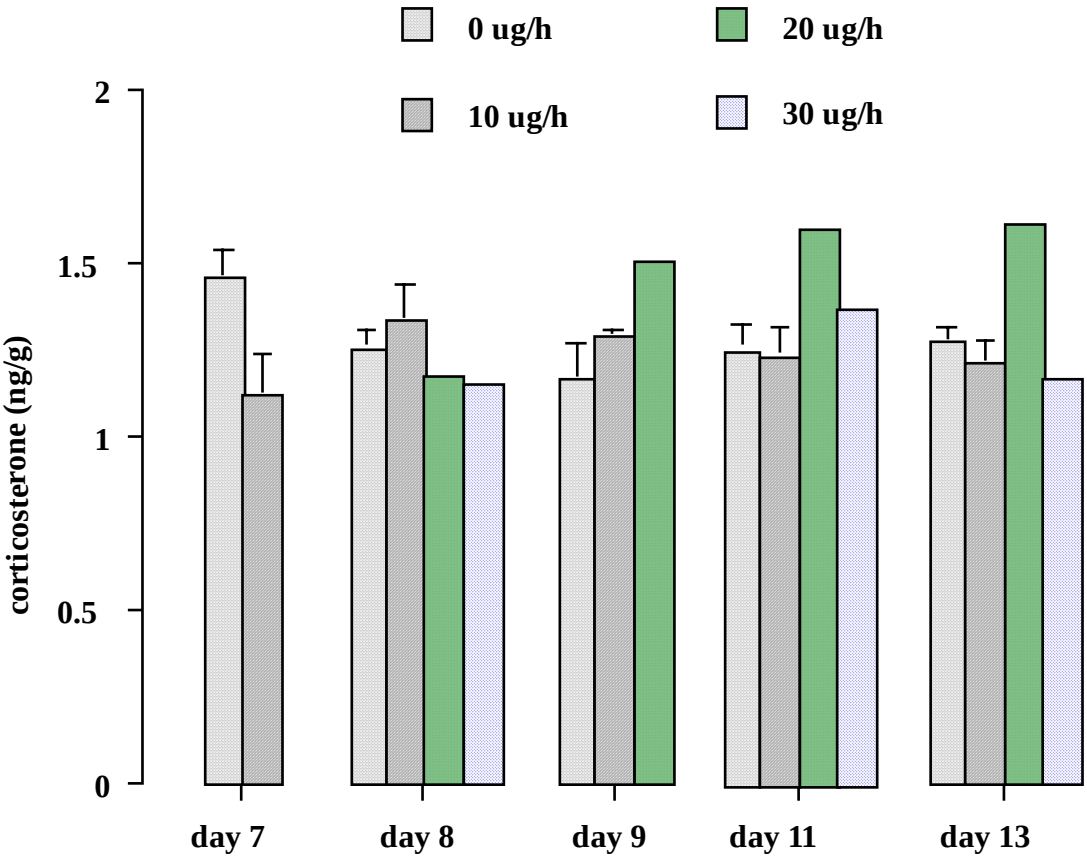



**Figure 3:** The mean (± SEM) egg albumen corticosterone concentration for hens infused with corticosterone at 0, 10, 20, 30 or 40 ug/h using subcutaneous mini osmotic pumps. Pumps were implanted on day 0 and eggs collected on days 7, 8, 9, 11 and 13. Only the groups infused with 0 or 10 ug/h had sufficient egg numbers to calculate a mean level.

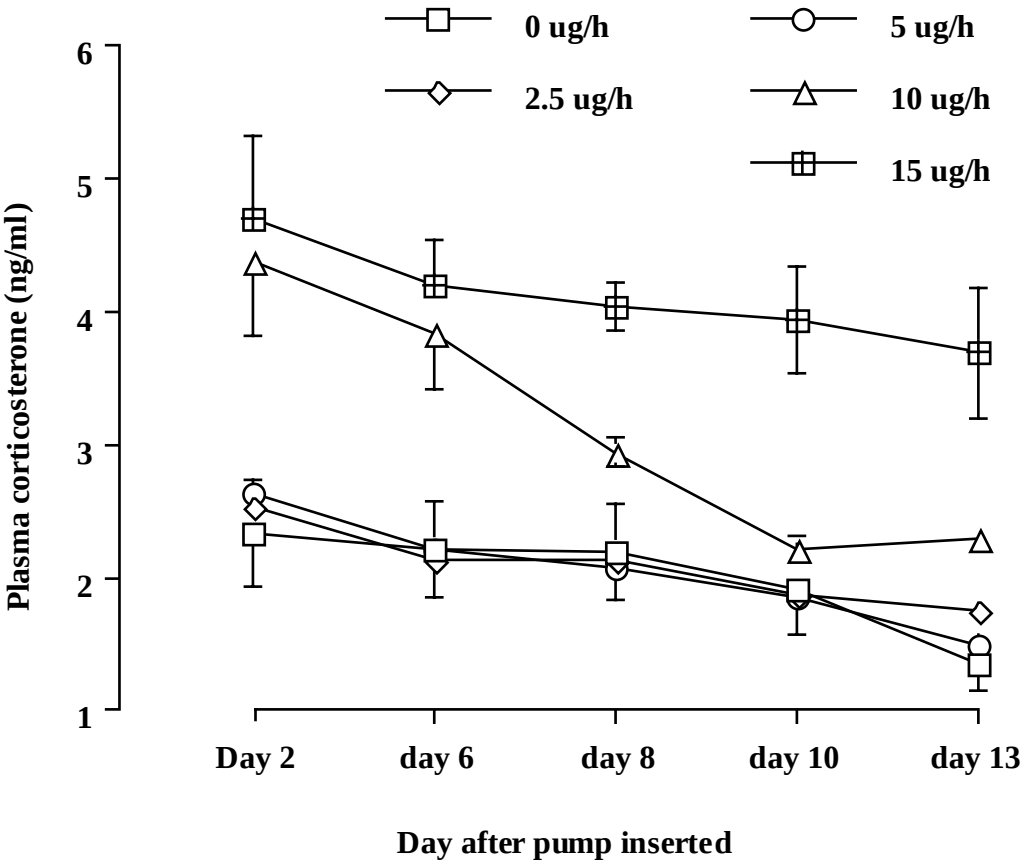



**Figure 4:** The mean (± SEM) plasma concentration of corticosterone for hens infused with corticosterone at 0 2.5, 5, 10 or 15 ug/h using mini osmotic pumps. The pumps were inserted subcutaneously on day 0 and remained in place until day 14.

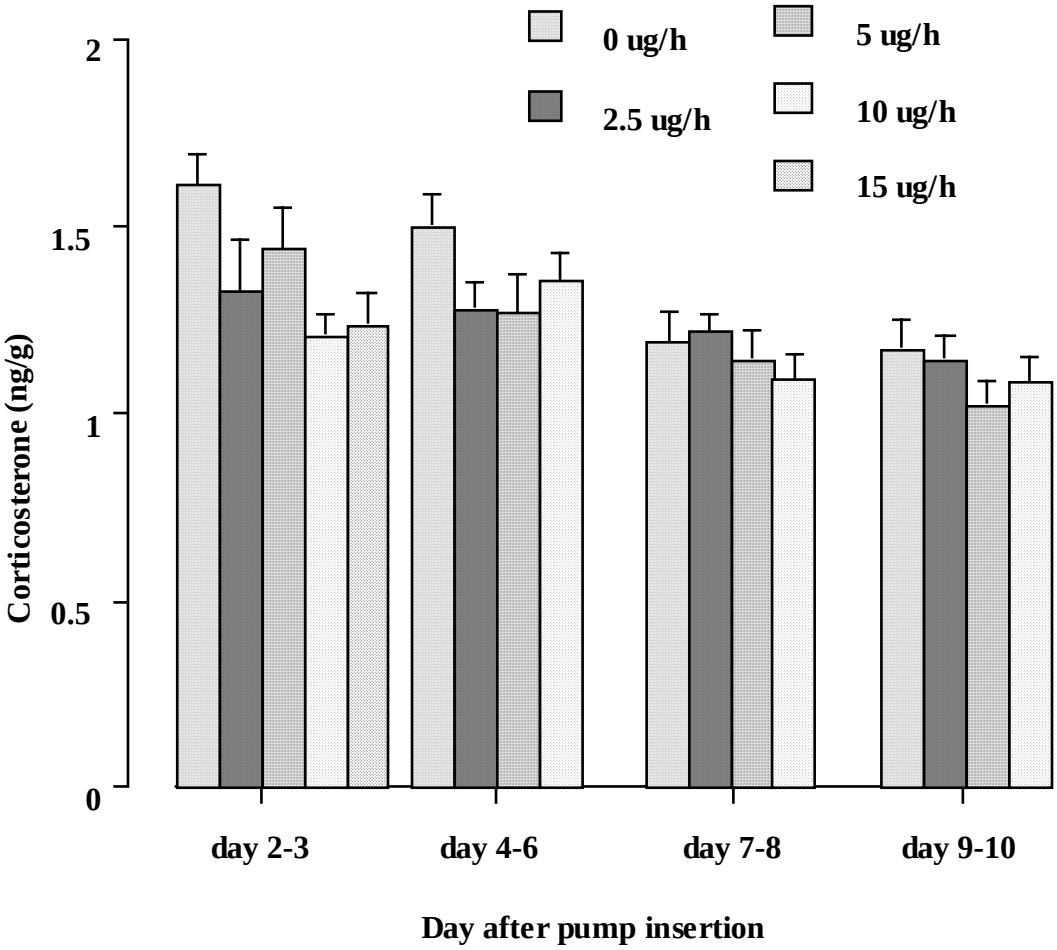



**Figure 5:** Egg albumen corticosterone concentrations for hens infused with corticosterone at 0, 2.5, 5, 10 and 15 ug/h for 14 days using mini osmotic pumps inserted subcutaneously. The pumps were implanted on day 0. Values are the means (±SEM) for all eggs collected on days 2-3, 4-6, 7-8 and 9-10 for each of the treatments.

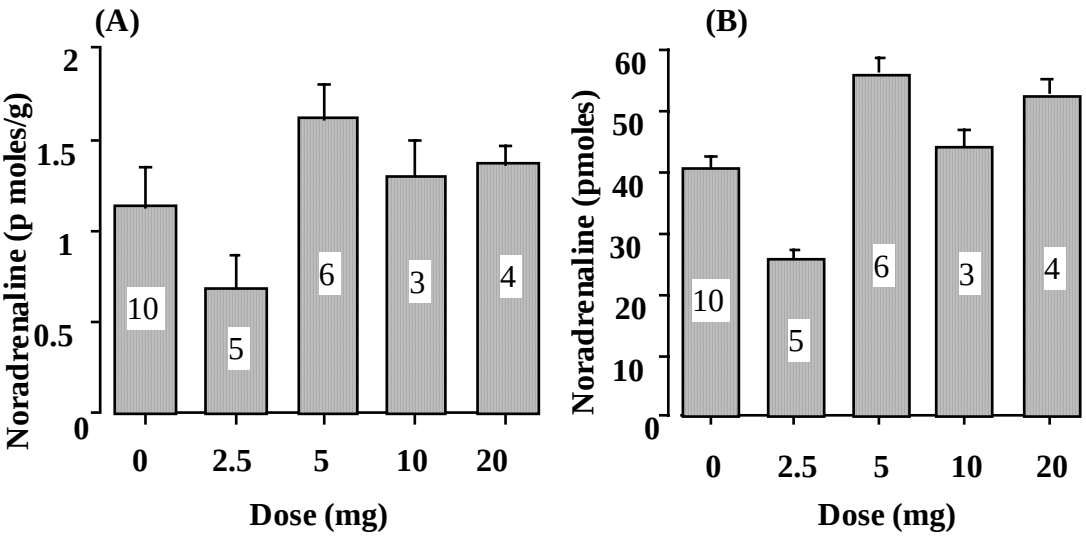



**Figure 6a.** The mean (±SEM) noradrenaline concentration (A) and total noradrenaline (B) in egg albumen for hens treated with subcutaneous injections of adrenaline. The number of eggs collected for each treament is given by number within the appropiate columns.

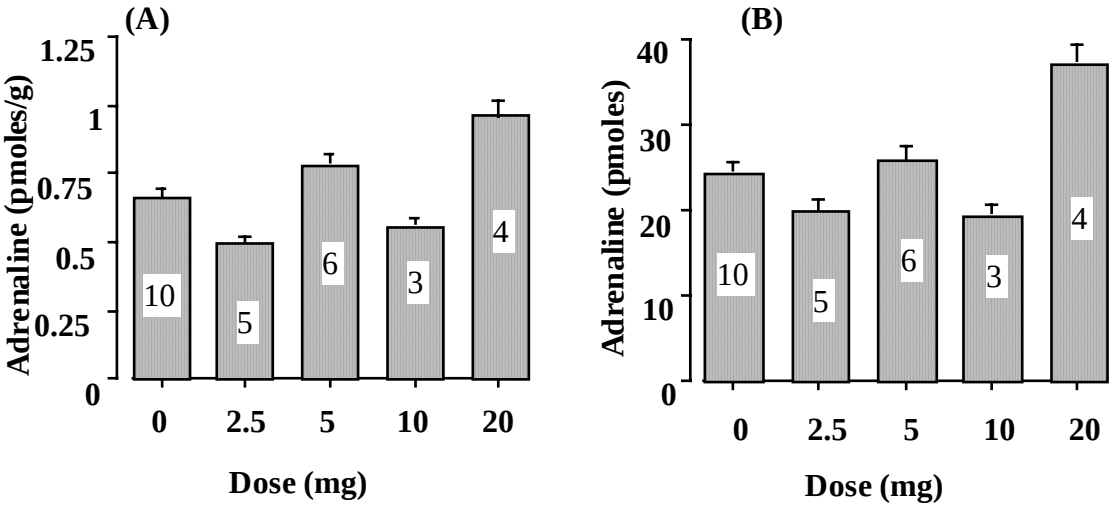



**Figure 6b.** The mean (±SEM) adrenaline concentration (A) and total adrenaline (B) in egg albumen for hens treated with subcutaneous injections of adrenaline.The number of eggs colected for each treament is given by the number within the appropiate columns.

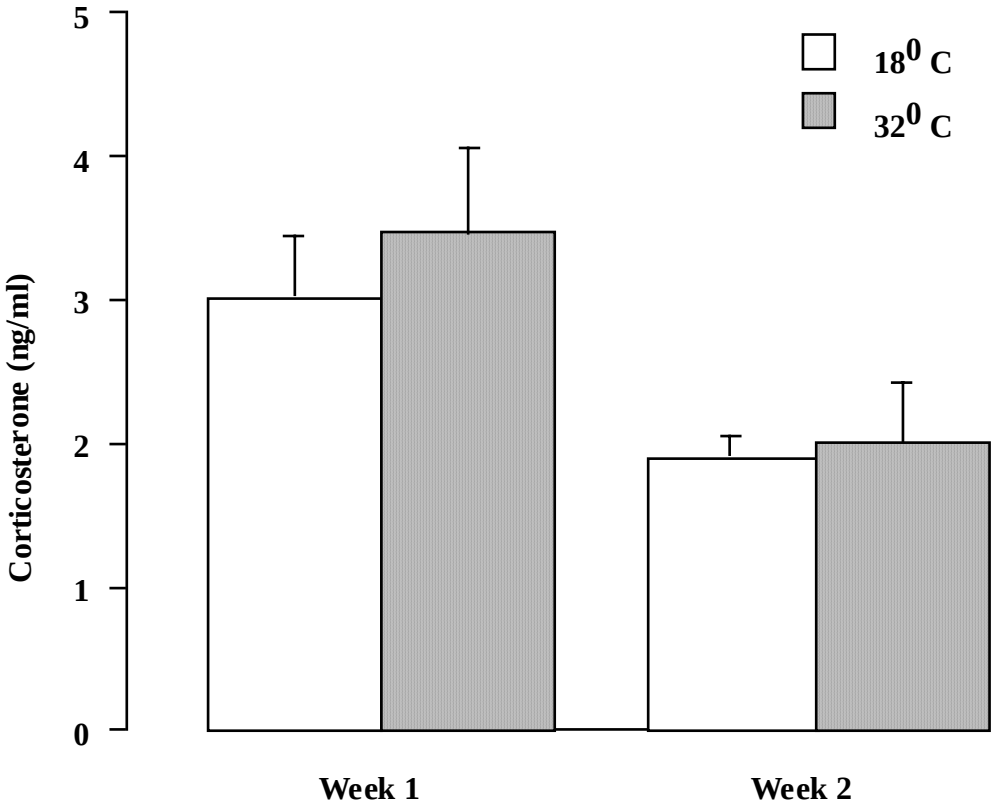



**Figure 7**. The mean (±SEM) plasma corticosterone concentration for hens housed at a temperature of $18^0$ C or $32^0$ C. The blood samples were taken at the end of the first or second week after the temperature was elevated to $32^0$ C. There were no significant differences between treatments.

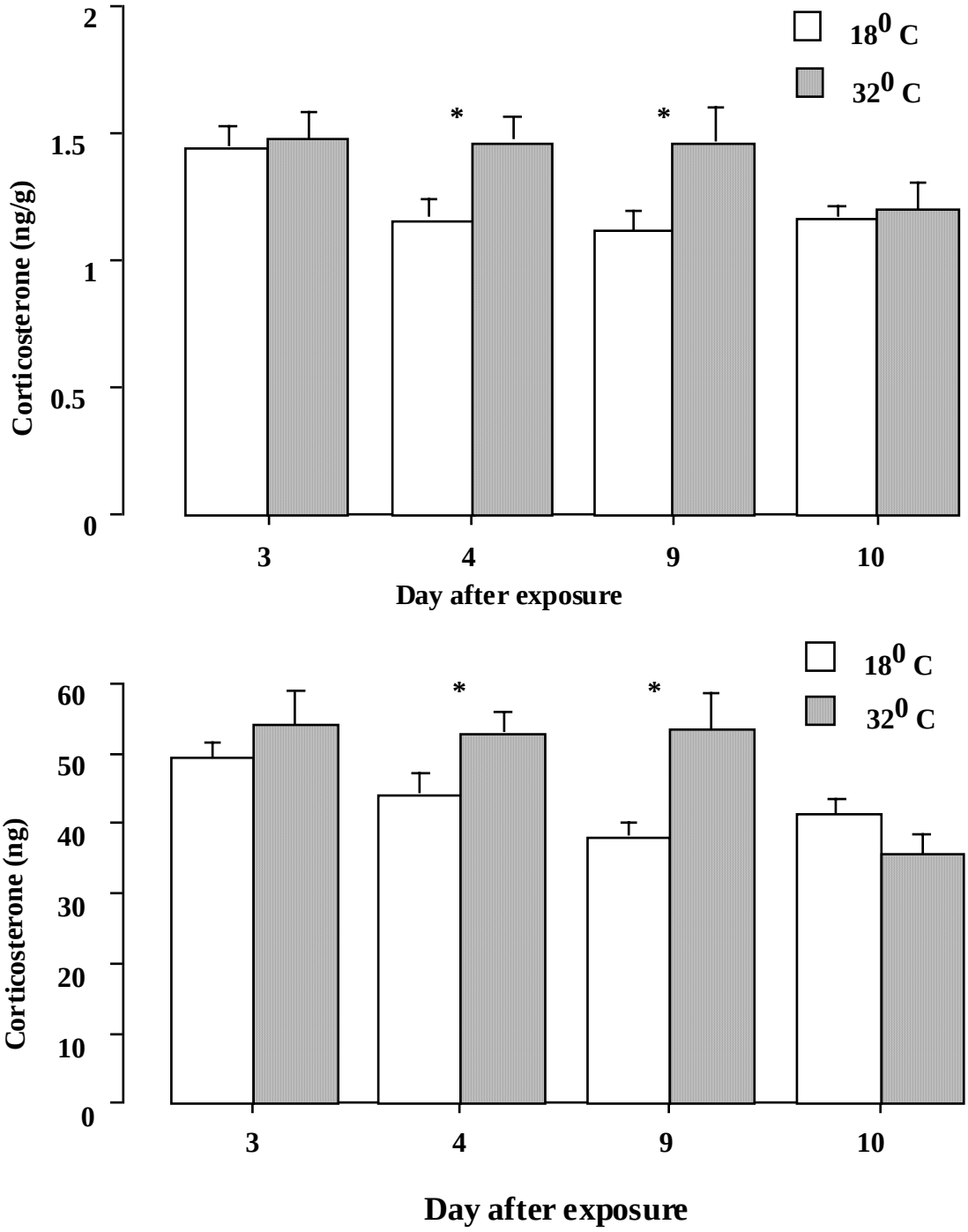



**Figure 8**. The mean (± SEM) corticosterone concentration (upper panel) and total corticosterone (lower panel) in egg albumen for hens housed at $18^0$ C or $32^0$ C for 14 days. Samples were collected on days 3, 4, 9 and 10 after exposure to the treatment temperature. Significant differences between treatments are shown by *.

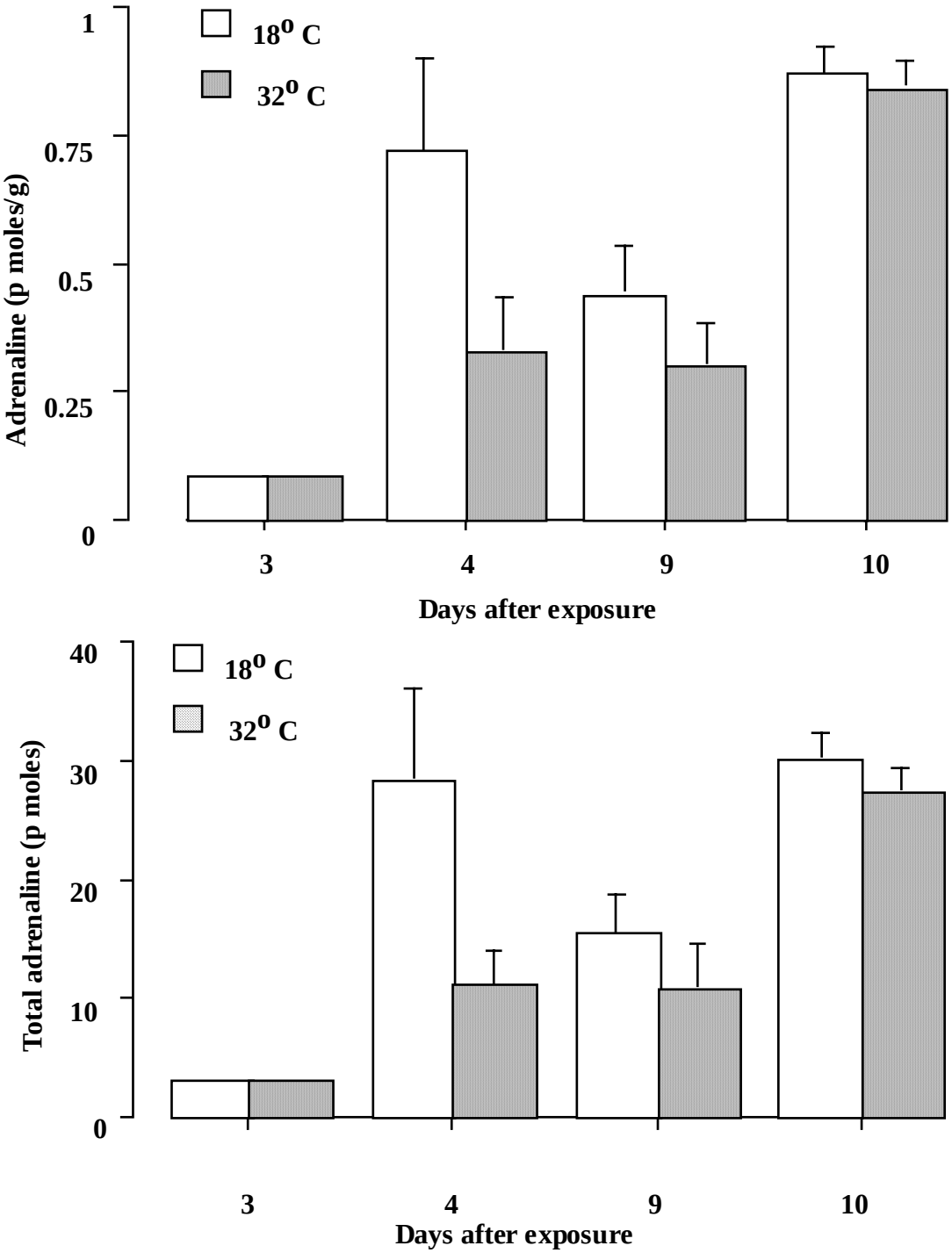



**Figure 9:** The mean (± SEM) adrenaline concentration (upper panel) and total adrenaline (lower panel) in egg albumen for hens housed at $18^0$ C or $32^0$ C for 14 days. Samples were collected on days 3, 4, 9 and 10 after exposure. The values on day 4 are significantly different.

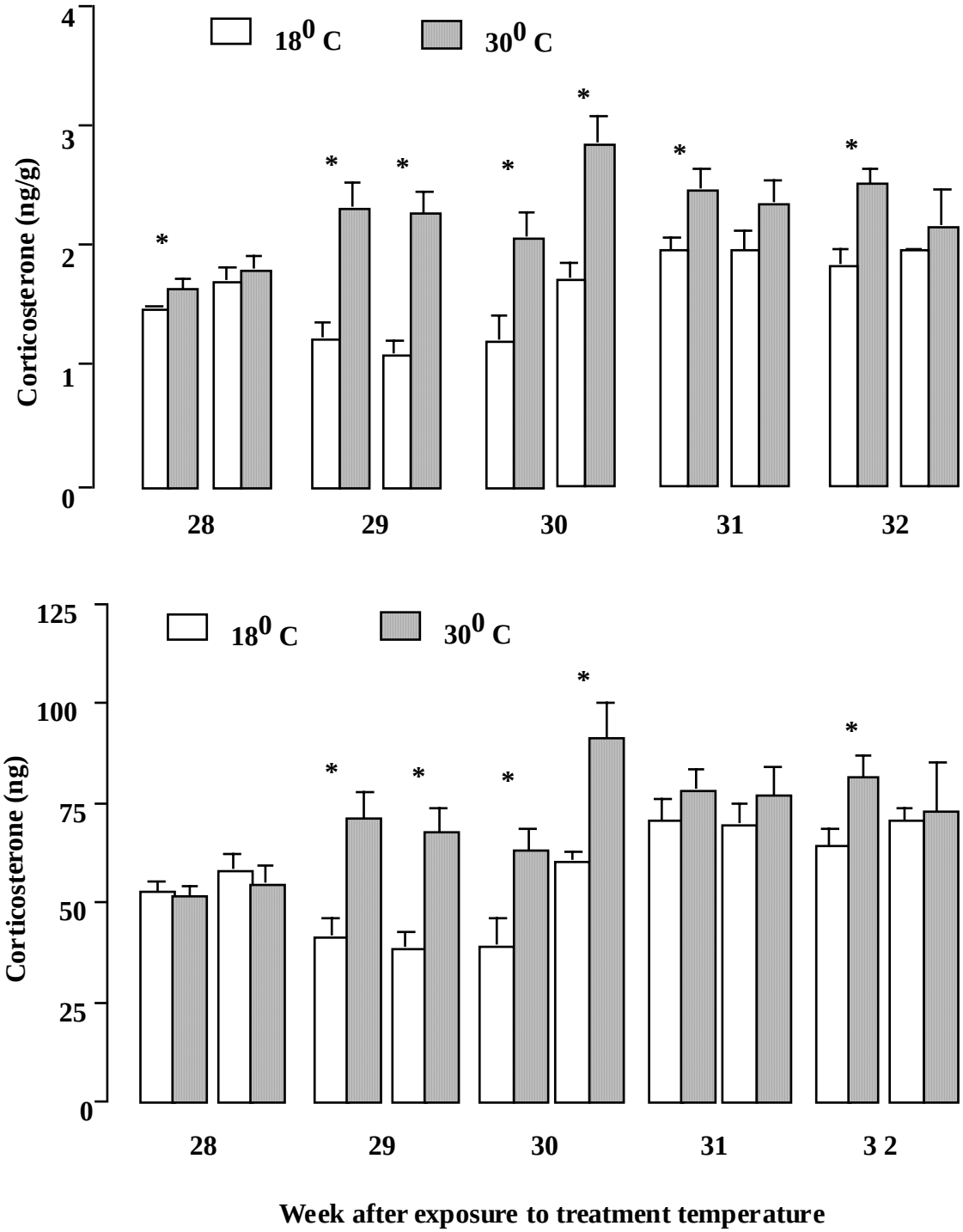



**Figure 10:** The corticosterone concentration (upper panel) and total corticosterone (lower panel) in egg albumen for hens exposed to $18^0$ C or $30^0$ C. Eggs were collected twice weekly during weeks 28 to 32 after initial exposure to the treatment temperature. Significant differences between treatments on individual collection days are highlighted by *.

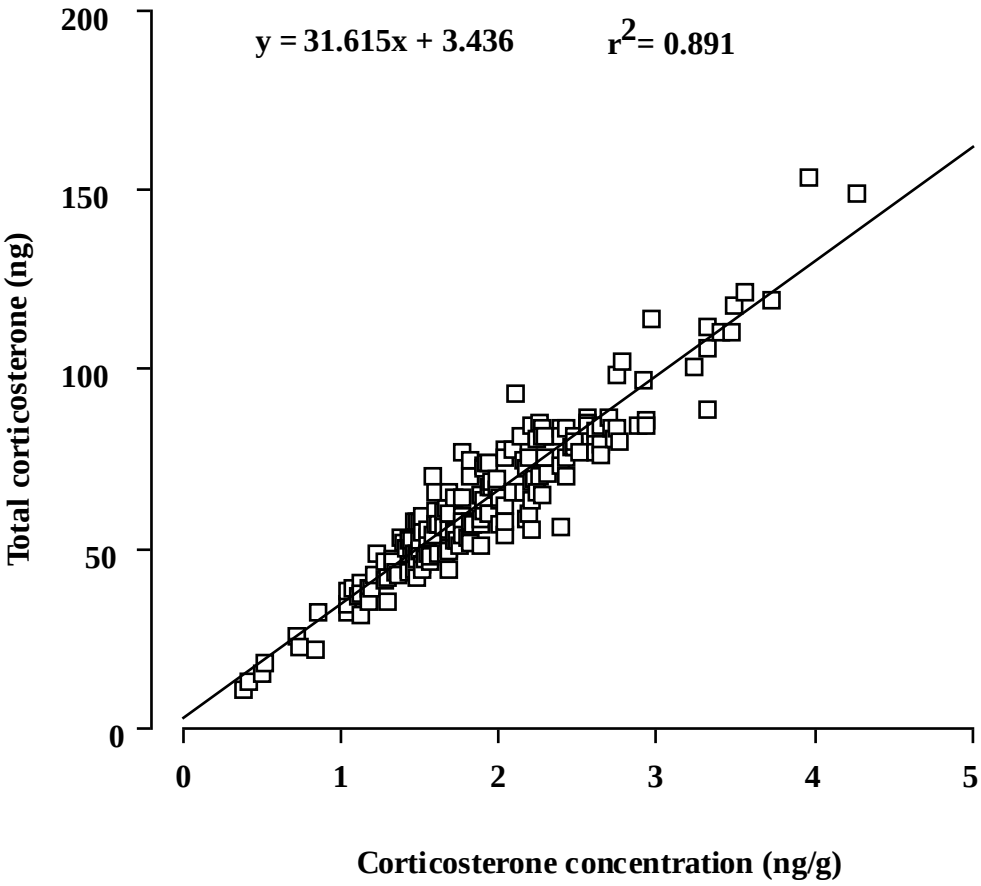



**Figure 11:** The relationship between the concentration of corticosterone in egg albumen and the total amount of corticosterone in albumen for all hens housed at $18^0$ C or $30^0$ C.

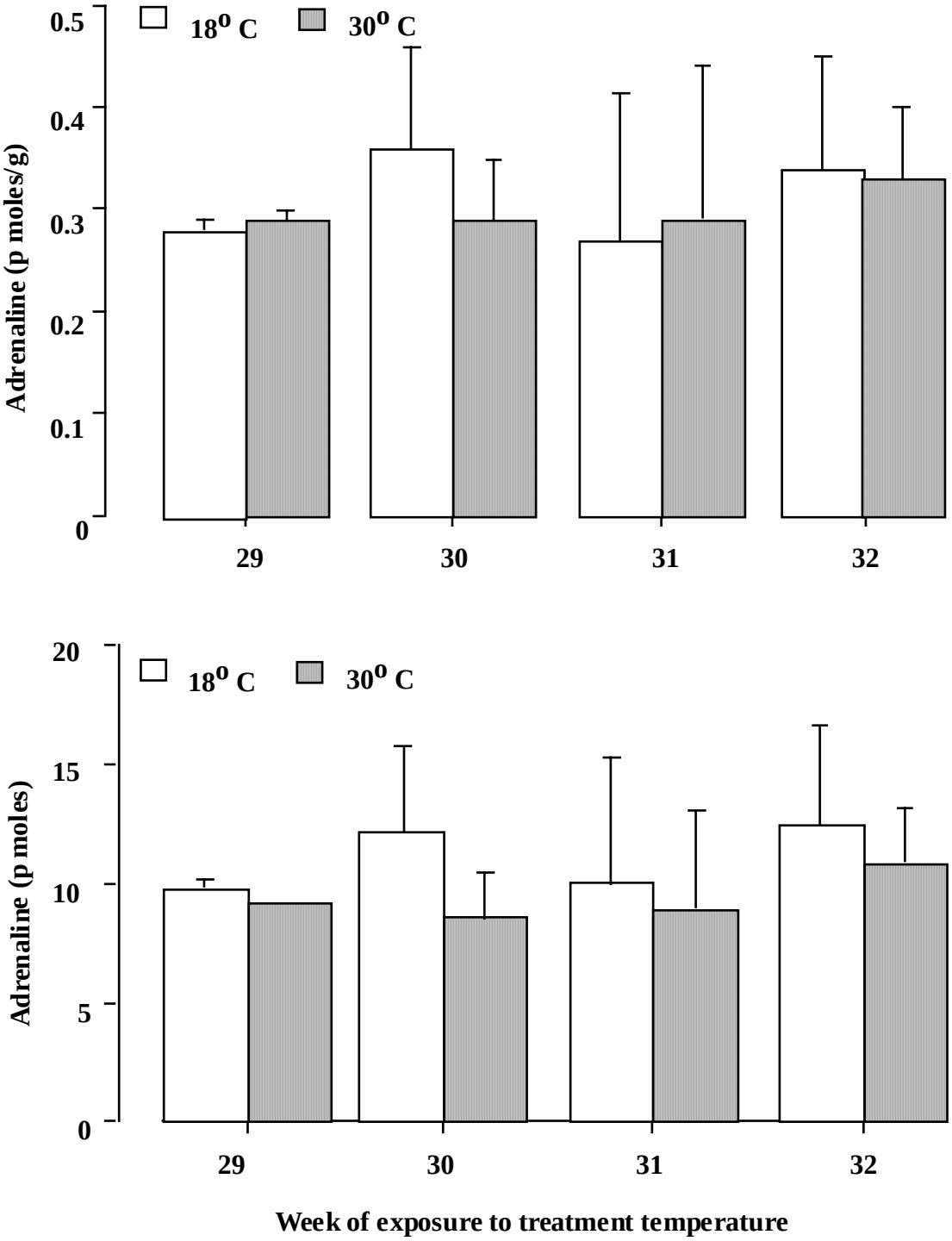



**Figure 12:** The mean (± SEM) adrenaline concentration and total adrenaline levels in egg albumen for hens exposed to $18^{o}$ C or $30^{0}$ C. Eggs were collected during weeks 29, 30, 31 and 32 weeks after initail exposure. There were no significant differences between treatments.

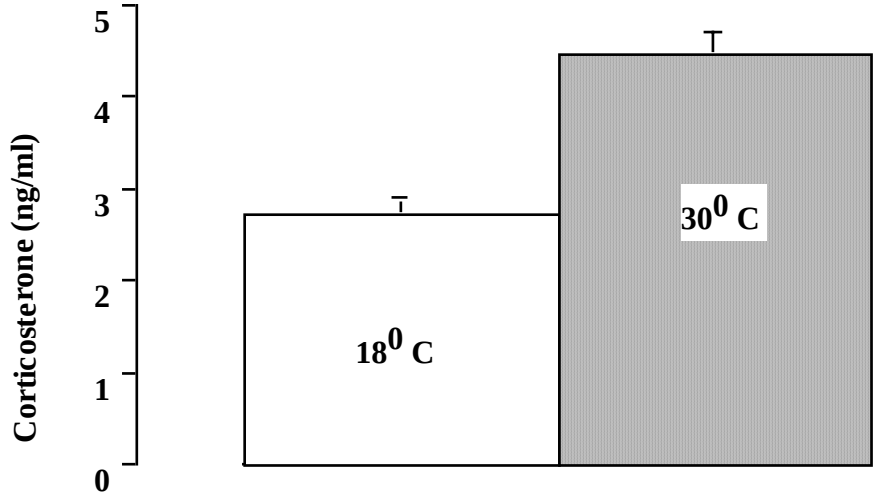



**Figure 13a**. The mean (±SEM) plasma concentration of corticosterone for hens exposed to $32^0$ C or $18^0$ C. Samples were taken at the end of week 32 after the initial exposure to the treatment temperature. The difference between the groups was significant.

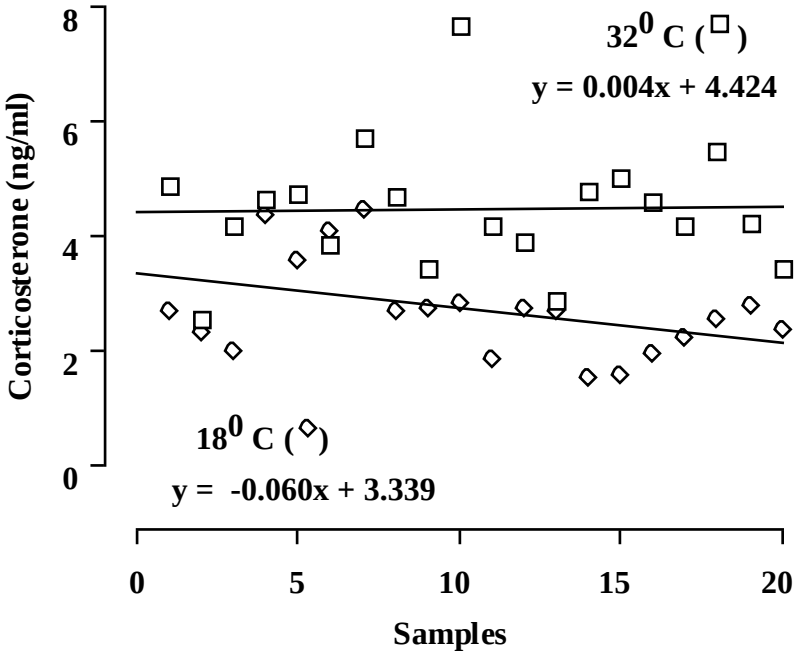



**Figure 13b**: The scatter plot of plasma corticosterone concentration for hens exposed to $18^0$ C (n=20) or $30^0$ C (n=20). The samples were taken at the end of week 32 after the initial exposure to the treatment temperature. Linear curve fits are given for each treatment.

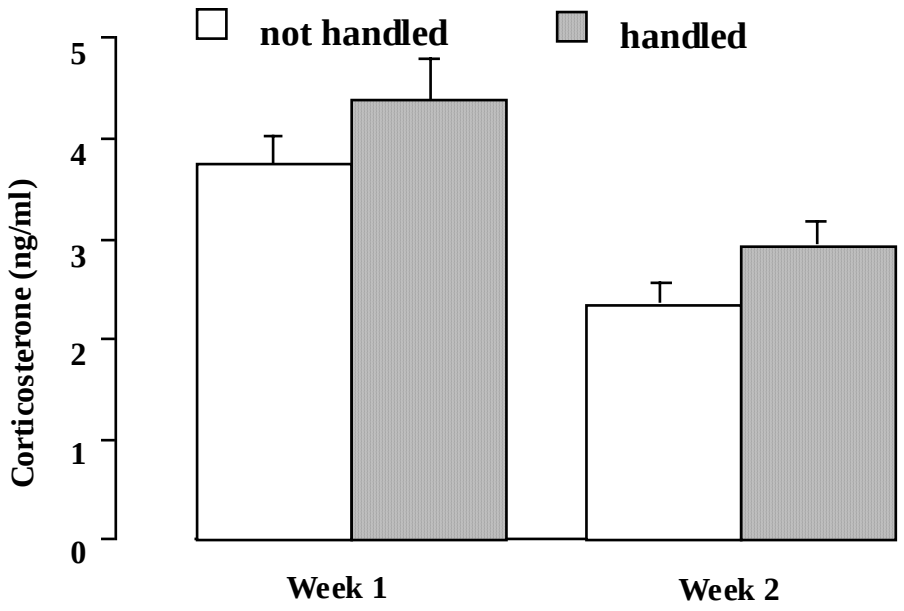



**Figure 14a**. The mean (±SEM) plasma corticosterone concentration for hens handled or not handled for 1 min every h for 6 h. The sample was taken 45 min after the last handling eposide.

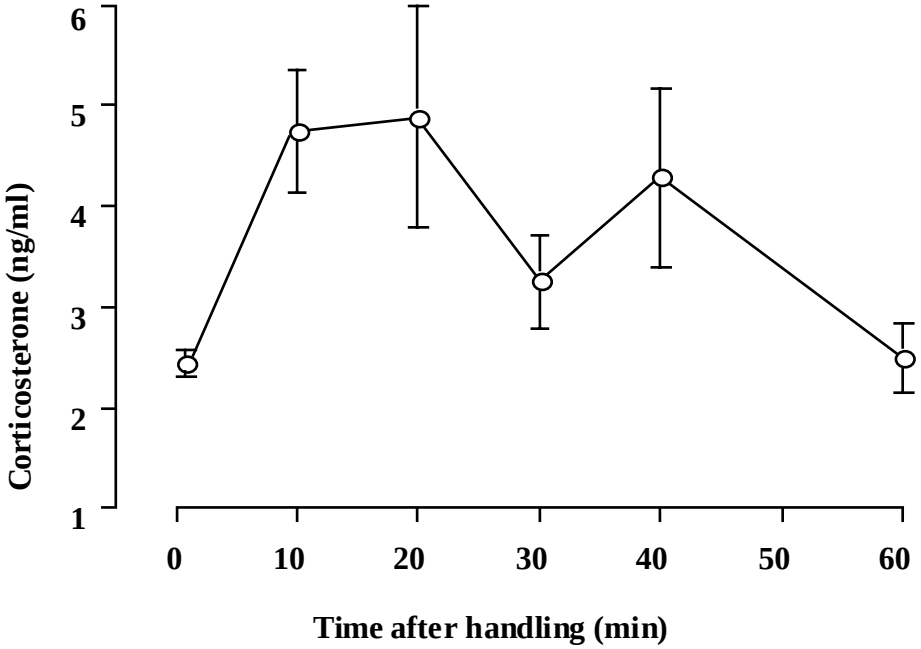



**Figure 14b**. The mean (± SEM) plasma corticosterone concentration for hens handled for 1 min and then bled at 10, 20, 30, 40 and 60 min later. Thirty six hens were used, 6 being bled at each sampling time.

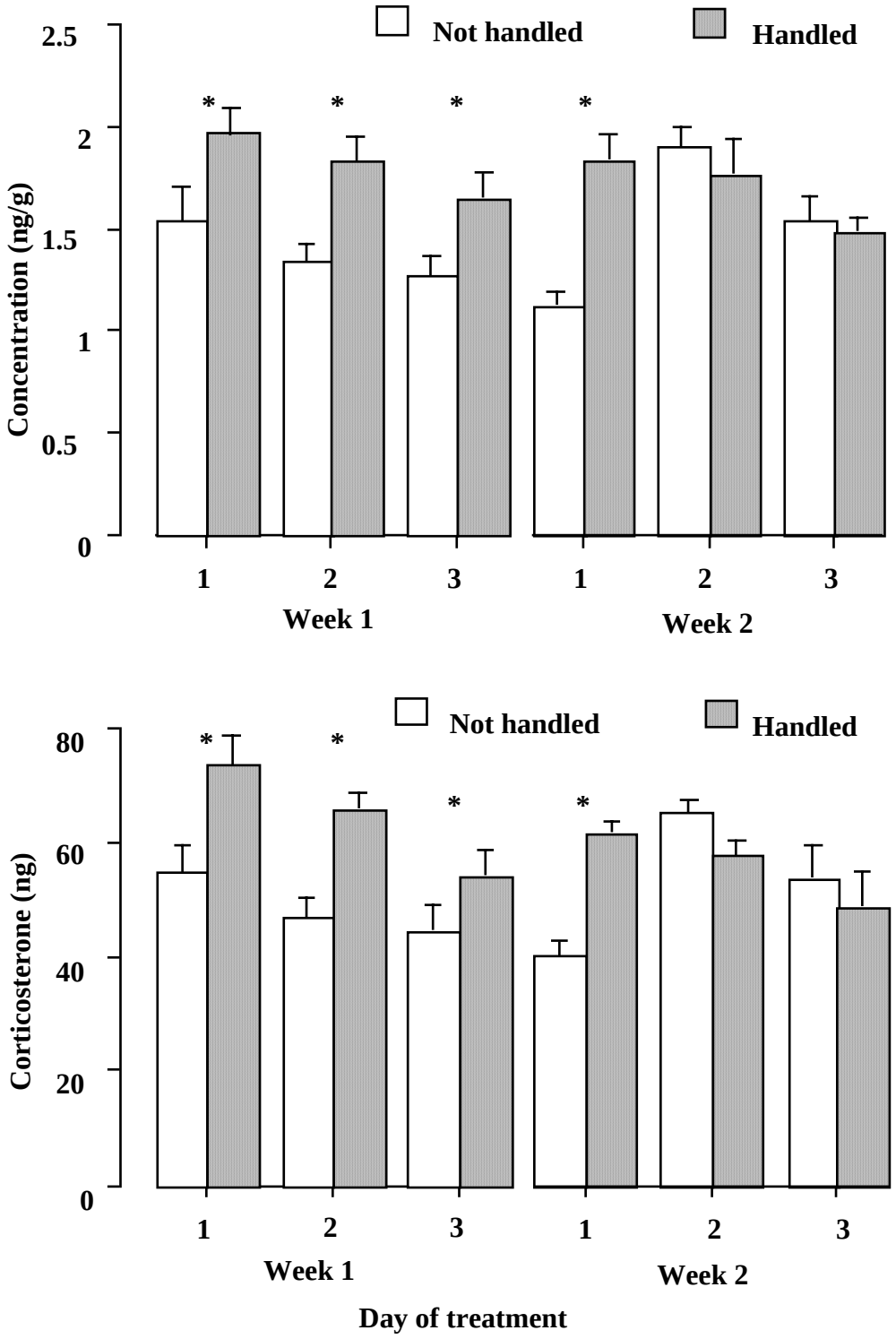



**Figure 15:** The mean (± SEM) concentration of corticosterone (upper panel) and total corticosterone (lower panel) in albumen for eggs collected from hens not handled or manually handled for one minute each hour for 6 hours each day starting at 0700h. Hens were treated on three consecutive days each week for two weeks with a one week break between these two periods. Significant differences are denoted by *****.

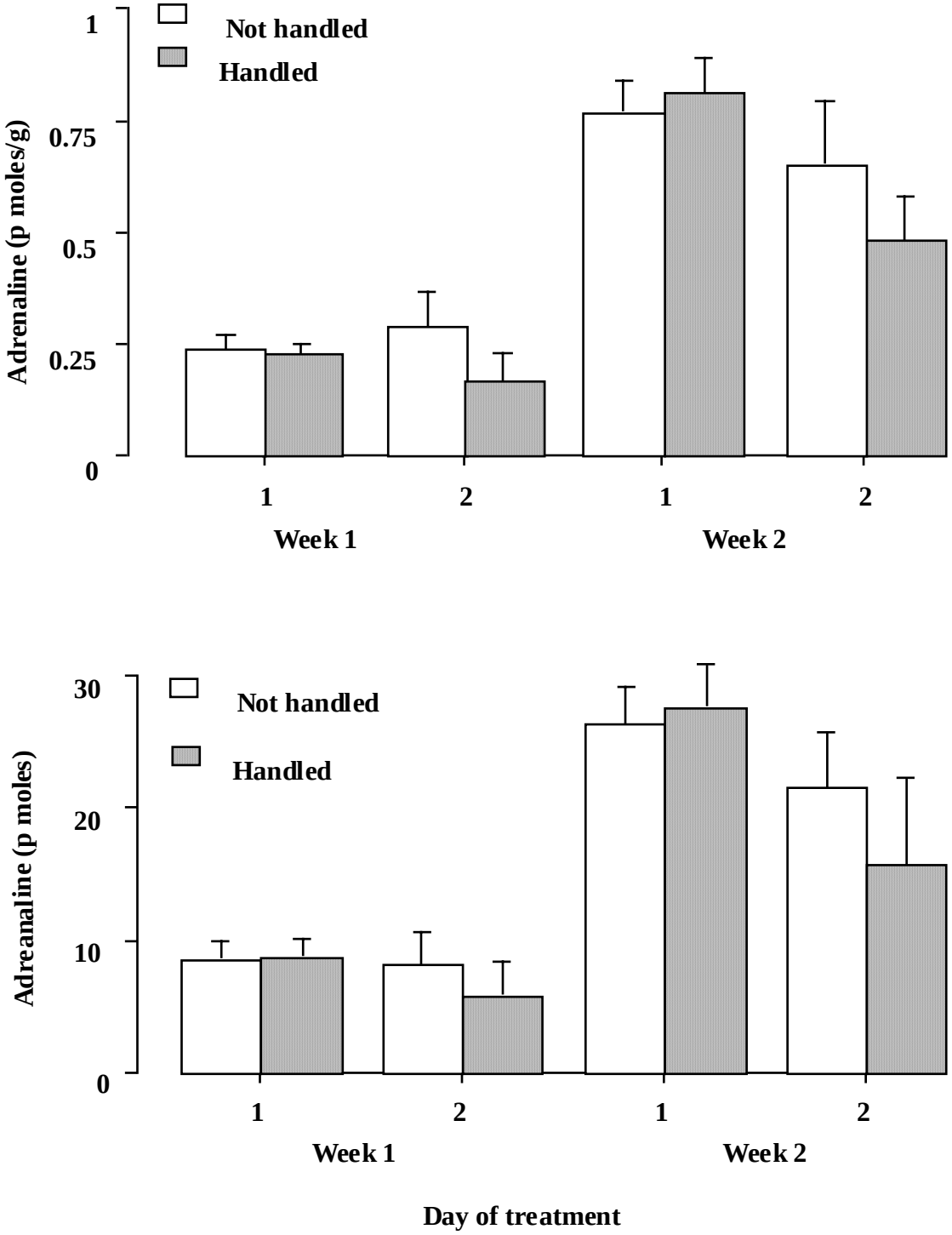



**Figure 16:** The mean (± SEM) adrenaline concentration (upper panel) and total adrenaline (lower panel) in egg albumen for hens not handled or manulally handled. Hens were handled for one minute each hour for 6 hours. Hens were treated on 3 consecutive days for two weeks with a one week break between the two treatment periods. There were no significant differences between treatments.

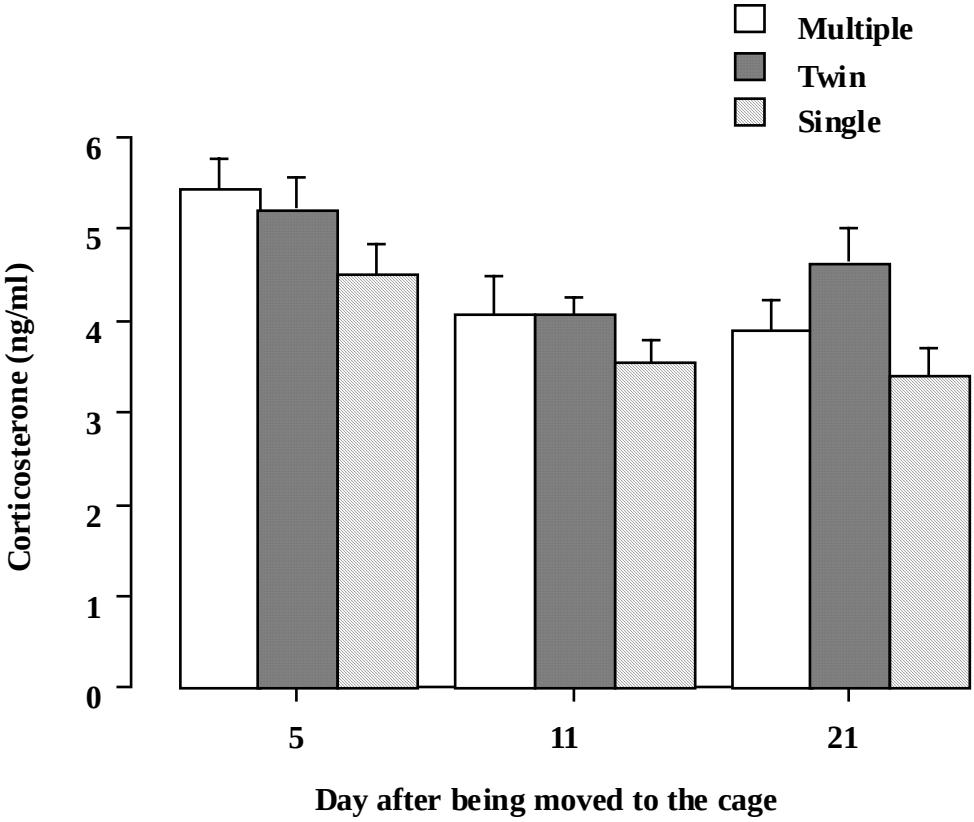



**Figure 17**. Mean (±SEM) plasma corticosterone concentration for hens housed in single, twin or multiple cages. Samples were taken on day 5, 11 and 21 after being moved to the cages. There were no significant differences between treatments.

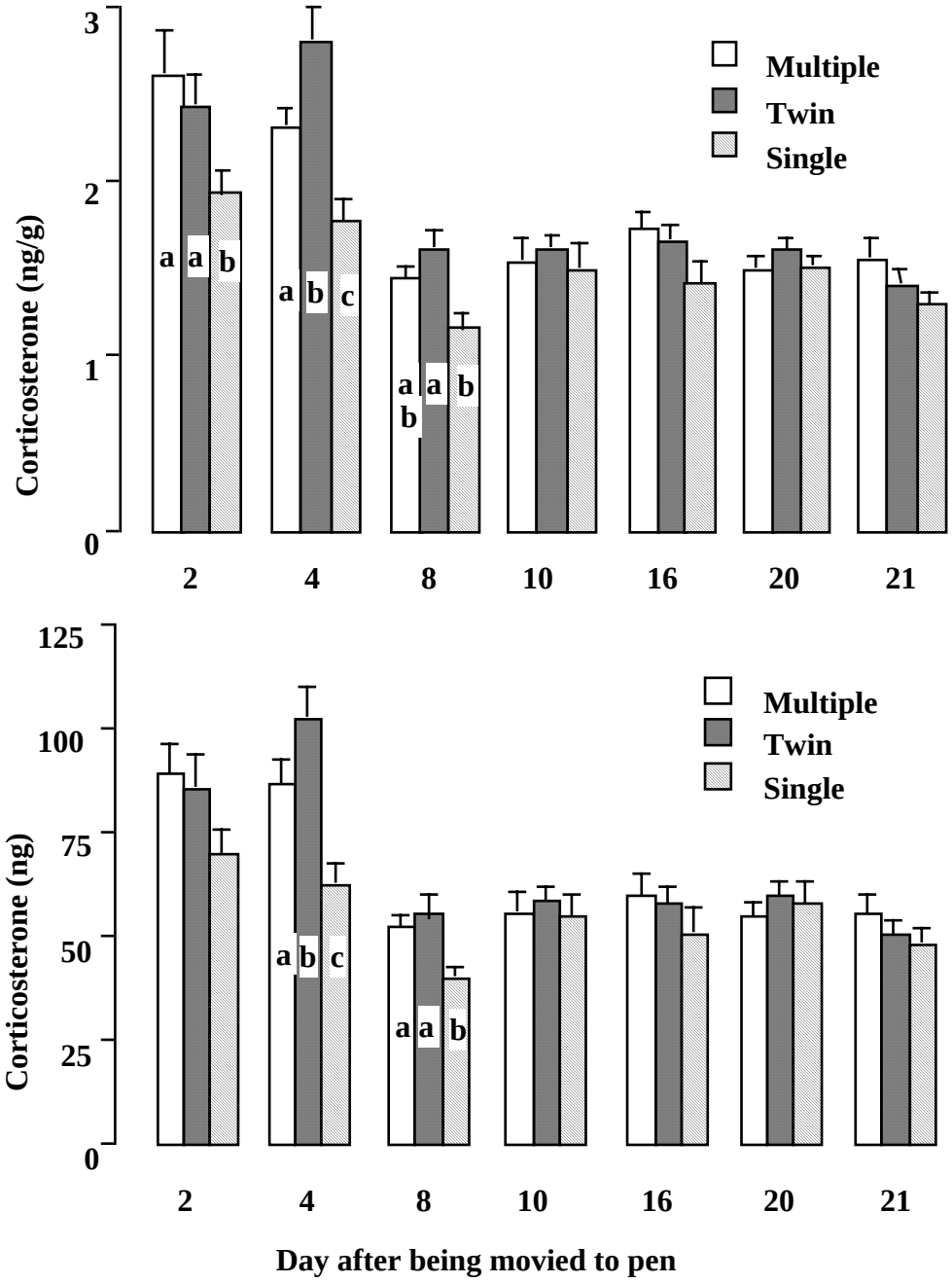



**Figure 18.** The mean (± SEM) corticosterone concentration and total corticosterone in egg albumen for hens housed in single, twin or multiple cages. Egg were collected on days 2, 4, 8, 10, 16, 20 and 21 after the hens were moved to the pens. On individual days, columns with different letters are significantly different.

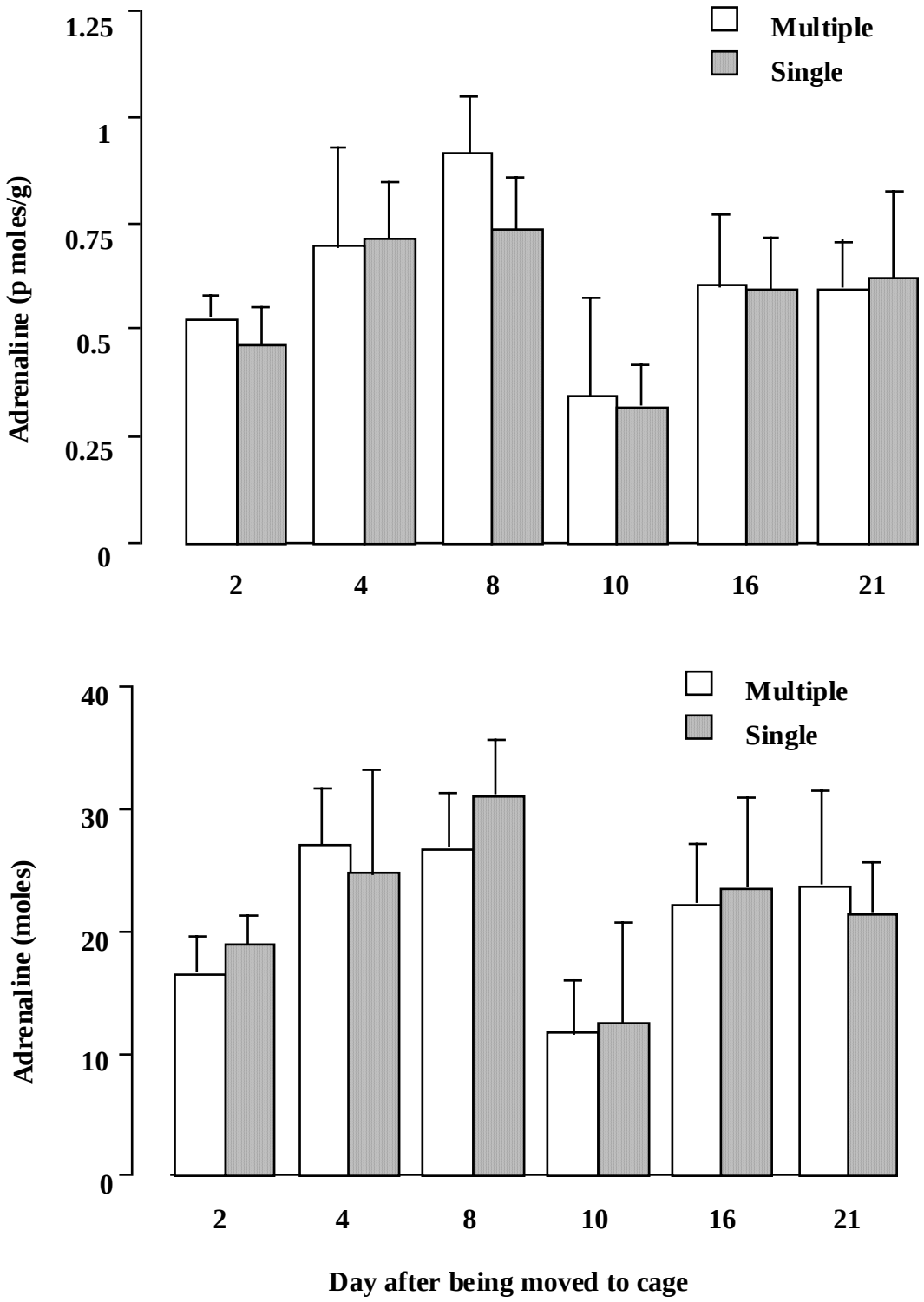



**Figure 19:** The mean (±SEM) adrenaline concentration (upper panel) and total adrenaline in egg albumen for hens housed in multiple or single pens. The eggs were collected on days 2, 4, 8, 10, 16 and 21 after the hens were moved to the cages. There were no significant differences between treatments.

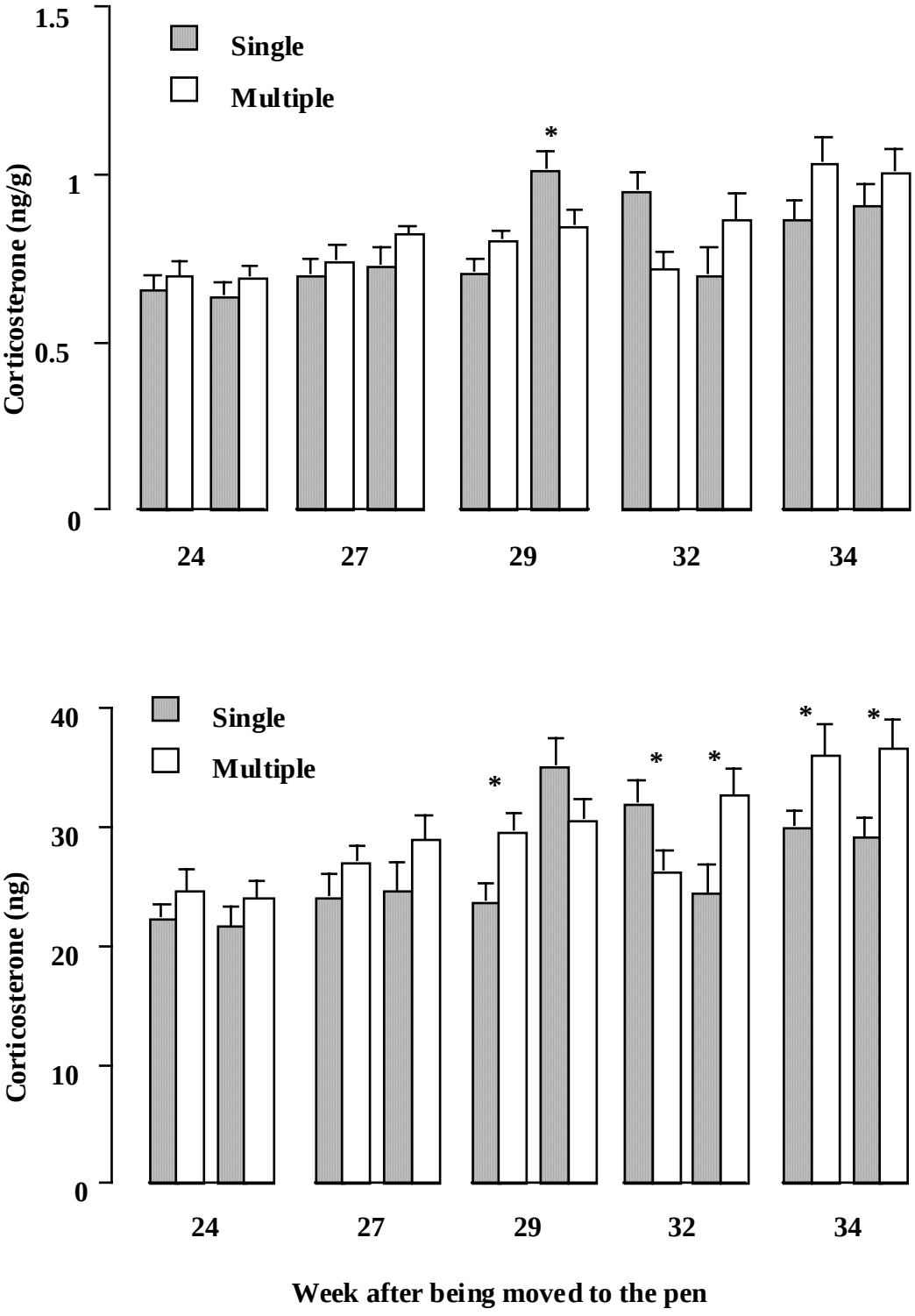



**Figure 20:** The mean (±SEM) corticosterone concentration (upper panel) and total corticosterone (lower panel) in egg albumen for hens housed in single pens or in multiple cages. Any significant differences between treatments are designated by *.

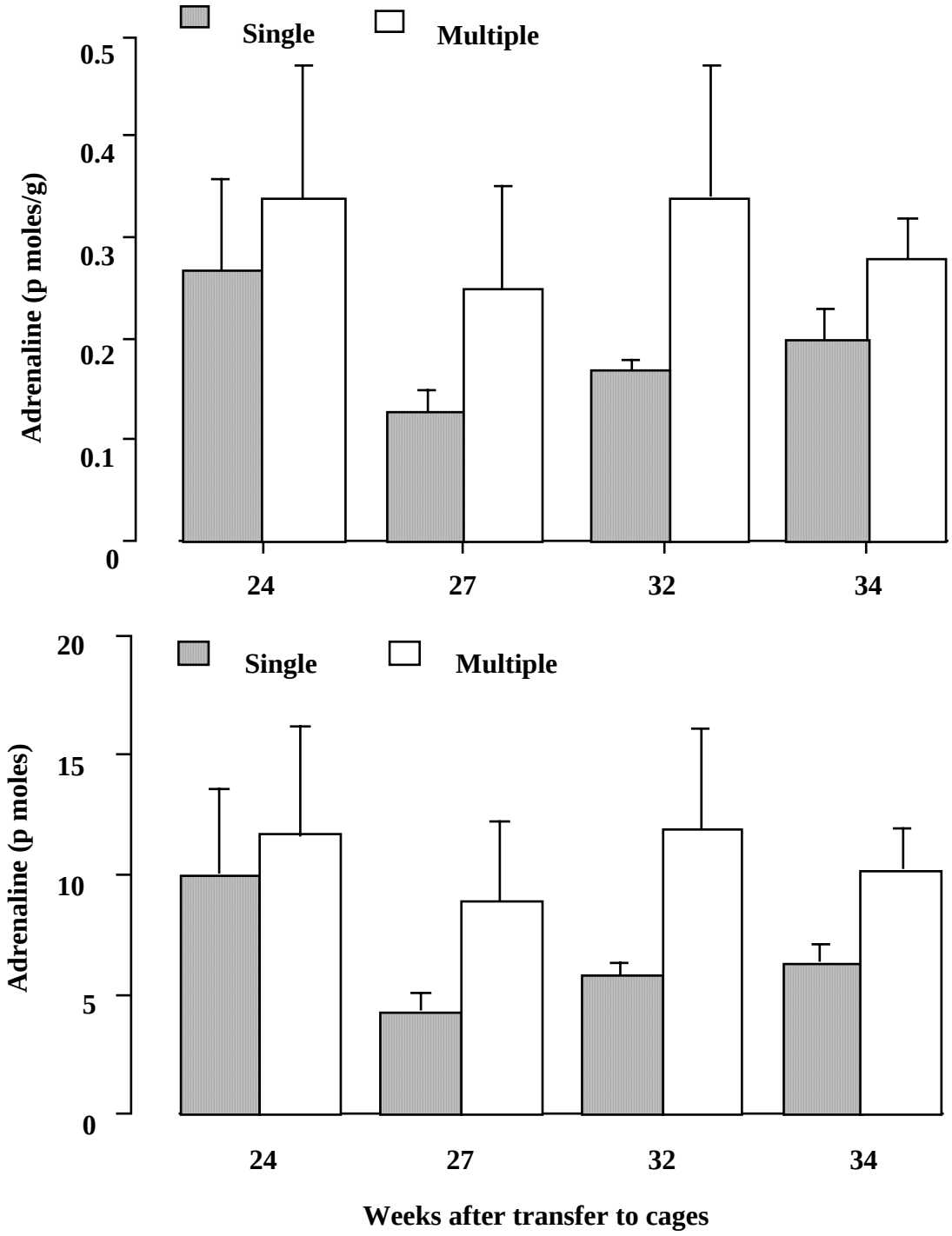



**Figure 21:** The mean (±SEM) adrenaline concentration (upper panel) and total adrenaline (lower panel) in egg albumen for hens housed in single pens or multiple cages. There were no significant differences between treatments.

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# Appendix 1. Stress, Hen Husbandry and Welfare – A literature review of stress in poultry

# Stress, Hen Husbandry and Welfare

**A literature review of stress in poultry**

**Undertaken as part of the RIRDC Project US-71A "Development of a non-invasive test of stress in laying hens"**

**Principal Investigators: Dr J. A. Downing**
**Associate Professsor W. L. Bryden**

**The University of Sydney**

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# Stress, Hen Husbandry and Welfare

## 1: Introduction

While the concept of stress is understood, a definition remains difficult. Stress as a term describes the way an organism responds to environmental stimuli it perceives as a threat, real or anticipated, to its survival or well-being (Harvey *et al*., 1984). Selye (1936) described a syndrome in response to a variety of 'noxious agents". For this syndrome there was an acute response, occurring within a few minutes and termed the 'alarm reaction' and a chronic response occurring over days, the 'general adaptation syndrome', which was an overall attempt by the organism to adapt to its changing environment. A simplistic attempt to categorize the stress response, has divided it into a reflex, 'alarm or emergency reaction" component, principally mediated by adrenomedullary activation, followed by a period of adaptation, accompanied by increased adrenal function, then by a stage of exhaustion which results in death if adaptation fails (Selye, 1950).

Stressors are events internal or external that elicit a defence response by the hen aimed at maintaining homeostasis. The ability to respond depends on the severity of the stressor and the inherent ability of the hen to respond. Responses can be specific or non-specific (generalized). While exposure to a stress evokes a range of physiological responses many highlight the significance of the adrenal gland in these responses. This and other aspects of the stress response in poultry are reviewed in the following comprehensive overview of this topic. Husbandry practices used in the egg industry and their welfare implications are also discussed.

## 2: The adrenal gland

There are paired adrenal glands located anterior and medial to the cephalic lobe of the kidney. They receive blood via braches from the renal artery and each gland has a single vein returning blood to the vena cava. The adrenal glands consist of chromaffin and cortical (interrenal) tissue and unlike in mammals, the glands are not divided clearly in to a distinct outer cortex and inner medulla. Chromaffin tissue is intermingled throughout the cortical tissue but concentrated around blood spaces and is more abundant towards the centre of the gland. Chromaffin cells account for about 15-25% of the adrenal tissue. Two distinct types can be identified, those releasing adrenalin and those noradrenaline (Ghosh 1980).

The cortical cells are arranged in cords which radiate out from the centre of the gland. The arrangement of specific cell types along the cords does give rise to a level of structural zonation and function. The cortical tissue is divided into the subcapsular zone, 20-

40 cells thick and producing aldosterone, and an inner zone which produces corticosterone (Holmes and Cronshaw, 1980).

The adrenal provides an example of the co-operation existing between the endocrine, nervous and immune systems in the control of a variety of functions (Ganong, 1963: Siegel, 1985; Hendricks *et al.*, 1991).While there is no division of the avian adrenal into distinct regions as in mammals, it is still appropriate to consider the hormones synthesised and secreted from the adrenal as two groups. The cortical hormones, those secreted by the cortical tissue and the medullary hormones, those secreted by the chromaffin cells.

# 3: The adrenal hormones

## 3.1 The cortical hormones

The hormones of the cortical tissue are mainly steroids. In mammals, over thirty steroids are found in the adrenal gland and most have a carbon skeleton of either 19 or 21 atoms. The steroids of the adrenal are divided into the glucocorticoids and the mineralocortcoids. For mammals corticosterone, cortisol, cortisone and 11-dehydrocorticosterone are the principle glucocorticoids while 11-deoxycorticosterone, 17-hydroxy-11-deoxycorticosterone and aldosterone are the principle mineralocorticoids. The situation is less clear with hens but the main corticosteroids are corticosterone, cortisol, cortisone and aldosterone with the principle glucocorticoid being corticosterone and aldosterone the principle mineralocorticoid (Holmes and Phillips, 1976). Unlike in mammals, where there is fairly clear distinction between glucocorticoid and mineralocorticoid functions, the avian corticosteroids have overlapping activities. Corticosterone is the main steroid released from the avian adrenal and the ratio of corticosterone to aldosterone is around 14:1 (Kalliecharan and Hall, 1977). Other steroids found in varying amounts include oestradiol, progesterone and androgens.

### 3.1.1. Biosynthesis of corticosterone

The major steps in the biosynthesis of the cortical hormones are shown in Figure 1. Adrenal steroids are synthesized *in vivo* from either acetate or cholesterol derived from acetate. Adrenal cholesterol is esterified, with these esters having a high long-chain fatty acids content. Preformed cholesterol is converted to pregnenolone which is the precursor for the synthesis of other steroid hormones. Subsequent biosynthesis consists of the progressive hydroxylation of precursor molecules by specific enzyme systems. The sequence of hydroxylation normally occurs in the following order, carbon C17 followed by C21 and C11.

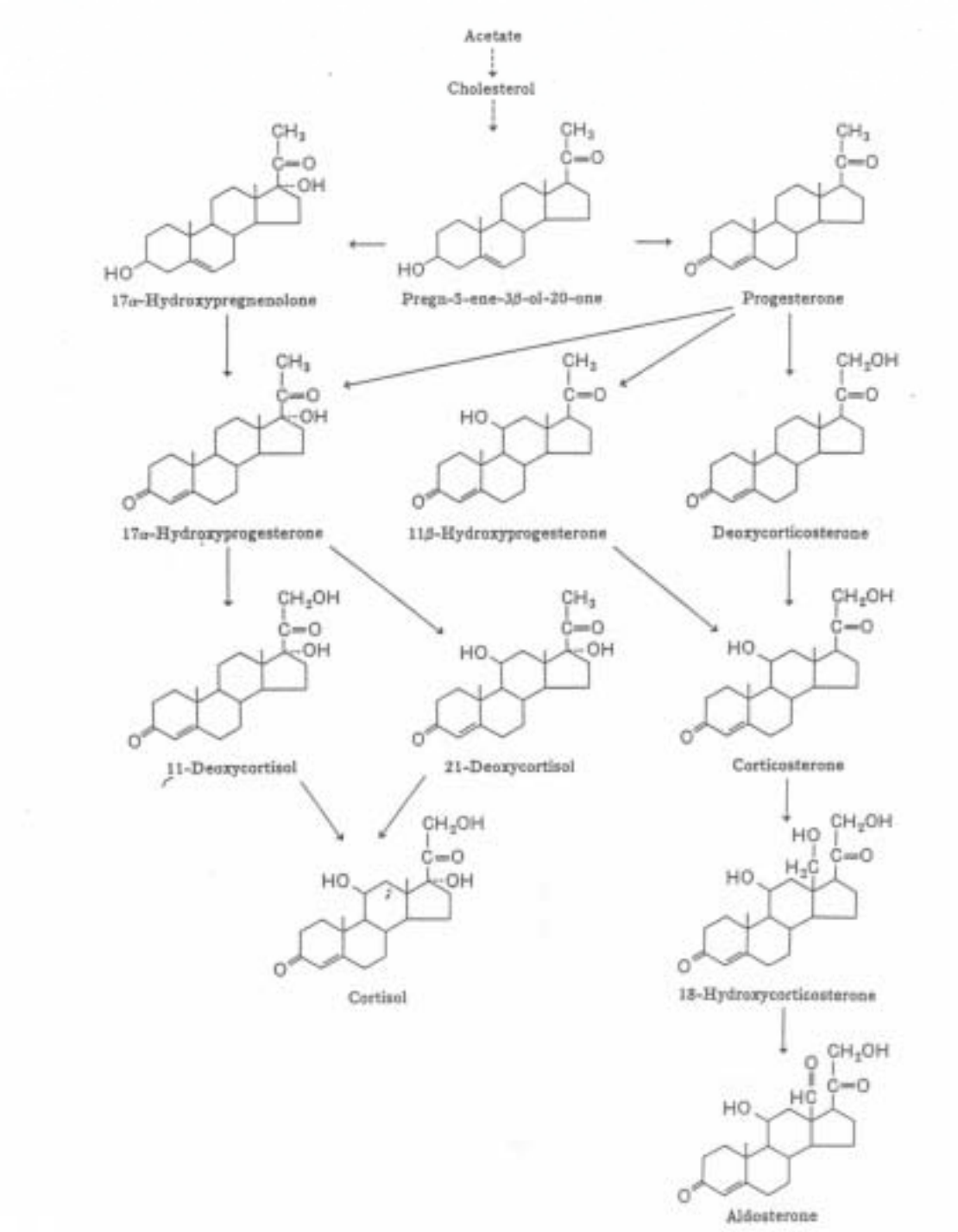


**Figure 1: The biosynthetic pathways of corticosterone and aldosterone**

The conversion of pregnenolone to progesterone is affected by 3ß-hydroxysteroid dehydrogenase. The hydroxylation of progesterone at C21 forms 21-hydroxyprogesterone and the further hydroxylation at C11 gives rise to corticosterone. Hydroxylation of pregnenolone at C17 produces 17-hydroxypregnenolone and this or progesterone acts as the precursor for 17-hydroxyprogesterone. Further hydroxylation at the C21 carbon results in 11-deoxycortisol which is the precursor of cortisol formed after hydroxylation at C11. The

avian adrenal gland has the ability to convert progesterone into 11ß-hydroxyprogesterone, and this appears to be a secondary pathway for synthesis of corticosterone in birds (Nakamura *et al*., 1978).

### 3.1.2. Biosynthesis of aldosterone

The biosynthetic pathways leading to aldosterone synthesis are given in Figure 1. While there are alternative routes for synthesis, the generally accepted route is cholesterol to pregnenolone to progesterone to dexoycorticosterone to corticosterone to 18-hydroxycorticosterone and then aldosterone. Most corticosterone synthesized in the adrenal is secreted, although some acts as a precursor for aldosterone synthesis (Aupetit *et al*., 1979).

The most distinctive feature of cortical cells is the presence of lipid droplets or liposomes, presumably containing cortical secretions or precursors such as cholesterol. The level of glucocorticoids in the gland is low and so there is a reliance on hormone synthesis before significant secretion into the blood can occur. Cortical cells contain high concentrations of ascorbic acid which is associated with glucocorticoid synthesis and the levels decrease following stress-induced glucocorticoid release.

## 3.2. Regulation of corticosterone secretion

One way of defining stress is to base the definition on the neuroendocrine response to stressors, that is, activation the hypothalamic-pituitary-adrenal axis (HPA). The consequence of this action is the secretion of glucocorticoids from the adrenal cortical tissue (Harbutz and Lightman, 1992) and the secretion of catecholamines from the chromaffin cells. The regulation of corticosteroid release involves a sequence of events starting with the release of hypothalamic factors, then ACTH (Adrenocorticotrophic hormone) from the pituitary and eventually corticosteroid from the adrenal.

### 3.2.1. The Hypothalamic-Hypophyseal axis

The pituitary gland (hypophysis) forms an integral connection with the brain. The pituitary tissue is derived from the adenohypophysis and the neurohypophysis. In birds, the adenohypophysis forms the pars distalis (anterior pituitary) and the pars tuberalis; the pars intermedia is absent in mammals. The neurohypophysis forms the pars nervosa (posterior pituitary), the infundibular stalk and the median eminence (ME).

The anterior lobe of the pituitary connects with the ME by means of hypophyseal portal vessels. Factors released into the portal system regulate the secretion of the anterior pituitary hormones. There is little or no nervous innervation of the anterior pituitary. The posterior gland consists of neurosecretory terminals which are responsible for the release of arginine vasotocin and mesotocin. A diagrammatic representation of the mammalian structures is shown in Figure 2.

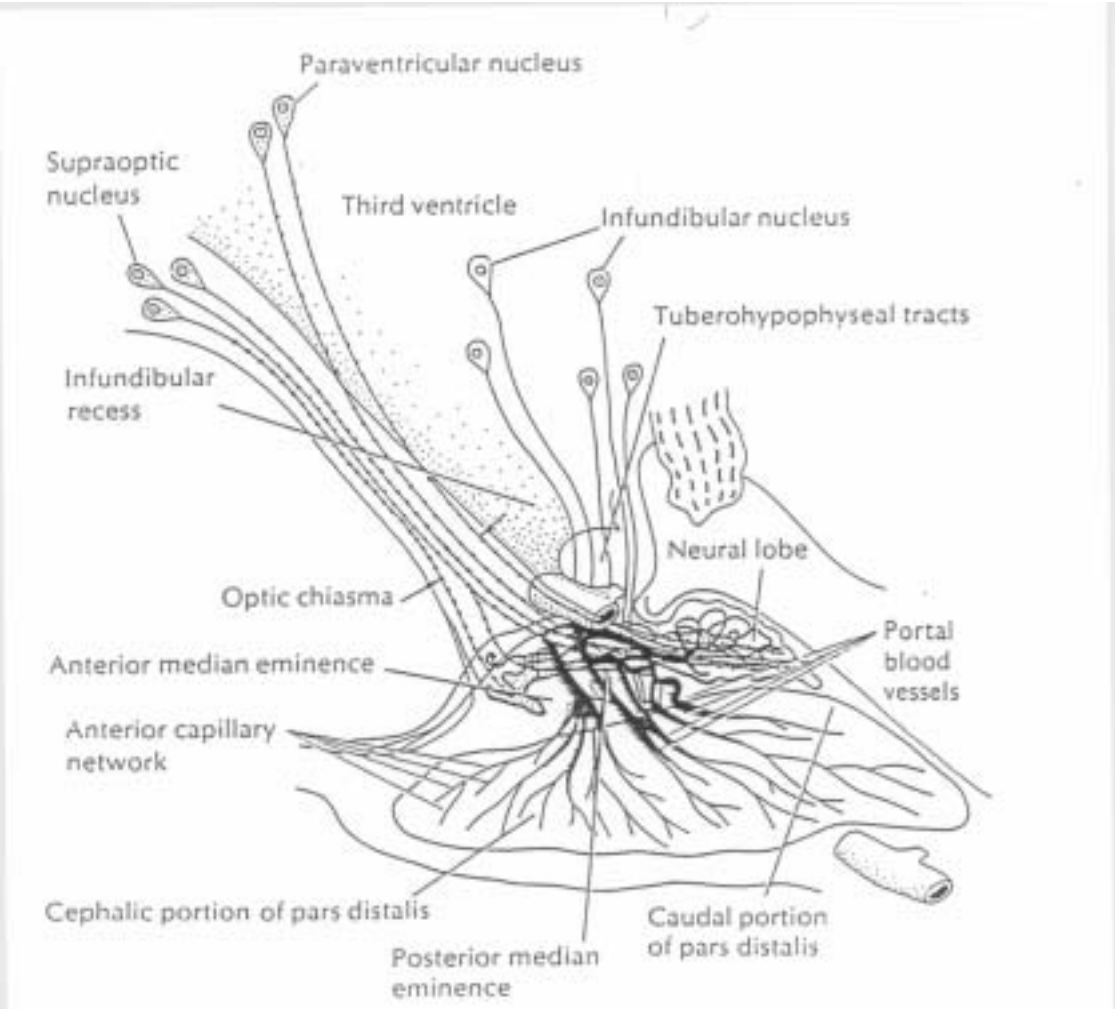


**Figure 2: Schematic diagram of the infundibulum and hypophysis.**

While the regulation of ACTH secretion is less well defined in birds than mammals, it has many similarities. In mammals, ACTH release is regulated by corticotropin releasing factor (CRF) and vasopressin (VP). Both CRF and VP are synthesized and secreted by the paravocellular cells of the paraventricullar nucleus (Whitnall, 1993) and thus a mixture of CRF and VP is presented to the pituitary and they act to augment one another (Caraty *et al*., 1990; Alexander *et al*., 1991; Jacob and Minton, 1993). Vasopressin augmentation maybe a strategy to elevate ACTH action during stress. Stimulation of aminergic activity in the hypothalamus is probably responsible for the release of CRF and VP (Yamashiro *et al*., 1984). In sheep, VP is a more potent stimulator of ACTH release than CRF (Pradier *et al*., 1986) but the reverse is the case in pigs (Liu *et al*., 1990). *In vitro* studies in rats have shown that oxytocin, angiotensin II, adrenalin and noradrenaline have limited capacity to stimulate ACTH release (Watanabe and Orth, 1988). It is also known that cytokines are involved in

regulating the HPA (Whitnall, 1993). Interleukin-1, interleukin-6 and TNF have been implicated and probably increase ACTH release during infection and inflammation.

Stress-induced responses of the HPA can be considered in two domains, the effects of acute stress and the effects of chronic or repeated stress. A variety of acute stressors have been shown to elicit increases in plasma ACTH and corticosterone. Acute stress results in an increase in bioassayable and immunoreactive CRF in the ME and lasts about 2-3 minutes (Murakami *et al*., 1989). The ME, CRF levels decrease after about 30 minutes, probably indicating release from the ME (Suda *et al*., 1988). There is a further peak at around 60 min, probably reflecting increased synthesis (Moldow *et al*., 1987).

There can be differential activation of the HPA with different stressors stimulating synthesis of particular mRNAs. Physical stress increases CRF mRNA and proenkephalin mRNA while psychological stress stimulates CRF mRNA only (Lightman and Young, 1987, 1988; Harbuz *et al*., 1991). Increases in proenkephalin mRNA suggest the involvement of endogenous opoids in the control of ACTH release. Arginine vasopressin (AVP) coexists with CRF in approximately 50% of the parvocellular CRF neurones (Whitnall *et al*., 1987). Hypertonic-saline stress increases CRF mRNA and AVP mRNA (Lightman and Young, 1988). The level of CRF mRNA remains unchanged after cold stress (Harbuz and Lightman, 1989) or ether stress (Watts, 1991). The differential release of ACTH-releasing factors into the hypothalamic-pituitary portal system is confirmed by blood sampling and immunoneutralization studies (Gibbs, 1984; Linton *et al*., 1985; Plotsky *et al*., 1985).

Studies into chronic stress are difficult because of the absence of a good experimental model. Many investigators have attempted to emulate chronic stress by using an acute stress applied over several days. With such an approach there is habituation which results in attenuated responses (Kant *et al*., 1985; Spencer and McEwen, 1990). Habituation seems to be specific to the stress imposed as alternative acute stressors can elicit a normal response (Kant *et al*., 1985) or heightened response (Hashimoto *et al*., 1988; Scribner *et al*., 1991). Plasma ACTH levels do not remain elevated in chronic stress (Hashimoto *et al*., 1988). Chronic stress is associated with near normal circulating ACTH and corticosterone levels. There has been the suggestion that AVP plays an important role in chronic stress. The proposal is that endogenous AVP is essential for the pituitary to remain responsive to stress during times it has become refractory to CRF stimulation (Scaccianoce *et al*., 1991). A study by Hauger and colleagues (1990) showed that restraint-stress results in loss of anterior-pituitary CRF receptors but that this did not effect the release of ACTH in response to another acute stressor. During chronic stress the pituitary could become hypersensitive to AVP.

### 3.2.2. Pituitary-adrenal axis

Glucocorticoid secretion from the adrenal is under the primary influence of adrenocorticotrophic hormone (ACTH), synthesized and released from the pituitary. Synthesis and release of corticosterone is stimulated by ACTH. Corticosterone levels in adrenal effluent blood increase 250% following an intravenous ACTH injection. The response is realised in 2-3 min but is not maximal until 15-30 min (Harvey *et al*., 1980). The ACTH is synthesized as part of the larger protein, proopiomelanocortin (POMC). After post-translation processing, POMC yields ACTH and ß-Lipotrophin which is further processed to ß-Endorphin and alpha-melanophore-stimulating-hormone ( MSH). As with mammalian ACTH, the avian equivalent is a simple polypeptide of 39 amino acids (Li *et al*., 1978). Ostrich ACTH differs from mammalian ACTH at positions 15,27,28,29,31 and 32 (Li *et al*., 1978). ACTH is synthesized by cells in the cephalic lobe of the anterior pituitary (Yamashiro *et al*., 1984).

There are also non-pituitary sources of ACTH or ACTH-like materials (Krieger, 1983). Rodent leukocytes (Smith *et al*., 1982) and non-stimulated avian leukocytes (Siegel *et al*., 1985) secrete ACTH-like material and this increases following antigen stimulation. Avian leukocytes are stimulated by CRF *in vitro* to produce assayable ACTH (Hendricks *et al*., 1995).

## 3.3. Regulation of aldosterone secretion

In mammals, aldosterone secretion is controlled by the renin-angiotensin system. Renin from the kidney capsules is released in response to low sodium or low blood volume. Renin in turn acts to stimulate the conversion of angiotensin-1 to angiotensin-II. It is angiotensin-II that acts to stimulate aldosterone release from the adrenal. There is evidence that the same system operates in birds (Radke *et al*., 1984). In birds atrial natruretic peptide (ANP) released from the heart is also involved in the regulation of aldosterone release. There is also evidence that ACTH stimulates release.

## 3.4. Corticosteroid transport

Glucocorticoids are transported in the blood bound to protein carriers in a reversible association. Corticosterone is bound to a specific binding protein, corticosterone-binding globulin (CBG) or to non-specific-binding protein (probably albumen). Corticosterone-binding globulin, has high-affinity low binding capacity whereas the non-specific-binding protein has high capacity but low binding affinity (Wingfield *et al*., 1984). Circulating concentrations of CBG are determined by endocrine status, most likely through an influence on liver synthesis (Kovacs and Peczely, 1983). Normally CBG is around 50-80% saturated and only requires small amounts of corticosterone to become fully saturated. A small increase in steroid blood concentration will increase the free circulating concentration greatly. Binding to the protein is probably important in controlling availability and in

stabilization of the free blood concentration. In contrast to mammals, avian CBG has affinity for dexamethasone (Gould and Siegel, 1978) and this could account for the different metabolic effects of dexamethasone seen for the two groups.

**3.5 Glucocorticoid receptors:**

Corticosterone receptors have been found in many body tissues (Hylka and Doneen, 1983: Bellabarb *et al*., 1983; Tu and Moudrianakis, 1973). The receptors are of the steroid/thyroid hormone nuclear receptor type which control differential gene expression by enhancing specific gene expression (Evans, 1988; Beato, 1989). There are two types of receptors in the CNS. Type-1 receptors bind aldosterone, corticosterone, cortisol and deoxycorticosterone equally *in vitro*. Type-11 receptors bind dexamethasone, cortisol, corticosterone, deoxycorticosterone and aldosterone with decreasing affinity (Reul and de Kloet, 1985). Type-1 receptors are thought to regulate basal CRF gene expression at the nadir of diurnal ACTH secretion (Dallman *et al*., 1987) and regulate peak ACTH secretion (Dallman *et al*., 1989). Type-11 receptors are involved in stress-induced ACTH release (Reul and de Kloet, 1985). In mammals the receptor is a polypeptide chain folded into three distinct domains; the carboxyl-terminal domain binds to the hormone, the middle domain binds DNA and the amino terminal end activates gene transcription. Most of the activated receptors bind to sites which do not initiate transcription (Alberts *et al*., 1989).

Selectivity of gene expression is achieved by restricting the expression of different receptors in specific cells. Different sets of genes in different cells will be accessible to the hormone receptor complex (Evans, 1988). Transcription of specific mRNAs result in proteins which elicit the specific cellular response attributed to corticosterone. The duration of the corticosterone response is determined in part by the rate of degradation of the receptor /hormone complex.

The hippocampus contains moderate to high glucocorticoid receptor numbers (De Kloet *et al*., 1987). In rodents changes to glucocorticoid receptor occupancy leads to altered interpretation of the environment and could determine an animals emotional state and its adoption of a coping strategy to stress (Korte *et al*., 1995; 1996).

**3.6. Factors stimulating corticosterone release**

Any stress imposed on animals and perceived as a threat will stimulate corticosterone release. Corticosterone release is thought to be a non-specific stress response. Factors reported to cause corticosterone release are feed and water deprivation (Scanes *et al*., 1980), heat or cold (Freeman and Manning, 1982; Edens and Siegel, 1975), infection (Curtis *et al*., 1980), restraint (Beuving and Vonder, 1978) and fear (Harvey *et al*., 1985).

### 3.7. Clearance and metabolism

Basal concentrations of plasma corticosterone for chickens range from 0.4-12 ng/ml using radioimmunoassay (Radke *et al*., 1984: Satterlee *et al.*, 1980). Corticosteroids are cleared from the circulation with a simple exponential decay. Estimates of the half-life of corticosterone range from 10-22 min for different species of domesticated birds (Birrenkott and Wiggins, 1984; Kovacs and Peczely, 1983: Thomas and Phillips, 1975). The liver is the main site of degradation.

### 3.8. Diurnal rhythm

Plasma corticosterone and CBG levels show a distinct diurnal rhythm (Beuving and Vonder, 1977; Kovacs and Peczley, 1983: Wilson *et al.*, 1982) with CBG levels lagging approximately 4 h behind (Siegel *et al*., 1976). Maximum concentration is observed at the end of the dark, start of the light, periods. The rhythm relates to changes in CRF, ACTH and hypothalamic activity. Shifts in the diurnal pattern occur in response to reproductive and nutritional status and in hens also to the ovulatory cycle (Wilson *et al*., 1982: Wilson and Cunningham, 1981). There are also seasonal variations in the pattern of corticosterone secretion. In birds generally this pattern is influenced by such factors as feed supply, weather patterns, migration and territorial behaviour.

### 3.9. Feedback regulation and habituation

Plasma corticosterone regulates brain corticosterone receptor numbers (Sapolsky *et al*., 1984) and feedbacks to regulate synthesis and release of CRF and ACTH (Kamstra *et al*., 1983: Vale *et al*., 1983; Sapolsky *et al*., 1984). Corticosterone also acts on the adrenal to decrease it's responsiveness to ACTH and on the liver to depress CBG synthesis (Etches, 1976; Malek, 1981).

The habituation of the corticosterone response that occurs in response to prolonged stress, heat (Siegel and Latimer, 1984), cold (Siegel and Latimer, 1970), underfeeding (Freeman *et al*., 1981) and exercise (Rees *et al*. 1983), could be accounted for by this feedback mechanism. Avian corticosterone response does not show habituation to exogenous ACTH (Rees *et al*., 1983). So adaptation to prolonged stress may result from a decrease in ACTH release, a consequence of habituation of the central nervous system and the control it has over CRF release. Adaptation probably increases the threshold required for a particular stimuli to elicit ACTH release. There are probably situations where there is adrenal compensation and stress-induced ACTH release results in an exaggerated adrenal response to a persistent stress or another unfamiliar stressor (Vernikos-Danellis, 1965). This could be the situation where there is continued elevation in plasma corticosterone (Beuving and Vonder, 1978) or adrenalin (Freeman and Manning , 1979) following handling stress.

The response from the adrenal can be modified by experience (Mason, 1971; Dantzer and Mormede, 1983). It is possible that an animal can develop an expectancy and cognitive

appraisal. Failure of expectancy may stimulate the HPA. For hens food deprivation results in elevated plasma corticosterone, however the response is greater in hens previously fed *ad libitum* than in hens fed intermittently (Rees *et al*., 1984). The involvement of the nervous system is critical to the intensity of the adrenal response. Physical stressors are unable to activate the HPA if emotional stimulation is absent. Since responses to stress are related to behavioural changes, then habituation of nervous input stimulus could be responsible in part, for suppressed adrenal function.

Neonatal exposure to thermal stressors appears to improve tolerance to heat in later life (Reece *et al*., 1972; Arjona *et al*., 1988). Acclimatising chickens to high temperatures later in life can also be achieved by exposing them to feed restriction during neonatal growth (Zulkifli *et al*., 1994a). Does this habituation occur without the presence of the glucocorticoid during the initial stress? This is a question that Zulkifli and colleagues (1994b) have attempted to answer. In their work, neonatal chickens were feed 60% *ad libitum* and were then treated with or without metyrapone. Fasting increases corticosterone in fowls (Zulkifli *et al*., 1993) and metyrapone inhibits the conversion of deoxycorticosterone to corticosterone (Dominguez and Samuels, 1963). For those chickens treated with metyraprone later growth under heat stress was compromised. Also, those not treated with metyraprone had superior disease resistance. The authors concluded that short-term disruption to homeostasis during the neonatal stage without any associated change in corticosterone levels may not be helpful to the animal in subsequent responses to stressors. Their suggestion is that corticosterone plays a role in habituation and prepares an animals for later disruptions to homeostasis.

There is some evidence that habituation can effect growth and egg production. Egg production is depressed by handling hens but only for those hens not accustomed to the procedure (Hughes and Black, 1967). Twice a day handling during the three weeks of brooding, increased growth rate in broilers and in female layer chicks but not male layer chicks (Jones and Hughes, 1981). Similar effects were reported by Thompson (1976) but not McPherson *et al*., (1961) and Reichman *et al*., (1978). However, in the later two studies, chicks were only handled once weekly. Irregular handling could be detrimental to growth whereas regular handling may enhance the chicks ability to cope with novel stresses.

Adaptation to the caretaker increases antibody response in hens (Gross and Siegel, 1979). The authors attributed this effect to a decrease in corticosterone. Heat exposure for 1 h increases corticosterone levels in lymphatic tissue (Siegel and Gould, 1982), however, the levels decreased over seven subsequent exposures to heat. A suggested reason for this could be, that prior stress increases the corticosterone binding to hypothalamic receptors and that this acts as a negative feedback to suppress CRF secretion (Davidson *et al*., 1968).

# 4. Hormones of the chromaffin cells

## 4.1. Biosynthesis of catecholamines

The synthesis of catecholamines, noradrenaline and adrenalin is shown in Figure 3. L-tyrosine, an amino acid which is derived from the diet or from the essential amino acid L-phenylalanine, is the precursor of the catecholamines. Hydroxylation of phenylalanine to tyrosine occurs in the liver, catalysed by phenylalanine hydroxylase. The oxidation of tyrosine to dopa (3,4-dihydroxyphenylalanine) occurs in the adrenals and is catalysed by tyrosine hydroxylase which resembles phenylalanine hydroxylase and uses the same co-factors. The next step is the decarboxylation of dopa to hydroxtryramine (3,4-dihydroxphenylethylamine) under the catalytic influence of aromatic L-amino acid decarboxylase. Hydroxtryramine is converted to noradrenaline under the influence of the enzyme  -hydroxylase. Methylation of noradrenaline to adrenalin is catalyzed by phenylethanolamine-N-methyl transferase. In the synthesis of the adrenal medullary hormones, hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine is the rate limiting step.

## 4.2. Control of catecholamine release

Noradrenaline is an adrenergic neurotransmitter and is synthesized and stored in nerve endings. When released it acts locally on adrenergic receptors and does not normally enter the plasma in sufficient quantities to act as a circulating hormone. Noradrenaline is released from all post-ganglionic nerve terminals but the response differs according to the type of adrenergic receptor present and post-receptor events. Tissue response will depend of the relative numbers of receptors present. Adrenalin is a circulating hormone released from the adrenal is response to pre-ganglionic impulses in the splanchnic nerves. Both the adrenal and peripheral nerves are controlled by pre-ganglionic sympathetic nerves originating in the spinal chord.

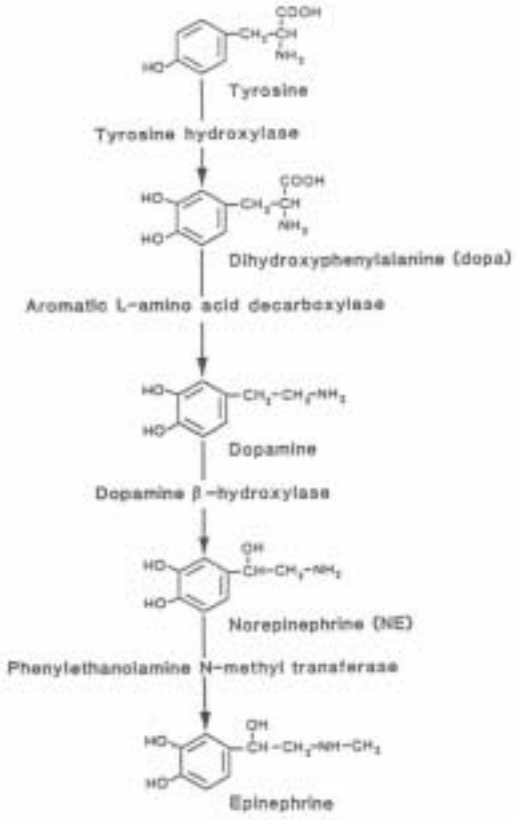


**Figure 3: The biosynthetic pathway of adrenalin (epinephrine) and noradrenaline (norepinephrine)**

The chromaffin cells, homologous to neural tissue, contain adrenalin and noradrenaline in granules or membrane-bound vesicles. Adrenalin and noradrenaline are synthesized in distinct cell types, the proportion of each varies with different bird species (Gosh, 1980). The association of chromaffin cells with cortical tissue is a development in phylogeny. In mammals the tissues are divided into two distinct areas, the cortex and the medulla. Further down the evolutionary scale there is an increasing level of randomization of the two tissues. In birds the chromaffin cells are distributed fairly evenly within the cortical tissue. Chromaffin cells are found in many parts of the body and are associated with sympathetic nerves, being in close contact with nerve endings. Stimulation of the sympathetic nervous system results in release of catecholamines from the chromaffin cells. The response is apparently unrelated to the severity of the stress imposed (Jurani *et al.*, 1980: Lahiri, 1982). The catecholamines can be released relative to their individual adrenal concentrations or adrenalin can be released preferentially because the conversion of noradrenaline to adrenalin can take place by the induction of phenylethanolamine-N-methyltransferase (PNMT). The chromaffin cells are also influenced by blood-borne factors and hormones.

Factors stimulating catecholamine release are varied. Environmental stressors such as heat and cold (Lin and Sturkie, 1968), restraint (Zachariasen and Newcomer, 1975) and forced exercise (Rees *et al*., 1984) can be potent stimulators of release. There are also many chemical and hormonal stimuli including insulin (Pittman and Hazelwood, 1973), ACTH (Zachariasen and Newcomer, 1974), corticosterone (Zachariasen and Newcomer, 1975) anaesthesia (Sturkie *et al*., 1970), and propranol (Nishumura *et al*., 1981).

**4.3. Catecholamine receptors**

Adrenalin and noradrenaline interact with specific cell surface receptors. Adrenergic receptors were first classified as either being of or ß subtypes and were identified according to adrenergic agonist effects on smooth muscle, contraction ( -effect) or relaxation (ß-effect) (Alquist, 1948). The response in the tissue will depend on the relative numbers of each receptor type and the ability of the agonist to interact with these receptors. More recent work has established that there are three distinct types of ß-receptors, these being named $ß_1$, $ß_2$ and $ß_3$ (Caron and Lefkowitz, 1993). There are two distinct classes of -receptors, $_1$ and $_2$ (Lands *et al*., 1967) and within these classes, three distinct types of $_1$ and $_2$ receptors (Caron and Lefkowitz, 1993).

Activation of adrenergic receptors and membrane signalling events involves three components, the receptor with its seven transmembrane segments, a guanine nucleotide regulatory protein (G protein) and an effector component that can be either adenyl cyclase or an ion channel ($K^+$ or $Ca^{2+}$) (Lefkowitz and Caron, 1988: Casey and Gilliman, 1988). The N-terminus end of the receptor is on the extracellular side and contains the catecholamine binding site with glutamate and aspartate residues forming part of the binding site. Activation of the receptor leads to an interaction with it's appropriate G protein, this promotes nucleotide exchange and activation of the G protein, this in turn modulates activity of the effector system. Both - and ß-receptors bind ligands with different pharmacological profiles and are coupled to different signal transduction mechanisms. Stimulation of $_1$-receptors and $ß_1$-receptors is by noradrenaline released from sympathetic nerve endings while $ß_2$ and $_2$ are stimulated by adrenalin released from the adrenal gland.

The $ß_1$ and $ß_2$ receptors are linked to the effector, adenyl cyclase via the transducer Gs-protein and results in increased cAMP. The $_1$ receptors activate phospolipase-C through a transducer. Phospolipase acts on phospoinositides to catalyse formation of diacylylglycerol and inositol triphosphate. The diacylglycerol activates protein kinase C which initiates phosphorylation of intercellular proteins. Inositol triphosphate promotes release of calcium ($Ca^{2+}$). The $_2$ receptors act to inhibit adenyl cyclase acting via protein transducer Gi.

# 5. The effects of corticosterone

## 5.1. General effects on metabolism

Corticosterone administration increases food intake, suppresses growth rate, increases carcase and liver fat levels (Baum and Meyer, 1960; Nagra and Meyer, 1963; Freeman and Manning, 1975; Bartov *et al*., 1980; Bartov, 1982). Some of these effects are seen after as little as 4 days of treatment (Bartov, 1982). Corticosterone increases plasma glucose and liver glyconeogenesis in birds (Snedecor *et al*., 1963: Stamler *et al*., 1954). The glucose is largely derived from protein catabolism (Nagra and Meyer, 1963) and the shift in metabolism favours fat deposition as there is an increases in plasma free fatty acids (FFA) and an increase in the saturated fat to unsaturated fat ratio (Nagra and Meyer, 1963).

Growth of broilers is impaired in a dose-dependent manner by corticosterone administration (Saadoun *et al*., 1987; Tur *et al.*, 1989; Siegel *et al*., 1989). The depressed growth rate in birds (Davison *et al*., 1983: Siegel and Van Kampen, 1984) is often in spite of an increase in feed intake (Bartov *et al.*, 1980: Siegel and Van Kampen, 1984). There are a least three factors contributing to the decreased growth rate (Siegel and Van Kampen, 1984). Firstly there is an increase in protein catabolism as indicated by increased nitrogen excretion, uric acid excretion and water intake. Secondly, a reduction in absorptive efficiency as indicated by similar energy retention but increased food intake. Thirdly, an increase in energy retention in the form of fat.

### 5.1.1. Effects on carbohydrate metabolism

Corticosterone is involved in glucose homeostasis and carbohydrate metabolism. A prominent action of corticosterone is to promote glucogenesis from non-carbohydrate sources such as amino acids and fatty acids (de La Cruz *et al*., 1981). For chickens, corticosterone administration results in hyperglycaemia and glycogenolysis in liver and muscle (Joseph and Ramachandran, 1992). Elevated plasma glucose levels (Saadoun *et al*., 1987; Simon, 1984) and increased hepatic glucose-6-phosphate activity (Joseph and Ramachandran, 1992) following corticosterone treatment supports a role in gluconeogenesis. An increase in muscle and liver levels of phosphorylase after corticosterone treatment (Joseph and Ramachandran, 1992) suggests that some of the effects are glucagon mediated. There is evidence that corticosterone influences glucagon release (Macro *et al*., 1972) and action (O'Neil and Langstow, 1978) as well as antagonizing insulin action (Natarajan *et al*., 1987). So the effects on carbohydrate metabolism could be related to changes in the insulin/glucagon ratio.

Dexamethasone, a synthetic corticosteroid, inhibits ACTH release in mammals (Carnes *et al*., 1987) and chickens (Smoak and Birrenkott, 1986). Adrenocortical insufficiency caused by dexamethasone treatment has the opposite effects to those resulting from adrenocortical

excess as seen by giving exogenous corticosterone (Joseph and Ramachandran, 1992). Thus in chickens dexamethasone does not mimic corticosterone action on carbohydrate metabolism as it does in mammals (Harrelson and McEwen, 1987).

### 5.1.2. Effects on lipid metabolism

Exogenous corticosterone is a potent lipogenic agent in chickens (Nagra and Meyer, 1963; Bartov, 1982). A similar effect has been reported for endogenous corticosterone (Saadoun *et al*., 1986). When hens belonging to a fat or lean selection line were treated chronically with corticosterone there was a dose-dependent decrease in body weight and increases in abdominal and liver fat (Bartov, 1982; Simon 1984; Wiliiams *et al*., 1985; Saadoun *et al*., 1987). There was no significant differences in the way both lines responded. Liver concentrations of triglycerides and cholesterol were elevated and phospholipids lowered (Saadoun *et al*., 1987). There was no effect on food intake and the increase in fattening is attributable, in the main, to an increase in protein catabolism as is suggested by elevated plasma uric acid levels in corticosterone-treated hens. Changes in circulating levels of insulin, glucose and growth hormone (GH) were also observed, however the changes were dependent on the corticosterone level and whether given during feeding or upon refeeding.

Corticosterone has a duel effect on insulin release, an immediate inhibitory effect and a longterm stimulatory effect (Simon 1984). A dose of 5 mg/d corticosterone increased blood glucose and insulin, suggesting insulin resistance or corticosterone-induced glucogenesis (Saadoun *et al*., 1987). The elevated insulin could account for the observed decrease in GH seen by Saadoun *et al*., (1987). Exogenous insulin depress GH in fasted chickens (Picaper *et al*., 1986).

For broilers injected with 0.5 mg/kg corticosterone for 5 days, significant changes in energy utilization were observed (Siegel and Van Kampen, 1984). Growth rate in corticosterone treated birds was lower for the first 3 days but similar on days 4 and 5 compared to untreated birds but food intake was greater. Energy intake was higher in the corticosterone-treated broilers as was energy excretion, resulting in lower efficiency when estimated on an energy-intake basis. While gross energy retention was greater in corticosterone treated broilers the consequence was an increase in fat deposition as indicated by the change in respiratory quotient (RQ). The pretreatment RQ was 0.88 which remained unchanged in the control but increased to 1.0 after 4-5 days of corticosterone treatment. An increase in RQ is indicative of an increased rate of fat deposition (Dukes, 1958). Fat deposition requires more energy than protein deposition, 65.4 kJ/g vs 32.4 kJ/g (Kielanowski, 1965). This would in part be the reason for the increased food intake because there is no increase in absorptive efficiency (Siegel and Van Kampen, 1984).

Abdominal fat pad weight is increased following corticosterone treatment (Siegel *et al*., 1989; Akiba *et al*., 1992; Hayashi *et al.*, 1994). The reported decrease in thyroxine (T4) following feeding of corticosterone (Hayashi *et al*., 1994) could be related to the fat changes as there is a relationship between metabolism, fat deposition and T4. When T4 is administered to chickens on diets with a high grain content there is a decrease in the abdominal fat weight and triiodothyronine (T3) levels are significantly depressed (Suthama *et al*., 1991). Adding trilostane (steroid synthesis inhibitor) to the diet overcame the effects on protein breakdown observed when corticosterone is given (Hayashi *et al.*, 1994). Trilostane itself, had some suppressive effects on protein synthesis, which might suggest that a low level of corticosterone could be required for maximum protein synthesis. Growth responses of adrenalectomized rats administered cortisol depend on the level given. Low levels promote growth while high levels induce weight loss (Waterlow *et al*., 1978).

### 5.1.3. Effects on protein metabolism

For production animals changes in protein metabolism are apparent after chronic stress (Klasing and Austic, 1984a,b; Imms, 1967; Richards, 1980; Beisel, 1977). The manifestations are retarded growth rate in the young and loss of body weight in adults. The weight loss can be in body water, fat or protein. In many cases these effects are not the consequence of a decreased feed intake but a decrease in feed utilization. In birds, protein catabolism is indicated by increased uric acid excretion (Adams 1968), this in turn results in increased urine flow and water intake. Corticosterone treatments increase water intake by up to six fold (Siegel and Van Kampen, 1984). High corticosterone concentrations cause atrophy of skeletal muscle (Bartov, 1985). When glucocorticoids are administered to animals the effects on protein metabolism are similar to those seen for stress; atrophy of skeletal muscle and lymphoid tissue and decreased body weight (Rousseau and Baxter, 1979). Adrenalectomy reduces loss of muscle protein during many types of stress, suggesting a role for glucocorticoids in the loss of muscle protein (Odedra and Millward, 1982).

Changes in protein metabolism reflect changes in the relative rates of protein synthesis and degradation (Tomas *et al*., 1979; Hayashi *et al*., 1992). Skeletal muscle protein degradation is increased in a dose-dependent manner by feeding corticosterone but there is no effect on protein synthesis (Hayashi *et al*., 1994). Corticosterone acts at the liver to increase glucose production and induce synthesis of enzymes involved in amino acid catabolism (Kenney, 1969). Varying reports indicate that net liver protein synthesis increases or remains unchanged (Chertow *et al*., 1973; Millward *et al*., 1976; Kim and Kim, 1975). In addition, corticosterone acts to amplify the actions of other hormones involved in protein metabolism. Psychological stress increases catecholamine and glucagon release (Bloom *et al*., 1972; Freeman and Manning, 1976). Glucagon will depress liver protein synthesis (Woodside *et al*., 1974).

Inflammation and infection are accompanied by large changes in protein metabolism and the pattern of change is similar irrespective of the agent. The major change is the mobilization of amino acids from skeletal muscle to the liver (Beisel, 1977; Richards, 1980). In general there is a decrease in plasma amino acids as they are utilized for energy, gluconeogenesis and protein synthesis in the liver (Wannemacher, 1977). In chickens, the amino acids levels in liver, spleen and plasma decrease and levels in muscle and thymus increase (Klasing and Austic, 1984b).

**5.2. Corticosterone effects on reproduction**

Ovarian regression is associated with increased plasma corticosterone (Etches and Williams, 1983; Etches *et al.*, 1984: Moudgal *et al.*, 1991). The rate of ovulation is also interrupted in a dose-dependent manner (Moudgal *et al.*, 1991) by corticosterone. Corticosterone injections will delay oviposition with higher doses needed towards the period of peak egg production, around 35-40 weeks of age. There are reports showing that corticosterone treatment induces ovulation (Etches and Cunningham, 1976: Etches and Croze 1983). Injections of ACTH will induce ovulation (Etches and Cunningham, 1976) and LH release (Wilson and Sharp 1976a; Etches and Croze, 1983), however, the levels needed are outside the normal physiological range.

The true effects of corticosterone can only be assessed by giving a constant infusion over an extended period. A normal physiological range (mean plasma level of around 3 ng/ml) can be maintained by subcutaneous infusion of 30 ug/h of corticosterone (Etches *et al.*, 1984). When this level of corticosterone was infused into hens, egg production dropped by day 3 and completely ceased by day 8 (Etches *et al.*, 1984). Similar effects can be achieved by dietary restriction or feeding diets deficient in calcium or sodium (Douglas *et al.*, 1972; Summers and Leeson, 1977; Whitehead and Sharp, 1976; Williams *et al.*, 1985). These dietary effects may be mediated by changes in corticosterone as levels increase with a reduction in food intake (Etches *et al.*, 1984).

There is a suggestion that the nutritionally-induced effects on egg production are the consequence of changes at the level of the pituitary. Reduction in food intake causes an immediate lowering of plasma LH, progesterone and oestradiol (Tanabe *et al.*, 1981). Nutritionally-induced ovarian regression is also associated with a greatly reduced pituitary responsiveness to LHRH which is not the case in corticosterone-induced ovarian regression (Etches *et al.*, 1984). Both oestradiol and progesterone (Bonney and Cunningham, 1976; Wilson and Sharp, 1976b) are required to maintain LH release. After the infusion of corticosterone, ovarian regression is associated with a decrease in progesterone, oestradiol and LH (Etches *et al.*, 1984) with the sequence of hormonal changes suggesting that corticosterone acts directly at the ovarian level.

In the study of Etches and colleagues (1984) there were differences but not significant differences in the total number of ovarian follicles in corticosterone-infused hens compared

to control hens (Etches *et al*., 1984). However, there was a major difference in the distribution of follicle sizes. There were more atretic follicles in the corticosterone treated hens and a smaller number of large follicles. Although there are less large follicles in corticosterone-treated hens the total number of follicles is maintained by recruitment of more small follicles.

Adrenalin induces follicle atresia *in vitro* (Moudgal *et al*., 1985) and *in vivo* (Moudgal *et al*., 1990a) and affects ovulation rate (Moudgal and Razdan, 1981). Adrenalin also delays oviposition (Sturkie, 1976), however this effect is dependent on the dose and stage of the laying cycle (Moudgal *et al*., 1990a). Any effects corticosterone has on reproduction could be mediated through its effects on catecholamine synthesis. Corticosterone stimulates the conversion of noradrenaline to adrenalin by inducing PNMT. When alpha-methyl-p-tyrosine, a blocker of catecholamine synthesis, is administered with corticosterone any detrimental effects corticosterone has on ovulatory events are prevented (Moudgal *et al*., 1991).

The daily rhythm of plasma corticosterone in laying hens (Etches, 1979) can be influenced by ovulatory factors. Corticosterone is elevated at the time of oviposition (Beuving and Vonder, 1978). Levels are depressed at night when preovulatory surges of progesterone, oestradiol and LH occur (Wilson and Cunningham, 1981). Progesterone has been reported to inhibit corticosterone release.

### 5.3. Corticosterone effects on the immune system

The immune system consists of immunocompetent cells and accessory cells. Accessory cells are phagocytic or adherent cells. In birds, immunocompetent cells are those derived from the thymus (T-cells) and those from the bursa of Fabricius (B-cells). Differentiation of T-lymphocytes gives rise to subpopulations of T-helper cells, T-suppressors cells and T-cytotoxic cells and all function within the humoral and cell-mediated components of immune responses. Acting as effectors to modulate immune function, they produce soluble lymphokines, known as interleukins or interferons, that regulate B- and T-cell proliferation and macrophage integration (Lillehoj *et al*., 1992). The B-cells differentiate in the bursa and are the cells responsible for antibody (Ig) production.

It is a widely held view that the immunosuppressive effects of stress are mediated by glucocorticoids. Glucocorticoid receptors are located on lymphocytes and monocytes and the number increase in response to immune stimulation (Comsa *et al*., 1982). Stress increases the incorporation of corticosterone into lymphoid cells (Gould and Siegel, 1981), inhibiting glucose uptake, protein synthesis and causing lymphocytosis, resulting in decreased cell number and antibody production. Immunosuppression has been reported after stress or ACTH injections (Thaxton and Siegel, 1970; 1973: Siegel 1987). Pharmacological doses of corticosterone can suppress cell-mediated immunity (Gross *et al*., 1980; Edens *et al*., 1983).

Regression of thymus, spleen and bursa occurs following ACTH or corticosterone injections (Siegel and Beane, 1961; Siegel, 1962).

The effects of environmental stress on the lymphatic tissues are similar to the effects of ACTH. Corticosterone infusion decreased thymus weight by 71% bursa and spleen weight by 57% and 35% respectively (Donker and Beuving, 1989). These changes in tissue weight are consistent with the idea that stressors and corticosterone have catabolic effects on lymphoid organs (Selye, 1976). Leghorn hens raised in crowded conditions show evidence of chronic stress and this is associated with increased adrenal weight and lymphatic tissue regression (Siegel, 1960).

Hens in stressful environments produce lower antibody response to a variety of antigens (Gross, 1972; Gross and Siegel, 1975; Thompson et al., 1980; Edens *et al*., 1983). Corticosterone causes a reduction in lymphocyte number (Davison and Flack, 1981) and an increased susceptibility to disease (Gross *et al*., 1980). After challenging hens with Marek's disease the severity is increased by continuous corticosterone infusion (Powell and Davison, 1986). When cockerels from lines selected for a high or low antibody titre to sheep red blood cells (SRBC) (Vander Zijpp and Nieuvland, 1986) were infused continuously with corticosterone to physiological levels (Webb and Mashlay, 1985; Beuving and Vonder, 1986) and then challenged with SRBC there was a suppression in total antibody production for the high line (Donker and Beuving, 1989). For the two lines there was a consistent difference in response to corticosterone suggesting that there was no genotype/environmental interaction which could be important when considering selection programs based on immune responsiveness. Davison and Misson (1987) reported a dose-dependent depression in titres to SRBC following subcutaneous corticosterone treatment. Injecting chickens with SRBC increases corticosterone secretion (Siegel *et al*., 1985). It has been suggested that stimulated leucocytes secrete ACTH and that this is the reason for the increase in corticosterone (Trout *et al*., 1988).

Within 12 h of heat treatment or ACTH injections antibodies to 3 specific antigens decreased (Thaxton and Siegel, 1972). Pretreatment with suppressor corticosterone compounds modified this effect (Siegel and Latimer, 1974). Exposure to heat decreases cell-mediated immunity in hens (Regnier and Kelley, 1981) as will a decrease in actual body temperature (Siegel, 1971). Any reduction in antibody production could increase the susceptibility to disease.

A feature of corticosterone is it's anti-inflammatory action. In general, inflammation is a defence mechanism involved in increasing blood supply and leucocytes at a site of injury. Inflammation is not always desirable, as in arthritic conditions, and corticosterone’s anti-inflammatory effects are beneficial in such conditions. There could be an internal feedback mechanism where activated lymphocytes secrete cytokines which act to increase

corticosterone release which then acts to suppress lymphocyte activity (Munck *et al*., 1984). This could be a mechanism to help maintain homeostasis.

The observation that in response to stress, adrenalectomized rats suffer immunosuppression, strongly suggests the involvement of factors other than the glucocorticoids (Keller *et al*., 1983). The releasing factor CRF has been implicated (Jain *et al*., 1991). Stress in adrenalectomized or normal rats decreases T-cell proliferation and natural killer cell cytotoxicity (Jain *et al*., 1991). These effects can partly be suppressed by administration of CRF antibodies or antagonists. Local effects of CRF could be important in inflammation as administering CRF antibodies reduces inflammatory responses and CRF is found in the area of inflammation (Karalis *et al*., 1991).

Activation of the immune system results in the release of cytokines. Interleukin-1 (IL-1) released from activated macrophages and monocytes stimulates T-cell proliferation, antibody production and causes fever. Interleukin-1 increases plasma corticosterone and/or ACTH (Besedovsky *et al*., 1986; Ovadia *et al*., 1989; Matta *et al*., 1990; Harbuz *et al*., 1992) and stimulates CRF release (Sapolsky *et al*., 1987; Navarra *et al*., 1991). With the gross effects of corticosterone being to suppress immune cell function, the ability of IL-1 to influence corticosterone release could act as a feed back mechanism to control the immune response. Interleukin-2 and interleukin-4 stimulate and inhibit PMOC mRNA levels respectively (Brown *et al*., 1987; Harbuz *et al*., 1992). Interleukin-6 (IL-6) increases ACTH release (Lyson and McCann, 1991) and could have a centrally-mediated effect as it evokes CRF release from hypothalamic explants (Navarra *et al*., 1991).

### 5.4. Corticosterone and psychological stress

In general, egg production decreases (Dorminey *et al*., 1972; Roush et al., 1984) and mortality increases (Marks *et al*., 1970: Grover *et al*., 1972) as cage density increases (Dorminey *et al*., 1972). Corticosterone levels are elevated as cage density is increased (Mashaly *et al*., 1984). A similar effect was reported for broilers where increased floor density elevated corticosterone (Pesti and Howarth, 1983). Adrenal weight has been used as a measure of stress and adrenal weight has been reported to increase as stocking density increases (Siegel, 1980). It has been suggested that these responses are to psychological stress.

# 6. The effects of catecholamines

## 6.1. Catecholamine effects on metabolism

### 6.1.1. Effects on carbohydrate metabolism

Catecholamine levels are raised following exercise (Rees *et al*., 1984), blood sampling (Rulofson *et al*., 1988) and immobilization (Zachariasen and Newcomer, 1974; 1975). Plasma catecholamine levels are closely associated with glucose (Cramb *et al*., 1982) and lipid (Campbell and Scanes, 1985) metabolism. Noradrenaline and adrenalin promote hyperglycaemia and this is associated with increased glycogenolysis, increased gluconeogenesis and decreased glucose utilization by peripheral tissues, with adrenalin being more potent than noradrenaline. Both hepatic and muscle rates of glycogenolysis increase following activation of the ß$_2$-adrenergic receptors (McDowell and Anninson, 1991). Activation of ß-adrenergic receptors on hepatocytes by catecholamines, stimulates adenylate cyclase leading to increased cAMP levels which inturn activate protein kinase, resulting in phosphorylation of phosphorylase kinase and glycogen synthetase. Phosphorylase kinase enhances glycogen breakdown. Adrenalin stimulates hepatic gluconeogenesis by increasing the availability of substrates, lactate and glycerol, these being generated during lipolysis. Catecholamines are also responsible for a decrease in the metabolic clearance rate of glucose (Himms-Hagen, 1967).

Catecholamines influence insulin and glucagon release. They act to inhibit insulin release, an effect mediated via  2-adrenergic receptors on the ß-pancreatic cells (Clutter *et al*., 1980) and this is independent of blood glucose levels. Activation of ß$_2$-adrenergic receptors stimulates insulin release (Remie *et al*., 1989). Both adrenalin and noradrenaline stimulate glucagon release (Steffens and Strubble, 1983). Glucagon is released from the pancreatic  -cells and acts to stimulate hepatic glycogenolysis and gluconeogenesis. Adrenalin has similar effects as does glucagon but is less potent. Adrenal and noradrenaline are released in response to the flight or fright syndrome, where as glucagon is released in response to nutritional stress. Because of the effects on insulin and glucagon, catecholamines alter the insulin/glucagon ratio. A decrease in the ratio results in mobilization of metabolic fuels such as glucose and FFA's. Basal levels of catecholamines but not transient levels associated with activity (ie oviposition) are closely related to FFA concentrations.

### 6.1.2. Effects on lipid metabolism

In domestic fowl, lipogenesis occurs in the liver with the adipose tissue functioning primarily for lipid storage and mobilization (Goodridge and Ball, 1967; O'Hea and Leveille, 1968; 1969). In most mammals, adipose tissue is the  major site of fatty acid synthesis. Hormonal regulation of lipogenesis is also different in mammals and birds with glucagon the most potent lipolytic agent in chickens (Goodridge, 1973).

The sympathetic nervous system is the main regulator of lipolysis in man (Webber and Macdonald, 1993) and sheep (Bassett, 1970). Adipocytes have both ß-adrenergic (stimulatory) and -adrenergic (inhibitory) receptors. Stimulation of the ß-adrenergic receptors by catecholamines activates GTP-binding protein which acts to stimulate adenylate cyclase resulting in an increase in cAMP and this stimulates protein kinase A to phosphorylate hormone-sensitive lipase which results in the release of FFA's and glycerol from stored triglycerides. Growth hormone and glucocorticoids will also stimulate adipocyte ß-adrenergic receptors. Growth hormone could act to increase B-adrenergic receptor number (Vernon, 1992). In ruminants, catecholamines inhibit lipogenesis (Vernon, 1980) which is associated with decreased acetyl CoA carboxylase activity, and stimulate lipolysis (Blum *et al*., 1982; Mersmann *et al*., 1974).

Mobilization of fat stores involves lipolysis and changes in blood supply. Sympathetic innervation of white adipose tissue is mainly of the vasculature and not the adipocytes (Fredholm, 1985). Stimulation of the -adrenergic receptor causes vasoconstriction and the ß-adrenergic receptor causes vasodilatation. Catecholamines act mainly on the adipocytes while the neural effects act on blood supply. Lipolytic effects are regulated via the $ß_1$-receptors and vasodilatation by the $ß_2$ receptors (Lands *et al*., 1967) with noradrenaline acting on the ß1 and adrenalin on the $ß_2$ receptors. This would suggest that the effects on lipolysis are largely due to noradrenaline and vasodilatation effects due to adrenalin.

In isolated chicken hepatocytes adrenalin inhibits lipogenesis in a dose-dependent manner and noradrenaline has a similar effect but with a lower potency (Campbell and Scanes, 1985; Capuzzi *et al*., 1975; Cramb, *et al*., 1982). The antilipogenic effects of adrenalin can be reversed using both - and ß-adrenergic antagonists. The evidence suggests that inhibition of lipogenesis involves both 1 and $ß_1$-adrenergic receptors (Campbell and Scanes, 1985). Adrenalin stimulates glycerol release from adipose tissue explants maintained in culture (Campbell and Scanes, 1985). This lipolytic effect was also observed using $ß_1$/$ß_2$-adrenergic agonists. Lipolysis is inhibited by 2 agonists similar to the situation in many mammals (Mersmann, 1984; Burns *et al*., 1981).

### 6.1.3. Effects on protein metabolism

Adrenalin infusion has been reported to decrease proteolysis but not to stimulate protein synthesis (Castellino *et al*., 1990; Mathews *et al*., 1990). Clenbuterol, a $ß_2$-adrenergic agonist, increases carcase protein content in chickens (Takahashi *et al*., 1993) and this is in line with similarly reported effects of $ß_2$ agonists on skeletal muscle (Buttery and Dawson, 1987). The clenbuterol effect on protein accretion in chickens is due to a decrease in the rate of protein degradation as the ß-agonist is reported to have no effect on rates of protein synthesis in chickens (Muramatsu *et al*., 1991). Decreased rates of uric acid secretion in clenbuterol treated chickens supports this position (Takahashi *et al*., 1993).

The effects of glucocorticoids on protein metabolism can be antagonized by ß-agonists. Feeding corticosterone to chickens (10 mg/kg of diet) increased carcase fat and abdominal fat and decreased carcase protein (Takahashi *et al*., 1993). When clenbuterol was also added to the diet (0.33 mg/kg) there was a significant effect on the corticosterone reduction in carcase protein but no effect on the carcase fat levels. In the chicken, ß2-agonists could act predominantly on protein deposition.

### 6.2. Catecholamine effects on Reproduction

The ovarian follicle is intensively innervated and contains adrenergic fibres (Gilbert 1969). A role for catecholamines in ovulation is suggested by the report that anti-adrenergic drugs inhibit and agonists induce ovulation in birds (Kao and Nalbandov, 1972; Moudgal and Razdan, 1981; 1985). As the largest ovarian follicle advances towards ovulation the levels of adrenalin and noradrenaline increase and the dopamine levels decrease (Moudgal and Razdan. 1983). These changes are not seen in the second largest follicle where levels remain low. Adrenalin *in vitro* (Moudgal *et al.*, 1985) and *in vivo* (Moudgal *et al.*, 1990a) causes atresia of ovarian follicles and *in vivo*, reduces egg production (Sykes, 1955). Adrenalin levels in the egg are a good measure of stress in hens (Moudgal *et al.*, 1990b) and poor egg shell quality (Moudgal *et al*., 1990c). These deleterious effects can be prevented if an inhibitor of catecholamine synthesis is administered (Moudgal *et al*., 1991).

In groups of hens laying at either 40% or 70% egg production, the high layers had significantly higher noradrenaline and lower adrenalin levels in the eggs compared to the low layers (Moudgal *et al*., 1992). The values suggest that there was greater conversion of noradrenaline to adrenalin, greater PNMT activity, in the poor layers. There is an increase in PNMT activity during stress or after corticosterone treatment (Zachariasen and Nemcomer, 1975). Dopamine levels in eggs do not appear to be an indicator of stress (Moudgal *et al*., 1992).

## 7. Stress

### 7.1. Definitions

An understanding of the relationship between animal production characteristics and stress is important in issues of animal welfare and animals rights. As the keeping of poultry progressed from small flocks for home consumption, to larger commercial enterprises, there were major improvements in production traits. Housing costs were reduced by increasing stocking densities, eventually culminating in the housing of hens in single or multiple cages. As this occurred there was a real or perceived increase in behavioural problems associated with housing layers.

The United Kingdom Animal Welfare Council (Webster and Nicol, 1988) stated that production systems should provide hens with:

1. freedom from hunger and thirst
2. thermal and physical comfort
3. freedom from pain and disease
4. freedom from fear and distress
5. sufficient space to exercise

The last of these has presently become a very controversial tropic. Intensive farming practices often deprive animals access to conditions that allow for the performance of normal behaviour. It has been considered that such deprivation leads to stress and a decline in the animals well-being. The critical question becomes, does an animal suffer when prevented from exercising a particular behaviour even when there appears to be no biological significance to performing the behaviour? There is no clear criteria to show that the failure to perform a behaviour is stressful to an animal. In any analysis a number of indicators should be evaluated in determining the level of stress suffered.

A simple means of defining stress is to base it on the neuroendocrine response to stressors, the final response of which is the release of glucocorticoids. Corticosterone is widely used as a indicator of stress (Rushen, 1991). Corticosterone and the thyroid hormones are physiological indicators of stress in the fowl (Etches, 1976; Beuving and Vonder, 1978: Siegel, 1980). It has been argued that environmental stressors exert their effect on plasma corticosterone through psychological influences (Dantzer and Mormede, 1983) and thus might provide a measure of an animals wellbeing.

Emotional stressors appear to be a more potent stimulator of stress than many physical stressors (Mason, 1975). The two extremes in the reaction to fear are the active behavioural response (fight or flight) or the passive response (conserve and withdraw). The active behavioural response is associated with high neurosympathetic activity (catecholamines) and low glucocorticoids, while glucocorticoids are high during the passive response (De Bore *et al*., 1990; Fuchs *et al*., 1993). In two lines of hens selected for either high or low levels of feather pecking (Bolkhuis *et al*., 1992), distinct differences in both the basal and restraint initiated levels of catecholamines and corticosterone were observed (Korte *et al*., 1997). The low feather pecking line had significantly higher basal corticosterone levels which were further elevated after manual restraint. After restraint the high feather pecking line had significantly higher noradrenaline levels. Hens with the shortest tonic immobility response, a test for fearfulness, also had the lowest corticosterone response (Beuving *et al*., 1989).

Attempts to provide a totally stress-free environment may not be in the lifetime best interests of the hen. An 'optimal' exposure to stress, especially in early life may be necessary to provide a coping mechanism to handle environmental stresses later in life. An optimal level maybe advantageous but there is no doubt that extreme stress is deleterious (Gross,

1983; Creel and Albright, 1987). Limited daily exposure to heat early has been shown to help protect hens during subsequent exposures in later life (Deaton *et al*., 1986).

**7.2 Responses to stress**

There are specific responses and nonspecific responses to stress. Specific responses depend on the stressor. For example the response to cold involves huddling, increased metabolic rate and changed blood flow patterns. Nonspecific responses are independent of the stressor. There is a stereotypic pattern of physiological responses to stress involving many organ systems. Non-specific response include increased blood flow, production of glucose from glycogen which prepares the animal for 'fright-flight' reaction and these effects occur in minutes and are catecholamine-dependent. A consistent non-specific response is an increase in corticosterone which is an acute response and responsible for glucose production from non-carbohydrate sources, principally protein. These changes are beneficial to the hen and result in some level of energy expenditure. If the stress is continued for a chronic period, however, then the corticosterone induced changes are detrimental to the hen. Pathological consequences include; ulcers, hypertension, immunosuppression and effects can be permanent after removal of the stress but if continued can result in death. Short term stressors such as heat (Beuving, 1980), food and water deprivation (Beuving, 1980) transport (Broom and Knowles, 1989) and fear (Beuving *et al*., 1989) give rise to elevated corticosterone levels.

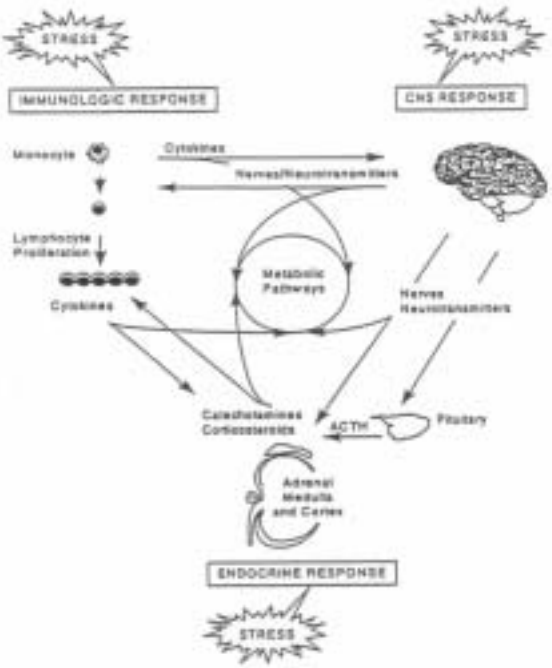


**Figure 4: The neuroendocrine-immune network. The response to stressors (physical, social or microbiological) provokes an integrated reaction involving the central nervous system (via nervous output), the endocrine system (via hormones) and the immune system (via cytokines) each of which influences the output of the other (Husband and Bryden, 1996).**

Recent evidence suggests that the endocrine, immune and central nervous systems interact and respond to stressors (physical, social or disease) in a coordinated manner. While the interactions between the brain and endocrine system have been long recognised, the participation of the nervous and endocrine systems in the regulation of immune-related responses has only recently been appreciated. In turn, the immune system influences neural activity and endocrine secretions. The bidirectional communication between the neuroendocrine and immune systems appears to be mediated by neurotransmitters, hormones, cytokines and receptors common to the three systems (see Kelley, 1988; Blalock, 1989; Arkins *et al.*, 1993; Johnson, 1997; Johnson *et al.*, 1997). Furthermore, leukocytes not only synthesize and secrete cytokines but also growth hormone, prolactin and insulin-like growth factors. In addition to the cross-talk between the endocrine and immune systems, there is also a dense innervation of lymphoid tissues allowing changes in central nervous system output following environmental change to directly impact on immune function (Felton *et al.*, 1988; Ottway & Husband, 1994). The coordinated response of these three systems during stress provides a primary example of how the brain-pituitary-immune axis serves to integrate the homeostatic responses of the animal (Husband, 1995).

A key question for those concerned with poultry production is how well birds cope with modern systems of animal management. Within its genetic capacity, an animal adjusts continually to changes in environmental conditions or stress, but at a metabolic cost. The effect of stress is an increase in endocrine-immune output resulting in the release of hormones (particularly those of the pituitary-adrenal axis) and cytokines and a change in the so-called “endocrine-immune gradient” (Elsasser, 1993). Metabolic changes following alterations in the gradient represent a homeorhetic response which alters nutrient partitioning away from growth and skeletal muscle accretion to metabolic processes which, during immunological stress, support the immune response and disease resistance. Many cytokines, such as interleukin-1, tumour necrosis factor- and interleukin-6, act directly on target tissues such as skeletal muscle, adipose, liver and bone and indirectly alter the circulating levels of hormones such as growth hormone, insulin, glucagon and cortisol. In so doing, they orchestrate glucose homeostasis, increase net protein oxidation, muscle proteolysis, nitrogen excretion and net hepatic anabolism (Klasing, 1988; Johnson *et al.*, 1997). The net result is reduced growth rate and increased fat deposition. The impact of these interactions on egg production has not been examined.

Amino acid redistribution occurs during immunological stress, with muscle being the major source of nitrogen. Some of the amino acids are used for B-cell proliferation and synthesis of immunoglobulins, others provide the carbon skeleton for increased gluconeogenesis by the liver whereas large quantities are incorporated into acute-phase proteins. These aspects of protein turnover are accompanied by a significant nitrogen loss. Calculations by Reeds *et al.* (1994) show that the amount of muscle protein mobilized is considerably in excess of the quantity of acute-phase proteins synthesized due to demands for phenylalanine, tryptophan and tyrosine. The net loss of body nitrogen occurs following the oxidation of amino acids remaining after completion of acute-phase protein synthesis (Reeds *et al.*, 1994). Research conducted with broiler chickens (Klasing & Barnes, 1988) and pigs (Stahly, 1996) demonstrate that animals subjected to greater immunological stress require less essential amino acids to achieve their decreased protein accretion rates than animals not stressed. Similar studies have not been conducted with laying hens.

# 8. Animal welfare

### 8.1. Definitions

Animal welfare is a ".... term that embraces the physical and mental well-being of the animal' (Brambell committee, 1965). An interest in welfare can stem from numerous factors and include economics, culture, philosophical attitudes, scientific, aesthetics, knowledge and religion (Craig and Swanson, 1994). Attitudes to animal welfare vary greatly. Kellert (1988), found that the attitude had a lot to do with the relationship that different professions had with

animals. Farmers were utilitarian (interested in animals as resources), white collar professionals were humanistic (animals as companions), naturalistic (concerned with wildlife) and ecological (concerned with wildlife and environment). Organizations related to animal matters have been categorised as concerned with exploitation, use, welfare, protection, rights and liberation (Morgan, 1986).

While there are many possible definitions according to the individuals interest, perhaps an appropriate general definition suitable to all interests is that of Hurnik (1988). "Animal wellbeing (welfare) is a state or condition of physical and psychological harmony between the organism and its surroundings characterized by the absence of deprivation, aversive stimulation, overstimulation or any other imposed condition which adversely affects health and productivity of the organism".

**8.2. Indices of welfare**

An assessment of welfare is difficult because it encompasses many factors, having positive and negative input to the final state of the animal. It is a sum of all the factors that impinge on the animal. Most definitions of welfare have been based of physiological assessments and measurements based on health, production, behaviour and physiology (Mench and van Tienhoven, 1986; Broom, 1991). Assessments of welfare rely on some measure of change and change itself may not be a measure of stress as an animals behaviour and physiology changes to maintain homeostasis. Any parameter provides only prima face evidence that the animals welfare is compromised. The big question is how much change signifies a risk to welfare? Measurements are often criticised with this question in mind and also for the difficulties that measuring techniques cause in interpretation of the measurements.

Health and productivity are not necessarily good indicators of welfare as they are often based on a unit or group basis. While unit production or health may be high, individual welfare could be poor for some hens. Based on an individual basis these indices can be good measures of welfare. While stress can decrease production it doesn't mean that a decrease in production is the responsibility of stress related causes.

Physiology and behaviour can be sensitive measures of welfare but with limitations. Physiological responses are related to changes in the stress-axis. The results are elevated heart rate, increased plasma corticosterone and catecholamine levels, adrenal hypertrophy and atrophy, immunosuppression, changes in growth and reproductive hormones and neurochemical changes (Freeman, 1976; Seigel, 1980). The difficulty with such measures is, what is normal? An increase in stress hormones could be beneficial or harmful depending on the duration and level of responses. One major difficulty is that often measurements are made as point samples and this can lead to misleading interpretations of data.

The concept that behaviour is a good indication of welfare is based on the premise that it reflects how a hen feels (Dawkins, 1991; Duncan, 1993). This is extended to the notion that if a hen has positive emotional experiences then its welfare is good and naturally the reverse indicates poor welfare. It could be an inappropriate measure of welfare as the absence of any behavioural pattern seen in the wild may not impinge on the welfare especially where other activities could be a suitable substitute (Hughes 1980; Dawkins , 1988). Assessing emotional state is difficult. There are three commonly used experimental means of assessing emotional state (Mench, 1992); (i) deducing effective states during pharmacological or environmental manipulation, (ii) preference testing: providing animals with choices and then assessing the choices made and (iii) motivation testing: assessing an animals desire to perform particular behaviours.

One of the major problems in assessing physiological responses to stress is that the collection of information is often stressful in it's own right (Freeman, 1985). Non-invasive measures could be helpful in identifying conditions responsible for poor welfare. Solomon (1991) has stated that shell quality is a very good indicator of a hens harmony with it's environment. Misshapen eggs or ones with calcium carbonate deposits are probably evidence of disturbances to the hen. Microscopic observations of shell changes could be useful indicators. Physical appearance, noise level and general behaviour are measures of a hens wellbeing. Poor comb, ruffled or loss of feathers are indicative of stress.

It is unlikely that any single indice of welfare is adequate. Multiple indicators involving health, behaviour, immune function, physiological measures of stress, productivity, indicators of pain, fear and frustration would be a better assessment (Craig and Swanson, 1994).

### 8.3. Fear responses

The fear response can range from mild avoidance to extreme hysteria (Duncan, 1985; Jones 1987). It would be only the extreme responses that are indicative of poor welfare as the 'flight-fight' syndrome is part of the normal response of adjustment to a changing environment. Frustration can be mild but if prolonged can lead to increased aggression (Duncan and Wood-Gush, 1971 and stereotypic behaviour (Duncan and Wood-Gush, 1972a).

### 8.4. Restricted feeding

As a general husbandry practice broiler breeder hens are restrict-fed during growth and laying. This improves egg production, fertility and lowers mortality (Hocking *et al*., 1987; Hocking, 1990; Katanbaf *et al.*, 1989). Also, the probability of multiple ovulations decreases in proportion to the level of reduction in body weight at the onset of lay (Hocking and Whithead, 1990). This practice has been questioned on welfare grounds.

Hocking and colleagues (1993) studied the effects of feed restriction in broilers on various physiological indices of welfare. By 12 weeks of restriction there was a significant increase in basophils, heterophils and the heterophil/lymphocyte ratio (HLR) and a decrease in lymphocytes. These changes were also associated with higher corticosterone levels at some sampling periods. Corticosterone levels increase following feed restriction (Freeman *et al*., 1981). There exists a positive relationship between the HLR and plasma corticosterone levels (Gross and Siegel, 1983). It could be that the elevated corticosterone may not be indicative of poor welfare but a metabolic response to feed deprivation.

Plasma activities of creatine kinase (CK) and aspartate transaminase (AST) are a measure of muscle tissue damage (Lumeij *et al*., 1988) and can increase during stress (Meltzer 1968; Tripp and Schmitz, 1982; Mitchell *et al*., 1992). The rapid growth rate of broilers is associated with elevated CK and AST activity and this is probably associated with an increase in muscle protein turnover. The effects are suppressed by restrict feeding (Hocking *et al*., 1993). These workers found no evidence that limiting access to water was stressful as long as water was available at the time of feeding.

Restrict feeding to 75% of *ad libitium* values resulted in a 73% increase in plasma corticosterone levels but these were normal by 5 weeks of treatment. This suggests that the birds could be adapting to the restricted intake (Freeman *et al*., 1981). However, restrict-fed broilers show increased fearfulness (Van Niekerk *et al*., 1988) and increased rate of pecking stereotypes (Savory *et al*., 1992). The effects of restrict-feeding might more stressful in young birds (Nir *et al*., 1975; Freeman and Flack, 1980; Scanes *et al*., 1980).

# 9. Hens' behavioural needs

Hens have behavioural needs (Hughes and Duncan, 1988) and the welfare components of production systems are often assessed according to the opportunity for hens to express these needs. The problem therefore becomes which behavioural activities need to be performed for proper hen welfare. All behaviour is internally or externally stimulated. For example nesting is internally stimulated because whatever the environment it needs to be performed. Response to predators in externally stimulated, it is only preformed if their is a perceived danger from a suspected predator. Dust bathing is stimulated by a complex interaction between internal (Vestergaard, 1982) and external visual stimulation (Petherick *et al*., 1995). A further complicating factor is that behaviour shows plasticity and so changes in behaviour do not necessarily indicate poor welfare.

## 9.1. Nesting

Prior to egg laying, hens show a particular sequence of behaviour (Duncan, 1980). Having access to some form of nest appears important to hens as they will work hard to gain

access to nests (Duncan and Kite, 1987; 1989). Hens housed in cages without nests display stereotypic pacing and increased aggression (Hughes, 1979) in the hour before oviposition.

### 9.2. Dust bathing

When supplied with appropriate material, hens will dust bath daily for around 30 min (Vestergaard, 1980), the reason appears to be removal of excess oil from the feathers. Preferences tests indicate that hens prefer environments with dust baths but they fail to exert any great effort to gain access to litter (Dawkins and Beardsley, 1986).

### 9.3. Maintenance behaviour

Tail wagging, scratching, feather ruffling, wing-flapping and stretching are all behaviours displayed by hens. The importance of not being able to perform these is difficult to assess. Sleeping and individual rest is essential (Blokhius, 1984) with perching being the normal position for such behaviour, however, hens seem to readily adapt to other positions. Exploration is performed in extensive systems but the importance of such behaviour has not been defined.

### 9.4. Feeding and drinking

It is obvious that both are essential. Cages limit the degree of foraging and other elements associated with it. Hens appear to forage for at least some of their food (Duncan and Wood-Gush, 1972b). There is no real indication that hens suffer if foraging is limited but aspects of it especially pecking, may be directed towards other hens (Bolkhius, 1986). A problem that can occur with drinking is if there is a sudden change in the water supply system.

### 9.5. Social behaviour

Hens show evidence of being social animals. Factors associated with decreased welfare include mixing unfamiliar birds, rearing single sexes and limiting space for large groups. In a study where birds were mixed daily there was increased immunosuppression, decreased reproductive rate and body weight (Siegel and Gross, 1965; Gross and Siegel, 1973; 1981). Social status could be important but there is no clear evidence for a relationship between social status and corticosterone levels (Mench and Ottinger, 1991).

### 9.6. Space

Legislation to control space has mostly been on the basis of the space occupied by the hen. This space is too small to perform all behaviours. Dawkins and Hardie (1989) estimated that this requires areas of 893-1826 $cm^2$/bird depending on the activity. Choice tests indicate that hens prefer increased space allocation (Nicol. 1986). In general crowing decreases

growth and egg laying, increases mortality, feather loss and is associated with adrenal hypertrophy and increased corticosterone.

# 10. Husbandry influences on welfare

### 10.1. Housing

In recent times there has been an increased effort to assess the benefits and disadvantages of various housing systems especially for egg producing layers. This has occurred because of the public perceptions that housing hens in cages is detrimental to their welfare. Public concern has directed administrators and researchers to assess the effects of housing on bird welfare. Assessments of the effect housing-type has on welfare have been based on measures of hen behaviour, comfort, health, environment, production, mortality and a number physiological measures of stress.

### 10.2. Physical comfort and behaviour

Crowding will restrict hen behaviour by limiting the space available to perform activities viewed as normal behaviour (Craig and Milliken, 1989). The allocation of space is minimal in caged-systems. A medium size hen occupies a space of around 600 $cm^2$ (Dawkins and Hardie, 1989) and so any system that recommends space allocation less than this would cause some measure of feather compaction. Alternative floor systems allow for an increase in bird space, deep litter 11/$m^2$ (Appleby *et al.*, 1988), strawyards 6/$m^2$ (Gibson *et al.*, 1988). While these alternative floor systems have a lower mean density, the actual density can be high at various times. Clustering can result in densities of 30 birds/$m^2$ (Gibson *et al.*, 1985). Cage height is also of importance, the often recommended height of 35-40 cm is insufficient.

The importance to a hens welfare of free movement to perform behaviours associated with egg laying, dustbathing, scratching, pecking, wing flapping and stretching is difficult to measure. The activities of hens removed from cages suggest that the space restriction of cages causes frustration (Nicol, 1987). To Wood-Gush (1972) the prelaying behaviour of some hens in cages suggests a level of frustration.

Fearfulness is greater in caged birds (Rutter and Ducan, 1989) but the incidence of aggressive episodes is greater in floor systems (McLean *et al.*, 1986). Aggression tends to decrease as the stocking density increases (Hughes and Wood-Gush, 1977). Feather pecking is more prevalent in barren environments and so higher in cage systems (Blokius, 1989). Feather pecking can progress to cannibalism which is more prevalent in floor systems (Appleby, *et al.*, 1989). Floor systems made of total slats or wire frequently are associated with a high incidence of feather picking and hysteria (Blokhius, 1989).

The absence of dust bathing, scratching and pecking associated with cages is often assessed as a welfare problem (Vestergaard, 1982). The non-cage systems allow hens a greater degree of expression for these behaviours (Gibson *et al.*, 1988; Appleby *et al.*, 1988). Litter systems allow for dustbathing, scratching, improved foot condition and decreased levels of feather picking and provide nest boxes. Free range systems provide a variety of stimuli although some hens never leave the hen house. Perches of an appropriate design can improve foot condition and bone strength and allow vertical space to be used more effectively (Duncan *et al.*, 1992).

### 10.3. Group size

This is different to density. For cage systems stress level has been reported to increase as the group size increases (Mashaly et al., 1984; Roush *et al.*, 1984). Small group sizes show increased production levels, less aggression and hysteria (Hughes, 1975: Robertson *et al.*, 1989). For large group sizes in floor systems subordinate hens are often attacked (Gibson *et al.*, 1988). This could involve failure to recognise individual hens in large groups as introduction of strangers into a group results in increased aggression (Craig *et al.*, 1969). Appleby and colleagues (1985) suggest that individual recognition is limited to groups of around 80 hens.

### 10.4. Beak trimming

Break trimming is an important welfare issue. Cannibalism can be a major cause of mortality and trimming has been reported to decrease mortality rates (Glatz, 1990). For untrimmed hens, mortality rate of 4-9 % for litter housing and 4-5% for caged systems have been reported and these decrease to 2-3 % following beak trimming (Appleby *et al.*, 1988). Trimming does not decrease the incidence of pecking but does reduce the injury resulting from pecking (Craig and Lee, 1990; Craig and Muir, 1991; Lee and Craig, 1990). There is evidence to indicate the trimming causes chronic pain (Cunningham, 1992; Breward and Gentle, 1985) and short- and long-term behavioural changes suggestive of pain (Lee and Craig, 1990; Duncan *et al.*, 1989b).

## 11. Hen health and husbandry

### 11.1. Environment and disease

As the environment in which hens are maintained changes, the pattern of disease changes (respiratory diseases are more prevalent in intensively managed systems). Disease problems associated with extensive systems are often husbandry problems rather than health problems (Ashton, 1988). Stressors such as air quality increase a hens susceptibility to primary infection, especially from viruses. Air borne contaminants become more critical in floor

systems where increased dust, bacteria and ammonia levels can have detrimental effects on health. Hens breathing contaminated air have increased levels of lung lesions (Maxwell *et al*., 1989), fluid accumulation in the lungs and lower blood oxygen levels (Oyetunde *et al*., 1978 ).

### 11.2. Tissue and bone damage

Feather loss is greater in caged systems than floor systems (Appleby, 1988). This is partly due to an increased incidence of abrasion but like all systems mainly due to pecking (Hughes, 1985).

Old hens housed in cages, have low bone strength and can have high levels (30-50%) of bone breakage during catching, handling and transport (Gregory and Wilkins, 1989). Tibia bone strength can be 19-42% greater in hens housed on the floor compared to those in cages (Rowland *et al*., 1972; Meyer and Sunde, 1974). Perches placed in conventional cages increased bone strength (Hughes and Appleby, 1989). The production costs associated with using perches include increased numbers of broken eggs and reduced egg mass (Tauson, 1984). Damage due to trapping of body parts is greater in caged systems (Tauson, 1985). Wet litter can cause increased foot problems in floor systems (Hill, 1986).

### 11.3. Nesting

In cages hens often display increased frustration at prelay and this suggests that there is inhibition of behaviour (Mills *et al*., 1985). Cages provide inadequate opportunity for nest building and this may be more important than actually having a nest site. Attempts have been made to modify cages so they incorporate a nest but these have had limited success (Wegner, 1990; Appleby, 1990).

### 11.4. Dustbathing

Wiepkema (1989) strongly believes hens should have access to dustbaths. When hens are prevented from using dust baths their motivation to use dust baths increases (Vestergaard, 1982, Van Liere and Bokma, 1987). Hens in cages display dustbathing as a vacuum activity. It is stimulated by the presence of litter but it is unclear whether the activity is performed satisfactorily without substrate being present.

### 11.5. Stockmanship

Handling is a potent stressor with corticosterone levels raising within 60 sec after immobilization by hand (Craig and Lee, 1990). Physical handling can have a positive or negative affect on growth (Gross and Siegel, 1979; Freeman and Manning, 1979; Collins and Siegel, 1987). The likely effects are related to the nature of the relationship, level of fear

and the consequent effects on production. Fear of humans has been reported to account for 21% of the variation in hen day egg production (Hemsworth and Barnett, 1989).

## 12. Physiological measures of welfare

Management conditions influence plasma corticosterone levels (Edens *et al.*, 1982; Mashlay *et al.*, 1984; Koelkebeck and Cain, 1984; Gibson *et al.*, 1986). Of three different housing systems, cages, strawyards and free-range, hens in cages had the highest circulating plasma corticosterone concentrations (Gibson *et al.*, 1986). In the same study levels of T4 were not affected by the housing system but T3 levels were significantly lower for the free-range hens compared to the caged hens. The T3 levels maybe a response to temperature differences in the different housing systems because T3 is a regulator of metabolic activity in hens (Klandorf *et al.*, 1981). During cold exposure T3 levels are elevated (Sharp and Klandorf, 1985). Alterations in floor space or 'personal space' can elevate plasma corticosterone (Mashaly *et al.*, 1984; Crompton *et al.*, 1981).

Short term stressors such as heat (Beuving, 1980), food and water deprivation (Beuving, 1980) transport (Broom, 1989) and fear (Beuving, 1989) give rise to elevated corticosterone levels. Corticosterone levels are elevated when the space allowance is below 400 cm2/bird (Craig *et al.*, 1986) The effects of space allowance can be influenced by temperature (Edens *et al.*, 1982). When space allocation is adequate there appears to be no difference with individual or group housing (Koelebeck and Cain, 1984).

## 13. General summary

There have been great improvements in livestock production in general but especially so in the intensive industries associated with poultry production. Over the last 30 years improvements in genetic selection, nutrition, management, and disease prevention and treatment have had a positive influence on the well-being of hens. The intensive confinement systems used in egg production are associated with perceived welfare problems and these include disruption of social attachments, restriction of movement and the consequent failure to perform behavioural traits, human-animal interactions and lack of environmental stimulation (Wood-Gush *et al.*, 1975).

The intensive management of hens for egg production has caused a sector of the public to become increasingly concerned with the welfare of hens. These concerns need to be considered and it is important to identify and define what constitutes a contented hen. The consequences of deprivation or overstimulation are often quite subtle and difficult to observe and quantify (Ewbank, 1988). Animal behaviouralists have made efforts to improve methods of assessing behavioural needs of hens and to determine how particular production systems

interact to provide or deprive hens of these needs (Hughes and Duncan, 1988). However good these determinations are, they still have limitations. What is required is an entirely objective, quantifiable indicator which unambiguously reflects a hens well-being.

It is clear that stress has many physiological consequences and for some of these there are definable changes in plasma constituents. Stress results in elevated heart rate, increased secretion of catecholamines and glucocorticoids, adrenal hypertrophy and atrophy, immunosuppression, changes in levels of reproductive and growth hormones and neurochemical changes (Wiepkema and Van Adrichem, 1986). Assessment of these changes could provide a means of measuring stress levels in hens but there are inherent problems associated with making such measurements and include:

1. Changes in chemical indicators are responses to noxious stimuli and the assumption is that the absence of any change is indicative of the hen not being subjected to stress. This is not necessarily correct.

2. Alterations in chemical indicators could be a response by the hen to normal environmental changes and reflect adjustments to diurnal influences or a hens efforts to maintain homeostasis.

3. Handling birds when collecting samples may induce a response to a noxious stimuli which is not indicative of the background well-being of the hen.

4. The difficulty of measuring some of the changes.

Many of the stresses to which a hen is subjected result in activation of the HPA-axis. The final consequences are, changes in plasma and tissue levels of glucocorticoids and catecholamines, secreted by the adrenal gland (Harbutz and Lightman, 1992). Co-ordination of neurosensory stress signals in the brain results in the release of neurotransmitters that inturn stimulate the release of hypothalamic releasing factors which act at the pituitary to stimulate the release of ACTH. In the circulation ACTH acts at the adrenal to stimulate corticosterone release. As part of the general adaptive response to stress, corticosterone increases gluconeogenesis and blood glucose, causes catabolism of muscle tissue, increases fatness and decreases immunological function. The last of these effects results in increased susceptibility to diseases and this is especially important in intensive housing systems. As part of the short-term ‘flight or fight’ response of hens to acute stress, the catecholamines are released from the adrenal gland. These hormones are released in high concentration in a matter of seconds following perception of a noxious stimulus. The major function of the catecholamines is to mobilise energy reserves to assist the hen to evade the stressor.

Not much is known about the physiological levels of the “stress” hormones in the hen. There are difficulties with the interpretation of circulating concentrations of hormones because of diurnal patterns and the rapid changes that occur in response to handling and blood sampling. These problems have been reduced by using non-invasive means of measuring levels of stress hormones. For some species, hormone levels are assessed in milk

and saliva. It is apparent that the secretory products of animals contain a wide range of growth factors and hormones which vary in concentration according to the physiological status of the animal (Prosser *et al*., 1991). Saliva contains cortisol among other hormones and the concentration is closely related to the degree of stress (Feel *et al*., 1985).

Of increasing concern to the egg industry is the growing public perception that the laying hen exists in a state of chronic stress for the duration of its productive life. At present there are no practical means of accessing stress in hens other than behavioural observations, which do not provide accurate determinations of well-being as discussed earlier. Adrenal hormone levels can be determined in blood but the actual blood sampling procedure compounds the results. Could the egg provide a non-invasive means of measuring acute stress levels in hens?

The diffusion of plasma constituents into egg white has received little attention but as the degradative metabolites of vitamin D are found in albumen (Fraser and Emtage, 1976) it is likely that other plasma solutes are also sequested into the albumen. The gradual accumulation of albumen over 6 hours during egg formation potentially provides a very accurate and integrated reflection of circulating hormones over this period. Determination of stress hormone levels in egg albumen could provide a non-invasive measure of acute and chronic stress in hens.

The new understanding now emerging regarding the integration of endocrine and immune responses to stressors suggests opportunities for intervention by nutritional strategies or by manipulating hormone and/or cytokine responses (Husband & Bryden, 1996). Only through a more complete delineation of the stress-induced perturbations in metabolism will we be able to determine which interventions are consistent with enhanced animal production and welfare. This will not be easy given the difficulties associated with developing appropriate stress paradigms as duration of stress is often brief and animals may encounter a number of stressors concurrently. Moreover, the task is more complicated as a change in voluntary food intake is an initial response as animals adjust to new environmental conditions or stress. Despite many years of research (see Forbes, 1995) our understanding of food intake control is incomplete. Since the acquisition and assimilation of nutrients is critical to survival, it is not surprising that this well regulated process is complex and subject to multiple levels of control.

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