Effect of heat and physiological stress on the growth performance, physiology and welfare of broiler chickens

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ABSTRACT

Broilers can be faced with a several stressful conditions during their production cycle which can have implications for both growth performance and animal welfare. Animal welfare encompasses the physical and mental well-being of animals, assessed from the biological functioning and subjective experience. The aims of this thesis were i) to develop and validate non-invasive means of assessing the welfare of broilers under physiological and episodic heat stress conditions, ii) to investigate the impact of episodic heat stress, physiological stress and light wavelength on the growth performance, physiology and welfare of broiler chickens and, finally, iii) to investigate a novel means of alleviating heat stress in broilers. Endogenous corticosterone measured in the urate sphere was suppressed by dexamethasone administration. In a cognitive bias task, birds offered mealworms injected with corticosterone to mimic chronic stress were pessimistic in their judgement about ambiguous positions. A positive correlation was established between physiological indicators of stress and cognitive bias. Although light wavelength was confounded with light intensity in our study, there was no difference in growth performance and cognitive ability of birds reared in the blue and red light, except for increased activity of birds in red light. Under simulated episodic heat stress, the change in CBT measured from a temperature-ID chip (ΔCBT-chip) and a data logger (ΔCBT-logger) was positively correlated. Significant positive correlations were found between the change in surface body temperature (SBT) under wing (ΔWT) and ΔCBT-chip, and between ΔWT and ΔCBT-logger. Significant positive regression equations relating change in CBT and RR with apparent equivalent temperature (a factor which combines environmental temperature and RH) were also developed. High temperature coupled with high RH aggravated the respiratory rate (RR) of broilers and this was accompanied by suppression of peening behaviour. High heat stress for 3 hours had a greater impact on birds than moderate heat stress for 6 hours. For broilers exposed to moderate heat stress, the provision of additional cup drinkers reduced the rise in CBT and the proportion of time spent in wing drooping behaviour, but enhanced SBTs suggesting increased heat dissipation.

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DECLARATION

This thesis has been composed by myself and has not been accepted in any previous application for a degree. All sources of information have been specifically acknowledged by means of referencing

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PUBLICATIONS AND CONFERENCE ABSTRACTS ARISING FROM THIS RESEARCH

CONFERENCE ABSTRACTS

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Chapter 1. Introduction

Heat stress in broiler chickens is of global concern to poultry farmers in both temperate and tropical regions of the world (Balnave, 2004). Heat stress is a condition caused by high environmental temperature and relative humidity (RH) which reduces heat loss and consequently results in the accumulation of heat within the body, and subsequently an increase in core body temperature (CBT) (Jensen and Toates, 1997). A rise in CBT by 4^{o}C leads to death among birds (DEFRA, 2005). Heat stress negatively also affects feed intake, growth rate, meat quality, egg production, immune function and so can lead to higher mortality (Sandercock *et al*., 2001; Quinteiro-Filho *et al*., 2010 and Mashaly *et al*., 2004). Collectively, these negative effects could reduce the sustainability of commercial broiler chicken production and have severe economic implications for the broiler industry (St-Pierre *et al*., 2003).

Birds could experience heat stress within the microclimate in commercial rearing conditions especially when birds are densely stocked as can occur at the end of the growing period, during the summer which can be characterised by high and variable environmental daily temperatures (Yahav, 2009), or during a heat wave lasting continuously for an average of 2.7 days (Vale *et al*., 2010). The impact of heat stress on birds depends on the environmental temperature in combination with the level of RH and the duration of exposure (Widowski, 2010). Given the predicted increase in frequency, duration and intensity of heat waves due to global warming (Robinson, 2001) thus it could be speculated that the degree of thermal discomfort and related mortality experienced by meat birds in the future could increase.

Estimating the CBT could be a useful index of the degree of heat stress experienced by the bird. Although there are reports in the literature about the possibility of temperature-ID chips to estimate the CBT of animals, there are some concerns about the reliability of these devices which warrant further investigation.

It has been reported that the total world poultrymeat production increased by 39% between the year 2000 and 2010 (59 and 82 million tonnes eviscerated weight, respectively (Terry, 2010). Growth in output of poultrymeat is seen in both temperate and tropical regions of the world. For example, Al-Fataftah and Adu-Dieyeh (2007) reported a 33% increase in poultry production in Jordan between 1985 and 2005,

whereas (Kale, 2011) reported a 7.7% increase in production in Nigeria between 2006 and 2010. However, expansion of global poultry production may be hampered by the effects of global warming on the incidence of heat stress in broiler chickens. Steps to promote sustainable poultry production in tropical regions could include genetic improvement of the indigenous breeds which are already adapted to the prevailing environmental conditions, or providing genetically-improved broiler genotypes with structures inside the poultry house to alleviate conditions of heat stress (Sonaiya, 1993).Unfortunately, installation of such features in developing countries may not be feasible because of the high capital cost and lack of constant power supply (Gous and Morris, 2005). Therefore, development of strategies to alleviate the effects of heat stress should be explored.

Clearly heat stress can have a significant impact on broiler chickens and their welfare is compromised if they experience thermal discomfort. Broom (1986) defined welfare as 'the state of an animal regarding its attempts to cope with its environment' while FAWC (1992) proposed that animals have good welfare when they are provided with the five freedoms, namely freedom from hunger, thirst, disease, discomfort, pain and distress and the opportunity to display normal patterns of behaviour. Welfare is compromised if these freedoms are not provided, such as in the case of exposure to heat or physiological stress. Welfare can be assessed through the study of biological functioning, subjective feelings and the display of natural behaviour (Fraser, 2003). Biological functioning depends on the measurement of physiological changes stimulated during stress, whereas subjective feelings are more concerned with the emotional state of the animal since animals are sentient beings and therefore have the capability to experience pleasure or pain (Duncan, 2006). Broiler chickens are known to have cognitive abilities in learning tasks (Boks, 2010), where cognition is described as the process of gathering and processing information to give an output (Keeler and Robbins, 2011).

Broilers can be faced with a number of stressful conditions during their production cycle namely extreme heat or cold, high humidity, bright light, wet litter or poor ventilation (Rosales, 1994) which can have implications for animal welfare. Stressors can affect biological functioning by stimulating both the sympathetic-adrenal medullary axis (SAM) and the hypothalamic-pituitary adrenal axis (HPA) (Shini *et al*., 2008), enhancing the production of glucose needed for survival during stress (Ognik and Sembratowicz, 2012) for fight or flight, and the release of specific hormones into the

bloodstream. In birds, the 'gold standard' of estimating the level of corticosterone in birds is through a blood sample which requires the bird to be restrained. Blood sampling is an invasive procedure which can affect the levels of corticosterone (Mormede *et al*., 2007), so that alternative non-invasive methods are needed. Measuring corticosterone from the urate sphere, the solid white component of urine, could be an alternative, non-invasive method although this approach requires validation for broiler chickens.

Another means of assessing the welfare of animals is by estimating their affective state which gives a measure of the positivity or negativity of a stimulus. Affective state can be defined as a 'neurophysiological state consciously accessible as the simplest raw (non-reflective) feelings evident in moods and emotions' (Russell, 2003). Since animals cannot report their feelings verbally, biases in information processing (known as cognitive bias) observed in humans have been extended to animals as a tool for assessing the emotional state of animals (Harding *et al*., 2004). Animals are trained to perform a particular task, and the interpretation that the animal gives to an ambiguous cue during the cognitive bias task indicates whether the animal is in a positive or a negative affective state. For example, a spatial task was developed to train laying hens to discriminate between a rewarded (corn in a bowl) and an unrewarded location (an empty bowl), however the birds were slow to learn to discriminate between the two locations and so an improved spatial task could be developed to assess the affective state of broiler chickens subjected to different stressors.

1.1 Thesis aims

Therefore the aims of this thesis were i) to develop and validate non-invasive means of assessing the welfare of broilers under episodic heat and physiological stress conditions, ii) to investigate the impact of episodic heat stress, physiological stress and changes in light wavelength on the growth performance, physiology and welfare of broiler chickens and, finally, iii) to investigate a novel means of alleviating heat stress in broiler chickens. These aims will be achieved through the following six objectives:

i. To validate three different methods of estimating core body temperature (CBT) in broiler chickens.
ii. To validate an improved cognitive bias task for broilers.
iii. To validate the use of the avian urate sphere as a non-invasive measure of stress in broilers.

iv. To evaluate the cognitive ability, growth performance and welfare of broilers reared under two different light wavelengths.

v. To investigate the impact of episodic heat stress and physiological stress on the growth performance, physiology and welfare of broilers.

vi. To investigate the potential for additional water provision to alleviate the effects of heat stress.

The outline of this thesis is as follows:

Chapter 2 presents a detailed critical review of the literature on animal welfare, how it can be measured and the effects of heat stress, physiological stress and light wavelength on the growth performance, physiology and welfare of broiler chickens.

Chapter 3 reports an experiment on the impact of light wavelength on the growth performance and welfare of broiler chickens, with the development of a new cognitive assessment test to estimate cognitive ability of broilers.

Chapter 4 describes the potential use of the avian urate sphere as a non-invasive measure of endogenous levels of corticosterone and the sensitivity of the Hypothalamic-Pituitary-Adrenal axis of broilers.

Chapter 5 reports the development of an improved cognitive bias task for broilers, which was validated under conditions which mimic chronic stress.

Chapter 6 describes a method to simulate episodic heat stress conditions in broiler chickens, including the validation of estimates of core body temperature from three different devices.

In Chapter 7, the relative effect of high levels of heat stress for 3 hours compared to moderate levels of heat stress for 6 hours on the growth performance, physiology and welfare of broilers is investigated. In addition, the responses from two groups of birds exposed to episodic moderate heat stress either for the first or second time is examined.

Chapter 8 describes a novel strategy to alleviate the effects of heat stress on broiler chickens through provision of additional cup drinkers.

Finally, Chapter 9 provides a general discussion of the main findings from each experiment and the implications for commercial broiler production.

Chapter 2. Literature Review

2.0 Introduction

This review will analyse the various definitions of animal welfare, with particular emphasis on the biological functioning and subjective feelings approaches of assessing animal welfare. Within each of these two approaches, the various indicators of welfare will be explored, with the aim of elucidating non-invasive means of assessing the physiology and welfare of broiler chickens. Finally, a critical review of the impact of heat stress, physiological stress and light wavelength on the growth performance, physiology and welfare of broilers will be undertaken, alongside possible strategies to alleviate the effects of heat stress.

2.1 What is Animal Welfare

It can be argued that the welfare of farm animals is of growing importance in many countries (Koknaroglu and Akunal, 2013), due to the acceptance that animals are sentient beings and the recognition of the link between good animal health and welfare with good animal growth performance and efficiency. There are a number of definitions of animal welfare including 'the state of an animal regarding its attempts to cope with its environment', (Broom, 1986). Bracke *et al*. (1999) defined welfare as 'the quality of life as perceived by the animal itself'. Webster (2001) defined welfare as 'the capacity to avoid suffering and sustain fitness'. FAWC (1992) defined animal welfare as the provision to animals with five freedoms, namely:

- Freedom from hunger and thirst, by the provision of ready access to fresh water and diet.
- Freedom from discomfort, by the provision of a comfortable environment.
- Freedom from pain, injury and disease, by the prevention or rapid diagnosis and treatment of injury, disease and infestation.
- Freedom to express natural behaviour, by the provision of sufficient space, proper facilities and company of the animal's own kind.
- Freedom from fear and distress, by prevention of mental suffering.

The five freedoms can be summarised into two broad groups namely physical and mental. In the list above, the first four freedoms are related to the physical category while the fifth freedom is related to mental well-being of an animal. So the definitions of Dawkins (2004) and Koknaroglu and Akunal (2013) that animal welfare consists of

the physical and mental well-being are not out of place. The words welfare and well-being are used interchangeably because they both refer to the state of the animal with regards to its environment, but the term welfare is commonly used in Europe whilst well-being is typically used in North America (Ewing *et al.*, 1999).

The definitions of animal welfare given by (Broom, 1986) and Bracke *et al*. (1999) reflect the state of the animal within its environment. It is not enough for the animal to simply perceive the environment as being stressful and not to be able to adjust to such conditions. The ability to adjust to stressful conditions could then be determined from how the animal copes with the conditions. Webster, 2001's definition of welfare (capacity to avoid suffering and sustain fitness) could be more applicable to animals in the wild or those that have access to open environments such as free range poultry. In such environments an animal has the opportunity to avoid stressful conditions, by seeking shade if the weather is hot. However, animals under confinement are limited in their capability to avoid stressful conditions. In such a case, domesticated animals survive under stressful conditions by devising means of coping with the situation. In one particular experiment, wild adult blue tits and pied flycatchers were less affected by harsh environmental temperatures because they could adopt behavioural responses such as seeking shade, whereas their nestlings were confined in the nest and could not escape the high temperature (Lobato *et al*., 2008).

Furthermore, FAWC (1992) definition of animal welfare as based on the provision of the five freedoms was developed for domesticated animals, where the farmer has the responsibility of ensuring that the animal is comfortable. The FAWC (1992) definition of animal emphasises the absence of hunger, thirst, disease, discomfort, pain and stress. On the other hand, Boissy *et al*. (2007) were of the opinion that welfare should not be based only on the absence of negative affective state but also on the presence of positive affective state. One common way of producing positive affective state in animals is through the provision of housing or cage enrichment. Environmental enrichment has been reported to be associated with positive affective states in starlings and pigs (Bateson and Matheson, 2007; Douglas *et al*., 2012) further details are provided in Section 2.4). The enriched environment offers greater possibility for exploratory behaviour than a barren environment. Examples of enrichment in starlings include the provisions of perches, water baths and tray of bark chipping (Bateson and Matheson, 2007). In addition to improving the welfare of birds, environmental enrichment has

economic benefits. The provision of a wooden cover panel to reduce aggressive interactions between male breeder cocks over a female for mating in 5 commercial farms increased egg production by 2.1%, increased the level of fertility and hatchability, estimated to an economic benefit of $3 million (Leone and Estevez, 2008).

2.2 Assessment of animal welfare

Research into methods of assessing animal welfare could be sub divided into three main approaches, namely biological functioning, subjective experience and naturalness, with each category having a different approach to welfare (Fraser, 2003). The biological functioning category focuses on the normal function of the physiological and behavioural process of the body system i.e. physical well-being (detail provided in Section 2.3) by quantifying productivity, stress response or suppression of immune function (Duncan and Fraser, 1997). The subjective experience approach focuses on the mental well-being of the animal (i.e. based on feelings and behaviour, Section 2.4). Previously, the subjective feelings were determined through methods such as qualification of stereotypic behaviour or a preference test (Duncan and Fraser, 1997), such that the preference for a stimulus indicates its degree of pleasantness to the animal. With recent developments in methodology, three main methods of assessing subjective feelings have emerged namely anticipatory behaviour, appraisal theory and cognitive bias (See section 2.4 for details). The naturalness approach focuses on allowing animals to perform their natural behaviour and live a life close to natural (Duncan and Fraser, 1997). People who hold to this approach are interested in animals living in the wild environment.

Although behavioural studies can be undertaken in the laboratory, the environment in the wild differs from that in the laboratory; thus there might be some restrictions on the full display of natural behaviour. Changes in behaviour observed between animals in captivity from those in the wild could be attributed to changes in the availability of resources such as feed, water, shelter and predators (Price, 1999). In the wild, animals need to search for these resources while under domestication these resources are normally provided in order to ensure that their welfare is not compromised (FAWC, 1992). Nevertheless, an understanding of the natural behaviour of an animal can assist in designing an appropriate housing environment under captivity. However, that does not mean that domesticated animals are totally exempt from experiencing stress. A study undertaken by Künzl *et al*. (2003) comparing the behaviour of wild and

domesticated guinea pigs showed that domesticated species displayed a lower frequency of aggressive behaviour such as head thrust, attack, chase and bite ($P<0.001$) and showed a greater frequency ($P<0.001$) of socio-positive behaviours such as social grooming and nudges compared to two groups of guinea pigs originating from the wild (1^{st} and 30^{th} generation of captivity). They suggested that domesticated animals are in a better welfare condition, as they are more tolerant and socially tuned to one another compared to their counterparts in the wild. Thus a complete study of animal welfare encompasses the assessment of both the biological and emotional states of the animals.

Since there is no holistic definition of animal welfare, in this thesis a suitable definition of welfare that will be adopted is one that includes the definitions of Bracke *et al*. (1999) and Broom (1986).Thus animal welfare is the quality of life as perceived by the animal and its attempts to cope with its environment.

2.3 Biological assessment of welfare under stress

2.3.1 The stress response

According to Selye (1976) stress is defined as a nonspecific response of the body to any demand. In agreement with this, Lupein *et al*. (2007) stated that stressors are things that trigger the stress response. Stressors stimulate the sympathetic-adrenal medullary axis (SAM) and the hypothalamic-pituitary adrenal axis, HPA (Shini *et al*., 2008), axes which enhance the production of glucose needed for survival during stress Ognik and Sembratowicz (2012), see Figure 2.1. Cockrem (2007) claimed that the main distinguishing factor between a stressor and non-stressor is the activation of the HPA axis. However, with the several limitations of measuring corticosterone from the blood (discussed subsequently in Section 2.3.1.2), this claim may not fully differentiate between a stressor and non-stressor because corticosterone can be increased under negative and positive stimuli such as the presence of a mate or an intruder.

The sympathetic-adrenal medullary axis (SAM), also referred to as the fight or flight syndrome, allows for a very rapid response because it involves the transfer of signals from the hypothalamus to the adrenal gland through nerve impulses (Ewing *et al*., 1999). The SAM secretes epinephrine such as adrenalin, which triggers rapid responses to stressors in the form of increased heart rate, blood pressure and also the production of

glucose (Dickens *et al*., 2010) Figure 2.1. The rapid rate at which adrenalin is secreted during stress has made it difficult for its use in the assessment of stress.

The primary glucocorticoids are cortisol and corticosterone (Frandson *et al*., 2003). While corticosterone is secreted in birds and rodents, cortisol is secreted in cattle, sheep, fish and pigs (Mormede *et al*., 2007).On the other hand, corticosterone secretion from the HPA axis depends on signals from the hypothalamus (the corticotrophin releasing hormone, CRH) and the pituitary gland (adrenocorticotrophic hormone, ACTH) (Dickens *et al*., 2010 and Mormede *et al*., 2007). Compared to adrenalin, the secretion of corticosterone is slower and has a more prolonged effect (Mormede *et al*., 2007), hence levels of corticosterone have been widely adopted in the assessment of stress. A simplified summary of the process involved in the secretion and metabolism of corticosterone is presented in Figure 2.1.

Once secreted, corticosterone is transported through the blood to the target organs which have receptors for binding with the hormone Nelson (2005). Corticosterone receptors are present in the brain, liver, kidney, lung, intestines, thymus, bursa of Fabricus and the nasal gland (Hess, 2006). The two main corticosterone receptors are mineralocorticoid (Type I) and glucocorticoid (Type II) receptors. Type I receptors have about 6-10 times more affinity for corticosterone than Type II receptors (Lupein *et al*., 2007).Under basal corticosterone levels, the mineralocorticoid receptors are occupied but under conditions of stress where further corticosterone is secreted, both the mineralocorticoid and glucocorticoid receptors are occupied (Lupein *et al*., 2007). Corticosterone receptors are intracellular, which implies that before corticosterone binds to its receptors, it has to penetrate through the cell membrane. The corticosterone-receptor complex then binds with deoxyribonucleic acid (DNA), which subsequently promotes gene transcription and synthesis of proteins or enzymes that consequently, produce the changes in physiological responses (Nelson, 2005).

The concentration of corticosterone in the blood is the difference between the amount of corticosterone secreted from the adrenal gland and that cleared (removed) from the body by the liver and kidney tissues (Hess, 2006). Metabolism of corticosterone takes place in the liver and also in the gut through bacterial deconjugation (Möstl and Palme, 2002), as shown in Figure 2.1. The clearance of corticosterone from the body system decreases as the bird ages (Hess, 2006); hence adult birds may be more likely to have a higher circulating corticosterone concentration than younger birds presumably because of

decreased activity of the liver. Beuving and Vonder (1978) reported that after an injection with porcine ACTH (1.2 IU/kg), the increase in plasma corticosterone was greater in young (36 weeks of age) than older (126 weeks of age) laying hens. This elevation in plasma corticosterone persisted for a longer time in the older than the younger birds (50-60 minutes versus 40 minutes respectively, $P<0.001$).

There is a diurnal change in basal levels of corticosterone, specifically levels are greater in the early morning (just after the dark period) than the evening due to increased energy required during the dark phase to compensate for the lack of feeding and locomotor activity (Hess, 2006). In laying hens, the adrenal sensitivity for ACTH in the morning was approximately double the level for the evening (Beuving and Vonder, 1986). The secretion of corticosterone is initiated by a wide range of stressors (Cockrem, 2007) which could either be from an internal or an external source (Borell, 2001). External stressors listed for broiler breeders are extreme heat and cold or high humidity, bright light, wet litter, poor ventilation, rapid growth, catching, transport and overcrowding (Rosales, 1994). It is likely that a similar list of stressors is applicable to broilers. For instance, in a heat stress study, exposure of broiler chickens to either 31 or 36^{o}C for 10 hour/day from 35 to 42 days of age resulted in a 110% or 147% increase in serum corticosterone levels respectively compared to control birds kept at 21^{o}C (Quinteiro-Filho *et al*., 2010).

In a simulated chronic stress study on broilers, Puvaldopirod and Thaxton, (2000) induced stress in broilers by implanting an ACTH (adrenocorticotropic hormone) mini-osmotic pump which delivered 1μL/h for 7 days (equivalent to 8IU/kg BW per day). Birds with the ACTH implant had greater corticosterone levels (20.53 vs 0.66 ng/mL, $P<0.05$) and glucose (828 vs 261 mg/dL, $P<0.05$) compared to the control birds on the 4th day of treatment. Of the top ten responses required for adaptation during stress, the level of corticosterone was topmost. The other nine stress responses in order of decreasing magnitude were glucose, liver lipid, relative liver weight, levels of total white blood cells, levels of nitrogen containing compounds, amount of total excreta, level of serum high density lipoprotein, water intake and cholesterol levels in the blood. This shows that increase in blood glucose during stress was next to that of corticosterone, thus the need for an increased energy supply to cope with stress as discussed earlier. The relative increase in liver weight has been associated with an increase in liver lipid due to the process of gluconeogenesis Puvaldopirod and Thaxton, (2000).

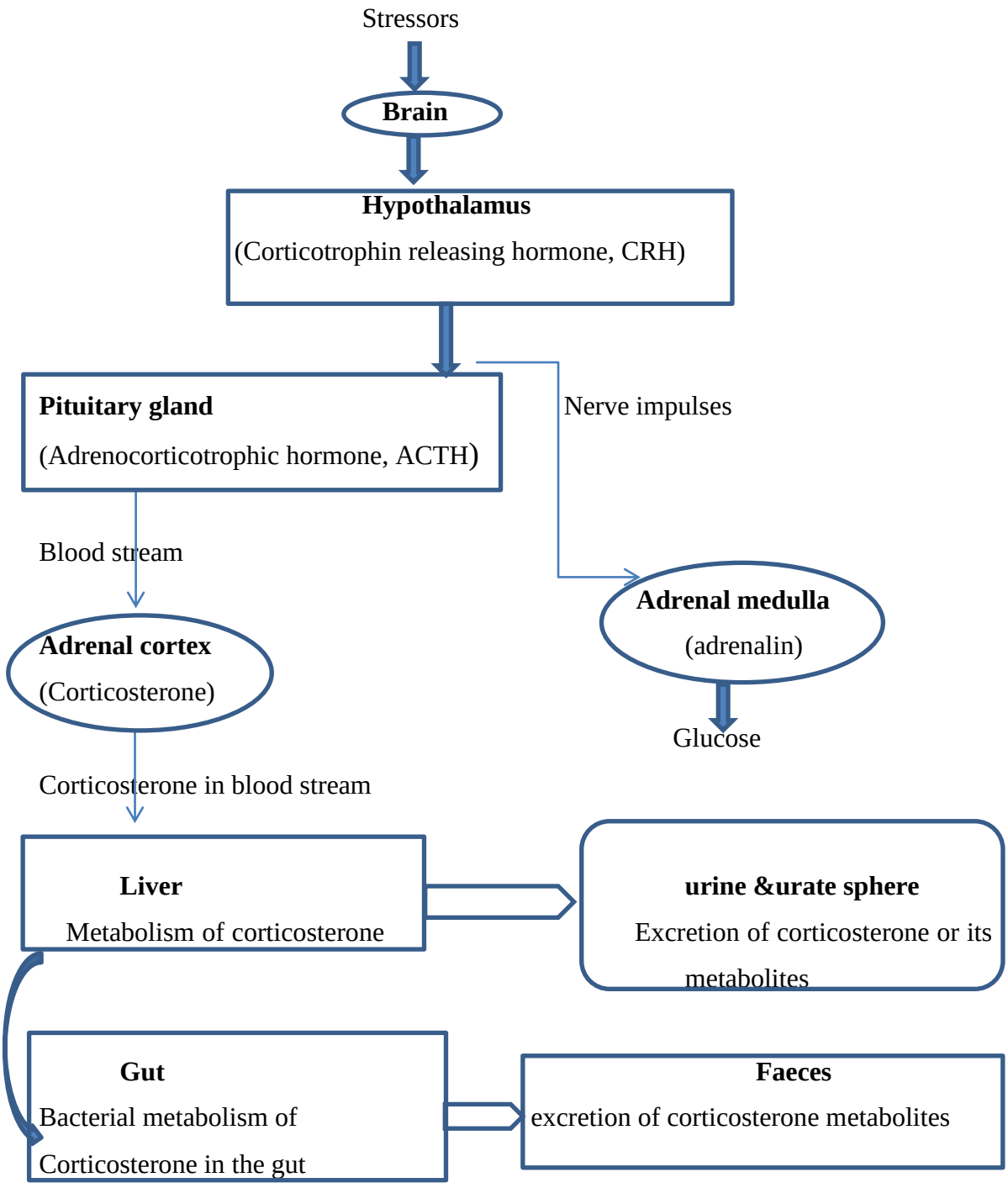

Figure 2.1. Schematic diagram showing the effect of stressors on the secretion, metabolism and excretion of corticosterone (adapted from Ewing *et al*., 1999 and Möstl *et al*., 2005)

2.3.1.1 Acute vs chronic stress

Acute stress can be defined as a single exposure to a stressor (Schoenfeld and Gould, 2012), whereas chronic stress is induced if either the stressor itself or the after-effect of the stressor lasts for a long term (Wiepkema and Koolhaas, 1993). No specific duration

has been reported as to when chronic stress is experienced, however studies have used a range of duration from 7 to 49 days (Post *et al*., 2003; Olanrewaju *et al*., 2006; Shini *et al*., 2008; Vahdatpour *et al*., 2009). It has been argued that acute stress is beneficial to the animal (Dickens *et al*., 2010) because the initiation of the stress response is associated with an increase in blood glucose, increased behaviour directed at fleeing or freezing, increased vigilance and increased cognition (Romero *et al*., 2009). Examples of some of the studies which have explored the effects of chronic stress in different species are summarised in Table 2.1. These studies show that prolonged secretion of corticosterone during chronic stress can have damaging effects on the animal, such as suppression of the immune system, breakdown of muscle due to gluconeogenesis, cardiovascular problems, fear, suppressed cognition and induced depression (Romero *et al*., 2009).

A study in adult male Wister rats (10-11 weeks) showed that treatment with corticosterone (10 mg/kg BW) for an acute (1 day) or chronic (10 consecutive days) period of time had similar effect on the amount of dendritic (neuron) hypertrophy (54 versus 79% respectively), Mitra and Sapolsky (2008). In fact, acute stress in this study resulted in the development of anxiety 12 days post treatment as evident from the reduced ($P<0.05$) exploration in an elevated plus maze compared to control rats Mitra and Sapolsky (2008), whereas the percentage entries into the open arm of an elevated plus maze was similar in rats subjected to chronic stress treatment and their corresponding control. Taken together, this suggests that acute stress is not totally beneficial to the animal and some of the negative consequences could manifest themselves later in the animal's life.

Table 2.1: Illustration of experiments in different species reporting the effects of chronic stress on the animal

Species	Chronic stress paradigm	Duration	Effects on the animal	Reference
Rats	Water deprivation, continuous lighting, cage tilting, grouped housing, soiled cage, water deprivation, stroboscopic illumination and white noise	4 weeks	↓ running activity, ↓ sucrose preference , ↑ heart rate	Grippo *et al.* (2003)
Mice	Stressors included inversion of day/night light cycle, tilted cage, restraint, overnight food and water deprivation & pairing with another stressed animal	6 weeks	↓ sucrose preference by 85%, ↑immobility time by 20%, ↓ body weight by 11%, ↓ mitochondrial membrane[s] in the cortex, hippocampus and hypothalamus by 20, 30 and 23% respectively	Gong *et al*., 2011
Starlings	Loud noise, cage tapping, cage rolling, human voice & restraint	18 days	↓ plasma corticosterone level, ↓ body weight, no effect on immune response to T-cell mitogen challenge	Cyr *et al*. 2007
Quails	Cage tapping, blasts of air and water, restraint, cage rocking, reduced ventilation, feed withdrawal & sound	14 days	↑ resting and preening behaviour, ↓ foraging behaviour, ↑ conspecific calls, ↑ shivering in an emergence test, ↑ learning in a spatial task	Laurence *et al*., 2012

2.3.1.2 Methods and effects of inducing physiological stress in broilers

To understand the stress responses and their effect on animals under a controlled environment, researchers have simulated conditions to mimic stress through exogenous administration of corticosterone in different forms, namely injecting the hormone directly into the animal (Dehnhard *et al*., 2003; Gao *et al*., 2008) adding the hormone to the animal's feed or water (Post *et al*., 2003 and Shini *et al*., 2008) or implanting the hormone into the body (Puvaldopirod and Thaxton, 2000; Olanrewaju *et al*., 2006).

One limitation of the administration of corticosterone through drinking water is the inability to regulate the dosage consumed by the birds, since this depends on the amount of water consumed. However, a regulated dose can be administered by injecting the animal directly or offering birds mealworms which had been previously injected with a known dose of corticosterone.

The indirect feeding of corticosterone to the bird remains the only non-invasive method of administering corticosterone (Müller *et al*., 2009) since it does not involve piercing through the skin (Dawkin, 2004) as is the case with injection, therefore preserving the body integument and minimising pain and discomfort to the animal. In their work with White-crowned Sparrows, (Breuner *et al.*, 1998) used a non-invasive method of inducing acute stress by injecting corticosterone into mealworms before offering them to the sparrows. Their results showed a peak in plasma corticosterone level seven minutes after the birds ingested the corticosterone-injected mealworms, a level which persisted for 60 minutes before returning to baseline levels.

In a study where chronic stress was mimicked, Olanrewaju *et al*. (2006)used exactly the method of Puvadolpirod and Thaxton (2000) to induce physiological stress in broiler chickens. Their aim was to extend the work of (Puvadolpirod and Thaxton, 2000) by investigating the effect of chronic stress on acid-base balance. Olanrewaju *et al*. (2006) showed that corticosterone level of birds implanted with the ACTH mini-osmotic pump (designated to deliver 8IUkg/BW per day) was elevated on the 4^{th} and 7^{th} day post implant. This was accompanied with an increase ($P<0.05$) in pCO_2, HCO_3^-, haematocrit, haemoglobin and glucose levels whereas pO_2, Na^+, K^+ and Cl^- levels were suppressed ($P<0.05$). Thus, apart from the elevated corticosterone causing an increased energy supply (glucose) through gluconeogenesis, there was the loss of electrolytes through the urine. While physiological stress caused increased pCO_2 and decreased pO_2 levels in the blood, the reverse was observed under heat stress where there was an increase in CO_2 loss during panting (Hocking *et al*., 1994).

For most commercial broiler producers, attainment of high body weight within a specific period is of an important objective to maximise throughput of the housing system. However, rapid weight gain may not be achieved if the flock is exposed to conditions that could result in chronic stress. In a study by (Vahdatpour *et al.*, 2009) designed to simulate chronic stress conditions in broilers, from day 1 to 49 days of age,

birds were offered corticosterone in their drinking water (10, 20 or 30 mg of corticosterone per litre). Results showed that the greater the concentration of corticosterone intake by the birds, the lower their final body weight (2121 g vs 1968 g vs 1868 g vs 1670 g for 0, 10, 20 and 30 mg of corticosterone respectively) indicating that chronic stress can have a direct effect on growth performance as nutrients are diverted away from growth.

2.3.1.3 Methods of measuring corticosterone

Corticosterone is mostly measured from blood plasma or serum (Mormede *et al*., 2007). However, there are several limitations to this method, including high variability of corticosterone levels, procedures such as catching and restraining the bird elevates corticosterone level itself if blood is not sampled within 2-3 minutes of the bird being caught (Mormede *et al.*, 2007). Another shortcoming of blood corticosterone is that under chronic (prolonged) stress, corticosterone levels in the blood can be reduced to baseline levels by the negative feedback mechanism, which assists in the regulation of the concentration of corticosterone in circulation (Pariante and Lightman, 2008) such that it becomes difficult to differentiate between a chronically stressed animal and a control. Destrez *et al*. (2013) found no difference in cortisol level of chronically stressed sheep (for a 6 week duration) and control sheep. 'In a negative feedback, the production of a product by the target tissue feed back to the source of the hormone and causes it to stop producing the hormone', Nelson (2011). In one particular study, two hours after injecting 9 day old chicks with 0.2μg/g body weight corticosterone, there was negative feedback through the corticotrophin releasing hormone precursor in the hypothalamus, thereby reducing the amount of corticosterone in circulation (Vandenborne, 2005). Corticosterone can also exert negative feedback at the pituitary level of the HPA axis (Lumeij, 1994a).

Nevertheless, chronic stress could be detected by conducting an HPA axis test such as the dexamethasone suppression test (Mormede *et al.*, 2007). Dexamethasone is a synthetic glucocorticoid, which suppresses the plasma corticosterone by inhibiting the pituitary gland's involvement in the secretion of corticosterone (Cole *et al*., 2000). Lack of suppression of corticosterone by dexamethasone indicates the hyperactivity of the HPA axis (van Praag, 2005) which is common under conditions of chronic stress.

To overcome these limitations of measuring corticosterone from the blood, there is increasing interest in the development of alternative methods. In birds, a number of non-invasive methods of assessing corticosterone exist including measuring levels in faeces, feathers, yolk and albumin of eggs (Cook, 2012). Measuring corticosterone metabolites from the faeces of small birds could be useful due to the limited amount of blood that can be sampled from such birds (Lobato *et al.*, 2008). Faecal corticosteroid metabolites have been useful in assessing the effect of ecological conditions such as ectoparasite infestation, ambient temperature and brood size in blue tits and pied flycatchers (Lobato *et al.*, 2008) and evaluating different capturing systems for great cormorants (Dehnhard *et al*., 2003). Level of corticosterone metabolites measured from egg is known to be positively correlated (R^2 = 0.47, P<0.05) with the stress status of the mother hen, with hens exposed to predators having greater levels of corticosterone metabolites in the egg laid the following day (Pitk *et al.*, 2012). This emphasises the usefulness of non-invasive means of measuring corticosterone under a wide range of environments.

However, there is a lag period between the peak of corticosterone level in the blood following an acute stress and when it can be detected in faeces, yolk and feathers, a lag which ranges from hours, days or weeks respectively (Cook, 2012). Starlings injected with ACTH showed a greater (P<0.0001) level of faecal corticosteroid metabolites after two hours post injection compared with control birds injected with saline (Cyr and Romero, 2008). The use of yolk and egg albumin to measure corticosterone level is obviously limited to broiler breeders or laying hens.

The droppings of birds comprise of faeces, urine and urate sphere which are the brown, liquid and solid white components respectively (Lobato *et al.*, 2008). Recently, the detection of corticosterone metabolites in the faeces and urine of Japanese quail and chickens was investigated by Hirschenhauser *et al*. (2012). After an intravenous injection of radio-labelled corticosterone, a peak of excreted metabolites (averaged for males and females) was observed in the urine of quails (50 mins) and chickens (97 mins), which were earlier than the peak observed in the faeces which came later (195 minutes and 190 minutes in the quail and chicken respectively). Since acute stress responses cannot be rapidly detected in faeces then the use of urine or urate spheres could be a better option for broilers. Furthermore, the collection of urine samples in birds requires the use of metabolism cages. In a commercial setting, broilers are reared on littered floors so that the urine is absorbed, whereas the urate sphere is solid in nature and not readily absorbed by the litter. Equally, under conditions of dehydration, 15 % of

the urine is reabsorbed from the colon (Lumeij, 1994b) and this could limit the amount of urine available for collection. Since the uric acid component is not dependent on the level of hydration (Lumeij, 1994b) and with 65% of uric acid present in the urate sphere (Braun and Pacelli, 1991), then urate sphere production is unlikely to be affected by level of hydration.

Urate spheres are formed in the renal tubules of the bird's kidney and contain uric acid, albumin and inorganic ions (Frandson *et al*., 2013).The main inorganic ions present in the urate spheres of domestic fowl are calcium (70%) and potassium (30%), (Casotti and Braun 1997). Using a freeze fracturing technique, Cassoti and Braun (2004) revealed that the proteins in the urate spheres are in the form of rings (3-4 rings), whereas the ions are randomly distributed in the urate spheres (Figure 2.2). Cassoti and Braun (2004) were the first to examine the composition of urate spheres in other avian species apart from domestic fowl. Their study involved 9 avian species namely; domestic fowl, Gray catfish, Wood thrush, Barn swallow, House sparrow, Chipping sparrow, Northern cardinal, Rufous-sided towhee and Oven bird. The size of the urate sphere ranged from 0.5-5µm in most of these species except the domestic fowl which had a diameter of 0.5-10µm.

Being a lipo-soluble substance, corticosterone and other steroid hormones form a bond with proteins in the blood such as albumin for transportation to the target organs (Nelson, 2011), Clapp (2010) therefore proposed that like other blood constituents, endogenous corticosterone could be passed into the solid white component of avian urine namely the urate spheres (see Figure 2.3). Clapp (2010) positively identified parent corticosterone (endogenous) in extracts of urate spheres of the Great tit. However, one limitation in his study was that the hormone assay used (ELISA) overestimated the level of corticosterone in the plasma and urate sphere. The use of urate sphere as a non-invasive tool for measuring stress levels in birds therefore needs to be further developed by adapting an appropriate hormone assay that would give an accurate measure of corticosterone level.

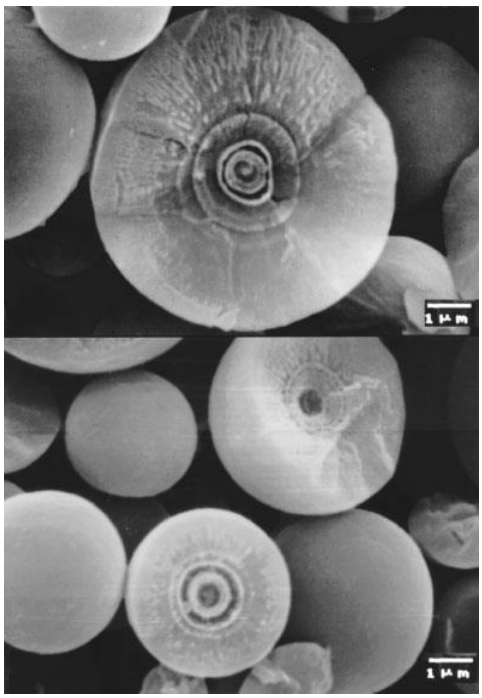

Figure 2.2: Cross section of urate sphere showing a central nidus and 3-4 rings of protein (Casotti and Braun, 2004)

Figure 2.3: The droppings of a broiler chicken showing the faeces (brown portion) and the urate sphere (white portion).

2.3.2 Heat stress

In this section explanation on the response of birds to different temperature zones will be discussed with emphasis on the temperature zone where birds become heat stressed

(upper critical temperature). Then various definitions and types of heat stress will be explored with the aim of developing non-invasive measures of core body temperature. Next, the impact of heat stress on the growth performance, physiology and welfare of broilers and finally existing methods of alleviating heat stress will be reviewed.

2.3.2.1 Homeothermy and temperature zones of broilers

Like all other avian species, birds are homeothermic in nature, meaning that they have the ability to maintain a stable internal (i.e. core body temperature, CBT) condition under extreme environmental conditions (Schwab and Schafer, 1972), which gives birds the opportunity to adapt through changes in their physiology or behaviour under environmental conditions that are outside the normal conditions. However, this may depend on the severity and duration of the environmental conditions.

One of the homeostatic processes involves the regulation of CBT (Sherwood *et al*., 2005) since the normal CBT of broilers is between 40.6-41.7^{o}C (Olanrewaju *et al*., 2010). In the thermoneutral or comfort zone, the range in environmental between point b and c in Figure 2.4, (typically between 18-22^{o}C, although this will vary depending on age, bodyweight etc.) laying hens and broiler chickens attain maximal growth and production (Keshavarz, 1990, Charles, 2002) because minimal effort is required to lose heat nor to produce extra heat to keep warm.

In fact, temperatures below or above the thermoneutral conditions are life threatening to broilers. For instance, there is a higher incidence of mortality in broilers transported during the winter and summer months than in spring and autumn periods (Vecerek *et al*., 2006), indicative of the detrimental effect of cold and heat stress on broilers. The same could apply for the lack of adequate control of the environmental temperature in poultry houses. Figure 2.4 from Hillman *et al*. (1985) shows the relationship between body temperature, metabolic rate, heat loss and ambient temperature. When the ambient temperature falls below the lower critical temperature (LCT, point a in Figure 2.4), the posterior hypothalamus triggers an increase in heat production by increasing the basal metabolic rate (BMR) and feed intake alongside the conservation of body heat through various means such as constriction of blood vessels (Sherwood *et al*., 2005).If ambient temperature keeps reducing below the LCT, and if the increase in metabolic heat production and suppression of heat loss is not enough to regulate CBT, then CBT begins

to drop due to the loss of heat from the body to the environment resulting in hypothermia (reduction in CBT) and subsequently death.

On the other hand, for temperatures above the upper critical temperature (UCT, point d in Figure 2.4), the temperature gradient between the bird and the environment is reduced and consequently heat loss by non-evaporative means is reduced. At this time, mechanisms that could help reduce metabolic heat production or increase heat loss are triggered such as increased water intake, reduced feed intake and increased respiratory rate for evaporative cooling (Hill *et al*., 2008). If these control measures fail, then the CBT would begin to rise leading to hyperthermia. An increase in CBT by 4^{o}C is life-threatening to birds (DEFRA, 2005).The upper critical temperature is the environmental temperature above which the bird is likely to experience heat stress. At the point where birds cannot control their CBT then welfare problems exist (DEFRA, 2005) thus an estimate of CBT could be a useful indicator of the welfare of broilers under conditions of heat stress.

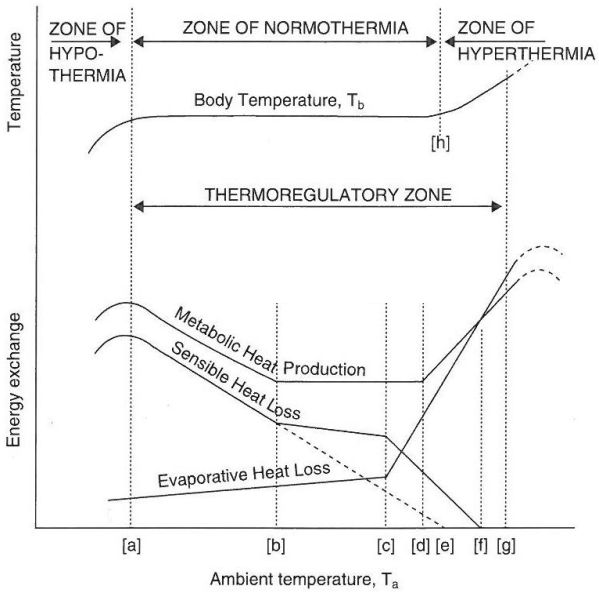

Figure 2.4: Effect of ambient temperature (Ta) on metabolic heat production, heat loss and body temperature (Hillman *et al*., 1985 as cited in Daghir, 1995). a=lower critical temperature, b= critical temperature, c= increased evaporative heat loss
d =upper critical temperature, e = ambient temperature equals body temperature
f = sensible heat loss equals zero because metabolic heat loss equals evaporative heat loss, g = thermal maximum, h = beginning of hyperthermia.

Given the opportunity, studies in broilers have shown that broilers can select their thermal environment depending on the need for heat gain or loss. Hooper and Richards (1991) used an operant conditioning test where birds were trained to peck at a disc to

receive a thermal reward in order to assess their preference for a particular thermal environment. Birds were subjected to three different temperature ranges (0-6, 22-24 or 32-36^{o}C). The least number of pecks by birds was in the thermal environment of 22-24^{o}C compared to the other two thermal environments. The increased number of responses by the birds in the warm and cold environments showed the need for a reduction or increase in the thermal rewards respectively. At temperatures of 22-24^{o}C, birds were more comfortable and so had no need to peck at the disc to obtain a thermal reward. The selection of the thermal environment based on the need for heat gain or heat loss was achieved because the birds were able to apply the knowledge in solving their problem. Operant conditioning tests depend on the use of behaviour and cognition by the birds to control its environment (Zebunke *et al*., 2013). This kind of opportunity is usually not available to commercially managed poultry where environmental conditions are mostly controlled automatically. However, birds reared with access to outdoors (free range) have the opportunity to seek shade if the weather becomes inclement (i.e. too hot or too cold).

2.3.2.2 Measurement of core body temperature

The body of an animal can be divided into the core and periphery. The body core consists of the central nervous system (brain and spinal cord), visceral organs and parts of the skeletal musculature, while the periphery includes the other parts of the skeletal musculature, skin, feathers, un-insulated extremities and subcutaneous fat layer (Schwab and Schafer, 1972). Although Schwab and Schafer (1972), did not specify which skeletal musculature belonged to the core or periphery, it is reasonable to suggest that deep skeletal musculature close to the visceral organs could be categorised as core while the peripheral skeletal musculature could be classed as the periphery. The main thermoregulatory centre is the hypothalamus which depends on signals sent from temperature receptors (thermo receptors) present on body surfaces (periphery) and in the core (central). The anterior hypothalamus controls heat loss while the posterior controls heat gain (Rastogi, 2007).

The standard means of measuring CBT is from a digital thermometer inserted into the rectum of the animal (Quimby *et al*., 2009). However, the procedure of handling and restraining the bird could interfere with core body measurement (Lowe *et al*., 2007). In addition, this method is laborious to use on a large population of animals (Lohse *et al*.,

2010). The use of thermometer probe to measure CBT in broiler chickens exposed to a 24 h period of heat stress (at 2 hour intervals) took about 4 mins per bird (Lin *et al*., 2004). Using a thermometer on a small number of birds may not be challenging, but it becomes very difficult and time consuming with large number of animals. Another limitation to the use of digital thermometers is the need to clean the probe before it is used in another animal (Quimby *et al*., 2009; Kort *et al*., 1998) to prevent the transfer of disease from one animal to another. Therefore methods that could be used on unrestrained animals were developed (Lowe *et al*., 2007).

For continuous CBT measurement from free moving animals without the need for restraint, data loggers (Dawson and Whittow, 2000) and telemetry devices (Lacey *et al*., 2000; Hamrita *et al*., 1998) have been developed. The use of data logger or telemetry devices often requires invasive surgery to be performed on the animal to place the device inside the body cavity, although some telemetry systems do not require surgery but need to be swallowed by the animal i.e. oral administration (Brown-Brandl *et al*., 2003). However, temperature readings obtained from orally administered telemetry devices are prone to high variability. This is probably because the measurement taken by this device is reflective of the gastrointestinal tract (GIT) temperature which can vary between meals, therefore extra effort is required to check for anomalies in the readings obtained. One advantage of telemetry devices over a data logger is that estimates of CBT are instantaneously passed to a computer (Lacey *et al*., 2000) whereas for a data logger, data of CBT can only be downloaded after retrieval of data logger from the animal (which requires further surgery or post mortem).

To overcome the challenges of digital thermometers, data loggers and telemetry devices in measuring core body temperature, recent advances in technology have led to the development of temperature-ID microchips that serve the dual purpose of giving electronic identification of individual animals alongside estimates of CBT (Chen and White, 2006). The temperature-ID chip requires a less-invasive means of implantation since it can be injected subcutaneously or intramuscularly, and does not require the animals to be restrained during data collection. CBT is estimated by scanning the chip with a designated scanner. Temperature-ID chips give an instantaneous reading of CBT as frequently as required without causing stress to the birds although they are more expensive than digital thermometers but less expensive compared to data loggers or telemetry devices (Chen and White, 2006).

In a comprehensive study, Torrao *et al*. (2011) implanted a data logger in the abdominal cavity and microchips in five different body parts of goats (retroperitoneum, subcutaneously in the groin, muscles in the hind limb, subcutaneous in flank and subcutaneous between shoulder blades) after which the goats were subjected to six different conditions each lasting for 7 h/day with an interval of one day between conditions. The conditions to which the goats were exposed to were thermo neutral, cold, cold plus radiant heat, hot, hot plus radiant heat and an induced fever conditions. Their results showed an effect of implantation site on the closeness of microchip temperatures to that of the data logger specifically microchips implanted in the retroperitoneum gave estimates of CBT closest (less by 0.2^{o}C) to that of the data logger, whereas temperatures measured from microchips implanted subcutaneously were less than that from the data logger by 0.5-1^{o}C. However, all microchip temperatures from the five body parts gave readings close to that of the data logger specifically under the hot conditions compared to the other five conditions, probably due to the reduced temperature gradient between the core and body surfaces under the hot condition arising from the increase in blood flow to the periphery.

Torrao *et al*. (2011) further reported a wide difference between CBT estimated from a data logger and microchips implanted intramuscularly and subcutaneously under thermoneutral conditions. This could probably serve as an explanation for the high limit agreement (1.5^{o}C) obtained between body temperature determined from microchips implanted subcutaneously between the shoulder blades and that from a digital rectal temperature in cats (Quimby *et al*., 2009) and rabbits (Chen and White, 2006). For accurate measure of CBT, it is of utmost importance for microchips to be implanted in an appropriate site. However, (Kort *et al*., 1998) raised concerns about the implantation of chips in the intraperitoneal region of rats, due to the need to avoid damage to internal organs. In a broiler chicken study involving the exposure of 16 day-old birds to heat stress (34^{o}C) for 18 h during which core and muscle temperatures were estimated alongside other physiological parameters, a similar trend was reported in the increase in core and muscle temperature being greater at the end of 6 and 18 h (Mujahid *et al*., 2009). This suggests that the response of muscle temperature under heat stress closely matches that of CBT. Giving this promising use of temperature-ID chips in previous studies it therefore suggests that temperature-ID chips implanted in the muscles of broilers could give accurate estimates of CBT under conditions of heat stress.

There are consistent reports about the validity of using temperature-ID chips against other devices in estimating CBT. Once implanted, microchips can function for a longer period of time than a telemetry device. One study reported that after two years of implanting microchips in wood buck, the chips still gave correct readings (Mrozek *et al*., 1995). In contrast, Hamrita *et al*. (1998) reported that battery of a telemetry device implanted in birds became weak after 100 days.

The use of microchips for measuring CBT of animal species such as cats (Quimby *et al*., 2009), mice, rabbits (Chen and White, 2006) has been validated against rectal temperature estimated from a digital thermometer. However, in goats, Torrao *et al*. (2011) validated three methods of estimating CBT namely a data logger, a microchip and a rectal temperature. The repeatability of using microchips was greater than that of digital thermometers. For instance, microchips implanted between the shoulder blade of rabbits gave repeatability variance of 0.038 compared to digital thermometers of 0.215 (Chen and White, 2006). Similarly, microchips implanted in between the shoulder blade of cats gave a repeatability variance of 0.22 compared to 0.40 from the digital thermometer (Quimby *et al*., 2009). Although the repeatability of the microchip was better in the study of Chen and White (2006) than that of Quimby *et al*. (2009), this could be due to the fact that different microchip models were used (IPTT-200 versus IPTT-300) and also species differences, since as 26 out of the 40 cats used in the latter study were reported to be restless during data collection. Nevertheless both studies have shown that CBT estimates from chips were more constant than those from the digital thermometer.

Few cases of problems with the use of temperature-ID chips have been reported. Lohse *et al*. (2010) and Chen and White (2006) reported malfunction of one and four chips, effectively meaning that 96.7% and 91.3% of microchips were reliable respectively. In the case of Lohse *et al*. (2010), the chip was not recognised by the scanner and post mortem exanimation could not detect the chip in the position where it had been implanted subcutaneously, probably because the chip had migrated to another position. Similarly, Chen and White (2006) suggested that the chip had migrated because it could not be detected by palpating the position where it had been implanted, neither did the scanner detect any reading from it. Additionally, a problem with another chip was underestimation of CBT, whereas the last two chips did not give estimates of CBT but could be palpated (Chen and White, 2006). Although the manufactures of these microchips report that they are anti-migratory, these studies have nevertheless shown

some level of chip migration when chips were implanted subcutaneously. Hence, the problem of microchip migration could be avoided if microchips were implanted into the deep muscle of the animal although this is more invasive.

2.3.2.3 Definitions of heat stress

Definitions of heat stress in the literature can be grouped into three themes, namely i) a definition based on the cause of heat stress, ii) a definition emphasising the effects of heat stress, and iii) a definition indicating both the cause and effect of heat stress. The third theme has the obvious advantage over the first two themes because of its completeness; hence it will be the focus for this thesis.

Some of the definitions which fall within the third theme are: 1) long exposure of birds to high temperature resulting in excess heat load more than can be dissipated by the birds (Chaiyabutr, 2004); ii) exposure of birds to high temperature and humidity at a point where thermoregulatory responses such as panting (increased respiratory rate) are observed (Ojano-Dirain and Waldroup, 2002); iii) a combination of temperature and humidity that causes the body temperature to rise above the birds' normal body temperature of 41.5^{o}C (Swick, 1998); and iv) high temperature and RH resulting in the inability of the birds to maintain body temperature such that body temperature increases out of the normal range (Jensen and Toates, 1997). Three out of these four definitions emphasise the cause of heat stress from high temperature and RH whereas just one attributed the cause to be from high temperature alone. Although high temperature can cause heat stress, its combination with high RH intensifies the intensity of heat stress experienced by an animal, a concept referred to as apparent equivalent temperature (AET, see Section 2.3.2.4). RH is a very important environmental factor, since high RH reduces the amount of heat loss through evaporative cooling, hence making it more difficult for regulation of CBT (Balnave, 2004). Two authors clearly state that heat stress causes a rise in CBT, This supports that report of DEFRA (2005) that welfare problems arising from heat stress exist when CBT begins to rise. Hence, a measure of CBT could indicate the welfare of the birds during heat stress. Chaiyabutr (2004) used the word '*excess heat load*', which implies storage of heat in the body because of the inability to dissipate heat which consequently results in an increase in CBT. Only one author (Ojano-Dirain and Waldroup, 2002), based the effect of heat stress on an increase in respiratory rate.

The definitions of Swick (1998) and Jensen and Toates (1997) are similar, except that Jensen and Toates (1997) were more specific in their definition by mentioning that heat stress was caused by high temperature and RH and not just any combination of temperature and RH as pointed out by Swick (1998). A criticism of the definition of Chaiyabutr (2004) is that whilst it mentioned long exposure to high temperature, there was no specific duration mentioned and this could mean hours or days or weeks or even months.

An illustration of the incidence of heat stress (high temperature, high RH or a combination of both) is shown in a survey from 10 commercial farms in the United Kingdom and Denmark undertaken by Jones *et al.* (2005). Results showed that at some points during the growth cycle, commercial broilers were exposed to temperature and RH levels which are above their thermoneutral zone (Jones *et al*., 2005). The percentage of time when broilers were exposed to temperature and RH above the recommended values throughout their growth cycle is presented in Figure 2.5, showing that the cumulative percentage of time when the temperature and RH rose above normal levels was higher in birds of age 3-6 weeks of age. At 3-4 weeks of age, most of the commercially reared birds had a greater percentage of time exposed to temperatures above UCT, whereas towards the end of the growth cycle (5-6 weeks of age), the percentage of time broilers experienced RH above 70% was greater. Coincidentally, the period of the growth cycle when birds experienced temperature and RH above the recommended values falls within the age when broilers have increased growth rates and fully developed feathers, both of which makes heat dissipation difficult. Hence most commercially-reared birds might experience heat stress conditions during some point in their growth cycle.

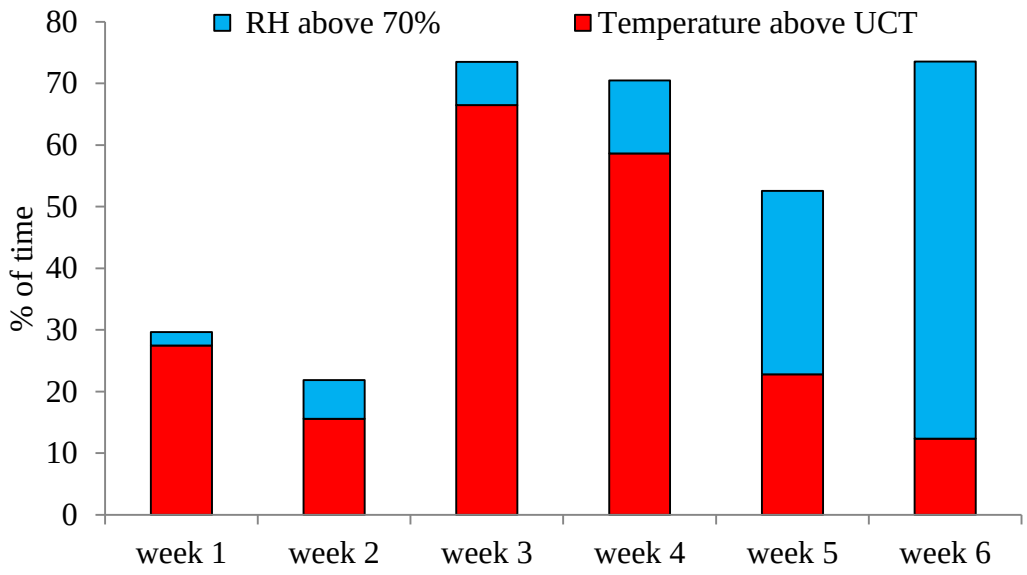

Figure 2.5: Percentage of time commercially reared broilers were exposed to temperatures above the upper critical temperature and RH above 70% throughout their growth cycle (Jones *et al*., 2005)

2.3.2.4 Types of heat stress

Environmental temperature and RH in both tropical and temperate regions of the world vary with season and this variation could lead to extremes of either low or high environmental temperature/RH. Acute heat stress is defined as the exposure of birds to short duration of sudden high temperature, whilst chronic heat stress is defined as the exposure of birds to a consistently high temperature for an extended period of time, ranging from days to months (Emery, 2004). In temperate regions, broilers are raised mostly in controlled environment buildings but a sudden high increase in temperature and humidity could lead to 50% mortality (Balnave, 2004). Conditions where broilers could experience acute heat stress include during a failure of power supply to control ventilation systems (Widowski, 2010), diurnal change in temperature/RH (Yahav *et al*., 1997) or transportation to the slaughterhouse (Mitchell and Kettlewell, 1998). In fact, a shortage of power supply resulting in ventilation failure for about 45 minutes during a heat wave killed 50,000 chickens housed in a commercially-run broiler unit in North Carolina, USA (Hegeman, 2011).

In an acute heat stress study broilers aged 33 days were exposed to 32^{o}C for 6 h, plasma corticosterone levels were similar in the heat stressed and control birds at the end of the sixth hour of heat stress, whereas rectal temperature was elevated compared to the control birds (42.8^{o}C versus 42.1^{o}C, $P<0.05$; Lin *et al*. 2006). The increase in CBT

indicates that the birds were unable to regulate their CBT and the increase in CBT of 0.7^{o}C implies that the birds experienced moderate levels of heat stress (Mitchell and Kettlewell, 1998). However, in this study corticosterone levels were not affected by exposure to heat stress. Thus, corticosterone secretion under conditions of heat stress might be a better measure of stress under a high heat stress rather than moderate levels of heat stress; however changes in CBT can be used to detect both moderate and high levels of heat stress.

On the other hand, chronic heat stress for a period of 2-3 months could be experienced in temperate regions during the summer (Renaudeau *et al*., 2012). The duration of chronic heat stress can be longer in tropical and subtropical regions, although in this case birds may have sufficient time to become acclimated to the harsh conditions (Swick, 1998), compared to acute heat stress conditions which demands rapid changes in the body system. Changes in biochemical, physiological and anatomical adjustments occur during acclimatisation (Hill *et al*., 2008). Therefore, the problem of heat stress in the tropics is usually that of reduced weight gain and food conversion, with mortality of only 10% typically recorded (Swick, 1998).

There are some variations in the duration reported for birds to become acclimatised to heat stress. While Hillerman and Wilson (1955) reported that it takes an adult fowl 3-5 days to become adapted to hot environments, Yahav *et al*. (1996) reported that 4-7 days was sufficient for broilers to control their CBT, when exposed to hot environmental conditions. The slight variation between the numbers of days required for acclimatisation could be due to genotype differences, hens versus broilers. In agreement with Yahav *et al*. (1996), a more recent study by Abdelqader and Al-Fataftah (2014), showed that exposure of 21-day old broilers to intermittent repeated heat stress (38^{o}C and 62% RH for 1, 2, 3 or 4 h) compared to control birds kept at 22oC, 65%RH daily for 14 days, improved their level of thermotolerance in a subsequent exposure to heat stress of 43^{o}C, 55% RH for 4 h when aged 36 days old. Specifically, during the heat challenge at 36 days old, birds which had previously been subjected to 4 h intermittent heat stress during acclimatisation had a lower rectal temperature (43.6 vs 45.7^{o}C, $P<0.05$), rate of increase in rectal temperature (1.74 vs 3.64^{o}C/h, $P<0.05$), evaporative water loss (5.5 vs 19.4 g/kg BW/h, $P<0.05$), mortality (0 vs 25%, $P<0.05$) and increased survival time (98.3 vs 58 minutes, $P<0.05$) compared to non-acclimatised birds (Abdelqader and Al-Fataftah, 2014). In this study, acclimatisation was achieved in 7 days, although the only consequence was a reduction in weight gain compared to birds

kept in the control conditions. Acclimation involves exposure to heat-stress conditions for several days or weeks in order to improve thermotolerance by increasing the safety margin of CBT and heat dissipation but reduces heat production (Yahav, 2009).

It is important however to note that an intermediate class of heat stress exists between acute and chronic forms, which is experienced during a heat wave when daily variation in environmental temperature/RH lasts continuously for one to five days with an average of 2.7 days (Vale *et al*., 2010). Robinson (2001) defined a heat wave as a period of extended high temperature and RH lasting continuously for a minimum of five days. In birds subjected to cyclic high temperature (21-30-21^{o}C) for 3.5h / day for 3 consecutive days, plasma corticosterone level was elevated ($P<0.05$) on the 1st and 3rd day with the 2nd day being intermediate (Mahmoud *et al*., 2004). This study shows that episodic high temperature is stressful to birds and the level of stress was similar on the three days. The experience of heat stress by broilers consecutively during a heat wave might be the reason for the high mortality. However, Mahmoud *et al*. (2004) did not consider the effect of RH nor did they estimate CBT under a simulated heat wave.

Due to global warming, there is an expected increase in the surface temperature of the earth, and from data analysis from the National Weather Service in the USA, Robinson (2001) predicted that the frequency, duration and intensity of heat waves would increase. Despite the great mortality of birds during heat wave, detailed investigation about the effects of episodic heat stress on the growth performance, physiology and welfare of broilers is scarce. Moreso, the duration of heat wave seems to vary in different regions of the world.

2.3.2.5 Apparent equivalent temperature (AET)

Since our definition of heat stress is that of a combined effect of high temperature and relative humidity, then it is necessary to introduce the concept of apparent equivalent temperature. Since the prevailing environmental temperature and RH interacts together and do not act independent of one another, then the cumulative effect of environmental temperature and water vapour density (derived from the prevailing RH) indicates the integrated thermal load (i.e. the amount of heat in the body) experienced by the bird. This integrated thermal load is referred to as the apparent equivalent temperature (AET), Mitchell and Kettlewell (1998). The effectiveness of heat loss is dependent on both the temperature and water vapour density gradient of the environment (Tao and

Xin, 2003a). The influence of RH and temperature on evaporative heat loss in adult fowl (hens) is presented in Figure 2.6, where changes in RH affected the efficiency of evaporative heat loss in warm (24^{o}C) and hot (34^{o}C) conditions, but had no effect at normal (20^{o}C) temperatures (Romijn and Lokhorst, 1966). The apparent equivalent temperature is derived from the formula: AET = T + e/γ*, where T is temperature, e is water vapour pressure, γ* is corrected psychometric constant (derived from the ratio of the resistances to heat and water vapour transfer) according to Mitchell and Kettlewell (1998).

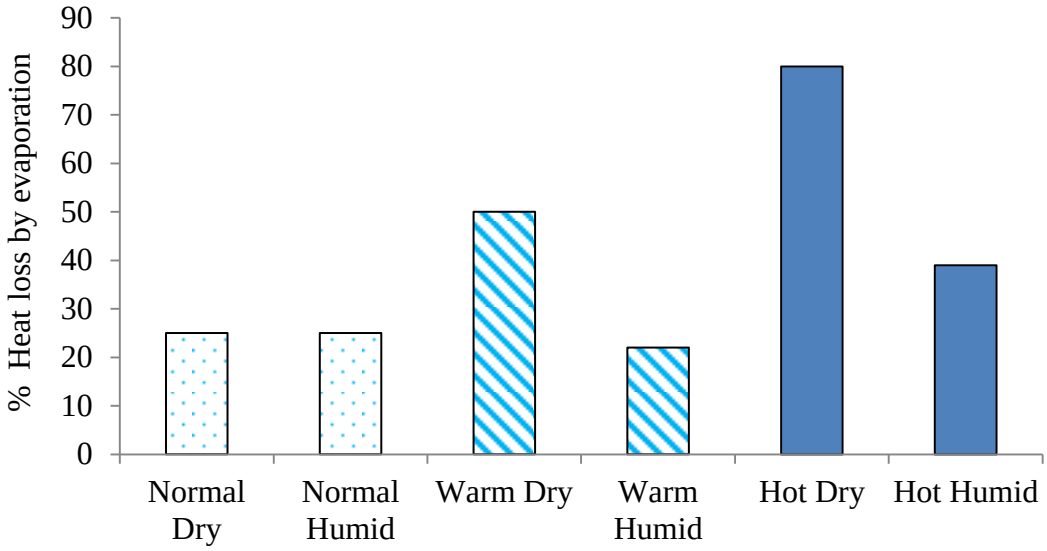

Figure 2.6: Effect of temperature and RH on evaporative heat loss (%) in hens (Romijn and Lokhorst 1966). Normal dry (20^{o}C, 40% RH), normal humid (20^{o}C, 87% RH), warm dry (24^{o}C, 40% RH), warm humid (24^{o}C, 84% RH), hot dry (34^{o}C, 40% RH) and hot humid (34^{o}C, 90% RH)

In this thesis, we adopted the definition of Jensen and Toates (1997) which states that heat stress is caused by high temperature and RH resulting in a rise in CBT above normal body temperature using a simulated episodic heat stress condition created by diurnal changes in the temperature/RH for a minimum of 3 days according to Vale *et al*. (2010).

2.3.2.6 Means of heat loss during heat stress

This section reports the various opportunities for heat loss in birds. A simple formula relating heat production and loss is S = H-E ± R ± C ± K (Dawson and Whittow, 2000),

where: S= heat gain or loss, H = metabolic heat production, E= evaporative heat loss, R = heat gain/loss by radiation, C = heat gain/loss by convection and K= heat gain/loss by conduction. If permitted to go outdoors as in the case of free range birds, heat can be gained from solar radiation especially when the ambient temperature is greater than the CBT (Chaiyabutr, 2004). So a bird can be said to be under a condition of thermal balance when the two sides of the equation are equal i.e. heat production = heat loss (Dawson and Whittow, 2000). CBT is maintained when S=0 but body temperature rises when S is positive i.e. more heat is gained than is lost, and falls when S is negative i.e. more heat is lost than gained.

If the CBT of the bird is greater than that of the ambient temperature, heat can be lost through four principal ways namely conduction (i.e. heat transfer through a medium which requires physical contact between the surfaces involved in the heat exchange), convection (i.e. heat transfer through a moving air or liquid), radiation (i.e. transfer of heat without a medium, such that heat is radiated from a warmer to cooler surfaces) and evaporation (i.e. heat loss through evaporation of water from body/respiratory surfaces), (Chaiyabutr, 2004). In a commercial poultry setting, broilers have physical contact with the litter and can lose heat to the litter through conduction. For instance, the heat lost from a 35 day old bird reared in temperature between 18.5-20.5^{o}C raised litter temperature by 10.72^{o}C (Gerken *et al*., 2006). This type of heat loss may not be available for caged birds so they will have to rely more on convection losses which could be enhanced by increased air velocity (Yahav *et al*., 2004).

The first three means of heat loss (through conduction, convection and radiation) are referred to as sensible means of heat loss (Chaiyabutr, 2004) and are highly dependent on the prevailing ambient temperature because heat flow can only be achieved when there is a temperature gradient, i.e. a temperature difference between two bodies (Chaiyabutr, 2004). Sensible means of heat loss are effective when the ambient temperatures are below or within the thermoneutral zone, as presented in Figure 2.4 (Chaiyabutr, 2004). The efficiency of sensible heat loss is controlled by two main factors. Firstly an increase in surface area of the bird, achieved by adopting positions such as sitting or standing with wings spread away from the body which is known as wing drooping (Etches *et al*., 2008). The second is through vasodilation of the blood vessels which is triggered by the hypothalamus to enhance heat transfer from the core to

the body surface (Teeter and Belay, 1996). Because of the feather covering in birds, heat transfer to the surface is directed towards less feathered body parts such as combs, legs and under the wings (Gerken *et al*., 2006).

Evaporative (also known as insensible) heat loss is greatly affected by the prevailing RH of the environment (Lin *et al*., 2006). Total evaporative heat loss is the sum of evaporation from the respiratory tract (primary) and the skin (secondary) (Dawson and Whittow, 2000) with the former greater than that the latter. Hence, an increase in respiratory rate in the form of panting is an indication of the need for evaporative cooling (Etches *et al*., 2008). During panting, evaporative cooling is achieved when latent heat of evaporation converts water from the liquid to the gaseous state. For every gram of water evaporated, 2.4 kJ of heat is lost (Renaudeau *et al*., 2012). The latent heat of evaporation comes from the tongue which is richly supplied with blood (Bligh, 1985).

2.3.2.7 Behavioural mechanism of heat loss in broiler chickens

Behaviour can be defined as the response of an organism to both internal and external stimuli. Keeling and Jensen, (2002) described behaviour as the best indicator of welfare for two reasons, namely it is easy to measure and it reflects an attempt to cope with a stressful condition. Some of the behaviours exhibited during heat stress include wing drooping, panting and drinking. As mentioned above, wing drooping behaviour involves the spreading of wings away from body to expose the less feathered parts of the body (Etches *et al*., 2008). This behaviour aids convective heat loss from the body surface especially under the wing (Etches *et al*., 2008), see Figure 2.7a. However, wing drooping behaviour declines as the body temperature begins to rise (Etches *et al*., 2008) presumably at the point where sensible heat loss is at its minimum level because the temperature gradient between the bird and the environment is reduced (See Figure 2.4), so the birds resort to panting.

One of the visible symptoms of a bird under heat stress is panting or increased respiratory rate (Etches *et al*., 2008) which is necessary for evaporative cooling (Menten *et al*., 2006) through the mouth and respiratory tract (Etches *et al*., 2008). The respiratory rate of birds is dependent on the age, ambient temperature and RH (Syafwan *et al*., 2011). DEFRA (2005) claimed that the presence of panting behaviour is an indication of the beginning of a welfare problem associated with heat stress (Figure

2.7b). Birds exposed to high heat stress conditions (32^{o}C) started panting after only 6.5 minutes compared to 7.5 minutes for birds exposed to moderate heat stress (28^{o}C), Gerken *et al*. (2006).

To increase evaporative cooling from body surfaces, birds can splash water on their combs and wattles (Dawson and Whittow, 2000).The effectiveness of evaporative cooling through panting is related to the increased frequency of breathing and saliva secretion (Chaiyabutr, 2004). One disadvantage of panting as an effective evaporative cooling mechanism is that it involves high muscular activity which consequently adds to the heat load of the bird, thus a greater energy requirement is needed to maintain homeothermy (Tao and Xin, 2003a). However, some behavioural mechanisms for heat loss can be inadvertently impeded by high stocking densities. For example, the opportunity for wing drooping behaviour can be limited during transportation to the slaughterhouse because of the reduction in space between birds (Mitchell and Kettlewell, 1998) which reduces the opportunity for heat loss by conduction and convection.

Figure 2.7a.Wing drooping behaviour **Figure 2.7b**. Panting behaviour (Iyasere, 2009)

Other prominent behavioural changes during heat stress are reduced feed intake and increased water intake (May and Lott, 1992). In a study that combined measures of feed intake and feeding behaviour during heat stress (32^{o}C), there was a reduction in the rate of eating within meals in domestic laying fowls (Rhode Island Red × Light Sussex) compared to those kept in normal condition (20^{o}C; Savory, 1986). Since behaviour exhibited during heat stress is directed at maximising heat loss and minimising heat production, an understanding of the behavioural needs of the birds during heat stress can serve as a pointer to strategies that could be explored to alleviate conditions of heat stress.

2.3.2.8 Effects of heat stress on growth performance, physiology and welfare of broiler chickens

Heat stress has a significant effect on the behaviour, physiology and performance of poultry (Chaiyabutr, 2004) as will be considered in detail in the following sections.

Effect of heat stress on core and body temperatures

The diurnal pattern (i.e. variations during a 24 h period) of CBT in broilers has been related to photoperiod (Lacey *et al*. 2000) because CBT is always at its maximum in the afternoon and minimum at night, probably due to increased feeding and locomotor activity of the birds during the day time. Core body temperature is considered to be a good indicator of welfare during heat stress as (previously discussed in Section 2.3.2.1) and the magnitude of its increase is highly dependent on the level of heat stress (Toyomizu *et al*., 2005). However, the increase in CBT when birds are exposed to high temperature can be aggravated by high RH. For instance, CBT in broilers aged 6 weeks was greater by 0.4^{o}C when kept at 28^{o}C, 80% RH compared to those kept at 28^{o}C, 50% RH (Lacey *et al*., 2000).

Age of the bird has a great role to play in thermoregulation of broilers (Lin *et al*., 2005). Using older birds, rectal temperature of broiler chickens at day 63 (43.8^{o}C) was greater than those at 35 days (43.1^{o}C) when these two group of birds were exposed to the same level of acute heat stress (32^{o}C and 75% RH for 2 h) in a study by Sandercock *et al*. (2001). So the bigger the bird, the more difficult it becomes to dissipate heat because of the higher heat production and lower heat loss, which in turn result in a greater increase in CBT.

During heat stress, there is an increased blood flow to the skin, comb, wattles and shanks due to peripheral vasodilation to aid heat dissipation to the environment (Widowski, 2010). Skin temperature is therefore dependent on both environmental temperature and heat loss from the core. A large volume of warm blood is transferred to the body surface through a vascular arrangement called arteriovenous anastomoses present in the legs (Dawson and Whittow, 2000).

A comprehensive study on the redistribution of blood flow in the body during heat stress in laying hens undertaken by Wolfenson *et al*. (1981) who showed that a 1^{o}C increase in CBT, which was considered as moderate heat stress, was associated with an increase in blood flow to organs active in heat dissipation such as the back (200%),

breast skin (679%), comb (427%), wattle (195%), tongue (429%), larynx (323%) and trachea (338%) compared to the control birds in which CBT remained relatively constant. The tongue, larynx and trachea are part of the upper respiratory tract and this increased blood flow to these areas emphasises the importance of heat loss from the respiratory tract. The results of Wolfenson *et al.* (1981) could be extended to broiler chickens, although broilers are more prone to heat stress than egg-laying strains (Sandercock *et al.*, 2006) because of their fast growth rate which is accompanied by a higher metabolic rate (Gous and Morris, 2005). The increased heat flow to the back may not be readily dissipated to the environment because of the increased feather covering; however the less feathered parts of the body such as the comb, wattles or breast are more efficient for heat loss. Body surfaces which are less feathered and allow for heat dissipation are referred to as 'thermal windows' e.g. comb, legs, under the wing (*apteria*) and around the cloaca (Gerken *et al.*, 2006), Figure 2.8. In this diagram the hottest parts of the body are those in red colour (comb, eyes, ear, wattle and leg/feet) while those in yellow colour are not as hot as those parts in red colour (wing, beak, nose).

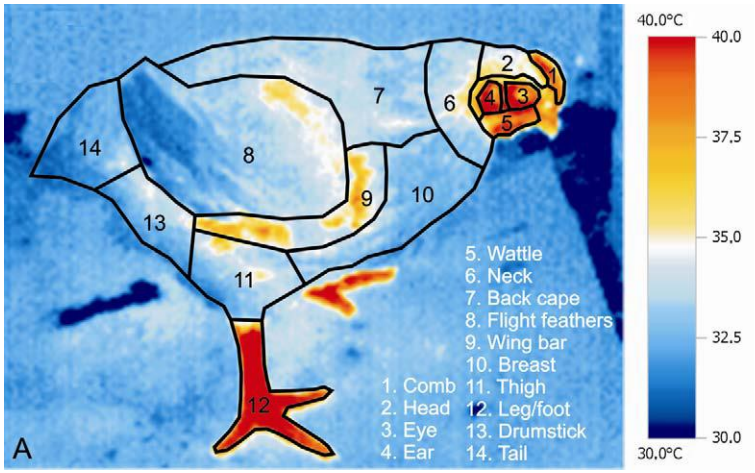

Figure 2.8: Thermograph image of the variation in surface body temperature of a 42 day old Cobb broiler chicken at an ambient temperature of 25.4-31.9^{o}C (Adapted from Nääs *et al.*, 2010)

Effect of heat stress on Physiological parameters

Blood is a liquid tissue made up of mainly of plasma (pale yellow in colour) and blood cells which flow through the body to perform several functions namely transportation (O_2, CO_2, food material, waste products, hormones and metabolites), regulation of body temperature, maintenance of acid-base balance and protection against disease (Rastogi,

2007). The circulatory system is very important for effective control of body temperature especially in altering O_2 supply and heat dissipation (Yahav *et al*., 1997). Heat stress could cause both quantitative and morphological changes in blood parameters (Lin *et al*., 2004; Borges *et al*., 2007) and this might in turn suppress the aforementioned functions of the blood system.

Some of the quantitative changes caused by high environmental temperature are an increase in plasma and blood volume, which consequently causes a decrease in whole blood viscosity (Zhou *et al*., 1999). An increase in plasma volume indicates increased flow of water molecules from the interstitial space into the blood needed for the transfer of heat from the core to the periphery Zhou *et al*. (1999), especially during acute heat stress. On the other hand, chronic heat stress is associated with dehydration due to increased moisture loss from the respiratory tract associated with increased evaporative cooling and increased water loss through the urine. Heat stress (35^{o}C, 50% RH) caused an increase in the number of heterophils and a decrease in the number of lymphocytes in blood, causing an increase in the H/L ratio in broilers after 4 weeks of exposure compared to control birds kept at 23.9^{o}C, 50% RH (Mashaly *et al*., 2004), implying reduced capacity of the immune function and hence following heat stress birds could be more susceptible to a disease challenge.

Blood glucose levels are increased under heat stress due to an increase in secretion of adrenalin, noradrenalin and glucocorticoids (Borges *et al*., 2007) to increase energy available to survive the stressful condition (Ognik and Sembratowicz, 2012). Apart from heat stress, simulation of stress through exogenous administration of corticosterone has also been shown to increase blood glucose levels (Puvaldopirod and Thaxton, 2000; Post *et al*., 2003 and Olanrewaju *et al*., 2006).

In response to heat stress birds may also increase respiratory rate, and a consequence of this is greater exchange in the lungs for CO_2, resulting in a decrease in pCO_2 which in turn leads to an increase in bicarbonate (HCO_3^-) excretion from the kidney and finally a rise in pH (Borges *et al*., 2007; Hockings *et al*., 1994). The change in acid-base balance is proportional to the change in core body temperature, and is especially greater in older birds due to their larger body size and concomitant greater susceptibility to heat stress (Sandercock *et al*., 2001). During heat stress proteins called heat shock proteins are produced which protect the body from damage (Tutar and Tutar, 2010).

Effect of heat stress on growth performance

Heat stress results in substantial economic loss to the poultry sector due to reduced productivity and increased mortality (Toyomizu *et al*., 2005). In a comprehensive study undertaken to estimate the economic losses due to heat stress from cattle, pigs, broilers and layers in the USA, the total loss across all three species was estimated to be $2.4 billion per annum (St-Pierre *et al*., 2003), equivalent to £1.5 million. This challenge of heat stress has resulted in poultry production in the tropics being inferior compared to that in temperate areas (Ojano-Dirain and Waldroup, 2002).

Regulation of feed intake in response to environmental conditions is referred to as thermostatic theory (Ferket and Gernat, 2006) such that environmental temperature above the UCT will lead to decreased feed intake of birds in order to reduce metabolic heat load. Heat stress can adversely affect voluntary feed intake, body weight gain, carcass characteristics and mortality (Mashaly *et al*., 2004 and Khan *et al*., 2012). In fact, a negative correlation ($P<0.05$) exists between body temperature and weight gain (R^2= -0.4), feed intake (R^2= -0.31) and feed conversion ratio (R^2= 0.24) in birds exposed to heat stress (32^{o}C) according to Cooper and Washburn (1998). The reduction in feed intake during heat stress could be related to decreased blood flow to the digestive system (Wolfenson *et al*., 1981), thus heat production associated with digestion, absorption and utilization of nutrients is suppressed (Syafwan *et al*., 2011). Hai *et al*. (2000) reported that during heat stress (32^{o}C) the amount of chyme (partially digested food passing from the stomach to the duodenum for extraction of nutrients) in the digestive tract of broilers aged 49 days increased ($P<0.05$), during heat stress conditions but the activities of the intestinal juice (trypsin, chymotrypsin and amylase) were suppressed ($P<0.05$) compared to control birds kept in 20^{o}C, 60% RH. The suppression of the intestinal juice could be related to the effect of heat on activity of digestive enzymes. Moreover, the degree of reduction in feed intake during heat stress is dependent on the severity and length of the heat stress (Mashaly *et al*., 2004). Heat stress also impairs meat quality of broilers by decreasing the pH of meat, increase plasma creatinine kinase levels which indicates a suppression of the integrity of the skeletal muscles (Sandercock *et al*., 2001).

A considerable amount of literature has been published on the effect of heat stress in broilers (Soleimani *et al*., 2011; Barbour *et al*., 2010; Quinteiro-Filho *et al*., 2010; Sayed and Downing, 2011). These studies have used different temperatures (30-38^{o}C) for different period of time (1.5 h to 24 h) to simulate heat stress conditions. Details

about the different levels of temperature, duration and methodology used and their effects on growth performance, physiology and welfare are summarised in Table 2.2.

In a comparative study of chronic heat stress, broilers aged 5 weeks were exposed to either constant high temperature (35^{o}C) or diurnal changes in temperature (15:35^{o}C for 12:12 h) or control moderate temperature (15^{o}C) for 3 weeks (Yahav *et al*., 1997). Although rectal and skin temperatures, blood pH, pCO_2 and haematocrit levels were similar in these two treatments, they were greater than those of control birds kept at 15^{o}C. The levels of haemoglobin and plasma volume were greater in birds exposed to diurnal heat stress than those exposed to constant heat stress (Yahav *et al*., 1997), indicative of acclimatisation. Increased plasma volume during heat stress suggests that the birds were not dehydrated. These haematological changes were absent in birds subjected to acute heat stress (35^{o}C for 6 h). Yahav *et al*. (1997) deduced that different types of heat stress have different effects on the broilers and this can account for differences in their ability to thermo regulate.

Table 2.2: Effects of various levels of temperature and relative humidity to simulate 'heat stress' on the growth performance, physiology and welfare of broiler chickens

Reference	Duration of heat stress	Type of heat stress	Heat stress conditions Temp. (^{o}C)	RH (%)	Control conditions Temp. (^{o}C)	RH (%)	Age of birds (days)	Effects of heat stress
Sandercock *et al*. (2006)	2 h	Acute	32	75	21	50	35	↑ CBT by 2.3^{o}C, changes in acid-base balance, skeletal damage
Sandercock *et al*. (2001)	2 h	Acute	32	75	21	50	35	↑ CBT by 1.7^{o}C, ↑ pH level by 0.54%, ↑ creatine kinase level by 31.8 and ↓ pCO_2 level by 39.3%
Soleimani *et al*. (2011)	3 h	Acute	36		26		30	↑CBT by 2.1^{o}C, ↑ heat shock protein by 30%, ↑ corticosterone level by 164%, ↑ heterophil/ lymphocyte ratio by 91.4%
Quinteiro-Filho *et al*. (2012)	10 h	Acute	31±1		21±1			↓ Feed intake by 31.4, ↓ body weight by 155%, ↑ corticosterone by 215%, ↑ mortality by 26%

Table 2.2 continue: Effects of various levels of temperature and relative humidity to simulate 'heat stress' on the growth performance, physiology and welfare of broiler chickens

Reference	Duration of heat stress	Type of heat stress	Heat stress conditions Temp. (^{o}C)	RH (%)	Control conditions Temp. (^{o}C)	RH (%)	Age of birds (days)	Effects of heat stress
Aengwanich and Simaraks (2004)	5h / day for 21d	Cyclic changes (Chronic)	33±1	60-70				↑ water: feed by 9:1, ↓ dry matter content of excreta by 12%, ↑ hypertrophy of heart, ↑ heart size, ↑ fat degeneration in the liver and ↑ oedema and haemorrhage in the kidney
Quinteiro-Filho *et al.* (2010)	6 days	Cyclic changes (Chronic)	31±1 10hr/day		21±1		35-42	↓ feed intake by 20%, ↓ body weight gain by 25%
Zhou *et al.* (1999)	4h	Acute	36		20		55	↑ plasma volume by 17.6%, ↑ blood volume by 16.8%, ↓ whole blood viscosity by 8.3% and ↓ haematocrit by 7.5%

2.3.2.9 Genetic selection of broilers and consequences for their ability to cope with heat stress

The process of genetic selection for improved growth rate (exotic breed with fast growth and lean tissue deposition) in broiler chickens was primarily aimed at increasing body weight to meet the food requirement of the growing population, but this process was not matched with a concomitant increase in size of the visceral organs such as lungs and heart which are relevant for thermoregulation (Havenstein *et al*., 2003). The heart assists in heat dissipation by increasing blood flow (Yahav *et al*., 1997) to organs that are actively involved in heat dissipation such as comb, wattles and respiratory tracts (Wolfenson *et al*., 1981). With the need to cope with increased cardiac output under heat stress, the heart enlarges due to blood accumulation which finally leads to heart failure (Aengwanich and Simaraks, 2004).

Comparison of broiler chickens of a 1957 strain (Athens-Canadian Random Bred Control) versus a typical 2001 breed (the Ross 308 genotype) showed that the modern broiler had a greater carcass weight but a relatively lower lung and heart weight compared to the ACRBC breed (Havenstein *et al*., 2003), see Figure 2.9. Increased growth rate of broilers genetically selected for faster growth is associated with an increase in metabolic heat production throughout their growth cycle (Figure 2.10a), therefore the modern broiler needs to be reared under a lower temperature compared to that of birds reared in the 1970's, Figure 2.10b (Gous and Morris, 2005). The modern day broiler chicken of an average weight of 1.8 kg, typically consume a daily energy intake of 2600 kJ Metabolizable energy of which 1600 kJ is lost to the environment (Gous and Morris, 2005). However, heat loss could be limited when the environmental conditions are high and so birds become heat stressed due to their relatively small lungs and heart compared to body weight, feather covering and lack of sweat glands.

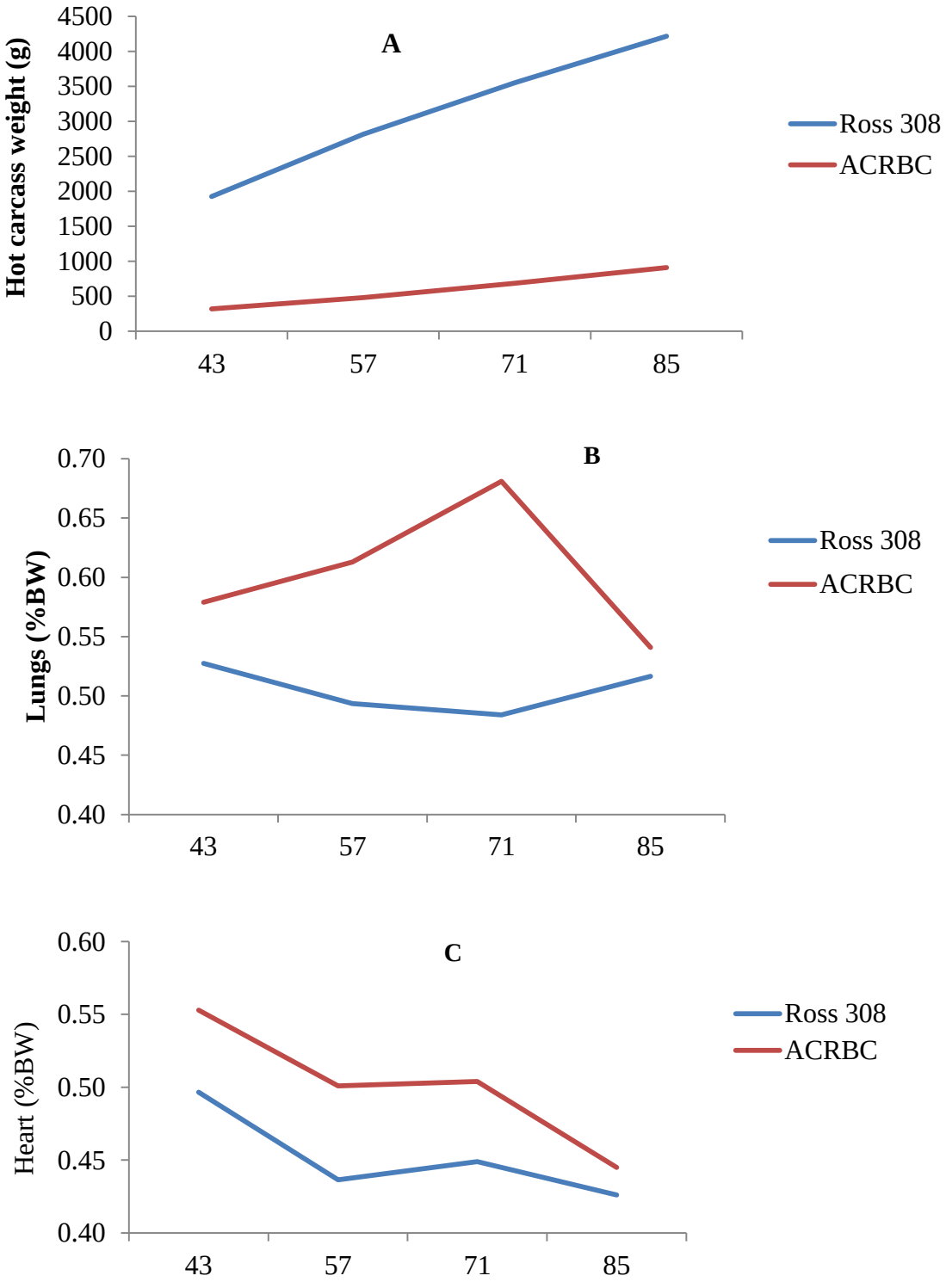

Figure 2.9: Carcass weight (A), relative lungs (B) and relative heart weights (C) of broilers typical of 1957 (ACRBC) and 2001 (Ross 308) breeds. Values were extracted from Havenstein *et al*. (2003).

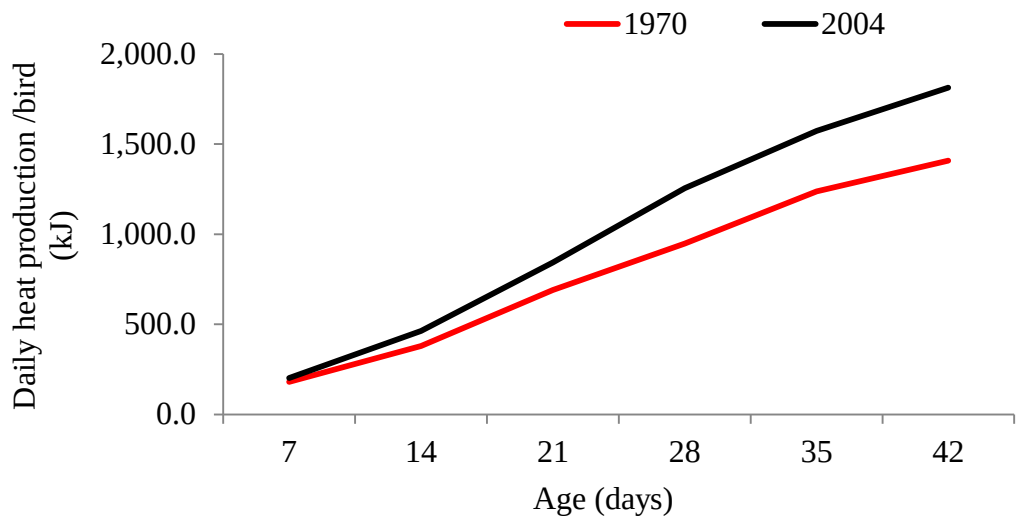

Figure 2.10a Effects of genetic selection for improved growth on heat production of broilers chosen to be representative of 1970 or 2004 (Gous and Morris, 2005)

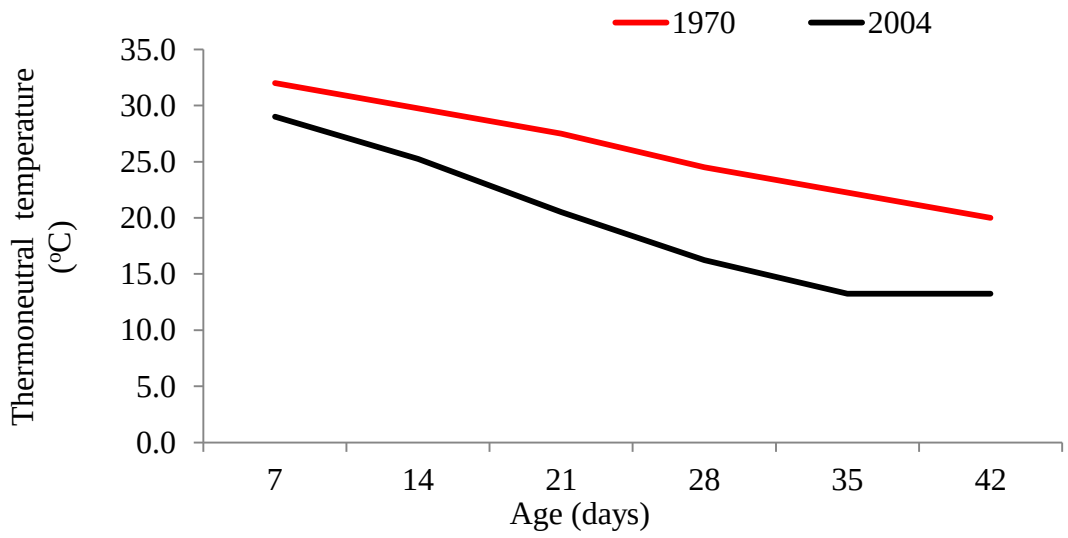

Figure 2.10b. Effects of genetic selection for improved growth rate on the predicted thermoneutral temperature of broilers chosen to be representative of 1970 or 2004 (Gous and Morris, 2005)

In a chronic heat stress experiment (exposure to 34^{o}C of broilers aged 5-8 weeks), Lu *et al*. (2007) reported a reduction in feed intake per degree rise in temperature between 21 and 34^{o}C of 3.4% and 1.7% in an exotic breed (Arbor Acres) and indigenous breed

(Beijing You) respectively compared to their corresponding counterparts in the control condition (21^{o}C). The weight gain of the Arbor Acre breed exposed to heat stress was lower (22.38 g/day) compared to its control (61.45 g/day), whereas the Beijing You breed exposed to heat stress (14.8 g/day) had a similar weight gain to its control (15.2 g/day). Lu *et al*. (2007) considered that the poor performance of the exotic breed could be due to their greater body weight (1876 g compared to the 713 g for the Beijing You breed) which leads to higher metabolic rate.

Yalcin *et al*. (1997) investigated the effect of heat stress on three genotypes of broilers originating from the USA, Germany or the U.K under summer (23-33^{o}C and 45% RH) and autumn (15-26^{o}C and 56% RH) seasons in Turkey. Their results showed that during summer, the body weight of the genotypes from the USA, Germany and U.K differed at 7 weeks of age (1.91 vs 1.82 vs 1.76 kg, $P<0.05$), with genotypes from the USA have greater body weight than those from Germany and UK. No differences in body weight between the three genotypes were observed in the autumn season. Average feed intake between 4 to 7 weeks of age for the three genotypes was reduced by 23% in the summer than autumn. Also, body weight at the end of 7 weeks was suppressed by 23% in the summer than autumn season for the three genotypes. It appears that broiler genotypes originating from the USA were able to perform better during the summer than those of the UK and Germany. Nevertheless, the performances of all three genotypes were suppressed by summer conditions. Therefore, for exotic breed to reach their full genetic potential in the tropical or subtropical regions of the world, they need to be kept under thermoneutral condition. This can be assisted by the provision of structures such as cooling systems to help alleviate conditions of heat stress in the poultry sheds (Sonaiya, 1993).

2.3.3 Alleviation of heat stress

Various management strategies have been used in an attempt to alleviate the symptoms of heat stress in broilers, including addressing aspects of feed and water provision and thermal manipulation.

2.3.3.1 Nutritional and feeding strategies

During a period of heat stress, feed intake is normally suppressed according to the thermostatic theory described previously (Section 2.3.2.8), hence withdrawal of feed deliberately might be unnecessary and is not in the keeping with the five freedoms of

animal welfare where animals are not be subjected to hunger. However, supplementation of electrolytes or the provision of a diet that would result in less production of heat energy during heat stress could be beneficial in some circumstances.

Optimization of feed intake by heat-stressed birds could be achieved by increasing dietary energy content. According to the study of Ghazalah *et al*. (2008), an addition of 5% poultry fat in the diet of heat-stressed broilers aged 29 days (29 - 36^{o}C and 50 - 60% RH) stimulated feed intake (2116 g vs 2061g, $P<0.05$) and bodyweight gain (962 g vs 870 g, $P<0.05$) compared with birds fed the control diet (0% poultry fat). In addition, inclusion of 5% poultry fat enhanced nutrient digestibility (crude protein, crude fibre, ether extract and organic matter). However, there was a higher abdominal fat (4.56%) in the carcass of broilers fed 5 % poultry fat compared with the control birds (2.67% ; Ghazalah *et al*., 2008), so that although growth rate was maintained there was some negative effect on carcass quality.

As described in Section 2.3.2.1, within the thermoneutral zone, water and electrolyte balance in broilers is at an optimum level but once panting begins in birds as a result of heat stress, there is an increased CO_2 loss and shift in the acid-base balance resulting in respiratory alkalosis. In a study by Sayed and Downing (2011), broilers exposed to heat stress and provided with either oral rehydration therapy, ORT + trisodium citrate or ORT+ sodium bicarbonate had greater body weight on the first four days of heat stress (32^{o}C, 80-100% RH) compared to birds offered ordinary tap water (261g vs 269g vs 236 g, $P<0.001$ respectively). Higher growth rate was attributable to the increase in water intake in birds offered the ORT treatment (489 vs 485 vs 399 ml/bird per day for ORT+ trisodium citrate, ORT+ sodium bicarbonate and control respectively). However, on the fifth to the tenth day of treatment, there were no significant differences in water intake or growth rate between treatments. It appears that intervention is more applicable for acute or during a heat wave than chronic heat stress conditions

Smith and Teeter (1992) undertook two trials to evaluate the effect of supplementing the drinking water of heat-stressed broilers with potassium chloride (0.2% KCl). In the first trial, birds were subjected to a cyclic temperature of 26 to 35^{o}C (temperature was held at 26^{o}C for 10 h, gradually increased until it peaked at 35^{o}C where it was held for 2 h after which it was gradually reduced to 26^{o}C). The second trial involved a cyclic temperature between 26.8^{o}C and 36.7^{o}C over each 24 h period (temperature below 33^{o}C for 12 h was the cool period, while the remaining 12 h above 33^{o}C was the hot period of the

cycle). In both trials, birds which had continuous KCl supplementation in their drinking water had a higher ($P<0.05$) water consumption and body weight gain per day than those without KCl supplementation. Smith and Teeter (1992) attributed the results to the role of KCl in promoting tissue synthesis and water retention.

Hurtwiz *et al*. (1980) proposed that since exposure of birds to environmental temperatures above the thermoneutral zone creates difficulty in dissipation of heat produced by metabolism, then feeding strategies that could alleviate heat stress should be based on the principle of reducing heat production associated with digestion. This could be achieved through reducing feed intake during hot periods, withholding feed prior to the expected period of heat stress and the use of pelleted feed rather than meal.

De Basilio *et al*. (2001) investigated the use of a dual feeding programme which involved feeding a high protein diet (26% CP and 3056 ME Kcal/kg) during the cool phase (26^{o}C, from 1600 to 0900h) and a high energy diet (8.6% CP and 3530 ME Kcal/kg) during the warm phase (30^{o}C, from 0900 to 1600h), in comparison with a control diet (20.70% CP and 3198 ME Kcal/kg) offered during the cool and warm phases. Male broilers aged 34 days were exposed to a heat challenge of 36 ± 2^{o}C and 40 to 58% RH for 7 h. The dual feeding system reduced body temperature by 0.77^{o}C and mortality rate by 27.7% ($P<0.001$). However, final body weight was lower in broilers offered the dual feed compared with broilers offered the control diet (2.3 versus 2.5 kg respectively, $P<0.05$). De Basilio *et al*. (2001) suggested that the higher energy content of the diet offered during the warm phase was not sufficient to compensate for the reduced protein content of the feed which is needed for growth. Perhaps with some refinement of energy/proteins, such a dual feeding programme might be worthy of further investigation.

Temporary removal of feed from broilers chickens is a method of alleviating heat stress which has been explored in a number of studies. Ahmad *et al*. (2006) examined the effect of three different feeding methods to alleviate heat stress in broilers, namely continuous (24 h feeding), intermittent (1 h feed available & 3 h no feed available) or complete feed withdrawal (no feed from 9:00am-5:00pm). The effect of these treatments on the respiratory rate of broiler during summer (hot conditions) was monitored. The results showed that the respiratory rate of broilers in the intermittent feeding and feed withdrawal treatments was lower in the afternoon (79.8 and 76.3

breath/min) and evening (66.3 and 64.6 breath/min) compared with continuous feeding (84.6 and 70 breath/min in the afternoon and evening respectively, $P<0.05$). The reduced respiratory rate achieved through intermittent and feed withdrawal was attributed to less heat production arising from reduced feed intake. Other beneficial effect of feed withdrawal during heat stress includes a reduced core body temperature

A number of studies have explored ways of manipulating the diet or drinking water of broilers to ensure that growth performance is not compromised by heat stress, taking steps to feed intake which is usually affected by heat stress so that growth rate is safeguarded under high environmental temperature and RH.

2.3.3.2 Installation of heat regulating structures

Despite the possibility of the use of nutritional strategies to alleviate heat stress previously discussed, Gous and Morris (2005) were of the opinion that installing heat-regulating structures offered a better means of reducing the effects of heat stress in broilers than nutritional strategies. Structures that can be installed in poultry houses to make birds more comfortable under heat stress include a ventilation system, installation of sprinklers and evaporative cooling systems. Although these structures can be effective in controlling heat stress, their use in most tropical countries is not always possible due to their high cost and requirement for constant power supply (Ojano-Dirain and Waldroup, 2002; Tirawattanawanich *et al*. (2010) In those areas then, nutritional strategies may be more applicable. Nevertheless, the installation of fans, evaporative cooling pads and even perches through which cool water is circulated (cool perch/roost) enhance heat loss from through sensible means (conduction, convection).

Consistently, reports show that the provision of cool perches has benefitted birds during heat stress. The percentage of time spent by broiler breeder hens on a cool roost (water-cooled roost made from metal pipes through which water at 20^{o}C is circulated) during heat stress (35^{o}C) was 36.8% greater than hens kept at thermoneutral temperature (25^{o}C), (Muiruri *et al*., 1991). In pens without this water-cooled roost, hens started panting at an ambient temperature above 30^{o}C, whereas hens that had access to water-cooled roost did not start panting until the temperature reached 40^{o}C. This result suggests that the hens used the cool perch to achieve thermoregulation. Estevez *et al*. (2002) also observed that 4-wk old broilers showed a preference for use of an ambient temperature perch placed 7.5 cm above the floor during the summer period (30.6-34^{o}C)

rather than water-cooled perch. However, when the birds reached 6 weeks of age, there was a shift in preference to the use of water cooled perch (circulation of water at 10^{o}C) or ambient temperature perch 15 cm above the floor rather than ambient temperature perch 7.5 cm above the floor. Hence broiler chickens above 4 weeks of age could be more susceptible to heat stress than younger birds and this could be aggravated by the reduced space between the bird as they become bigger, which could result in the transfer of heat from one bird to another. To avoid this happening, the authors suggested that birds preferred to use the cool perch or the ambient perch but at a greater height from the floor to enhance the opportunity for heat loss. With increasing body weight as the bird ages, it might have been expected that the birds would find it more difficult to access the perches. However, birds clearly showed their willingness to avoid heat stress by making the physical effort required to access either a cool perch or even an ambient perch at a greater height that would allow them to distance themselves from other birds and so dissipate heat more effectively.

Recently, Zhao *et al*. (2012) used a cooled perch consisting of 11 parallel galvanised steel pipes (length of 20-25 cm and internal diameter of 2 cm). The perches were made cool by the constant flow of 400 mL/min of tap water cooled to 10^{o}C. The use of cool perches in this particular study reduced the percentage of birds panting during the summer (maximum temperature of 31.1-34.1^{o}C) arising from the reduction in mean rectal temperature. In another study Zhao *et al*. (2013) also conducted during the summer months (temperature range of 30.8-33.9^{o}C) in China, cooled perches were validated as a means of alleviating heat stress. Using the same perches (galvanised pipes through which water of 10^{o}C flowed at 400 mL/min), reduced the percentage of birds panting from 24.4 to 8.45% ($P<0.001$). Birds displayed perching behaviour by sitting, standing or walking with both feet on the perch for more than two seconds. The use of the cool perch did not affect the percentage of birds exhibiting behaviours like feeding, drinking and standing (Zhao *et al*., 2013).

The fact that the provision of cool perches alleviated the effects of heat stress in broilers and hen evident from the reduction in panting behaviour and core body temperature suggesting the transfer of heat from the ventral region of the body when the birds are sitting, or from the feet when in a standing or walking position, to the cooled perch through conduction. This method could remain efficient providing that the temperature of the water circulating in the pipes could be maintained at a lower level that that of the

body temperature of the birds. Furthermore, sustenance of this method relies on a constant supply of power to cool and pump the water through the perches.

Wolfenson *et al*. (2001) attempted a different approach to alleviate heat stress by promoting heat loss through evaporation from the ventral region since this area is sparsely feathered. The protocol of ventral cooling during heat stress period (38^{o}C, 40% RH for 10 h) involved repeated wetting the ventral regions of the birds, firstly 3×30-seconds wetting using 43 L/h at 5 min intervals and then subsequent wetting for 10 seconds at 30 min interval until the end of the heat stress period. This strategy was reported to reduce the core body temperature of birds by 1.5^{o}C compared to control birds. However, temperature of the comb and feet were not affected by ventral cooling, neither was the level of corticosterone reduced in birds that underwent ventral cooling compared to the control birds. One limitation of the ventral cooling and intermittent sprinkling is that it could only be adopted in a battery cage system which is the main housing system for laying hens which is void of litter in and not for broiler chickens and with the move to stop the use of battery cages, the applicability of this strategy may be further restricted.

2.3.3.3 Thermal Manipulation to induce adaptation

Another method of alleviating heat stress is thermal manipulation during embryogenesis (development of embryo) or early post-hatch induce a lifelong adaptation of birds to subsequent heat stress exposure. Thermal manipulation during the early days of post hatch has also been used to successfully induce thermotolerance by taking the opportunity that brain and body development is completed at 10 days of age (Arad and Itsaki-Glucklish, 1991). In one study, Thermal manipulation using a temperature of 39.5^{o}C for 3 h daily during late embryogenesis (day 16-18 of incubation) improved chicks thermo tolerance by reducing body temperature (42.7^{o}C vs 44^{o}C) and corticosterone level (18.6 ng/mL vs 34 ng/mL) compared with control birds when exposed to heat stress of 41^{o}C for 6 h at 3 d of age (Yahav *et al*., 2004). The result of this particular study are supported by those of Collin *et al*. (2007), who reported that birds acclimated to 39.5^{o}C for 3 h/d at 8-10 or 16-18 d of incubation had a lower cloaca temperature (37.9 ^{o}C) than control birds (38.2 ^{o}C) which were incubated at 37.8 ^{o}C throughout the 21-day incubation period.

Thermal manipulation by exposure to 39.5^{o}C and 65% RH for 12 h/day from day 7 to 16 of incubation reduced the body temperature of broilers from hatching until 70 days (> 4 kg). This technique consequently resulted in a significant reduction ($P<0.05$) in feed intake of male and female broilers from day 14 - 70 of age compared to control birds (37.8^{o}C, 56% RH), Piestun *et al*. (2013). The reduction in feed intake was accompanied with a reduction in body weight of females compared to their control birds (4.37 kg vs 4.49 kg, $P<0.05$), but this same effect was not observed in males (Piestun *et al*., 2013). Other consequences of thermal manipulation recorded in this study were a 3% reduction in hatchability, which the authors estimated as being equivalent to a financial loss of $53.75 million during hatching and then and an additional $310 million from chicks that did not hatch and could not be available for rearing to market weight (Piestun *et al*., 2013).

Although these studies demonstrate the potential to induce thermotolerance by manipulating the incubation or post hatch environment, there should be caution on the use of thermal manipulation during embryogenesis and early post hatch since apart from causing mortality of a proportion of embryos, it could also induce early life stress in birds that could result in cognitive and emotional disorders later in life (Bingham *et al*., 2013). In birds, exposure to early life stress in Zebra finches was mimicked through the use of an oral dosage of 6.2 μg of corticosterone per day from 12 - 28 days after hatching (corticosterone was dissolved in peanut oil before administering to the birds). Subsequent survival of these Zebra finches over a three year period showed that early life stress resulted in suppression of survival (red lines in Figure 2.11) compared to the control birds which were fed peanut oil (black lines in Figure 2.11), Monaghan et al. (2011). Equally in humans, early life stress was responsible for 36% of the variation in the hippocampal response to positive emotional stimuli (Aust *et al*., 2013).

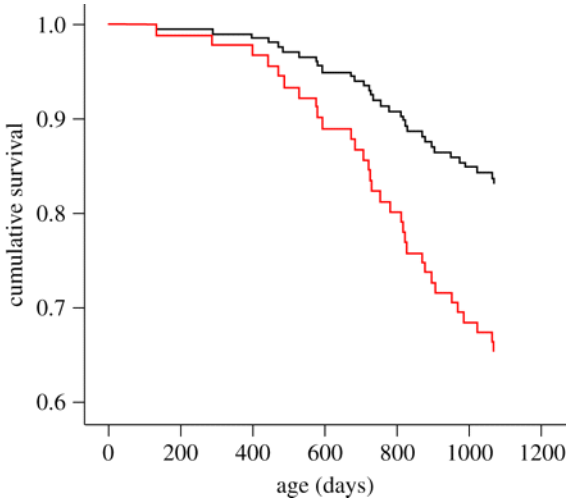

Figure 2.11: Effect of simulated early-life stress on days 12 to 28 (administration of corticosterone) on the survival of Zebra finches over a 3-year period (Monaghan *et al*., 2011). Corticosterone and control birds are represented by red and black lines respectively.

2.4 Effect of lighting conditions on growth performance, physiology and welfare of broilers

In addition to heat stress, another environmental condition that could be a potential source of stress to birds during their growth cycle is the lighting programme. Like other environmental conditions, light could affect the health, productivity and welfare of broiler chickens (Alvino *et al*., 2009; Deep *et al*., 2012; Prayitno *et al*., 1997a; Lewis and Morris, 1998; Olanrewaju *et al*., 2012). The three aspects of light are namely intensity, wavelength and source (Lewis and Morris, 2000) which collectively define the lighting conditions of the poultry house.

Poultry are better at colour discrimination than other livestock species since they perceive light wavelengths through their eyes (retinal photoreceptors) and photosensitive cells in the brain which serve as extra-retinal photoreceptors (Prescott and Wathes, 1999). In the retina, the rod receptor cells are more sensitive to blue and green light wavelengths and low light intensities (below 0.4 lux) whilst the cone receptor cells are more sensitive to higher light intensities (0.4-44 lux; Lewis and Morris, 2000). Apart from the rod and cone retinal receptors, birds can also perceive

light through the extra retinal photoreceptors (hypothalamic photoreceptors) present in the skull to regulate the secretion of serotonin and melatonin hormones from the pineal gland which in turn control physiological systems such as locomotor activity, core body temperature and reproduction (Lewis and Morris, 2000). Penetration of light through the eyes travels to the hypothalamic suprachiasmatic nucleus through the retinohypothalamic nerves then to the paraventricular nucleus, super cervical ganglion and finally to the pineal gland (Aral *et al*., 2006). However, the pineal gland produces melatonin at night or in the dark, and it is melatonin that regulates processes such as physiological and behavioural processes (Zawilska and Wawrocka, 1993) implying that these processes could be hampered when broilers are reared under continuous light conditions without any dark period.

2.4.1 Effect of light wavelength on growth performance, physiology and welfare of broilers

Attention should be given to the light wavelength provided in the poultry house because of its potential to affect behaviour, growth performance, level of stress and finally quality of meat produced. Prayitno *et al*. (1997a) created different light wavelengths by placing different light coloured filters over tungsten filament bulbs, namely white (no filter), blue, green and red. The wavelength of the blue, green and red wavelengths created were 450, 550 and 650 nm respectively with all lights adjusted to give an intensity of 30 lux. Prayitno *et al*. (1997a) reported that broilers reared in the white and red lights were more active than those reared in blue or green wavelengths. This was evident in the white-reared birds exhibiting a greater frequency of walking compared to birds kept in red, green or blue lights (1.18 compared to 1.04, 0.83 and 1.03 events/h respectively, $P<0.001$). However birds reared in the red light displayed greater ($P<0.001$) frequencies of floor pecking (0.91 events/h), wing stretching (0.69 events/h) and aggressive behaviour (0.07 events/h) compared to those in the other three light wavelengths. The authors reported an increase ($P<0.001$) in the gut contents (expressed as a % of carcass composition) of birds reared in green and blue (1.9% and 2.8%) wavelengths compared to birds reared in the white or red light (1.3 and 1.0%) and suggested that this might be due to reduced rate of feed passage arising from the reduced activity.

In a preference test in the same study, broiler chickens which had been previously reared in different light wavelengths (red, white, blue or green) migrated to the blue

light whereas those reared in the blue light chose to move to the green light, probably because it was of a similar short wavelength as the blue light (Prayitno *et al*., 1997a). Prayitno *et al*. (1997a) claimed that birds chose to migrate to the blue light because it had a calming effect which was evident in the reduction in the activity levels of the birds, and that the high stimulatory effect of red light could be aversive to broilers. After three hours of giving birds the opportunity to select their preferred light wavelength, only those birds previously reared in the red light had migrated to the blue light, whereas after a week before birds from other light wavelengths showed a preference for the blue light.

In another study, Rozenboim *et al*. (2004) investigated the effect of three different light wavelengths on growth and development of male broilers. From days 1 to 10 of age, birds were reared either blue, green or white light (light emitting bulbs, LED)), after which the light wavelength was switched from blue to green (BG) or green to blue (GB) wavelengths until the birds were 20 d of age. Rozenboim *et al*. (2004) reported that body weight of birds reared under green light was higher ($P<0.05$) compared to those reared in the other three lights from as early as 4 d of age. Switching light wavelength at 10 d had a significant effect on final bodyweight of broilers with those having GB light being heavier than those having BG light (2897 g vs 2709 g, $P<0.05$). To understand the mechanism behind the increased growth rate of birds reared under green or blue light wavelengths, Halevy *et al*. (1998) undertook a study which involved rearing broilers under green, blue, red or white lights (LED bulbs) from day 1 to 35. They found that birds reared in either the green or blue lights had a greater body weight and this was associated with greater number of satellite cells compared to those in the red or white lights. A positive correlation was found between breast muscle weight and satellite cell proliferation ($R^2=0.92$). Halevy *et al*. (1998) also found increased growth hormone gene expression in birds reared in green and blue. Although in their experiment the level of growth hormone was not measured, however Halevy *et al*. (1998) attributed the increased breast muscle growth to an increase in protein synthesis. This was supported in a latter study undertaken by Rozenboim *et al*. (1999), who reported that blue light enhanced growth by stimulating the production of plasma androgens as early as 9 days of age, hormones known to be involved in protein production, consequently resulting in an increase in body mass.

Increased growth of male birds under the green and blue light has attributed to increased levels of testosterone production, which in turn stimulates the myofibre growth of the

pectoral muscles. In another study conducted by Cao *et al*. (2008) to understand how green and blue light enhances growth in broiler chickens using another breed of male broilers (Arbor Acre) reared under one of four light wavelengths namely white, red, green or blue using an LED bulb adjusted to give equal light intensity of 15 lux from day 1 to 49 days of age. At 26 days of age, broilers reared under green light was heavier ($P<0.05$) than birds in the white and red light, with those reared under blue light being intermediate. From 26 days of age onwards, body weight of broilers in the blue light overtook ($P<0.05$) that of broilers in the other three light wavelengths. In this same study, green light enhanced the myofibre growth of pectoral muscle at 21 days (17.74 vs 54.01vs 23.2 %, $P<0.05$ compared to birds in the white, red or blue light respectively), but at 49 days of age birds in the blue light had greater myofibre growth (21.1vs 21.7 vs 9.92 vs 21.69%, $P<0.05$ compared to birds in the white, red and green lights respectively). At days 7 and 21, plasma testosterone levels were greater in birds reared in green and blue light (both $P<0.05$) compared to birds in the other lights. Hence, it seems that light of short wavelengths as in green or blue stimulates the release of hormones necessary for growth.

Light wavelength also has effects on the immune response, stress responses and meat quality of birds. Adopting a similar protocol of Cao *et al*. (2008), Xie *et al*. (2008) investigated the effect of four different light wavelengths namely white, red, blue or green all adjusted to give the same level of intensity (15 lux) on the immune response of broilers. After 21 days of rearing birds under one of these four light treatments, birds in the green light were heavier by 80.8 and 54.8 % respectively compared to birds in the red and blue light. However, at day 49 the lymphocyte proliferation was greater in birds reared under the blue light compared to those in red light ($P<0.05$), but levels in the green light were not dissimilar. Also at 49 days of age, the antibody titre following vaccination with Newcastle disease on day 3 and 20 was greater in birds reared under blue light by 62.8% ($P<0.05$) compared to birds in the red light, however the antibody titre of birds in the blue light was similar to those in the green and white light. Finally, Xie *et al*. (2008) reported that birds reared in the blue light had a 44.0 and 59.4% lower amount of interleukin-1β compared to those reared in red and white light respectively, implying that blue light assists in reducing the stress level in birds. From the studies of Cao et al. (2008) and Xie *et al*. (2008), it seems that the effect of light wavelength on growth and immune response were age related, with green wavelength enhancing these

parameters at an early and blue wavelength enhancing parameters at a later stage of the growth cycle.

Whilst farmers may be more concerned about producing heavy birds at the end of the growth cycle, consumers are likely to be more interested in the quality of meat available in the market. So, using the same protocol as Cao *et al*. (2008), Ke *et al*. (2011) explored the effect of light wavelength on meat quality of Arbor Acre broilers. They found that birds reared under a green or blue light for 7 weeks had an improved meat quality evident from the increase in protein content compared to birds in the other three light wavelengths. This could suggest that blue light was less stressful to the birds as previously reported by Xie *et al*. (2008) and further reports show that stress and the associated increase in level of corticosterone is associated with an increase in protein breakdown (Gao *et al*., 2008).

There may be occasions when producers might choose to keep birds under a particular light intensity and/or wavelength to address a particular objective. For example, catching birds prior to transportation to the slaughterhouse has been recommended to be undertaken under dim or blue light conditions in order to reduce the activity levels of birds and by so doing reduce the level of stress. In fact, exposure of birds (42 days) to a blue light wavelength (17-20 lux) at the point of being hung on shackles prior to slaughter reduced ante mortem stress evident from a reduction (39 versus 53%) in the occurrence of pale soft and exudative (PSE) breast meat compared to those kept under white light, although the intensity of the white light was greater (321-323 lux; Barbosa *et al*. 2013).

Collectively these studies illustrate that light wavelength can have a significant impact upon behaviour patterns, growth rate, immune capacity, level of stress and meat quality of broilers. In addition, the use of coloured bulbs reduces energy consumption for the commercial poultry producer (Lewis and Morris, 2000), hence the increasing popularity of blue or green coloured light.

2.4.2 Effect of light intensity on growth performance, physiology and welfare of broilers

It has been suggested that rearing birds under low light intensity reduces their activity level consequently resulting in an increase in growth performance compared to birds reared under high light intensity because less energy is expended on locomotion so

more available for full growth. However, reports on the effect of light intensity on growth performance are inconsistent. Blatchford *et al*. (2009) used one of three levels of light intensity either 5, 50 or 200 lux in an illumination programme of 16 hours light and 8 hours darkness (16L: 8D) from the first to the sixth week of age, and found no significant difference in body weight gain of birds reared in the three treatments. Although birds kept at 5 lux had suppressed activity levels, birds in the three light intensities had similar level of gait score. The differences in results could be attributed to the light schedule used and the duration of the study.

In a behavioural study using similar light intensities as that of Blatchford *et al*. (2009) but with a longer photoperiod (23h), Alvino *et al*. (2009) reported that birds reared under 5 lux were more inactive, sleeping for the majority of the time, compared to birds reared in 50 and 200 lux (0.75 vs 0.70, $P<0.001$).

In laying hens, the use of low intensity light of 5 lux between the 16^{th} to the 24^{th} week of age (which is the critical period of coming into lay) suppressed the level of egg production compared to birds exposed to light intensity of 150 lux (O'Connor *et al*., 2011), emphasising the role of light intensity in the regulation of reproduction. O'Connor *et al*. (2011) found no effect of low light intensity on levels of physiological stress assessed from the level of plasma corticosterone.

Commercial poultry farmers often rear birds under low light intensity in order to alleviate the incidence of feather pecking. Feather pecking is the damage to the feathers by other birds through pecking. The level of severe feather pecking such as feather pulling, was reported to increase by 2-3 times in birds reared in 30 lux compared to those reared in 3 lux (Kjaer and Vestergaard, 1999) probably because high light intensity is associated with an increased level of activity (Lewis and Morris, 2000). Although feather pecking is not a common problem with broiler chickens, nevertheless low light intensity can have an impact on the productivity of the birds.

So far, we have reviewed the literature on the biological functioning approach to assess welfare of broilers under heat stress, physiological stress and lighting conditions were welfare of animals could be based on the measurement of growth performance, physiology and behaviour of the animals. Next, is a review on the subjective feeling assessment of animal welfare.

2.5 Subjective feeling assessment of welfare under stressful conditions

2.5.1 Definition of affective state and emotion

With increasing awareness of the sentient nature of animals, animal welfare has progressed to the study of animal emotion (affective states). Sentience was defined by Duncan (2006) as 'the capability of an animal to experience positive and negative affective states'. Although the term affective state and emotion are commonly used interchangeably, affective state is a broad term that encompasses emotions, feelings and mood, hence emotion is a subset of affective state (Sloman *et al*., 2003). Core affect or affective state can be defined as a 'neurophysiological state consciously accessible as the simplest raw (non-reflective) feelings evident in moods and emotions' (Rushell, 2003). Another word for core affect (affective state) is the animals' feelings and it indicates the immediate condition of an individual (Russell, 2003). According to Désiré *et al*. (2002), emotion was defined as 'an intense but short-living affective response to an event accompanied with specific body changes'. Emotion can be defined as the mental state or feelings experienced by humans and animals (Breed and Moore, 2011). Emotion has the following characteristics, namely i) it is associated with an event, ii) it lasts for a brief period iii) it is accompanied by behavioural actions or facial expressions (Scherer, 2003). On the other hand, mood has the following characteristics, namely i) it is not related to an object/event, ii) it last for a longer period than that of emotions, and iii) it is cognitive in nature and lacks a distinct behavioural action or expression (Scherer, 2003). Although emotion and mood are different, emotion can be transformed into a mood if it lasts for a longer time and vis versa (Scherer, 2003).

Emotion has three main components, namely behavioural, autonomic and subjective feelings which respectively involve measures of changes in posture or activity, changes in visceral and endocrine responses and emotional experience or feelings (Dantzer, 1988). The behavioural and autonomic responses fall within the biological functioning approach of assessing welfare and they demonstrate the degree of arousal caused by a stimuli, whereas the measure of emotion indicates the level of valence of a stimuli i.e. positivity or negativity (Mendl *et al*., 2009). Animal welfare scientists have successfully developed techniques to estimate the autonomic responses of animals; however the measure of the subjective component of emotion is of a greater interest to the field of animal science (Mendl *et al*. 2010). In addition to these three components, Scherer (2001) proposed two further components of emotion, namely cognitive evaluation (appraisal of stimulus) and motivation. Scherer (2001) acknowledged that the

motivational and subjective components of emotion could easily be determined in humans who have the capability to express themselves verbally, thus regarded as the 'gold standard' of expressing emotions. Unlike humans, animals cannot express themselves verbally; nevertheless Scherer (2001) proposed that the subjective feeling and motivation of animals could be successfully understood by quantifying the behaviour, autonomic and cognitive evaluation. The relationship between these components yields a specific emotion (Rusell, 2003). The cognitive component assists in the processing of information relating to the stimulus (Scherer, 2003).

Mendel *et al*. (2010) proposed a dimensional framework for assessing emotion in animals, which include valence on the horizontal axis (ranging from positive to negative) and arousal on the vertical axis (ranging from high to low). The combination of arousal and valence of a stimulus produces different kinds of emotion. Different emotions are produced under high arousal and positive valence compared to those produced by a high arousal and negative valence, likewise for low arousal and positive valance or low arousal and negative valence. Fear and anger are two emotions that are similar in valence and arousals but differ in their behavioural expressions. Fear is associated with avoidance while anger involves attack (Mendl *et al*., 2010).

2.5.2 Assessment of affective states in animals

The study of emotion or affective state cuts across several scientific fields including animal welfare, affective neuroscience, psychopharmacology (study of the effect of drugs on the nervous system and behaviour), evolutionary zoology and comparative psychology, with each field of study having its own unique method of assessing emotions (Paul *et al*., 2005). The field of neuroscience involves the use of magnetic resonance imaging (MRI scanner) and electrophysiology techniques (Davidson and Irwin, 1999), both of which are very complex and cannot be used to determine the emotional states of animals that are unrestrained (Mendl *et al*., 2009). Nevertheless, these techniques have enhanced knowledge on the mechanisms through which emotions are developed. For instance, functional magnetic resonance imaging is used to detect changes in the brain when an individual is performing a specific task (Nelson, 2005).

Currently in animal welfare studies, three main approaches have been developed to assess emotion, namely appraisal theory (Désiré *et al*., 2002), anticipatory behaviour

(Spruijt *et al*., 2001) and cognitive bias (Harding *et al*., 2004). The appraisal theory approach evaluates emotions based on how the animal appraises the environment along with behavioural and physiological responses (Désiré *et al*., 2002). An animals' appraisal of a stimulus is based on suddenness, familiarity, predictability and pleasantness of the stimuli which subsequently determines the kind of emotion that is produced (Désiré *et al*., 2002). The anticipatory behaviour approach assesses emotion based on an animal's appraisal of a stimulus regarding its expectation for a reward (Spruijt *et al*., 2001). Thirdly, the cognitive bias task approach is based on the biases to information processing (Mendl *et al*., 2009). It appears that the appraisal and cognitive bias task approach of assessing affective states of animals are based on the relationship existing relationship between cognition and emotion, but whilst the appraisal theory believes that cognition influences emotion, the cognitive bias theory believes that emotion influences cognition.

The emotional state of humans results in biases of information processing, such that people in a negative emotional state interpret ambiguous cues pessimistically (MacLeod *et al*., 1986). Therefore, estimation of the level of bias in information processing has been extended to animals as a means of determining their affective states on an objective scale (Harding *et al*., 2004). Before an animal is presented with an ambiguous stimuli, such an animal must have been trained to differentiate between positive (rewarded e.g. with food or mealworms) and negative (non-rewarded or aversive stimuli) stimuli (Mendl *et al*., 2009) which involves cognitive processes. After this training, the animal is presented with an ambiguous stimulus and its response (optimism or pessimism) will indicate whether the animal is more likely to be in a positive or a negative affective state.

2.5.2.1 Cognitive bias task to assess the affective state of animals

The first attempt to investigate cognitive bias in animals was undertaken by Harding *et al*. (2004) on male Lister hooded rats. The rats were trained on a go/no-go (operant discrimination) task in which the rats had to press a lever to obtain a positive reward (a 45 mg food pellet) on hearing a particular tone, and not to press the lever on hearing another tone which was associated with a negative reward (a burst of white noise of 70 db for 30 s). After the task was successfully learnt, the affective states of the rats were manipulated by moving half of the rats to unpredictable housing, where the conditions were continuously changing (unpredictable) such as tilting the cage, changing the

light/dark cycle or keeping the bedding damp. These unpredictable conditions were intended to simulate a chronic stress paradigm (9 days duration), since exposure to a series of acute stressors is known to result to chronic stress (Dickens *et al*., 2010) because of the continuous elevation of corticosterone. After 9 days, the rats were then presented with three additional unreinforced tones which were intermediate in tone to that associated with food and that associated with white noise tone, and the responses of the rats in terms of their latency to press the lever was observed. Rats kept in the unpredictable housing conditions took longer ($P<0.05$) to respond when presented with positive and near-positive ambiguous tones compared with rats kept in the predictable housing. Harding *et al*. (2004) concluded that rats kept in unpredictable housing conditions were pessimistic in their judgement of ambiguous tones which was a symptom of depression or negative affective state. In humans, the frequency of being in a negative affective state was greater in those individuals having depression compared to those individuals judged as being in a normal state (Lawton *et al*., 1996).

Another study that used a cognitive bias task in birds was that of Bateson and Matheson (2007), where starlings were trained to associate palatable meal-worms (injected with 0.2 ml water) with a white-covered petri dish (0% shading) and unpalatable meal-worms (injected with 0.2 ml 2% quinine sulphate) with a dark grey-covered petri dish (80% grey-scale shading). The starlings were later given a judgement task by introducing three unreinforced probe trials (using 20, 40 or 60% grey scale shading). Birds that had recently been moved from enriched to barren cages, achieved by removing the enrichment items such as tree branches, the water bath, a tray of bark chippings from the enriched cage, were more likely to interpret the 20% shaded lid as a negative one, indicative of increased ‘‘pessimism’’ in birds that had recently experienced a degradation in their environmental quality. Pessimism has been defined as ‘a higher tendency of responding negatively’ (Brilot *et al*., 2010) and thus is widely regarded as a robust indicator of negative states (Mendl *et al*., 2009).

Since Harding *et al*. (2004) reported the suitability of assessing affective states in rats, other studies have validated its usefulness in other animal species such as starlings (Brilot *et al.*, 2010), capuchins (Pomerantz *et al*., 2012), dogs (Cassey *et al*., 2008), pigs (Douglas *et al*., 2012), sheep (Destrez *et al*., 2013), honey bees (Bateson *et al*., 2011), chicks (Salmeto *et al*., 2011) and laying hens (Wichman *et al*., 2012), although with modifications in the type of task used in training the animals. Cognitive bias tasks have also been useful under conditions of acute conditions (isolation or light intensity) to

differentiate between emotional states which are of close valence such as depression and anxiety (Salmeto *et al*., 2011; Burman *et al*., 2009).

In most cognitive bias tasks, the positive reinforcer (reward) was food while the negative reinforcer could be the presence of an aversive stimuli or nothing at all. The conditions that have been used to influence the affective state range from unpredictable housing conditions (Harding et al., 2004), environmental enrichment (Bateson and Matheson, 2007) and light intensity (Burman *et al*., 2009). Other factors that could influence the affective state of animals are previous experience, environmental conditions and individual differences (Mendl *et al*., 2009). Other environmental conditions which are yet to be investigated on the affective state of broilers include heat stress, light wavelength or intensity. Even though there are reports that blue light are preferred by broilers because it makes them calm, however, the impact of lighting conditions on cognition which is the basis upon which the cognitive bias task is undertaken is yet to be reported in chickens.

A cognitive bias task has been used by Salmeto *et al*. (2011) to evaluate the affective state of chicks subjected to isolation stress. Chicks isolated for 5 mins took longer to approach aversive ambiguous cues, whereas those isolated for a longer period of 60 mins showed an increased latency to approach both aversive (owl silhouette) and appetitive (chick silhouette) ambiguous cues. This suggests that chicks exposed to a 60 min isolation period were in a more negative state compared to those isolated for 5 mins (Salmeto *et al*., 2011). With laying hens, Wichman *et al*. (2012) reported no difference in the affective state of laying hens kept under basic or enriched housing conditions. The authors suggested that the lack of a difference in housing system was probably because the difference between the two housing environments was judged to be only mild. Birds in the enriched housing conditions were provided with three perches of different heights (20, 40 and 100 cm high) while those in the basic housing still had a 20 cm perch (Wichman *et al*., 2012). Thus it could be argued that even the basic housing conditions provided some degree of environmental enrichment to the birds. In this study, birds required more than 12 training sessions before they could learn to differentiate between the reward (corn) and unrewarded locations (an empty bowl) in a spatial task.

2.5.2.2 Relationship between lighting condition, cognition and affective state

In humans, cognitive function has been reported to be affected by light, by extension light conditions could affect emotion because cognition is one of the components of emotion. Beavan and Ekstrom (2013) reported that one hour exposure to blue light (40 lux) enhanced the accuracy of humans in a visual go/no go task (where participants have to press the space bar on a computer keyboard, Go or withdraw from pressing, No-Go, when a non-cued stimuli was presented). This result was similar to that of participants who ingested 240 mg caffeine capsule one hour prior to the test. In the same study, Beavan and Ekstrom, (2013) found that participants subjected to one hour exposure to blue light were less distracted ($P<0.05$) in an Eriksen Flanker task compared to those that consumed caffeine. Eriksen Flanker task is used to determine the level of vigilance of humans by monitoring the number of responses that are non-relevant to a particular context (Beavan and Ekstrom, 2013). The authors attributed the effect of light on cognitive function to a part of the brain called the amygdala, which receives light transmission through the retinohypothalamic tract and then stimulates alertness.

A two part mechanism of how light induces emotion was proposed by Rautkyla *et al*. (2012), namely the circadian and the limbic pathways which work in parallel. Light stimuli penetrates through the retinal hypothalamic tract (RHT) to the suprachiasmatic nucleus (SCN) which then sends a signal to the pineal gland for the control of the circadian system. The second pathway involves the transmission of light through the RHT to the amygdala (limbic system) which is involved in emotion. Both pathways meet at the lateral hypothalamic region (orexin neurons) where they elicit cortical arousal. Rautkyla *et al*. (2012) believed that the second pathway is more substantial because it explains why the alerting effect of light is observed during the day when there is no production of melatonin from the pineal gland.

In rats, the effect of light intensity on emotional state (anxiety) was investigated by Burman *et al*. (2009) using a cognitive bias test after the rats had learned to discriminate between a reward (containing four pellets of feed) and unrewarded (containing one 2 % quinine-soaked pellet) location in a radial maze arm (a maze with five radial arms). Switching light from low (10-15 lux) to high (65-100 lux) intensity resulted in a pessimistic judgement of ambiguous positions indicated from the longer latency of approaching the ambiguous locations, whereas rats in the treatment where they were switched from high to low intensity were faster in approaching the ambiguous locations,

hence were considered to be 'optimistic'. This study demonstrates that lighting conditions could affect the emotions of animals.

In broilers, it is not yet known whether light wavelengths have similar effects on the cognitive abilities. Although it has been shown from a preference test that broilers migrated from white, green and red light to blue light wavelengths (Prayitno *et al*., 1997a) which suggests that the birds might have found the blue light more pleasant, it is unknown how the affective state of birds might be influenced by lighting conditions.

2.5.3 Effects of stress on cognition

Since cognition is one of the components of emotion, it is necessary to relate stress to cognition. According to Shettleworth (1998), cognition is defined as 'information processing', which includes all the processes involved in the acquisition, storage, processing and interpretation of information. The process of gathering and processing information to give an output is referred to as cognition (Keeler and Robbins, 2011). Keeler and Robbins (2011) also stated that cognition involves the memory, language and executive functions such as decision-making. Although animals cannot communicate verbally, nevertheless they use other means of communication such as songs or alarm calls against predators (Hollén and Radford, 2009) to convey information to each other. Boks (2010) argued that broiler chickens have good cognitive ability because the birds were able to learn a spatial hole-board task by discriminating between those cups which had mealworms and those without mealworms.

Cognitive function or processes can be impaired by stress. Acute stress imposed before, during or after learning a task can affect the cognitive function of the animal by impairing attention, learning and recalling of an already learnt task (Mendl, 1999, see Figure 2.12). Imposing a stressor before/during learning (points A and B in the diagram) impairs the attention of the animal by causing a shift in attention from the task that is to be learnt to the need to search or to escape from the stressor. On the other hand, imposing a stressor after a task has been learnt (point C) can be used to evaluate memory formation, and the effect of this stressor on memory can be determined by carrying out a memory recall test. Finally, presenting a stressor prior to memory recall (point D) could be used to evaluate the effect of stressors on memory recall (Mendl, 1999).

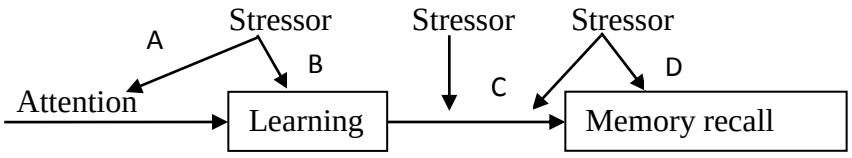

Figure 2.12. Effect of stressors imposed at different time points on cognitive functions in animals (attention, learning and memory recall) (after Mendl, 1999).

Memory formation or consolidation is affected by catecholamines and glucocorticoids (corticosterone) which are produced by the SAM and HPA axis during stress, which have different effects on memory (Mendl, 1999). Memory is the retention of information and contributes to the survival of the animal (Pravosudov, 2005) by avoiding aversive substances. Catecholamines such as adrenalin enhance memory by increasing the level of glucose available to the brain (Mendl, 1999) whereas glucocorticoids impair memory (Lupein *et al*., 2007). The mode of action of these two substances differs in that while adrenalin is not able to cross the blood-brain barrier, corticosterone can (Mendl, 1999) because of its liposoluble characteristics (Lupien *et al*., 2007).

The effects of stress on the brain are expressed both in the cellular and subcellular levels especially in the hippocampus (which controls memory), the prefrontal cortex (controls decision-making) and the amygdala (which controls emotion) all of which are densely populated with glucocorticoid receptors (Alkadhi, 2013). In the previous section, it was shown that regions of the brain such as the hippocampus and the amygdala are related to the development of emotions. Hence if stress brings about changes in these parts of the brain then there could be potential effect for stress to affect the emotion of animals. The prefrontal cortex is present in mammals but the corresponding structure in avian species is called nidopallium caudolaterale, NCL (Güntürkün, 2005), in Figure 2.13. In Zebra finches, mineralocorticoid receptors are limited to the hippocampus whereas glucocorticoid receptors are prominent in the hippocampus, paraventricular nucleus, nidopallium and cerebellum (Hodgon *et al*., 2007). The varied distribution of receptors in different parts of the brain and their corresponding affinities for corticosterone suggests that there could be differences in cognitive functions as a result of stress.

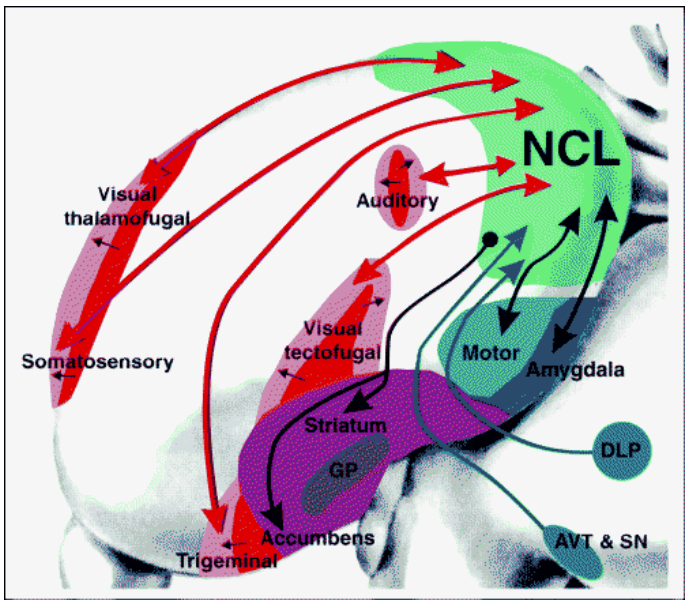

Figure 2.13: Simplified diagram showing the brain of a pigeon with connections of the nidopallium caudolaterale (NCL) sensory systems (depicted in red and pink) and the amygdala (Adapted from Güntürkün, 2005).

The level of damage to brain cells in response to increased corticosterone levels can be evaluated from suppression of the production of new cells in the hippocampus (hippocampal neurogenesis) or the dendrite (granule cell volume). In mice, continuous delivery of corticosterone from an implanted corticosterone pellet (40 mg/kg/day) for 14 days induced chronic stress which consequently resulted in a 50% reduction in hippocampal neurogenesis and granule cell layer volume and subsequently a display of anxiety-like behaviour (reduction of time spent in the light side) of a light-dark box (Murray *et al*., 2008). The light-dark box consisted of a test box partitioned into two unequal halves; the smaller side was coloured black and illuminated with red light whereas the larger side was coloured white and illuminated with white light of 450 lux to evaluate anxiety such that an animal was said to be anxious if it avoided the light section of the light-dark box (Murray *et al*., 2008).

In humans, the effect of heat stress on cognitive ability depends on the duration and intensity of heat exposure (Hancock and Vasmatzidis, 2003). In one study, 2% reduction in body weight through dehydration during heat stress or physical exercise reduced the performance of candidates in a cognitive task (Grandjean and Grandjean, 2007). Cognitive dysfunction during heat stress could be caused by changes in the brain namely alteration of the blood brain barrier, oedema, increase in level of amino acid

neurotransmitters (glutamate and aspartate) and decrease in levels of gamma amino butyric acid (GABA) and glycine (Sharman, 2006).

Although literature on the effect of heat stress on the cognitive ability in chickens is lacking, nevertheless, the effects of other stressors on the impairment of cognitive performance has been reported in several animal species. Mimicking chronic stress in sheep was explored by exposing them to several stressors typical of farm conditions, namely predators, exposure to odour of blood or urine, loud noise, wet straw bedding and switching on the light during the night for 7 weeks, reduced their performance in a Y-maze test so that they made fewer correct entries compared to control sheep (1.57 vs 1.95, $P<0.05$; Destrez *et al*., 2013). Quail (256 day old) selected for high or low stress response differed in their performance in learning a spatial task (one where the bird had to learn the position where they could get a food reward), in a study by Suhr *et al*. (2010). The results showed that birds from the high stress strain made few correct entries to the baited arm of an 8-arm radial maze compared to those from the low stress line (an approximately 2 vs 5 entries, ($P<0.05$), Suhr *et al*. (2010).

In another avian species, zebra finches (13–20 g body weight) were selectively bred to respond differently to stressors in terms of the level of corticosterone secreted i.e. either low or high levels of corticosterone secreted in response to a stressor. Finches were subjected to human restraint for 20 minutes before undertaking a spatial and visual task in a study by Hodgson *et al*. (2007). After the 20 minute restraint, birds from the high stress strain performed less well in a spatial task by opening approximately 3 out of the 7 flaps in search of food (the food was hidden in one well) compared to those of the low stress strain which opened approximately 2 flaps. Performance in the visual task was not affected by selection for corticosterone (Hodgson *et al*., 2007) which suggests that the effect of stress on cognitive ability is dependent on the type of task.

In summarising this section, there are numerous examples in the literature of how stress impairs cognitive performance of several animal species.

2.5.4 Relationship between stress and emotion

There seems to be a clear relationship between stress and emotion. Whilst stress involves the study of responses to situations that are considered aversive, negative affective states can be induced by stressful conditions (Paul *et al*., 2005) by altering the cognitive process involved in decision making (Salmeto *et al*., 2011). The indirect role

of stress on the affective state has been investigated by subjecting animals to conditions known to be stressful, before evaluating their affective state. As discussed previously (Section 2.5.2.1), in a study by Harding *et al*. (2004), rats subjected to a series of stressful housing conditions for 9 days to mimic conditions of chronic stress and a cognitive bias task showed that these rats kept in stressful conditions were in a negative affective state.

Although stress and emotion could trigger similar physiological responses, conclusions about the affective state of animals cannot be fully made based on the results obtained from physiological parameters of stress because situations likely to induce different affective states may give rise to the same physiological responses (Mendl *et al*., 2009). For instance, rats subjected to two different stimuli, namely social defeat and sexual behaviour, had a similar increase in corticosterone levels (>300 ng/ml; as reported by Buwalda *et al*., 2012). Although Buwalda *et al*. (2012) did not assess the affective state of the rats subjected to these two conditions. However, in a later study, Papciak *et al*. (2013) found that the 'loser' rat in a psychosocial stress daily for 3 week (fight between an intruder and a resident rat lasting for maximum of 3 mins per day) was pessimistic in its judgement of ambiguous cues indicative of a negative affective state. Hence welfare of animals encompasses the emotional state, in addition to behavioural and physiological indicators.

Several studies have demonstrated that the development of affective state is associated with changes in several parts of the brain. The basolateral amygdala controls the processing of both positive and negative affective states (Davis and Whalen, 2001; Siebert *et al*., 2003). Rats treated with noradrenalin-corticosterone to mimic acute stress conditions had significant increase in densities of c-Fos immunoreactivity (a measure for neural activity) in the lateral amygdala ($P<0.01$), basolateral amygdala ($P<0.001$) and dentate gyrus of the hippocampus ($P<0.01$) but no changes in the medial and central amygdala (Enkel *et al*., 2010). These changes were accompanied with a pessimistic judgement of ambiguous cues, which could be linked with the production of a negative affective state.

An electrophysiological study by Zald and Pardo (1997), involving the use of positron emission tomography (PET) demonstrated the activation of right and left amygdala and the orbitofrontal cortex in human subjects exposed to aversive olfactory stimulus (sulfoxide gas) which was considered arise from an increase in regional blood flow to

these regions of the brain. In the same study, regional blood flow to the orbitofrontal cortex increased when subjects were exposed to mild odorant whereas the left amygdala was activated under strong aversive odour.

The stimulation of the amygdala triggers other changes related to fear or anxiety such as the activation of autonomic responses (increased blood pressure, skin response and defecation), behavioural responses (freezing) and the release of stress hormone (Davis, 1992). Lesions in the amygdala suppressed the display of fear-related responses such as freezing behaviour, vocalisation, corticosterone release and defecation in rats subjected to conditioned stimulus of white noise (10 times exposure to 56 db for 5 s each) along with a 0.5 sec foot shock (Golstein *et al*., 1996). This implies that activation of the amygdala corresponds to the development of negative affective state and fear-related responses.

Sheep injected with 0.10 mg/kg of the drug diazepam to reduce the level of fear had a reduced cortisol level during an isolation test compared to control sheep (2.91 ng/ml vs 6.72 ng/ml, $P<0.05$) and were more positive in their judgment of ambiguous cues closest to the positive location in a cognitive bias task (Destrez *et al*., 2012). The authors suggested that a positive affective state could be promoted by reducing the level of fear in animals. This is in agreement with the study by Salmeto *et al*. (2011), who reported a display of anxiety and depression state in chicks subjected to 5 and 60 mins isolation stress respectively. Using a similar protocol to that of Salmeto *et al*. (2011), reported a reversal in the depressive behaviour of chicks subjected to 60 mins isolation stress following an administration of 15 mg/kg imipramine which is an antidepressant. The use of an antidepressant drug probably blocked the stimulation of the amygdala.

2.6 Summary

A critical review of the literature has found that heat stress and physiological stress are important issues that can affect the growth performance, physiology and welfare of broiler chickens. Stress can have a substantial impact on the sustainability of broiler meat production. Animal welfare encompasses both the physical and mental well-being (i.e. being in a good mental state) of animals (Dawkins, 2004), yet most research in broiler chickens has focussed on the physiological and behavioural measurements, with little attention given to the bird's affective state.

Environmental temperature is the main cause of heat stress in broilers which is exacerbated by high RH by reducing the opportunity for heat loss through evaporation. Changes in CBT are a primary response to excess heat load. Although the common method for determination of CBT is using a digital thermometer placed in the rectum, the benefits of being able estimate CBT from free-moving animals without the need for restraint has led to the development of assisted technologies such as temperature -ID chips. Temperature-ID chips are relatively less invasive compared to the data logger and do not require the animal to be restrained. Although the use of temperature-ID chips has been validated in a number of species, there are concerns of variability in measurement and migration around the body of chips implanted subcutaneously.

Under chronic stress there is a prolonged elevation of levels of corticosterone in the blood stream of birds. Unlike short-term secretion of corticosterone, chronic stress has detrimental effects on welfare such as impaired growth rates, alteration of the immune system, cognitive dysfunction and behavioural changes. The urate sphere could be an alternative of non-invasive method of measuring corticosterone in broilers.

These negative impacts of chronic stress can be averted to some extent through good management practices such as the proper regulation of the environmental conditions of temperature, RH and light conditions. An understanding of the behavioural response of broilers during heat stress might give an insight into the thermoregulatory needs of the birds and so to the development of relatively low-cost alleviation strategies. Behavioural changes such as increased water intake and splashing of water on specific body surfaces such as the combs and wattles confirm the important role of water in thermoregulation under heat stress. Hence, a strategy tailored towards enhanced water utilisation (assuming a ready supply of water) could help alleviate heat stress and so requires further exploration.

2.7 Conclusions

- Lighting conditions including intensity and wavelength have been shown to have a significant effect on growth, behaviour and physiology of broilers but there is also evidence that lighting conditions could affect cognition and affective state of animals.
- Levels of corticosterone can indicate the presence of stress in birds. However, the use of the urate sphere as a non-invasive alternative to blood sampling requires further development.

- Determination of the affective state of a wide range of animal species has been undertaken under different stressful conditions however; the direct effect of feeding corticosterone (simulating chronic stress) on the affective state of broiler chickens has yet to be explored.
- During heat stress, core body temperature is an important indicator of welfare, but there is the need to validate the use of temperature-ID chips as a less invasive means of measuring CBT.
- Reports suggest that the frequency, duration and intensity of heat waves would increase as a result of global warming, so research is needed to explore strategies to alleviate the effects of heat stress.

Chapter 3: Effect of light wavelength on the growth performance and welfare of broiler chickens

3.1 Introduction

In commercial poultry production the environmental conditions inside the building can have a substantial effect on the growth performance and welfare of the birds. Lighting conditions is an important environmental condition of poultry production (Alvino *et al*., 2009) which could affect behaviour, physiology and growth (Deep *et al*., 2012; Prayitno *et al*. 1997a,b). Light can be divided into three aspects, namely intensity, wavelength and source (Lewis and Morris, 2000) which collectively define the lighting conditions of an environment.

The broiler industry typically recommends keeping birds under relatively dim lighting of less than 5 lux (Deep *et al*., 2010) because the use of dim light reduces activity levels which in turn enhances energy utilisation and maximises growth rates (Alvino *et al*., 2009). However, in laying birds, the use of low light intensity (3 lux) reduces the incidence of feather pecking (Kjaer and Vestergaard, 1999) but on the other hand low light intensity of 5 lux suppressed the level of egg production especially when applied at the critical period of laying (16-24 weeks), according to O'Connor *et al*. (2011). In a large scale study using 950 Ross broilers, Deep *et al*. (2010) compared birds reared under one of four different levels of light intensities, namely 1, 10, 20 or 40 lux and reported that there was no effect of light intensity on body weight, feed intake, mortality or skeletal health which suggests that the birds were able to adapt to the low light intensities. However, birds kept at 1 lux had enlargement of several eye parameters, namely eye weight, corneal and dorsoventral diameter (all $P<0.05$), presumably as they were straining their eyes in order to see clearly. Not only that, but birds exposed to 1 lux spent greater time sitting indicative of less activity, resulting in a higher incidence of ulcerated foot pad lesions (39.17 vs 25.83 % for birds in the 1 and 40 lux treatments respectively, $P<0.05$; Deep *et al*. (2010). Hence, keeping birds in a low light intensity can have implications for reduced welfare both in both broilers and laying birds.

When given the opportunity, broilers have shown a preference to stay under blue wavelength light rather than white light because the calming effects arising from reduced activity (increased time spent sitting and sleeping) (Prayitno *et al*., 1997a). Thus the use of blue light has been advocated to reduce stress levels in broilers during rearing (Xie *et al*., 2008) or in a slaughter house (Barbosa *et al*., 2013). In a study in

humans, being in a blue wavelength was associated with reduced physiological arousal as indicated by reduced respiratory rate (19.8 vs 18.4 breaths per minute respectively before and after 30 minutes of exposure to blue light; Visweswaraiah and Telles, 2006). In contrast, Prayitno *et al*. (1997a) argued that red light had a stimulatory effect on broilers, which might prove useful when there is a need to increase levels of activity, e.g. to promote foraging behaviour.

Light is also an important regulator of brain function and cognition (Vandewalle *et al*., 2009; Chellappa *et al*., 2011). Cognition can be described as the processes involved in information processing which entails attention, learning, memory and decision-making (Mendl *et al*., 2009). In a study on humans, Smolders *et al*. (2012) showed that providing high light intensity (1000 lux) during the working hours of the day enhanced the level of alertness compared to people kept in low intensity light (200 lux). It is thought that the degree of alertness may affect cognitive processes such as attention and memory recall.

In rats, a cognitive bias task showed that rats switched from a high (65-100 lux) to low (10-15 lux) intensity were faster to approach ambiguous locations compared to rats which were switched from low (10-15 lux) to high (65-100 lux) light intensity (Burman *et al*., 2009). The longer latency of rats switched from low to high light intensity to approach the ambiguous positions was interpreted as an indication of pessimism whereas rats switched from high to low intensity were considered to be optimistic.
It might be that a similar effect of light wavelength on cognition could be observed in broilers exposed to different light wavelengths, and so by extension, this could affect their emotions. Therefore the aim of the current study was to develop a new cognitive assessment method to evaluate cognitive ability in broiler chickens exposed to one of two different light wavelengths, namely red or blue light. These particular light wavelengths were chosen according to previously-reported differential effects on growth performance, behaviour and immune response in broilers (Prayitno *et al*., 1997a; Cao *et al*., 2008; Xie *et al*., 2008). Since red light is made up of a high proportion of long wavelength rays such as that of white light, then a red wavelength light treatment might result in a similar effect and so stimulate the birds (Prayitno *et al*., 1997a). We hypothesised that birds reared under blue light would perform better in a cognitive test than birds reared under red light.

3.2 Materials and methods

3.2.1 Experimental design

The experiment was designed as a 2 x 2 factorial with two light wavelengths (red or blue) and two sexes of broilers (male, female) giving a total of four treatments with four replicate pens per treatment. The experiment was conducted in two identical climate chambers in the controlled environment suite at the Ridley Building. In each room, eight pens of broilers were used as shown in Figure 3.1. The study was conducted under close supervision of staff from the University's Comparative Biology Centre (CBC), who confirmed that this study could be conducted without the need for a licence from the Home Office since birds would be kept within the guidelines from the code of recommendations for the welfare of meat birds (SCAHAW, 2000). Birds would be unlikely to experience conditions that might be considered harmful. In this and subsequent studies on heat and physiological stress, the intention was to simulate conditions similar to that of commercial broiler production in terms of bedding material, temperature and light intensity, so that the results could be easily translated to the industry.

3.2.2 Set up of light wavelengths in climate chambers

Each of the climate chambers used for this experiment had four, white 58W fluorescent light bulbs (150×10 cm), one in each corner of the room. The red and blue light wavelengths were created by placing specific blue or red filters (Filter 120 and Filter 106 respectively, according to Prayitno *et al.*, 1997b; obtained from LEE Filters, Andover, England) over the fluorescent light bulbs. Filters came in sheets measuring 10 x 10 cm and were cut to size and fitted over the fluorescent bulbs and held in place with blue tac. Light intensity was measured using a light meter (RS 180-7133 light-meter, Lutron, UK). The aim was to create a light intensity at bird height of approximately 10-15 lux in keeping with DEFRA (2002) recommendations. To achieve this level of intensity, the red light filters had to be placed in double thickness over the bulbs to reduce the light intensity to about 10-14 lux. Thus as shown in Figure 3.1, the four corner pens adjacent to the source of fluorescent light had a light intensity of 10 lux while the four pens in the middle of the room had a light intensity of 14 lux. In contrast, a single thickness filter of blue gave a light intensity of only 2-3 lux. The four corner pens had a light intensity of 2 lux at bird height while the four pens in the middle of the room had an intensity of 3 lux.

Table 3.1. Experimental layout of pens showing the lighting conditions in each rooms and light intensity (lux) measured at bird height in each pen

Room 1: Red light (average of 12 lux)		Room 2: Blue light (average of 2.5 lux)	
Pen 7 (10 lux) male (n=8)	Pen 8 (10 lux) female (n=8)	Pen 7 (2 lux) male (n=8)	Pen 8(2 lux) female (n=8)
Pen 5 (14 lux) female (n=9)	Pen 6 (14 lux) male (n=9)	Pen 5 (3 lux) female (n=9)	Pen 6 (3 lux) male (n=9)
Pen 3 (14 lux) male (n=9)	Pen 4 (14 lux) female (n=9)	Pen 3 (3 lux) male (n=9)	Pen 4 (3 lux) female (n=9)
Pen 1 (10 lux) female (n=9)	Pen 2 (10 lux) male (n=9)	Pen 1 (2 lux) female (n=9)	Pen 2 (2 lux) male (n=9)

3.2.3 Animals and facilities

A total of 140 broiler chickens (70 birds of each sex) of a commercial genotype (Ross 308, Aviagen, Newbridge, Scotland) were purchased from a commercial hatchery at day-old (Vion Foods Ltd, Duns, Berwickshire, Scotland). At the hatchery, the chicks were vaccinated against infectious bronchitis (BN A118CN01). On arrival to the experimental site, the chicks were immediately placed into single-sex pens (six pens had 9 birds each while the remaining two pens had 8 birds per pen). The number of birds chosen was based on the total number of rings that could be accommodated in each room, along with the diameter of each pen. To ensure sufficient statistical replication, the aim was to have as many pens of a representative group size as possible. Identical floor pens were formed using adjustable plastic sheets clipped together to make a circular pen with diameter of 90 cm. The area of the pen was set to provide each bird with 0.053m^2 of floor space which, assuming the birds reached a mean weight of 1.65 kg, equated to a stocking density of 31 kg/m^2 which is below the maximum stocking density of 39 kg/m^2 permitted for meat chicken production in the United Kingdom (DEFRA, 2011). The pens were bedded with wood shavings to a depth of 5 cm (Goodwill's Wood shavings and Timber Products Ltd, Ponteland, UK) and contained a drinker and feed trough. Birds had *ad libitum* access to commercially manufactured

starter, grower and finisher rations appropriate to their age to (W.E. Jameson & Son Ltd. Masham, UK) along with drinking water.

Temperature and relative humidity (RH) was closely regulated to follow the commercial guidelines specified for this genotype of broiler chicken in the Ross Broiler Management Manual (Aviagen Ltd, Midlothian, Scotland). RH was maintained at 60% throughout whilst the temperature was set initially at 30^{o}C and subsequently reduced by 1^{o}C every three days. By day 27, the temperature was 20^{o}C and this was maintained until the end of the study at Day 35.

In keeping with commercial practice, for the first week the chicks were reared under a light regime of 23 h light: 1 h darkness (23L: 1D) to facilitate settling in and encouraging the birds to feed. The length of the dark period was subsequently increased so that from Day 8-32 the regimen was 18L: 6D and from Day 33-35 this was 23L: 1D. The daily routine included cleaning the drinkers and feed troughs and checking the health of the birds. Stale bedding was removed and replaced with fresh litter every three days. Although such a fastidious cleaning regimen is atypical of commercial practice, CBC staff considered this a necessary precaution in this first experiment to be conducted in the climate chambers using commercial broiler chickens. Cleaning the pens was undertaken carefully so as to minimise disturbance to the birds and was carried out on days when behavioural recordings were not undertaken.

3.2.4 Estimation of performance and welfare indices

The birds were monitored and weighed every week using a bucket placed on a weighing scale. The bucket was tarred to zero and then each bird placed individually in the bucket and its weight recorded. Feed intake per pen per week was also recorded along with any birds which died. From these data, production indices of mean daily feed intake, daily weight gain, feed efficiency and mortality were subsequently calculated as follows:

i) Daily feed intake per bird (g) = total feed consumed per pen day / number of birds per pen

ii) Weight gain per bird (g) = current week body weight per bird- previous week body weight per bird / 7 days

iii) Feed Efficiency = total body weight gain per pen per week / total feed intake per pen per week.

iv) Mortality (%) = (total number of dead birds per pen at the end of the study / total number of birds per pen) × 100

3.2.5 Behaviour observations

The behaviour of the birds was monitored by video recording using a series of video cameras (DCS-900 and DCS- 910, Ethernet cameras supplied by BT, London, UK) connected to a computer. Behavioural observation was undertaken during weeks 2 to 5 of the experiment for two days per week on two pens per treatment (pens 3 & 4, each pen having 9 birds) for two hours per day (one hour in the morning between 10-11am, and one hour in the afternoon between 2-3pm). Subsequently, an observer viewed the video footage and at 2 min intervals, scanned the pen and classified each bird into one of five mutually exclusive behaviour categories namely feeding, drinking, walking, sitting or foraging. An ethogram of behaviours monitored is presented in Table 3.2. These behaviour categories were chosen following observation of a sample of footage from a number of pens, selected to encompass the range of activities performed by the birds. Scan sampling was used to identify behaviour states since under dim light it was considered too difficult to identify individual birds necessary for focal sampling. For each pen, the total number of birds performing each behaviour per hour was expressed as a proportion of the total number of possible observation points.

Table 3.2: Ethogram of the five mutually exclusive behaviour categories monitored

Behaviour category	Definition
Feeding	Birds has beak in or above the feeder
Drinking	Birds has beak in or above the drinker
Walking	Bird is walking around the pen
Sitting	Birds is resting on its abdomen on the litter with raised head
Foraging	Birds is pecking or scratching in the litter

3.2.6 Procedure for training and cognitive ability tests for broilers

3.2.6.1 Overview

Since the training for the cognitive bias test had to be undertaken in an adjacent experimental room which did not have adequate temperature control, the birds had to be given sufficient time to reach an age when they were less dependent on supplementary heat. So, at 19 days of age, one bird from each pen was randomly selected and tagged for training on a visual discrimination task between palatable food (standard pelleted broiler feed sprayed with water) and unpalatable food (standard food sprayed with 4% quinine, see Appendix 1) in a test cage. Thus in total eight birds from each light treatment were recruited (four males and four females). Hodgson *et al*. (2007) reported no sex difference in the performance of Zebra finches in spatial or visual task, so the current study did not take the sex of the bird into consideration.

The feeds used for the discrimination test were identical to that feed given to the birds in their home pen (other than the addition of water/quinine). Previous studies have reported 2% quinine to be aversive to starlings and rats (Bateson and Matheson, 2007; Burman *et al*., 2009) and so this was considered to be an unpalatable but non-toxic additive. However due of the nature of broiler chickens being selected for fast growth rate and consequently large appetite, we decided to use a higher dose of quinine, namely 4%.

Birds randomly selected from pens 1- 4 were trained to associate palatable food with white cones and unpalatable food with black cones, while birds from pens 5-8 were trained to associate palatable food with black cones and unpalatable food with white cones. This was undertaken to counterbalance for colour of the cone to be associated with feed type. Each cone was made of paper and had a diameter of 7.5 mm and height of 25 mm (Figure 3.1).

Before starting the training session, birds were deprived of food for a period of two hours immediately after the six hour period of darkness to increase their motivation to subsequently feed in the discrimination task and thus increasing the chances of them successfully learning the task. Birds used for the training were taken out of their home pen to the test room which had a double decked cage placed on a table. One cage served as a holding cage and the other a test cage (see Figure 3.2 and 3.3 respectively). The holding cage was divided into four portions, one bird for each light wavelength/cone colour with which they were trained to associate with palatable feed. The test and

holding cages were adjacent to each other so that any bird undergoing training in the test cage could see other birds in the holding cage to avoid isolation. The test room was fitted with white light having an intensity of approximately 300 lux, thus a somewhat greater intensity than what the birds had in their home pen but necessary so that they could easily identify the different cone colours. To avoid any bias associated with training birds from one treatment for a longer time than another, birds were selected alternatively from each lighting treatment/colour of training cones in a clockwise manner as presented in Figure 3.4 below.

Figure 3.1: Example of white cone

Figure 3.2: Holding cage

Figure 3.3: Test cage set with black cones

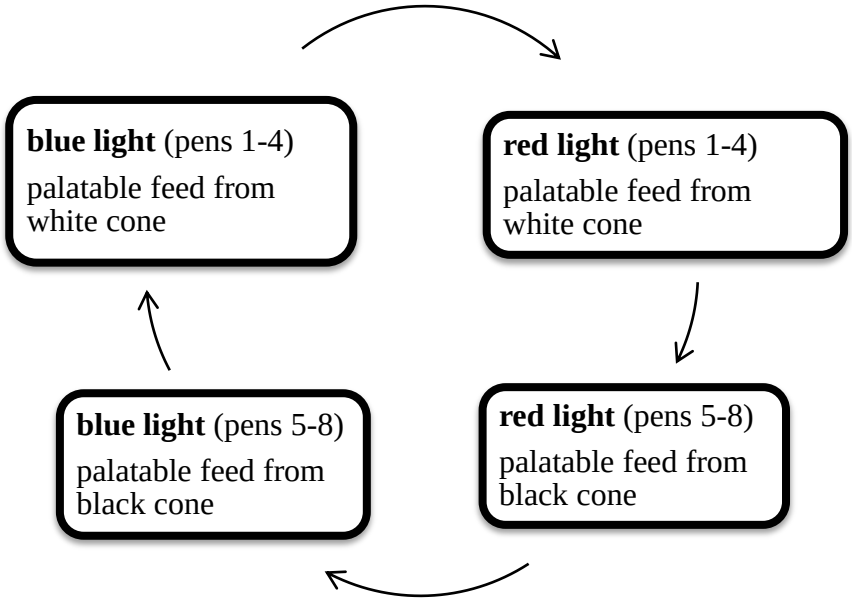

Figure 3.4: The order of selecting birds from the holding cage for training/cognitive tests

3.2.6.2 Training on a discrimination task and cognitive tests

Day 1 (acclimatisation): feed pellets identical to what the birds had in their home pen was scattered on a piece of brown paper spread on the floor of the test pen. The bird undergoing training was allowed to eat the pellets for a period of 5 minutes before it was returned to the holding cage. Each bird had two sessions of this acclimatisation (with an interval of 80 mins) to help them become habituated to feeding in the test pen.

Day 2 (pre-training): palatable feed pellets were placed inside each of six cones of the appropriate colour which had been positioned on their side so that the feed was visible to the bird. Each bird had two sessions of this pre-training per day, each session lasting for five minutes. The aim of this process was to enable the birds to associate either white or black cones with palatable feed. White cones were made of standard white A4 printer paper, whilst black cones were made from standard white A4 paper printed with 100% black shading.

Day 3 & 4 (pre-training stage 2): Palatable feed pellets were placed in each of six petri dishes (5 cm diameter) which were fixed to the floor of the test pen and covered with either a white or black cone depending on which cone colour the bird was being trained to associate with palatable feed. The feed was placed in the petri dish so that when the bird opened the cone the feed was not scattered over the floor of the test cage. Each bird

had two of these sessions per day with each session lasting five minutes and an interval between sessions of 80 minutes. This stage of the training was to teach the birds to flip open the cones to access the feed underneath.

Day 5, 6 & 7 (training): On each of these three days, each bird had two sessions with an interval of 80 minutes. On day 5 and 6, the birds were given eight cones in each session, four palatable and four unpalatable. On Day 7 the number of cones used in each session was increased to twelve (i.e. six palatable and six unpalatable) so that the birds were faced with a greater number of choices and also to prepare them for cognitive tests when they would be presented with equal number of the three cone types. Data from Day 7 was analysed and used for selecting birds for the cognitive test. The number of palatable cones out of the first six cones opened by each bird per session and the order in which the cones were opened (i.e. 1st palatable and 1st unpalatable) was determined. Birds that opened eight or more palatable cones across two sessions, i.e. birds which opened at least 66.6% palatable cones, were considered to have passed the learning criterion. Three birds had a score less than 66.6% (one bird from the red wavelength treatment and two birds from the blue wavelength), so 13 birds continued with the cognitive tests.

Day 8 (Cognitive tests): The aim of these tests was to explore how birds responded to an ambiguous (intermediate) cone when presented either alone (50% with palatable feed or 50% empty) or simultaneously with the palatable and unpalatable cones (at this time the ambiguous cone was empty). The ambiguous cone was grey in colour and of same dimensions as the palatable or unpalatable cones. For cognitive test 1, birds were presented with twelve ambiguous grey-coloured cones, formed from printing the standard white A4 paper with 50% shading. Six of these ambiguous cones had palatable feed while the other six were without feed, so that the birds might learn that opening the intermediate cone has only a 50% chance of obtaining a palatable food reward. Each bird had one session of this test which lasted for up to three mins but data was collected on the number of cones opened in the first minute. This test was carried out to assess the reaction of the birds to the ambiguous cone.

For cognitive test 2, birds were simultaneously presented with four cone types (palatable, unpalatable, ambiguous cone without feed) for a period of three minutes. Palatable feed was placed only under the palatable cones, whereas the ambiguous cones had no feed underneath them. This test was done to assess the decision of broiler

chickens about which cones to open. The number of each of cone type opened for the first six cones was recorded and used for data analysis. For cognitive test 2, the order in which the cones were opened was also recorded for the 1st palatable, 1st ambiguous and 1st unpalatable cones.

3.3 Statistical analysis

Data on growth performance, feed intake and scan behaviour sampling satisfied the conditions of the normality test (Shapiro-Wilks), therefore parametric tests were used for the analysis. LSD (Least square significance) was used for a post hoc test. However, data on the training and cognitive tests were not normally distributed so an appropriate non parametric test was used. Because of the few number of birds that died/culled during this study (three birds), no analysis was undertaken for mortality/culled birds (All statistical tests were undertaken using the SPSS statistical package (SPSS version 19, Rothampstead, UK) as follows.

i) Growth performance and feed intake

Feed intake, weight gain and feed efficiency were analysed using a repeated measures GLM having day (7 to 35) as the repeated factor and light wavelength and sex as the between subject factors in a full factorial model to take account of any interaction between light wavelength and sex. Start weight, total weight gain and final weight were analysed using GLM, having light wavelength and sex as factors.

ii) Behaviour

The mean proportion of total observation time the birds engaged in each of the five behaviour categories (sitting, walking, feeding, drinking and foraging) was analysed using a repeated measure general linear model (GLM). The within subject factors was day (14-35) while light wavelength and sex were the between subject factors in a full factorial model.

To account for any relationship between the five different behaviour categories, the proportion of time spent on each behaviour across the four weeks was calculated and values subjected to a principal component analysis (PCA). Bartlett's test of sphericity $\chi^2 = 484$, $P<0.001$, indicated that the correlations between the mean proportion of time engaged in the different behaviour categories were sufficient to justify a PCA. An initial

analysis was run to obtain Eigen values for each new component. Only factors which had eigen values greater than 1 were considered. This analysis produced two new factors, so the values of these two new factors (mean per pen per observation) were analysed using GLM with light wavelength and sex as between subject factors in a full factorial model.

Multiple comparisons for days (feed intake, weight gain, feed efficiency and proportion of observation time) were adjusted for using Bonferroni correction. If the test assumption of sphericity was violated, (Mauchly's test, $P<0.05$), then the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity.

iii) Training and cognitive tests

The data from the third day of training were analysed in order to establish whether the birds had learnt to discriminate between the palatable and unpalatable cones. Discriminant learning was assessed via two measures: the number of each of the cones opened and the order in which the cones were opened (i.e. 1st palatable and 1st unpalatable cones opened). Because data collected during the training and cognitive tests were count data and were not found to follow a non-normal distribution, non-parametric tests were used for the analysis. For both training and cognitive tests data, a Mann Whitney U test was used to test for the effect of light wavelength on the number and order in which cones were opened. If there was no significant effect of light wavelength, then data for birds from both light wavelengths were pooled together and a Freidman test was undertaken to test for differences between the palatable and unpalatable cones.

3.4 Results

3.4.1 Effect of light wavelength and sex on the performance of broilers

3.4.1.1 Daily feed intake

Overall, there was a significant main effect of light wavelength ($F_{1, 12} = 5.68$, $P< 0.05$) on daily feed intake/bird, with birds reared under blue light (85.3 g/bird/day) eating more than birds reared under red light (77.8 g/bird/day). There was no significant main effect of sex on daily feed intake nor was there a light wavelength × sex interaction

(Figure 3.5). Repeated measures analysis showed that there was a significant main effect of age ($F_{1.243,\ 14.920} = 543.96$, $P<0.001$), with mean feed intake increasing as the birds increased in age. There were no significant interactions between age × light wavelength, age × sex or age × light wavelength × sex for daily feed intake.

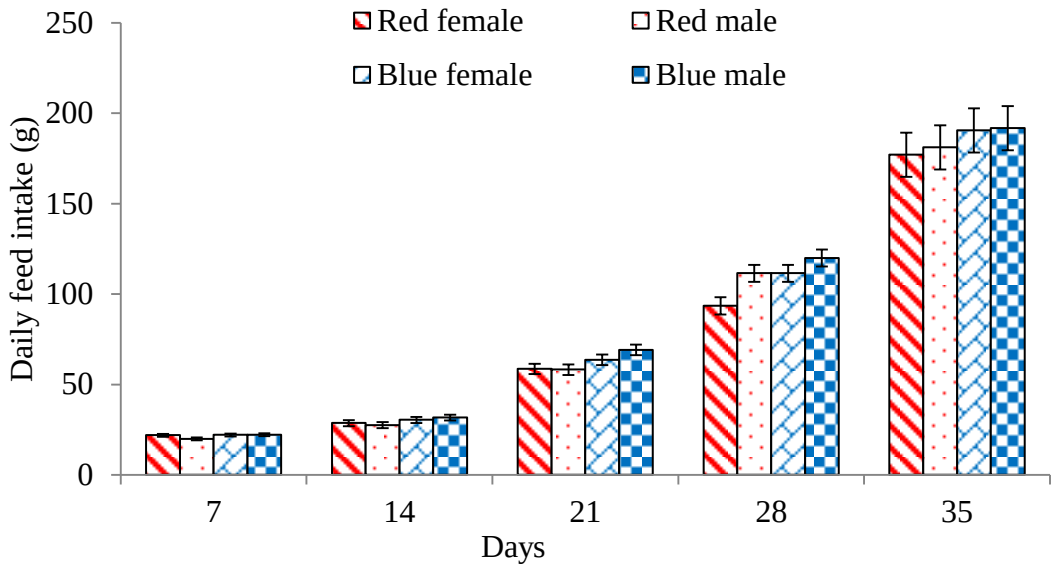

Figure 3.5: Effect of light wavelength and sex on mean daily feed intake of broilers from 7 to 35 days of age.

3.4.1.2 Body weight and mean weight gain

Table 3.3 shows that neither average start weight, final weight nor total weight gain were affected by light wavelength or sex and there were no treatment interactions. There was a marginal difference in start weight based on sex ($F_{1,\ 12} = 4.37$, $P = 0.058$). Equally, there was no effect of light wavelength or sex on mean daily weight gain, nor was there a significant light wavelength × sex interaction. However, there was a significant effect of age on daily weight gain ($F_{1.329,\ 15.945} = 145.51$, $P < 0.001$), with a greater daily weight gain in older birds; the daily weight gain was significantly greater at Day 28 and 35 compared to other ages. Although there was no significant interaction of age × light wavelength or age × sex on daily weight gain, there was a significant interaction between age × light wavelength × sex ($F_{1.329,\ 15.945} = 4.05$, $P<0.05$), specifically in broilers reared in the blue light where the females (84.4g) had a greater ($P<0.05$) weight gain than the males (69.1g) at 35 days of age (Figure 3.6).

Table 3.3: Effect of light wavelength and sex on start weight, final weight and total weight gain of broiler chickens from 7 to 35 days of age

Treatment	Start weight / bird (g)	Total weight gain / bird (g)	Final weight / bird (g)
Red × female	50.14	1454.81	1504.95
Red × male	48.63	1499.07	1547.70
Blue × female	50.11	1564.85	1614.96
Blue × male	48.68	1562.32	1611.00
SEM	0.703	51.932	52.301
	P value		
Light wavelength	0.988	0.121	0.123
Sex	0.058	0.695	0.717
Light wavelength × sex	0.949	0.660	0.663

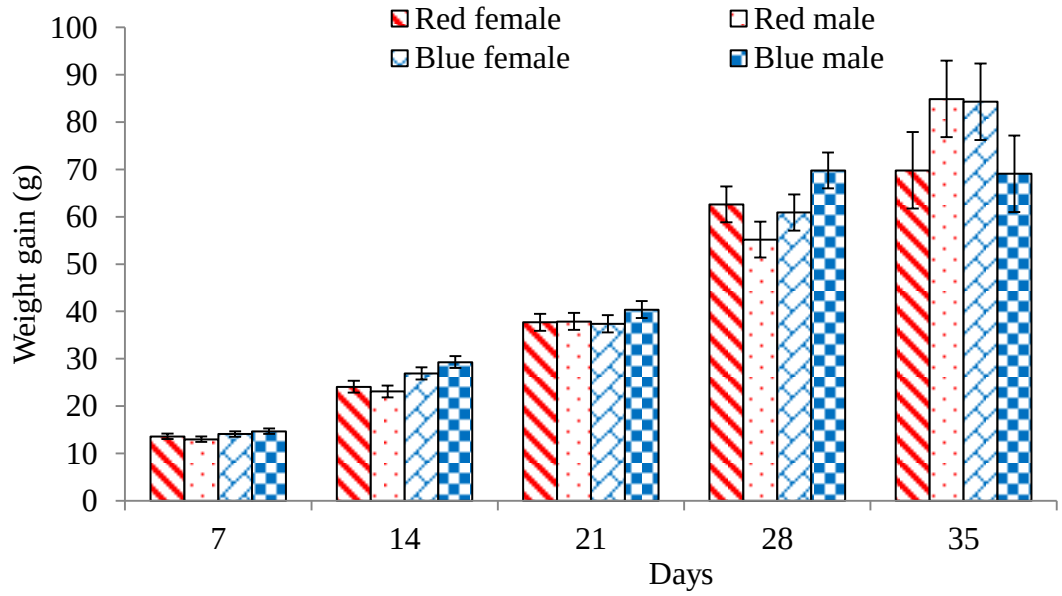

Figure 3.6: Effect of light wavelength and sex on mean daily weight gain of broilers from 7 to 35 days of age.

3.4.1.3 Feed efficiency

There were no significant effect of light wavelength or sex on feed efficiency nor was there an interaction between light wavelength and sex. However, there was a significant effect of age on feed efficiency ($F_{2.036, 24.437} = 75.31$, $P<0.001$) such that feed efficiency was greatest at Day 14 and lowest at Day 35 day with days 7, 14 and 28 having intermediate values. There was no significant interaction of age × light wavelength or age × sex on feed efficiency. There was a tendency towards significance for interaction of age × light wavelength × sex on feed efficiency ($F_{2.036, 24.437} = 3.24$, $P = 0.056$), with a tendency for female broiler chickens reared in the blue light wavelength to have a greater feed efficiency at 35 days of age than females (Figure 3.7).

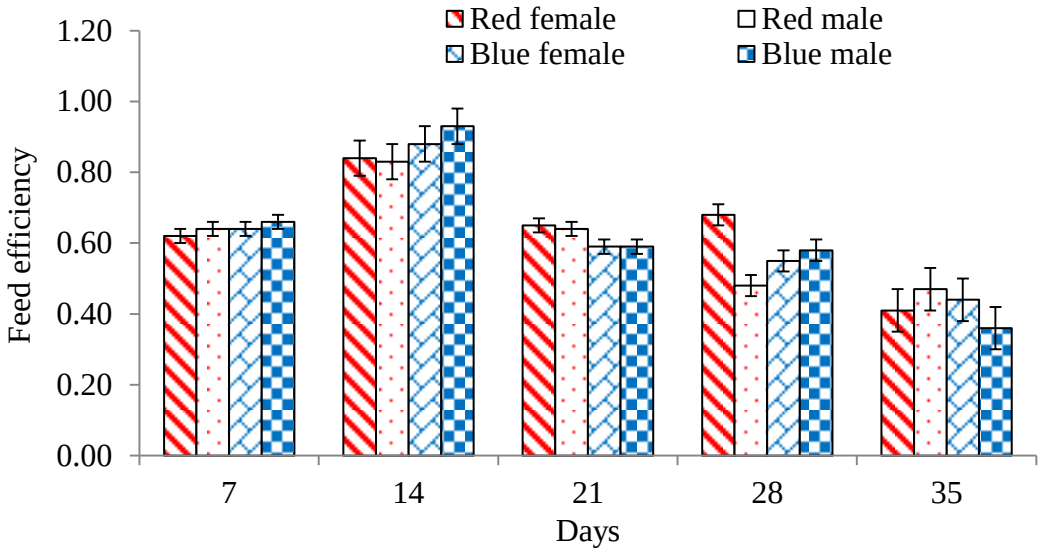

Figure 3.7: Effect of light wavelength and sex on feed efficiency of broilers from 7 to 35 days of age.

3.4.1.4 Mortality & culls

During the experiment one bird died and two birds were culled due to poor health thus the percentage mortality/culled was 2.14%. The only mortality recorded was a male chick from the blue light room on the third day of the experiment. The two birds culled were females, due to poor health conditions (i.e. one for swollen crop and the other for lameness), one from each light wavelength.

3.4.2 Effect light wavelength and sex on general behaviour patterns

With the exception of sitting and foraging behaviour, overall there was relatively little effect of light wavelength or sex on the proportion of total observation time engaged in five different behaviour categories as shown in Table 3.4.

Table 3.4: Effect of light wavelength and sex on the proportion of total observation time spent on five different behaviours. Values are means

	Age (days)							
	14	**21**	**28**	**35**	**Age**	**Age ×light condition**	**Age ×sex**	**Age ×light condition × sex**
Sitting								
Red female	0.013	0.013	0.023	0.020				
Red male	0.020	0.013	0.021	0.021				
Blue female	0.011	0.020	0.021	0.024				
Blue male	0.019	0.017	0.022	0.025				
Pooled SEM	0.001	0.002	0.002	0.002	**	*	*	NS
Walking								
Red female	0.007	0.006	0.003	0.004				
Red male	0.006	0.007	0.004	0.002				
Blue female	0.009	0.004	0.003	0.003				
Blue male	0.005	0.006	0.003	0.003				
Pooled SEM	0.001	0.001	0.000	0.000	**	*	**	*
Feeding								
Red female	0.004	0.002	0.003	0.003				
Red male	0.002	0.004	0.004	0.003				
Blue female	0.005	0.002	0.003	0.002				
Blue male	0.003	0.006	0.005	0.004				
Pooled SEM	0.002	0.001	0.001	0.001	NS	NS	0.07	NS

Table 3.4 continue: Effect of light wavelength and sex on the proportion of total observation time spent on five different behaviours. Values are means

	Age (days)							
	14	21	28	35	**Age**	**Age ×light condition**	**Age ×sex**	**Age ×light condition × sex**
Forage								
Red female	0.008	0.011	0.004	0.004				
Red male	0.005	0.008	0.005	0.005				
Blue female	0.006	0.006	0.006	0.003				
Blue male	0.004	0.003	0.002	0.000				
Pooled SEM	0.001	0.002	0.002	0.001	*	NS	NS	NS
Drinking								
Red female	0.001	0.001	0.001	0.001				
Red male	0.001	0.002	0.001	0.002				
Blue female	0.002	0.001	0.001	0.001				
Blue male	0.001	0.001	0.001	0.001				
Pooled SEM	0.001	0.001	0.001	0.001	NS	NS	*	NS

3.4.2.1 Sitting behaviour

There was a significant effect of light wavelength (P=0.050) on the proportion of observation time spent sitting, which was greater for birds in the blue light than those in the red light. There was no effect of sex nor was there an interaction of light wavelength x sex on the proportion of observation time spent sitting. However, there was a significant effect of age ($F_{3, 36} = 16.23$, $P<0.001$) on the proportion of observation time spent sitting which was greater at 28 and 35 days than at 14 and 21 days (Figure 3.8). Furthermore, there was a significant interaction of age × light wavelength ($F_{3, 36} = 3.19$, $P<0.05$), where birds in the blue light spent a greater proportion of time sitting at 21 and 35 (both $P<0.05$) days of age. The significant interaction of age and sex on sitting

behaviour ($F_{3, 36}$ = 4.53, P<0.05) showed that at 14 days only, males spent a greater (P<0.001) proportion of observation time sitting than females (Table 3.4).

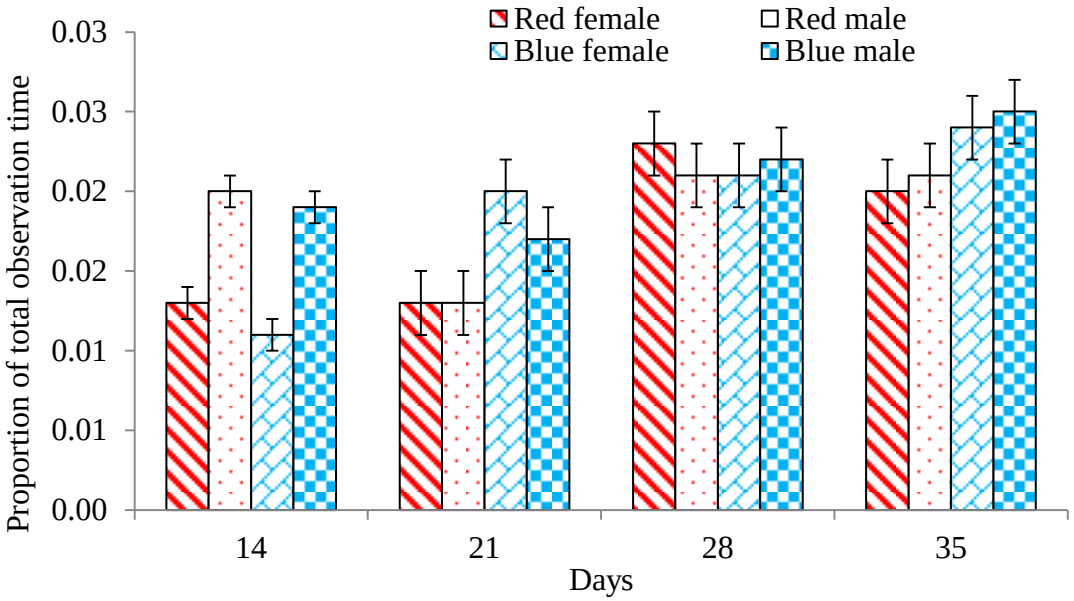

Figure 3.8: Effect of light wavelength and sex on proportion of total observation time spent sitting by broilers from 14 to 35 days of age.

3.4.2.2 Walking behaviour

The proportion of observation time spent walking was not significantly affected by light wavelength or sex nor was there an interaction between these two factors. However, age had a significant effect on the proportion of total observation time spent walking ($F_{3, 36}$ = 51.83, P<0.001), with a greater level of walking at 14 and 21days than at 28 and 35 days. Interaction of age and light wavelength was also significant ($F_{3, 36}$ = 4.57, P<0.05) especially at 21 days of age (P<0.05) where walking activity was greater for birds reared under the red wavelength. There was a significant interaction of age and sex on the proportion of time spent walking ($F_{3, 36}$ = 10.71, P<0.05) which occurred at 14 and 35 days (both P<0.05) of age with pullets showing a greater level of walking than cockerels (Table 3.4).

3.4.2.3 Feeding and drinking behaviour

The proportion of total observation time spent feeding or drinking was not significantly affected by light wavelength, sex or age nor were there any second or third order interactions Table 3.4.

3.4.2.4 Foraging behaviour

The proportion of total observation time spent by birds foraging was significantly affected by light wavelength ($F_{1, 12} = 10.36$, $P<0.05$) and sex ($F_{1, 12} = 7.07$, $P<0.05$). Birds reared in red light had a higher level of foraging than those reared in blue light and pullets spent more time foraging than cockerels. There was no interaction of light wavelength and sex on time spent foraging. There was a significant effect of age on time spent foraging ($F_{1, 36} = 4.34$, $P<0.05$), with greater levels of foraging behaviour seen at 28 and 35 days of age than at 14 and 21 days (Figure 3.9). There were no significant age ×light wavelength, age × sex or age ×sex × light wavelength interactions (Table 3.4).

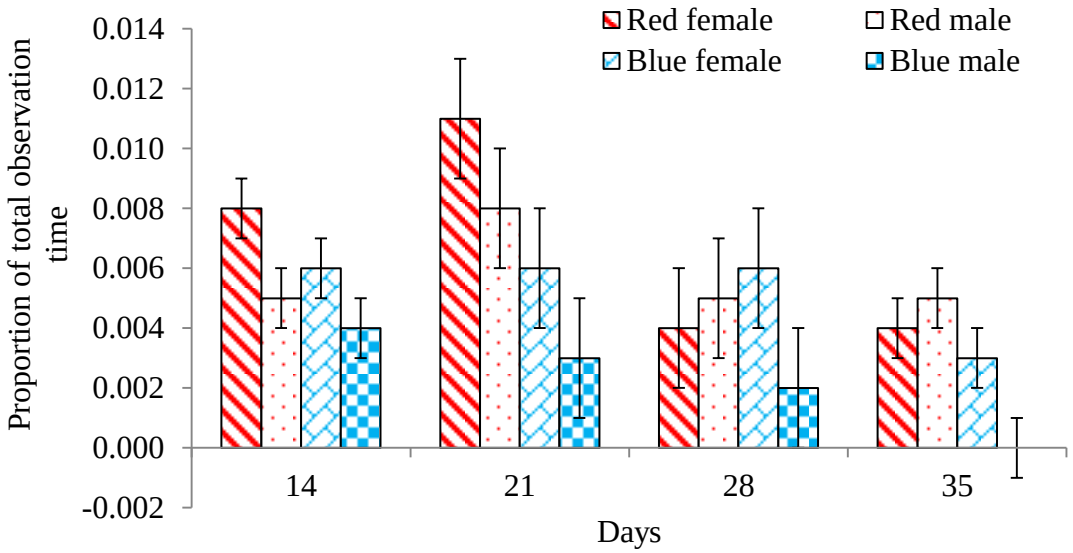

Figure 3.9: Effect of light wavelength and sex on the proportion of total observation time spent foraging by broilers from 14 to 35 days of age.

3.4.3 Principal component analysis for behaviour analysis

Two components were found to have Eigen values greater than Kraiser's criterion of 1.0 and, in combination explained 78.46% of the variance of the amount of this behaviour. Factor 1 had an Eigen value of 2.39 and explained 47.87% of the variance while Factor 2 had an Eigen value of 1.53 and explained 30.59% of the variance (Table 3.5). Table 3.4 shows the loading of different behaviour categories onto the two factors, with loadings below 0.4 regarded as being too low (Field, 2009).

Table 3.5: Loadings of each behaviour category on the two factors extracted by the principal component analysis (PCA) and the % variance in amount of that behaviour explained by each factor

Behaviour category	**Factor 1**	**Factor 2**
Sitting	-0.942	0.004
Walking	0.841	-0.165
Feeding	-0.181	-0.858
Drinking	-0.184	0.778
Foraging	0.795	0.510
% variance in behaviour explained by each factor	47.87	30.59

3.4.3.1 Effect of light wavelength and sex on Factor 1 (General activity pattern)

For Factor 1, large positive values were associated with a high proportion of observation time spent walking and foraging and large negative values corresponded to a high proportion of time spent sitting. Thus with more walking/foraging and less sitting, Factor 1 can be considered as 'general activity'. There was a tendency for light wavelength to have a significant effect on general activity of birds ($F_{1, 12} = 4.575$, $P = 0.054$), with broilers reared under the red wavelength being generally more active than

their counterparts reared under blue wavelength. There was also a tendency for a significant effect of sex on general activity of birds ($F_{1, 12}$=4.630, P=0.052), with female birds involved more in walking and foraging than males. There was no interaction between light wavelength and sex for general activity levels (Table 3.6).

3.4.3.2 Effect of light wavelength and sex on Factor 2 (General feeding pattern)

For Factor 2, large positive values corresponded with a high proportion of time spent drinking and foraging whereas large negative values corresponded to a high proportion of time spent feeding. Thus, with more drinking and foraging and less feeding from the trough, Factor 2 was termed general feeding behaviour. Neither light wavelength nor sex had any effect on the levels of general feeding behaviour as indicated by Factor 2 nor was there a significant light wavelength by sex interaction (Table 3.6).

Table 3.6: Effect of light wavelength and sex on the value of Factors 1 and 2

Treatments	**General Activity (Factor 1)**	**General Feeding (Factor 2)**
Red female	0.894	0.027
Red male	0.006	0.519
Blue female	0.011	0.368
Blue male	-0.911	-0.914
Pooled SEM	0.421	0.457
	P values	
Light wavelength	0.054	0.256
Sex	0.052	0.405
Light wavelength × sex	0.969	0.076

3.4.4 Effect of light wavelength on cognitive ability of broilers in a visual discrimination task

Training of birds on a visual discrimination task

It was observed that the birds opened all the cones when they were given a three minute period during the training. Presumably the birds did not find the unpalatable feed

(coated with quinine) sufficiently aversive because they have been fasted for a period of time. Therefore, data obtained during the training and cognitive test 2 was based on the first six cones opened.

There were no significant differences between birds from the two light wavelengths in the number of palatable and unpalatable cones or the order in which the 1st palatable and 1st unpalatable cones were opened. Therefore, data from birds from the two light wavelengths were pooled together and then subjected to a Wilcoxon sign rank test. The results showed that the number of palatable cones opened was greater ($P<0.001$) than the number of unpalatable cones, see Figure 3.10a. Also palatable cones were opened sooner than the unpalatable cones (Figure 3.10b).

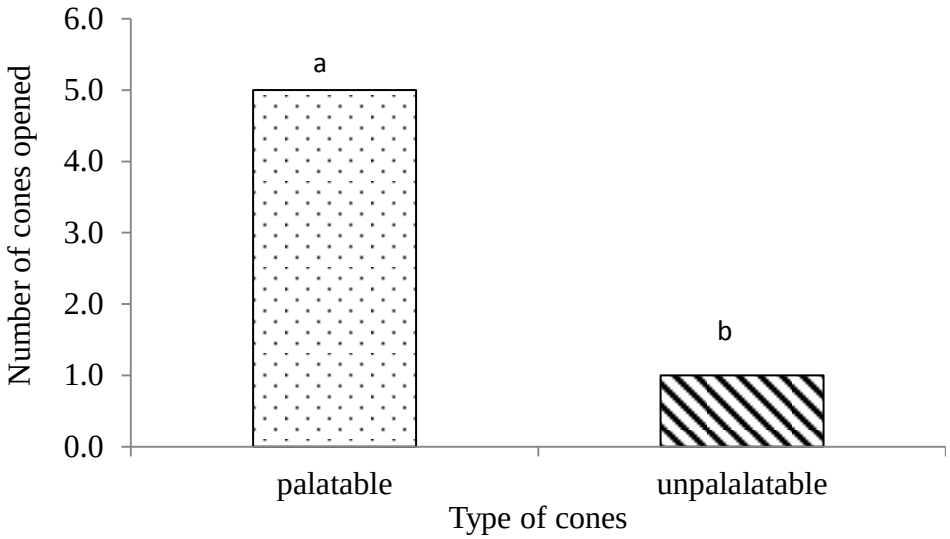

Figure 3.10a: Number of palatable and unpalatable cones opened by broilers (mean of two light wavelengths) on the third day of training (n=13). ab Medians with different letters differ significantly at $P<0.001$.

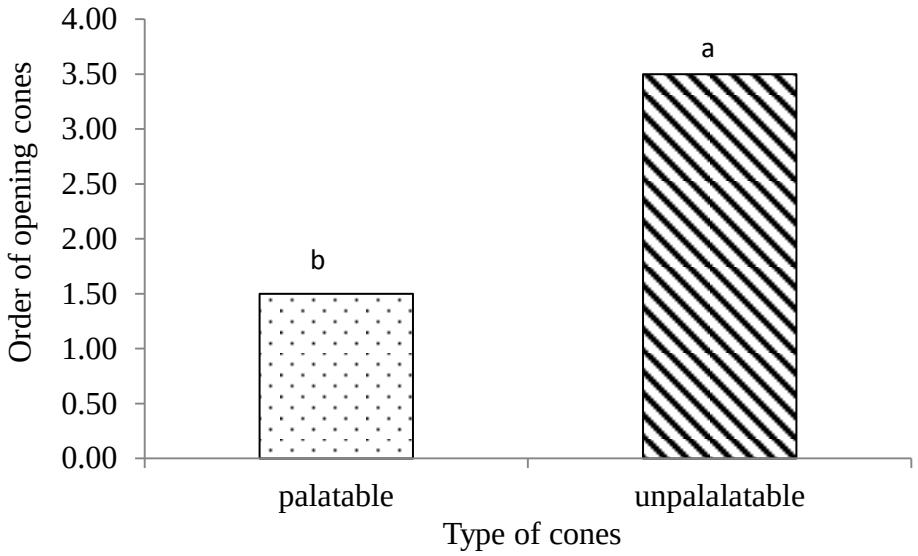

Figure 3.10b: The order in which palatable and unpalatable cones were opened by broilers (mean of two light wavelengths) on the third day of training (n=13). ab Medians with different letters differ significantly at P<0.05.

3.4.5 Tests with ambiguous cones

3.4.5.1 Cognitive test 1

There was no significant difference in the number of intermediate cones opened by broilers from the two light wavelengths (median of 7.0 vs 8.5 cones for the red and blue wavelengths respectively).

3.4.5.2 Cognitive test 2

The Mann Witney U test showed no difference in the order and number of the three cones opened between by birds from the two light wavelengths during cognitive test 2. Hence, data from birds in the two light wavelengths were pooled and subjected to a Friedman rank sum test to compare the responses of the birds to the three cones types. There was a significant difference between the number of the three cone types opened (χ^2= 11.45, P<0.05), with a greater number of palatable and intermediate cones opened than the unpalatable cones (Figure 3.11a). In addition, there was a significant difference in the order in which the three cone types were opened (χ^2= 12.46, P<0.05), such that the 1st palatable and 1st intermediate cones were opened earlier than that of the 1st unpalatable cone (Figure 3.11b).

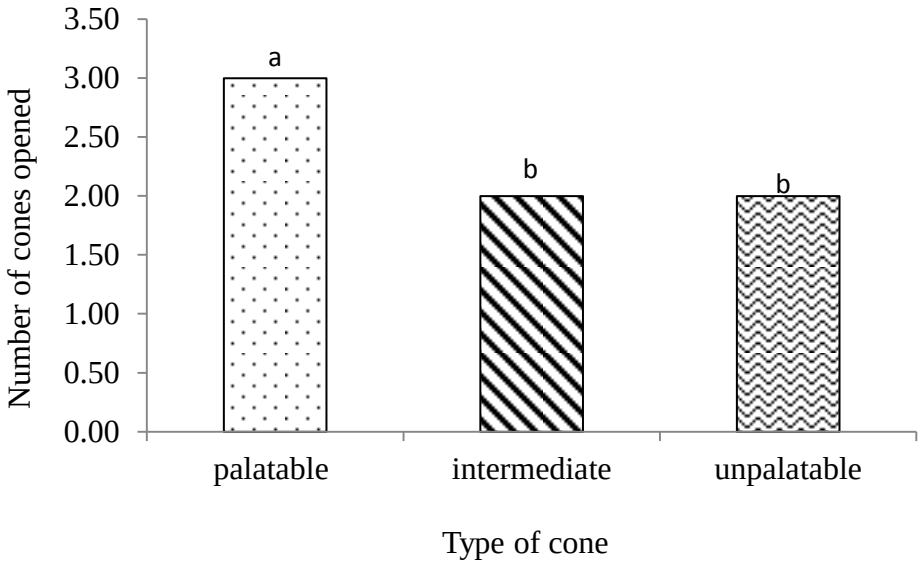

Figure 3.11a: Number of palatable, intermediate and unpalatable cones opened by broilers (mean of two light wavelengths) during cognitive test 2 (n=13). ab Medians with different letters differ significantly at P<0.05.

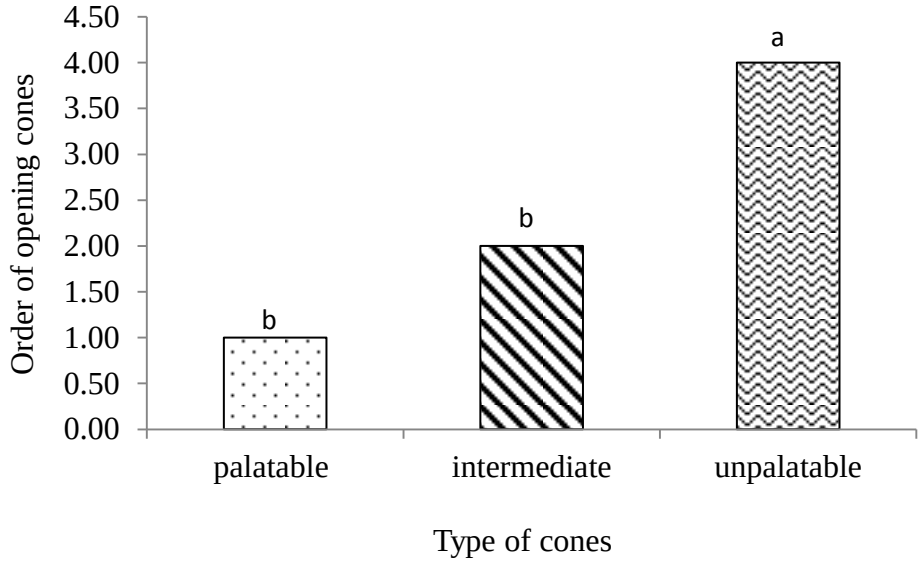

Figure 3.11b: The order of opening the palatable, intermediate and unpalatable cones by broilers (mean of two light wavelengths) during cognitive test 2 (n=13). ab Medians with different letters differ significantly at P<0.05.

3.5 Discussion

The aim of this study was to develop a new cognitive assessment method to evaluate cognitive ability in broiler chickens exposed to one of two different light wavelengths. In addition, the behaviour and growth performance of broilers were monitored.

In this experiment it is regrettable that the same light intensity was not achieved in the two light wavelength treatments, so that firm conclusions about the effect of light wavelength cannot be made due to the possibility of an interaction between light wavelength and intensity. In the blue light room, mean light intensity at bird height was 2.5 lux while in the red light room it was an average of 12 lux. Indeed, it should also be borne in mind that lux is not a totally relevant measure of light as perceived by chickens. Birds have different spectra sensitivity compared to humans, and so the intensity of light perceived by birds is different to that measured by a standard light meter which measures intensity in lux (Prescott and Wathes, 1999). Prayitno and Phillips (1997) reported that chickens perceived red and blue light as being equal only when the mean ratio of red to blue light intensity was set at 1:3.

There were a number of other limitations, such as variation in light intensity within a particular light wavelength treatment (see Figure 3.1), and the use of an extremely bright (300 lux) test room. Despite these shortcomings however, the results are still worthy of comparison to other studies reported in the literature.

3.5.1 Effect of light wavelength on the performance of broiler chickens

Daily feed intake was enhanced in broilers reared under a blue wavelength compared to those reared under a red light but this was not reflected in daily weight gain, total weight gain or final weight of the birds, or in the level of feeding behaviour observed. Prayitno *et al*. (1997a) found no difference in body weight of birds reared under white, red, green or blue wavelength but they did report a heavier bone weight in birds reared in green and blue light than those reared in red or white light. In the current study, the birds were reared from day 1 to day 35 in one of two different light wavelengths whereas in the study of Prayitno *et al*. (1997a), the birds were first reared under white light (from day 1 to day 7) after which the birds were randomly assigned to four different light wavelengths i.e. (from day 7 to day 28), nevertheless, the extended period of the current study did not result in a significant difference in body weight. Using two light wavelengths (red and blue) and three light intensities (low, medium and high),

Prayitno *et al*. (1997b) did not detect any interaction of light wavelength and intensity on final body weight, although birds reared in a red light with high intensity had a numerically lower body weight than those reared in blue light with low intensity (1381 vs 1435 g respectively).

The current study raised birds for 35 days, but it is possible that if the birds had been kept for longer, differences in body weight might have been seen. Cao *et al*. (2008) reported that levels of plasma testosterone, myofibre growth of the major pectoralis muscle and body weight were significantly greater for broilers reared under blue light than those in white, red and green lights from day 38 onward. Although direct comparison between our study and that of Cao *et al*. (2008) is difficult because of the confounding effect of light wavelength and intensity (2.5 versus 12 lux in the blue and red light treatments respectively) used in the current study compared to 15 lux used in the study of Cao *et al*. (2008). Previously, rearing birds in blue light has been reported to increase weight gain. In a study by Prayitno et al. (1997b), rearing broilers for 6 to 7 weeks under blue light increased weight gain of the birds by lowering activity level, but this was said to increase the chance of abnormal gait developing by 50% due the greater bodyweight. Rozenboim *et al*. (1999) also reported that broilers exposed to blue light were heavier from day 20 to 34 than those reared in red light, ($P<0.05$). The differences in results between studies could be due to the different sources of the light wavelength, different light intensities, or the interaction between lux and wavelength as mentioned previously. It could also depend on the breed of the birds, stocking density or other management practices such as duration of photoperiod. In the current study, light wavelength was created by the use of light filters and the light schedule was 18L: 6D for most of the rearing period (Day 8-32) whereas Rozenboim *et al*. (1999) used light emitting diode (LED) bulbs of different colours with a lighting period of 23L:1D for 35 days, so that again this extended exposure to blue light could have resulted in greater weight gain. However, rearing birds under continuous light for 24 h has been associated with an increase in duration of tonic immobility (130 s vs 84 s, $P<0.05$) and heterophil/lymphocyte ratio (1.53 vs 0.43, $P<0.05$) compared to birds reared under 12 h light, indicative of increased level of fearfulness and chronic stress respectively (Zulkifli *et al*., 1998). Reports on the effect of day length on level of fear in broilers are inconsistent , since a recent study by Schwean-Lardner *et al*. (2012) found no different in the duration of tonic immobility in birds exposed to one of four different photoperiods (14, 17, 20 and 23 h) . Lack of treatment effect therefore could be due to

the difference in day length used in each study, management system or breed. At day 27 and 28 of age which is a period when broilers begin to become less active (Alvino *et al*. 2009), Schwean-Lardner *et al*. (2012) found that 'optimal welfare' was achieved in broilers exposed to 17 h light compared to birds under 23 h light by promoting less inactivity (53.81 vs 77.01, P<0.05), more feeding (12.87 vs 7.61, P<0.05) and preening behaviour (3.15 vs 1.12, P<0.001).

Deep *et al*. (2010) and Deep *et al*. (2012) found no effect of light intensity (1, 10, 20 or 40 lux) on body weight gain of broilers up to 35 days of age. Similarly, the use of light intensities of 5, 50 and 200 lux had no effect on final body weight, even immune system such as B and T lymphocyte proliferation and gait score of broilers (Blatchford *et al*., 2009). Hence, the results obtained in the current study might be more of an effect of wavelength rather than intensity.

3.5.2 Effect of light wavelength on the behaviour of broiler chickens

The low sample size of behaviour recordings in the current study could have reduced the power in detecting a significant effect of light wavelength on time spent in different behaviour categories, or it could have been a confounding effect of wavelength/intensity. Nevertheless, at 21 and 35 days of age, birds in the blue light spent a greater proportion of time sitting than those in red light who spent more time walking. This indicates that red light made the birds more active at a younger age. Prayitno *et al*. (1997a) who also reported that time spent sitting was greater in birds reared in blue light compared to those in red light (13.5 vs 10.2 minute/hour, P<0.001), after adjusting the ratio of the light intensity of the red and blue light to 1.0:3.3 (to factor in the claim that birds perceive the these two light wavelengths to be similar at this ratio).

In the current study, level of feeding behaviour was not influenced by light wavelength. This is despite results of monitoring daily feed intake which showed that birds in the blue light had a greater feed intake than their counterparts in the red light. This could suggest that the blue light enhanced feed consumption per visit to the feeder as there was no difference in the proportion of time spent feeding by birds in the two light wavelengths. Prayitno *et al*. (1997a) also found no difference in the feeding behaviour of broilers reared in white, red, green or blue light wavelengths once adjustments to perceptions of the bird had been taken account of. It could also be argued that in the

current study the low light intensity (2.5 lux) of the blue light did not hinder the birds from feeding properly, perhaps because the birds adjusted to these dim conditions. Deep *et al*. (2010) reported that birds exposed to dim light (1 lux) had anatomical changes in their eyes compared to those in kept under light intensities of 10, 20 and 40 lux.

In fact, result of the principal component analysis (PCA) grouped the five behaviour categories into two main groups' namely general activity and feeding patterns. Exposure of birds to red light enhanced their level of activities in foraging and walking, which could be attributed simply to the greater light intensity in the red light treatment, since greater light intensity is associated with greater activity (Prayitno *et al*., 1997b). Increased activity of birds in the red light could be attributed to its long wavelength which in turn triggers a higher stimulatory effect on the hypothalamus (Lewis and Morris, 2000). The increased foraging behaviour under red light could also be the reason for the reduction in daily feed intake compared to birds in blue light, so that birds were more active in foraging in the litter instead of feeding from the feeder. Levels of behaviours such as standing, walking, drinking, aggression and wing stretching increased in birds reared under red light as light wavelength was increased whereas only wing stretching and aggression increased with increase in light intensity in birds reared under blue light (Prayitno *et al*., 1997b).

In the current study the proportion of total observation time spent sitting and foraging was greater when the birds were 28 and 35 days of age, which corresponds to age of greatest weight gain. On the other hand, between day 7 and 21 of age the weight gain was lower and birds at this period spent a greater proportion of their time walking. This suggests that with increase in age and body weight, there is a change in behaviour from walking to sitting indicative of decreased activity level. It has been reported that broilers spend 37% and 61% of their time sitting at 7 and 30 days of age respectively and throughout their growth cycle, 50% of the time budget of the birds was spent sitting (Kristensen *et al*., 2007). The switch from active to inactive behaviour at an older age might be attributed to increased body weight, meaning greater effort is required to move around and a reduced opportunity to move around because of the associated reduction in space available in the pen. This result is in agreement with Alvino *et al*. (2009) also who reported that during the photophase (light period), broilers were more active at 3 weeks of age than at 4 or 5 weeks of age (both $P<0.05$). Sultana *et al*. (2013) also found an age-related effect on the activity level of broilers. In the current study we had few behaviour categories and with the assistance of the PCA behaviour such as walking and

foraging were considered as active behaviour while sitting as inactive however in the study of Alvino *et al*. (2009) behaviour such as feeding, drinking, walking, preening and foraging were regarded as active while the inactive behaviour consisted of sitting, sleeping and standing. Lewis and Morris, 2000) reported that the response of retinal photoreceptors to light stimulates growth and causes changes in behaviour whereas the response to hypothalamic light receptors to light regulates reproduction in birds.

Thus despite the challenge of confounding light intensities, the current study found changes in behaviour patterns similar to previous experiments suggesting that the birds perceived the two light wavelengths differently.

3.5.3 Effect of light wavelength on the cognitive ability of broilers

Broilers from both light wavelengths were equally successful in learning the visual discrimination task by showing preference for the palatable feed. Studies on cognitive bias training typically present the animals with the trained cues one after the other (Harding *et al*., 2004 and Bateson and Matheson, 2007). However, in the current study, the birds were able to identify the palatable cones and flip them open to access palatable feed when they were presented simultaneously with the unpalatable cones, thus demonstrating the use of cognitive abilities (Boks, 2010). Although it was observed that if given a period of 1 minute, birds eventually flipped open all the cones (both palatable and unpalatable). This could be as a result of the high motivation of broiler chickens to feed coupled with the period prior to testing when the birds did not have access to feed. Hence the decision to concentrate on the first six cones opened during the training and cognitive test 2. Barnett *et al*. (2007) reported a similar findings that starlings which were feed restricted consumed more mealworms injected with 2% quinine compared to birds which were not feed restricted (23 vs 62, $P<0.05$). Perhaps a better method of training birds would be either with a food reward and no reward (Wichman *et al*., 2012) or a food reward and a punisher which could be an aversive environmental stimulus such as noise or an air puff (Edgar *et al*., 2013).

Nevertheless, when the two cone cones were presented simultaneously, the birds had to process the information associated with each cone colour before deciding which cone to open. This also shows that the birds had learnt to discriminate between cones. In confirmation of the learning task, birds from both light wavelengths opened the palatable cones earlier than unpalatable cones, and they also opened a greater number of

the palatable cones. The lack of hesitancy to open the intermediate cone when it was presented alone suggests a readiness to explore this ambiguous cone, probably due to their increased level of motivation to feed. Finally, when presented with three cone types simultaneously, representing palatable, intermediate (ambiguous) and unpalatable cones, birds from both light wavelengths opened the palatable and intermediate cones earlier than the unpalatable cones, but opened more palatable cones than intermediate cones. Deep *et al*. (2010) reported the anatomical changes caused by low light intensity (1 lux) could impair the vision of the birds however; bird in the current study reared under blue light with an intensity of 2.5 lux had no difficulty differentiating between the three cone colours. Probably vision was clear in the testing room which was fitted with a greater light intensity (300 lux).

The cognitive ability displayed by the birds in this cognitive test is called 'executive function' which is controlled by the prefrontal cortex of the brain; in place of prefrontal cortex birds have the nidopalliun caudolaterale (Güntürkün, 2005). Executive function implies the involvement of cognitive processes in taking the right decision under different situations (Güntürkün, 2005). The prefrontal cortex has been reported as one of the brain regions mostly affected by stress (Alkadhi, 2013), hence if the light wavelengths used in the current study had been stressful to the birds, then it would have reflected in an impaired cognitive ability as long term exposure to stressful conditions have been reported to decrease cognitive ability in humans (Hancock and Vasmatzidis, 2003). In the current study such an effect was not evident, so that it seems that neither blue nor red light wavelengths used in the current study had any deleterious effect on the birds. In humans, exposure to blue light enhanced cognitive functions (Beavan and Ekstrom, 2013). Hence investigation about the effect of light wavelength on cognitive function could be explored further, especially under conditions were light intensity can be closely regulated. Burman *et al*. (2009) reported that a change in light intensity from high (65-100 lux) to low (10-15 lux) or vis versa did not affect rats the ability of rats to discriminate between the already learnt rewarded and aversive locations during a cognitive bias test, but it did result in bias in information processing about the ambiguous locations.

3.5.4 Effect of sex on growth performance and behaviour of broilers

Female broiler chickens were heavier than their male counterparts in the blue wavelength at 35 days, although at the end of the experiment there was no difference in

total weight gain between male and female broilers. Rozenboim *et al*. (2004) attributed the stimulation of growth in male broiler chickens reared under blue light to an increase in testosterone production (Lewis and Morris, 2000). On the other hand, the growth rate of both male and female broilers in the red light wavelength was similar throughout the growth cycle indicative of flock uniformity. Overall, the growth performance of broiler chickens in both light wavelengths was similar at the end of the experiment. In the current study, females were more active (walking and foraging) than the males (sitting).

3.6 Conclusion

Although in this experiment light wavelength was confounded with light intensity and the sample size of behavioural observations was small, the results showed that light wavelength had a significant impact on the behavioural activity patterns of broilers. Red light enhanced the level of activity of the birds whilst blue light enhanced sitting behaviour. Broilers from both light wavelengths were equally successful in learning a visual discrimination task and displayed a similar high cognitive performance in the test of their cognitive ability. Neither blue nor red light wavelength appeared to impose any form of stress on the birds, based on the results of growth performance and cognitive ability, although the levels of stress hormones such as corticosterone were not estimated.

Chapter 4: Development of a non-invasive method of assessing stress in broiler chickens- estimation of endogenous corticosterone levels from the urate sphere

4.1 Introduction

During stress, corticosterone is secreted into the bloodstream (Rich and Romero, 2005) which consequently results in an increased level of blood glucose (Ognik and Sembratowicz, 2012) required to sustain metabolism. The secreted corticosterone is transported through the blood to the target organs/cells (Palme *et al*., 2005). For this reason, measuring levels of corticosterone in blood plasma/serum has remained the 'gold standard' of assessing stress (Mormède *et al*., 2007; Cook, 2012). However, procedures such as catching and handling required during blood sampling could be stressful to the bird, thus affecting the basal levels of corticosterone. To overcome this pulsatile (episodic change) increase in levels of corticosterone (Nelson, 2005), blood should be sampled within 2-3 minutes of handling the birds (Mormède *et al*., 2007).

Another shortcoming of measuring plasma corticosterone is its unreliability in detecting chronic stress (Shini *et al*., 2008; Mormède *et al*. 2007) because under stressful conditions for an extended duration, the negative feedback mechanism regulates the level of corticosterone in circulation (Pariante and Lightman, 2008). However, there can be situations when the negative feedback mechanism is reduced or impaired, so that the level of corticosterone is continuously elevated (Pariante and Lightman, 2008). At such times, abetter method of detecting chronic stress is through a HPA sensitivity test, otherwise referred to as a dexamethasone suppression test (Mormède *et al*. 2007). A dexamethasone suppression test assesses the function of the HPA-axis, by suppressing the release of corticosterone because dexamethasone blocks the effect of corticotrophin releasing hormone on the pituitary (Cole *et al*., 2000). Dexamethasone suppression test is used to detect those psychiatry disorders associated with a lack of suppression of endogenous levels of corticosterone in depressed patients (Cole *et al*., 2000) because of the continuously elevated levels of corticosterone in circulation. Therefore, a dexamethasone suppression test could be used to detect conditions of chronic stress in humans and its use has been extended to animal species. In birds, there are inconsistencies in the literature on dexamethasone suppression in the plasma or other non-invasive methods. Studies have shown that administration of dexamethasone suppressed plasma corticosterone levels in laying hens (Etches, 1976), pigeons (Westerhof *et al*., 1994) and Great tits (Clapp, 2010), although the protocol employed in

each of these experiments differed slightly which might account for some of the variation in the results seen. For example, Westerhof *et al*. (1994) reported that dexamethasone suppressed plasma corticosterone levels by 82% at the time of its diurnal peak (at night). On the other hand, Etches (1976) first stimulated an increase in plasma corticosterone by injecting birds with exogenous ACTH, and after administration of dexamethasone they reported a 67% suppression of levels of plasma corticosterone. Westerhof *et al*. (1994) used eight different doses of dexamethasone in pigeons (either 500, 100, 50, 10, 5, 1, 0.5 or 0.1µg/kg) and reported that with the exception of the 0.1µg/kg dose, all doses significantly suppressed levels of plasma corticosterone. However, Rettenbacher *et al*. (2004) and Dehnhard *et al*. (2003) found no effect of dexamethasone administration on the level of corticosterone in birds. Despite these contradictions, the use of a dexamethasone suppression test in avian species to detect chronic stress or assess the sensitivity of the HPA axis seems to have some promise, especially if an appropriate dose of dexamethasone can be administered.

Alternative means of measuring corticosterone in birds are from the faeces, feathers, yolk and albumin of eggs (Cook, 2012). Measuring corticosterone from the droppings (faeces or urine) is less stressful to the birds than taking a blood sample and there are no limitations regarding the amount that can be collected or the duration of collection, hence it could be a better means of collecting multiple samples to assess stress (Rettenbacher *et al*., 2004) compared to plasma samples. The delay in the production of excreted corticosterone after an increase in plasma levels depends on the transit time through the gut and for urine production (Hirschenhauser *et al*., 2012). In the literature, the lag period between a peak in corticosterone level in the plasma and that in the faeces is typically between 2 to 4.5 h in broilers (Dehnhard *et al*., 2003), 1.4 h in laying birds (Rettenbacher *et al*., 2004) and 2 h in starlings (Cyr and Romero, 2008).It was reported that the level of stress estimated from corticosterone metabolites could be detected earlier in the urine than in the faeces of broilers and quails (Hirschenhauser *et al*., 2012). However, the collection of urine samples from birds involves restricting the bird in a metabolism cage with a collecting board underneath, and even with a collecting board, the urine could dry up before the time of collection (Hiebert *et al*., 2000).

Urate is a product of nitrogen metabolism excreted from the urinary system by the kidney during the process of filtration (Goldstein, 2000). Reabsorption of water in the kidney further increases the concentration of urate in the urine thus, protein-bound hormones (albumin) could be involved in maintaining the colloidal nature of the urate

thereby enhancing the formation of urate spheres (Casotti and Braun, 2004).Since corticosterone is transported through the blood to the target organs, it was speculated by Clapp (2010) that the endogenous corticosterone in the blood could be passed into the solid white component of avian urine which is known as the urate sphere (Figure 4.1). Some advantages of sampling from the urate sphere over urine are its solid nature which prevents it from being readily absorbed by the litter. Thus birds does not need to be caged, and its applicability could be extended to free living birds (Clapp, 2010).The amount of urine produced by bird is dependent on its level of hydration (Hiebert *et al*., 2000; Cook, 2012) but the same is not true for the urate sphere. Thus, sampling corticosterone from the urate sphere could be a better non-invasive means of detecting stress in birds when compared to plasma, faeces or urine.

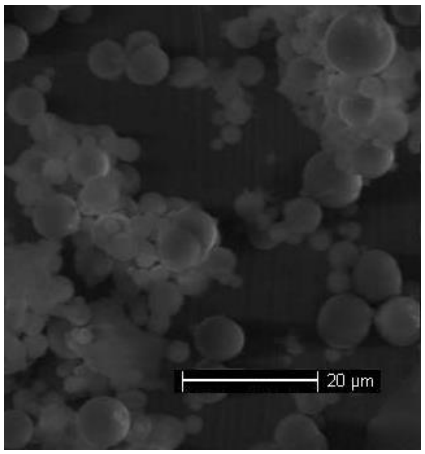

Figure 4.1. Electron micrograph scan of a urate sphere from a chicken (Clapp, 2010)

Clapp (2010) developed a method which successfully identified endogenous levels of corticosterone in the urate sphere of Great tits. He further validated the use of the urate sphere in assessing the sensitivity of the HPA axis using a dexamethasone suppression test. However, one major limitation of Clapp (2010) was an overestimation of the levels of corticosterone by the hormonal assays undertaken (ELISA) in that study. An improved hormone assay is required that would give a more accurate estimate of the level of corticosterone, so that the full potential of using the urate sphere as a non-invasive means of measuring endogenous levels of corticosterone and sensitivity of the HPA axis in birds can be fully harnessed. Since corticosterone metabolism is known to differ between avian species (Hirschenhauser *et al.*, 2012),then results from one species

cannot be generalised for other species. To track the peak of corticosterone in plasma and the urate sphere, we propose to offer birds a stable corticosterone compound (deuterated corticosterone) through mealworms after which serial collection of blood and urate sphere samples will be taken at intervals so that the excretory pattern of corticosterone in the urate sphere can be established.

Therefore, the aim of this study was to validate the use of the avian urate sphere as a non-invasive means of estimating levels of endogenous corticosterone specifically, by using a more sensitive assay, and to validate the suppression of endogenous levels of corticosterone in the plasma and urate sphere after the administration of dexamethasone.

To achieve this aim, three objectives were involved namely

1) To estimate basal levels of endogenous corticosterone in the plasma and the urate sphere.
2) To determine the time after administration when levels of a stable corticosterone compound (deuterated corticosterone) and dexamethasone would peak in the plasma and the urate sphere.
3) To determine the effect of dexamethasone administration on the suppression of levels of endogenous corticosterone in the plasma and the urate sphere.

It was hypothesised that 10 mins after ingestion of mealworms previously injected with deuterated corticosterone (Cort d8) and dexamethasone (Dex.) dissolved in DMSO, a significant peak in levels of Cort d8 and Dex. would be detected (Breuner *et al*., 1998) with similar peaks seen in the urate sphere some 30 mins after ingestion (Rettenbacher *et al*., 2004) . The second hypothesis was that a significant suppression of the levels of endogenous corticosterone in the plasma and urate sphere would be detected 2h after ingestion of treated mealworms (Westerhof *et al.*, 1994).

4.2 Materials and methods

4.2.1 Ethical considerations

This experiment was conducted under Project Licence number PPL 60/4270 of the Animals (Scientific Procedures) Act 1986. Approval for the project was given at both national (Home Office) and local (Ethical Revenue Committee) level, with the number

of birds used considered to be the minimum required to obtain a statistically significant difference between treatments.

4.2.2 Experimental design and overview of treatments

This study was conducted in an experimental room in the Comparative Biology Centre (CBC) using a cross-over design (each bird served as a control for itself) having two phases: in Phase 1, on Day 6 Cort-Dex birds were offered mealworms injected with deuterated corticosterone (Cort d8; $C_{21}D_8H_{22}O_4$; a stable isotope, 4-pregnen-11β, 21-diol-3, 20-dione; QMX Laboratories, Thaxted, UK) and dexamethasone (Sigma Aldrich, Gillingham, UK) dissolved in a non-polar solvent, namely DMSO (Sigma Aldrich, Gillingham, UK),whereas Control birds were offered mealworms injected with DMSO only. In the literature, it has been reported that an acute dose of corticosterone (4μg) offered to white crown sparrows elevated plasma corticosterone levels for 60 minutes, after which they returned to baseline values (Breuner *et al*., 1998). In a different study, a dose of 500μg of dexamethasone offered to pigeons required a period of 52 h for corticosterone levels to return to baseline values (Westerhof *et al*., 1994). Therefore, the decision was made to adopt a three-day interval before the second phase (Phase 2) of the study began on Day 9. The treatments were swapped over so that control birds now received mealworms injected with corticosterone/dexamethasone, and birds previously on the Cort-Dex treatment now received control mealworms (injected with DMSO only). There were four replicate pens with three birds per pen. The arrangement of the experiment and timing of sampling is presented in Table 4.1. To ease the workload, application of treatment was staggered over two days.

4.2.3 Animals and application of treatments

A total of 12female broiler chickens (Ross 308, 32 days old and approximately 1.1 kg body weight) purchased from Oakland Farms Ltd., York, UK. On Day 0, birds arrived at the laboratory and were allocated to four pens (each 30 cm tall and 90 cm in diameter bedded with wood shavings 5 cm deep; Goodwill's Wood shavings and Timber Products Ltd, Ponteland, UK) at three birds per pen. Female broilers were chosen because of their rapid excretion of radiolabelled corticosterone in the urine (Hirschenhauser et al., 2012). In each pen, two birds were randomly assigned to the control treatment and one bird to the Cort-Dex treatment. One dosage of Cort d8 equivalent to 4mg/Kg bodyweight(Post *et al*., 2003) was tested against the control. This

non-invasive method of administering corticosterone involved on Day 6 offering the birds mealworms which had been previously injected with either Cort d8) and dexamethasone both dissolved in DMSO) or DMSO only. On Days 1 to 3, the birds were offered standard mealworms (nothing injected) once a day in their pen so that they become accustomed to feeding on mealworms before the application of treatment.

4.2.4 Preparation of experimental mealworms

Hormone preparation

To prepare the deuterated corticosterone (Cort d8), 25mg of Cort d8 was dissolved in 0.25ml of DMSO and vortexed giving a concentration of 100 mg/ml. Dexamethasone was prepared by dissolving 50 mg of dex. in 1.0 ml of DMSO and vortexed giving a concentration of 50 mg/ml.

Preparation of mealworms

Before injecting mealworms they were made inactive by placing them inside a plastic bag which was then placed on ice for approximately 20 mins. According to treatment, Cort d8, dexamethasone and DMSO or DMSO only were injected into the mealworms using a 100µL Hamilton syringe (Hamilton Bonaduz AG, CH-7402 Bonaduz, Switzerland). To the control birds, a dose equivalent to 40 µl of DMSO was administered per bird by offering the birds four mealworms each injected with 10 µl of DMSO (Figure 4.2c). Cort-Dex birds were offered seven meal worms, the first two mealworms were injected with Cort d8 (20 µl of the corticosterone 100mg/ml preparation was injected into each mealworm to arrive at a dose of 4mg per bird) while the other five mealworms were injected with dexamethasone (20 µl of the dexamethasone 50mg/ml preparation was injected into each mealworm to arrive at a dose of 5mg per bird). As soon as the mealworm was injected, it was offered to the bird as shown in Figure 4.2d.

Figure 4.2a: Birds placed in pen awaiting the production of baseline guano samples

Figure 4.2b: Birds provided with feed and water after baseline drooping and plasma samples were collected

Figure 4.2c: Injecting mealworms with DMSO

Figure 4.2d: A broiler bird being offered a mealworm

4.2.5 Experimental procedure

Commercial-specification poultry feed was provided *ad libitum* to birds in a feed hopper placed on the floor of the pen (20% CP, 4% oil, 6 % ash and 13.0 MJ/kg ME, Poultry Pro Finisher Pellets, W.E. Jameson & Son Ltd, Masham, UK). Fresh water was provided daily in a bell drinker (Wells Poultry Equipment, UK). Daily checks on the health and welfare of the birds were conducted.

At the start of each Phase, a clean heavy duty waterproof plastic membrane sheet was spread on the floor of the experimental room. A plastic ring similar in size to the home pen (30 cm tall and 90 cm in diameter) was then placed on the plastic membrane sheet to be used as the holding pen. A transparent perspex divider was used to divide this holding pen into three equal sections and one bird was placed in each section (Figure 4.2a). The birds were left in this pen until they produced the first dropping (basal

sample), which was within 20 minutes of being placed in the holding pen. Immediately a dropping was produced, the bird was picked up and a blood sample taken within 2 mins to act as a basal sample. Then the bird was placed back in its section and offered the appropriate mealworms according to treatment. Feed and water was then provided to the bird (Figure 4.2b).

At 10 mins and 2 h post treatment, the 2nd and 3rd blood samples were taken from each bird, again within 2 mins of the bird being picked up. All droppings produced between 10 mins and 2 h post ingestion of mealworms were collected and labelled according to treatment and time post ingestion of mealworms.

4.3 Data collection

Blood and dropping samples were collected from each bird at different points as described previously. The order and time of day when each pen was tested was maintained to avoid order effect or diurnal changes in hormone level. Thus, Pens 1and 3 were sampled in the morning (am), while Pens2 and 4 were sampled in the afternoon (pm) on Day 6 and 7 respectively and again after crossover of treatments on Day 9 and 10 (Table 4.1).

4.3.1 Blood sampling

Three blood samples were collected from each bird as follows: the 1st blood was sampled before treatment was applied; the 2ndand 3rd samples were taken at 10 mins and 2 h post treatment respectively. Blood was sampled within 2 mins of handling the bird. In each case a 1 ml sample of blood was collected from the interstitial vein using a 23 gauge needle and immediately poured into an EDTA tube and placed in an ice box. Blood was then centrifuged for 5 min at 1,200 rpm within 1 h of sampling. Plasma was transferred to a 0.5 ml eppendorf tube and stored at −20°C for subsequent analysis.

4.3.2 Urate sphere sampling

All droppings produced prior to feeding mealworms and for up to 2 h post treatment were collected from each bird. Whole droppings were picked up from the plastic membrane of the pen floor and immediately the white urate sphere was separated from the droppings using a spatula and placed in plastic bag and frozen at -80^{o}C.

4.4 Laboratory analyses

All laboratory procedures and analyses were adopted from Clapp (2010).

4.4.1 Preparation of blood samples for hormone analysis

Plasma samples were allowed to thaw and then vortexed. A 100µl sub-sample was then taken for analysis. To each 100µl of plasma, an equal volume of 1% formic acid was added and then vortexed to mix. Next, 10µl of an equimolar 1ug/ml mixture containing two internal standards (IS: progesterone- and cortisol, Sigma Aldrich, Gillingham, UK) was added and the tube again vortexed. Internal standards were added to the plasma samples for adequate calibration of the amount of hormone present in the sample.

Endogenous corticosterone, dexamethasone and deuterated corticosterone were extracted from the 210µl mixture using a preconditioned 96-well Solid Phase Extraction plate (Varian Bond Elut Plexa 30mg, eluted with 100% MeOH). The 500µl eluate was evaporated to dryness in a 40^{o}C water bath under a nitrogen stream (Turbo Vap), for 20 minutes. The dry residue was re-constituted to 100µl using 10% MeOH in water. Each sample was then transferred into an auto-sampling vial and stored at -20°C until subsequent analysis.

Table 4.1. Arrangement of treatments and timing of experiment for data sampling from each of the 12 birds on Day 6 & 7 and Day 9 & 10

	Pen 1			**Pen 2**			**Pen 3**			**Pen 4**		
	Bird 1	Bird 2	Bird 3	Bird 1	Bird 2	Bird 3	Bird 1	Bird 2	Bird 3	Bird 1	Bird 2	Bird 3
Phase 1												
Day 6	DMSO	DMSO	Cort d8 + dex									
Day 6				DMSO	Cort d8 + dex	DMSO						
Day 7							DMSO	Cort d8 + dex	DMSO			
Day 7										DMSO	DMSO	Cort d8 + dex
Phase 2												
Day 9	DMSO	Cort d8 + dex	DMSO									
Day 9				DMSO	DMSO	Cort d8 + dex						
Day 10							Cort d8 + dex	DMSO	DMSO			
Day 10										DMSO	Cort d8 + dex	DMSO

4.4.2 Urate sphere samples

The urate sphere sample was allowed to thaw after which further separation of the urate sphere from any dropping particles was undertaken by careful dissection with a sterile spatula. The urate sphere was then dried by placing on a heat block covered with aluminium foil in a fume cupboard. After drying for 2mins, about 40-50mg of the urate sphere was weighed into a 2ml eppendorf tube. Hydrolysis and extraction of the hormone from the urate sphere was undertaken by adding 1 ml of 5% glacial acetic acid to the tube before it was vortexed and shaken for 20 mins. After removing the samples from the eppendorf shaker (Eppendorf shaker 5432), the samples were vortexed again before 0.5ml of methanol was added and then shaken for another 5 mins. The sample wasthen set into a centrifuge and spun at 12,000 rpm for 5 mins. The supernatant was decanted into a fresh 2 ml eppendorf tube. 0.5ml of methanol was added to the residue, vortexed and centrifuged at 12,000 rpm for 5 mins and the resulting supernatant was added to the previous supernatant in the eppendorf tube. The combined supernatant was kept at -80^{o}C for subsequent analysis.

The solid residue of the urate sphere was used for uric acid determination based on the combined method of Adeola and Rogler (1994) and the spectrophotometric method of Van Handel (1975). To the urate sphere residue in the eppendorf tube, 1.5ml of 0.5% $LiCO_3$was added, vortexed and then poured into a 10 ml test tube. The eppendorf was rinsed twice with $LiCO_3$into the test tube. The contents of the 10 ml test tube were made up to 10 ml by adding sufficient $LiCO_3$ solution, before being vortexed and placed in a boiling water bath for 10 minutes in a fume cupboard. After boiling for 10 minutes, the test tube was vortexed again before the contents were decanted into a 100 ml volumetric flask. The test tube was rinsed twice with 18 MΩ deionised water and added to the contents of the volumetric flask, which was then made up to 100 ml by adding sufficient deionised water.

The volumetric flask was shaken properly and then 1mL of a representative aliquot was taken into a 2 ml eppendorf tube and centrifuged at 12,000 rpm for 2 min. Then a 0.1 ml sample of the contents was taken and placed into another eppendorf tube containing 0.9 ml of water, before being vortexed to make 1/10th concentration of the sample. For spectroscopic analysis, 0.15 ml of this 1/10th concentration was placed into a micro titre

plate and then 0.5 ml of Neocuproine and 5 ml of $CuSO_4$were added (summarised in Figure 4.3). Ten minutes later, the micro titre plate was inserted into a plate reader (Spectramax plus plate reader, NB 950) set with an endpoint of LM1-LM2 as 450-650 nm. Figure 4.6 shows the flow chart of the procedure.

Reagent 1: 20g Na_2CO_3; 8g glycine; 250mg $CuSO_4.5H_2O$ in 500ml 18Mohm water; Reagent 2: 400mg Neocuproine HCl in 80ml water. Colour agent was made by taking 10 parts of Reagent 1 and 1 part of Reagent 2. The uric acid content of each sample was derived from the uric acid standard calibration curve using the dilution factors.

4.4.3 Preparation of urate sphere samples for mass spectrometry

The 2 ml sample of urate sphere extract was thawed and vortexed and a 1 ml sub-sample taken for analysis. It was not necessary to add formic acid because the samples were already acidified (2.5% acetic acid in 50:50 MeOH: water). Tothe 1 ml of urate sphere sample, 10µl of the internal standard (IS) mixture was added and then vortexed to mix. The hormones were extracted from this 1010µl mixture using Solid phase extraction (Varian Bond Elut Matrix C18, eluted with 500µl 0.1% formic acid in MeOH). The concentration of hormone in the urate sphere samples were standardised according to their uric acid content. All hormone standards and IS stock solutions were made up from powder using 75% MeOH in water to a concentration of 0.5 mg/ml. To produce the 1µg/ml IS standard mixture, each 500µg/ml IS stock solution was initially diluted 1:50 giving 10 µg/ml, then diluted 1:5 to give 2 µg/ml. Equal volumes of the two IS solutions were mixed together giving a final concentration of 1µg/mL for each IS. This equates to 10ng of each IS in the final 100µL volume i.e. 100 ng/ml.

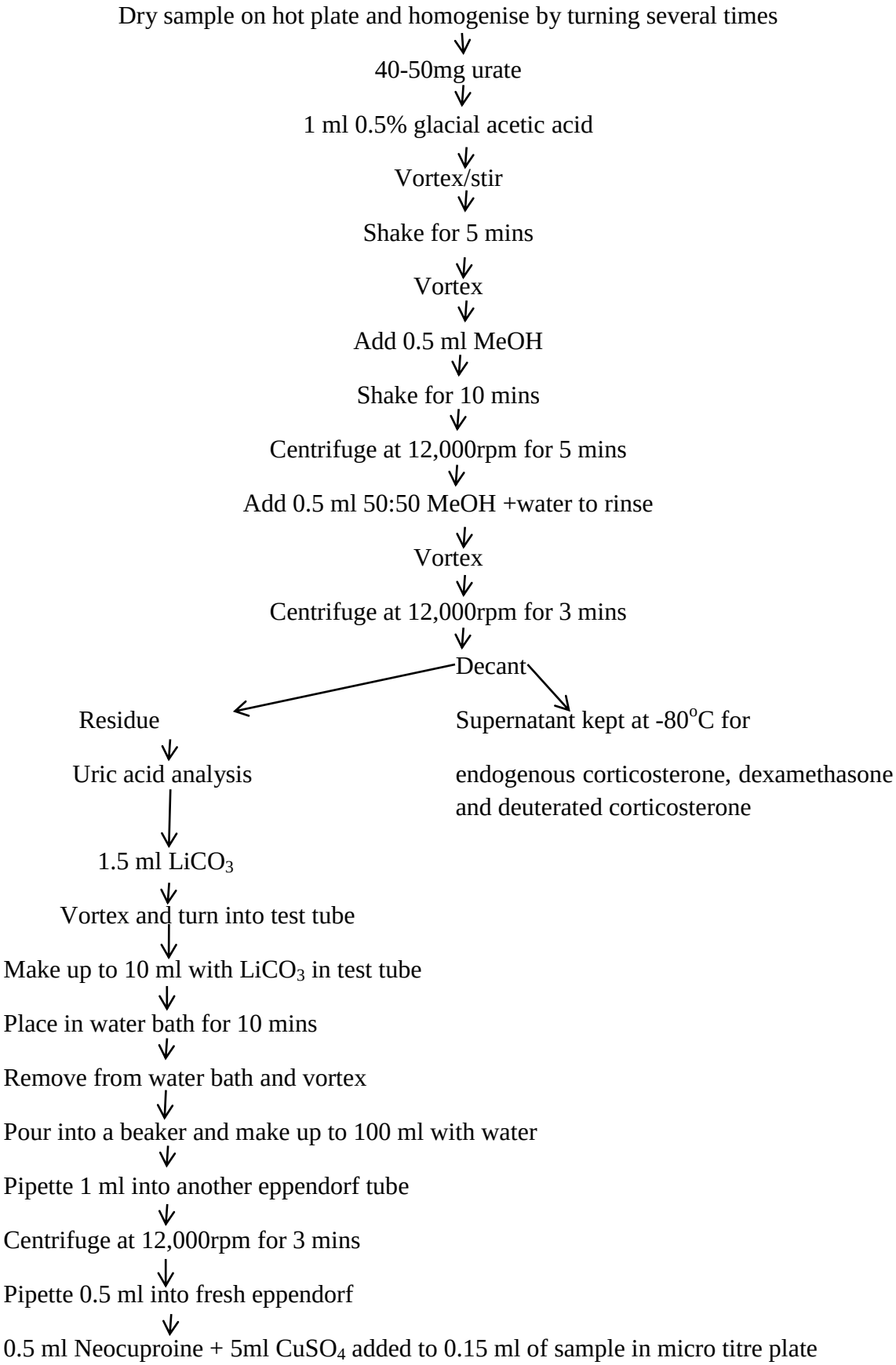

Figure 4.3: Flow chart for extraction of endogenous corticosterone from the urate sphere and for analysis of uric acid

Finally, the levels of endogenous corticosterone, deuterated corticosterone and dexamethasone from both the plasma and the urate sphere were estimated using a Thermo Quantum Ultra Triple Quadrupole mass spectrometer coupled to a Shimadzu Prominence XR UPLC.

4.5 Statistical analyses

Plasma levels of endogenous corticosterone satisfied the normality test undertaken using Shapiro-Wilks test in the SPSS statistical package (SPSS version 19, Rothampstead, UK). Hence, data was subjected to a repeated measures GLM having the three sampling times (baseline, after 10 mins and after 2 h) as the within subject factor and treatment as the fixed factor in the analysis.

The level of endogenous corticosterone in the urate sphere was adjusted using the concentration of uric acid for each sample. The adjusted data was checked for normality and since it was found that the distribution did not follow a normal pattern, then a $\log_{10}$ transformation was undertaken before proceeding with a parametric analysis in the form of repeated measures GLM. Levels of the baseline endogenous corticosterone in the urate sphere showed high variability, although they were not significantly different between birds before they were randomly allocated to treatment groups. Therefore, the data was standardized by subtracting the basal levels of endogenous corticosterone from subsequent values to generate the change in levels of corticosterone and this new parameter was analysed using a one way analysis of variance in SPSS.

Finally, basal levels of endogenous corticosterone measured from the plasma and the urate sphere were subjected to a Pearson's correlation to check for any relationship between the two samples. For this step, data obtained from the four days of data collection were pooled together.

4.6 Results

Although detecting the time when the levels of deuterated corticosterone and dexamethasone peaked in the plasma and urate sphere was one of our objectives, due to laboratory error, the levels of deuterated corticosterone and dexamethasone could not be analysed.

4.6.1 Levels of endogenous corticosterone in plasma

A scatter plot showing the mean and individual levels of endogenous corticosterone in plasma (ECP) is presented in Figure 4.4. Overall, there was no significant main effect of

treatment on levels of ECP, Figure 4.5. There was a significant effect of time of sampling on the levels of ECP ($F_{2, 40} = 3.22$, $P<0.050$) due to a greater level of ECP 10 mins post treatment compared to basal and 2 h post treatment levels. There was no significant interaction between treatment and time of blood sampling.

4.6.2 Levels of endogenous corticosterone in the urate sphere

A scatter plot showing the mean and individual levels of endogenous corticosterone in the urate sphere (ECUS) is presented in Figure 4.6. Overall, there was no significant main effect of treatment on levels of ECUS, Figure 4.7. There was no significant effect of time of sampling on the level of ECUS ($F_{2, 22} = 1.47$, $P>0.05$). However, there was a significant interaction between treatment and time of sampling on ECUS ($F_{2, 22} = 3.98$, $P<0.05$), specifically in Cort-Dex birds which had lower levels of ECUS at 60-90 mins after treatment. However, after the standardization of the levels of ECUS with respect to basal levels, there was a significant difference in level of ECUS at both 20-30 mins ($F_{1, 11}= 9.421$, $P<0.05$) and 60-90 mins ($F_{1, 11}= 8.38$, $P<0.05$) post treatment. At these two time points, the suppression in the level of ECUS was greater in Cort-Dex birds than in control birds (Figure 4.8). There was no significant correlation between the basal levels of endogenous corticosterone in the plasma with those in the urate sphere ($R^2= -0.322$, $P>0.05$).

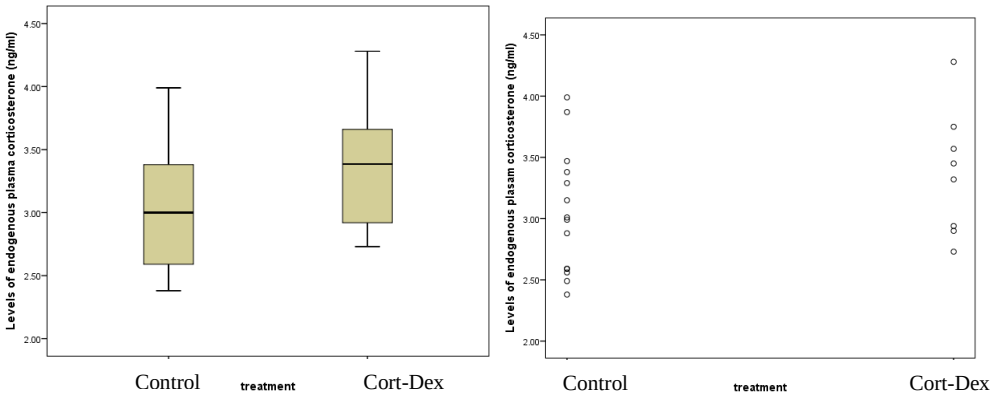

Figure 4.4. Mean (left) and individual (right) variations in basal level of endogenous plasma corticosterone (ng/ml) of broilers before offering treated mealworms in either Control (DMSO only) or Cort-Dex (deuterated corticosterone + dexamethasone) treatments

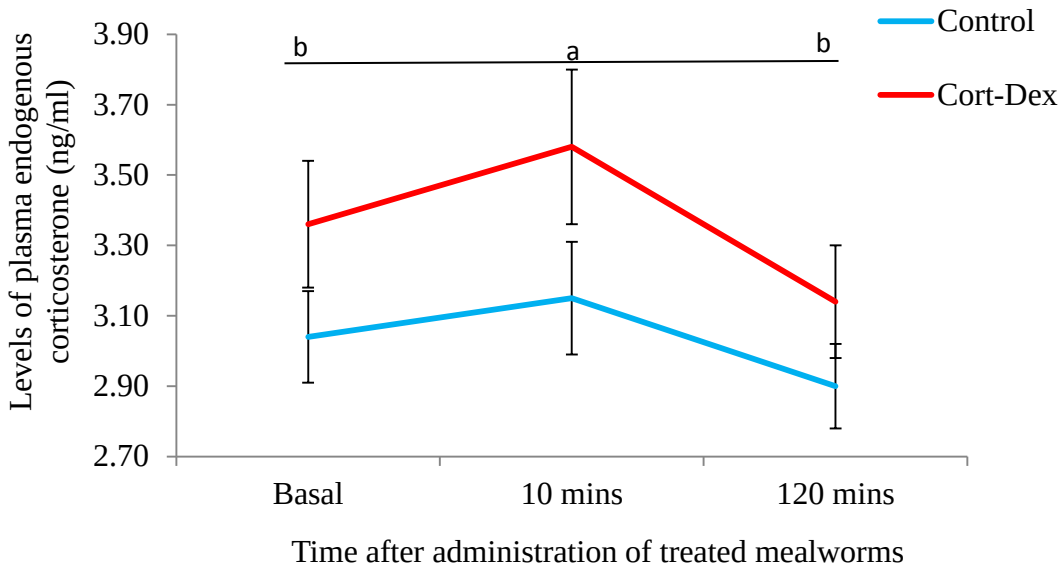

Figure 4.5: Levels of endogenous corticosterone in the plasma of broiler chickens offered mealworms injected with deuterated corticosterone + dexamethasone (Cort-Dex) or DMSO only (Control). Control (n=14), Cort-Dex (n=8). Values are means ±1SEM. ^{ab} means with a different superscript (overall mean for the two treatments at each sampling point) differ significantly at P<0.05

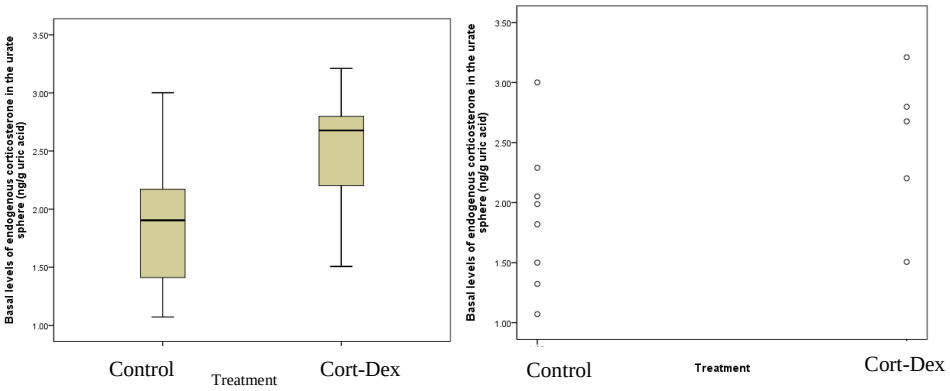

Figure 4.6. Mean (left) and individual (right) variations in basal levels of endogenous corticosterone in the urate sphere (ng/g uric acid) of broiler chickens before offering treated mealworms in either Control (DMSO only) or Cort-Dex (deuterated corticosterone + dexamethasone) treatments

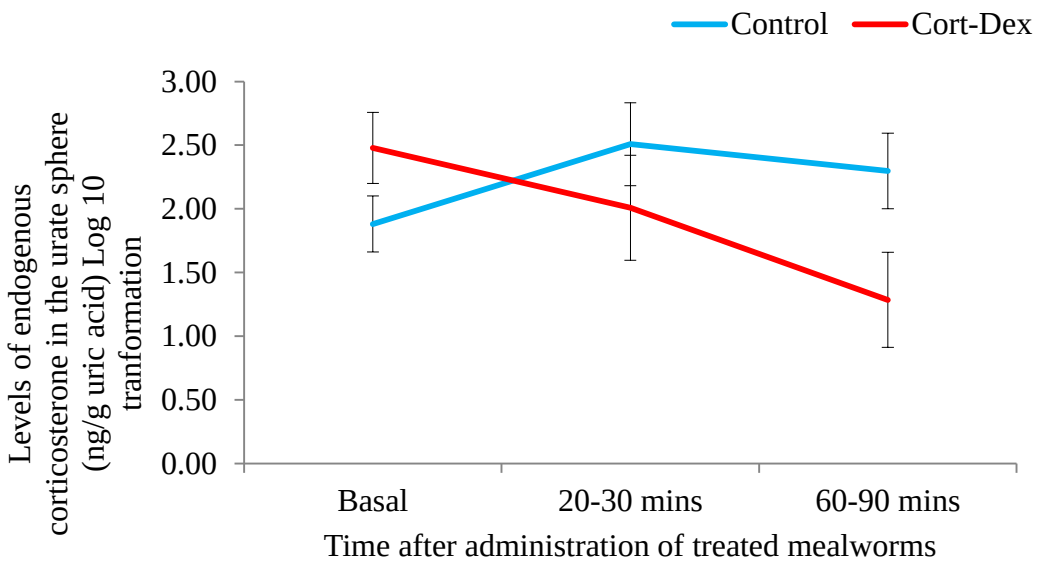

Figure 4.7: Levels of endogenous corticosterone in the urate sphere of broiler chickens in the Control (n=8) and Cort-Dex (n=5) treatments. Values are means ±1SEM.

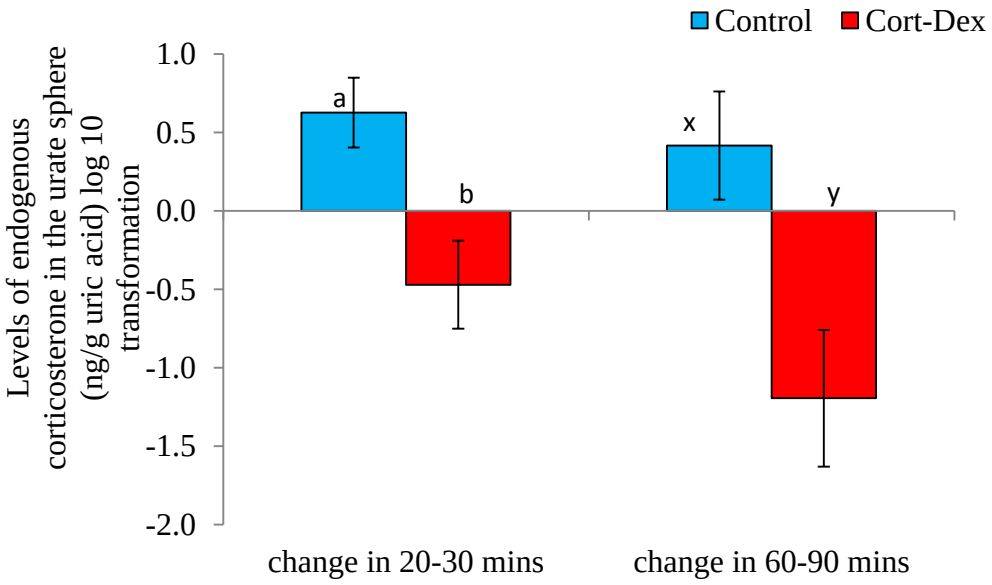

Figure 4.8: Change in level of endogenous corticosterone in the urate sphere relative to basal levels in the Control (n=8) and Cort-Dex (n=5) treatments. Values are means ±1SEM.[ab] means with a different superscript differ significantly at P<0.05, [xy] means with a different superscript differ significantly at P<0.05

4.7 Discussion

The aim of this experiment was to explore the potential of using the urate sphere component of avian urine as a non-invasive measure of levels of endogenous corticosterone in broiler chickens. In addition, investigation of the suppressive effect of dexamethasone on levels of endogenous corticosterone in the urate sphere and plasma was undertaken. Three objectives were outlined at the beginning of the chapter. However, due to laboratory error, the levels of deuterated corticosterone and dexamethasone were not analysed so one of the objectives could not be achieved. The levels of endogenous corticosterone in the plasma and urate sphere and the effect of dexamethasone suppression on endogenous corticosterone will therefore be discussed.

4.7.1 Basal levels of endogenous corticosterone in the plasma and urate sphere

The basal levels of endogenous corticosterone in plasma (ECP) and endogenous corticosterone in the urate sphere (ECUS) were widely variable between birds and between treatments, even though the basal samples were taken before birds were offered treated mealworms and the birds were handled for a minimum amount of time to move them from their home pen to the adjacent test pen. Since the birds were kept in the same environmental conditions with the same husbandry factors such as stocking density, lighting conditions that could be potential stressors, hence the variation in basal levels of corticosterone could be attributed to individual differences between birds (Cockrem, 2007). For example, there could be a difference between birds in their response to a change in environment from a familiar one (home pen littered with wood shavings) to another pen similar in size but free from litter (heavy duty waterproof sheet on the floor) and nevertheless novel.

Nevertheless, in the current study, the range in basal levels of corticosterone in the plasma was 2.4 - 4.0 ng/ml with an average of 3.2 ng/ml. These values are comparable to those reported in the literature. Wang *et al*. (2013) reported a mean basal level of plasma corticosterone of 3.8 ng from broilers selected for either short or long tonic immobility. Shini *et al*. (2008) reported basal levels of plasma corticosterone within the range of 3.1-3.7 ng/ml. However, Soleimani *et al*. (2011) reported a basal corticosterone level of commercial broilers of 2.3 ng/ml. The slight difference between our results and those of other studies could be due to the breed or age of the birds used. Chickens of different breeds are known to have different levels of basal corticosterone. In one study, the basal level of plasma corticosterone in the Red jungle and village fowl was 12.17

and 4.48 ng/ml respectively (Soleimani *et al*., 2011). Hence, it can be suggested that the LC-MS/MS assay used to determine corticosterone levels gave a close estimate of basal plasma corticosterone levels. For the urate sphere, the basal endogenous corticosterone level was estimated to be 2.1 ng per gram uric acid, but in the absence of any other reports in the literature, it is impossible to confirm whether this is an accurate value.

The increase in endogenous corticosterone levels in the plasma after 10 mins post ingestion of mealworms compared to other sampling periods could suggest the stress experienced by birds when subjected to several blood sampling (Cooks, 2012). In the current study, birds were sampled twice within a ten minute period.

The lack of a significant correlation between the basal levels of endogenous corticosterone in plasma and urate sphere could be due to the differences in the composition of the two samples. Due to the concentric rings of protein in the urate sphere, Casotti and Braun (2004) hypothesised that the formation of urate sphere occurs in a step-wise manner, which probably takes some time. Hence, the urate sphere is an integrated sample accumulated over several minutes (Clapp, 2010) whilst the plasma is of a single point sample (Nelson, 2005). Clearly further work is needed to fully validate the use of the urate sphere as a non-invasive measure of stress in broiler chickens.

4.7.2 Effect of dexamethasone on the levels of endogenous corticosterone in the plasma and urate sphere

In the current study, suppression of levels of plasma corticosterone was not observed some 2 h after the administration of dexamethasone. There are inconsistencies in the literature about the effect of dexamethasone on levels of corticosterone. While some studies (e.g. Westerhof *et al*., 1994; Etches, 1976) have reported suppression of plasma corticosterone levels 90 minutes after administration of dexamethasone, other studies have failed to detect an effect of dexamethasone on plasma corticosterone (Dehnhard *et al*., 2003, Rettenbacher *et al*., 2004) levels. However, in the current study there was significant suppression of levels of corticosterone levels in the urate sphere observed some 20-30 mins after administration of dexamethasone, an effect which persisted until 60-90 mins post treatment. After injecting rats with 50µg/kg of dexamethasone to rats, Cole *et al*. (2000) detected a significant peak of dexamethasone in the plasma after 30 mins and a greater peak after 120 mins. For a suppression to have occurred in the urate sphere as early as 20-30 minutes, it suggests that dexamethasone peak must have

occurred in the plasma earlier than 30 mins. Information on the time course for the excretion of corticosterone after a peak level in the plasma is scarce in chickens, however in a study by Rettenbacher *et al.*(2004) corticosterone metabolites were detected in the white uric acid component of the excreta, which the authors claimed to be the 'urine' in a single hen, some 36 mins after administration of radiolabelled corticosterone. It is possible that Rettenbacher *et al.*(2004) confused the use of urate sphere for urine because urine is known to be liquid and colourless while the urate sphere is solid and whitish in colour (Lobato *et al*., 2008). Often times, corticosterone metabolites are mainly measured from the feaces because corticosterone has undergone metabolisation in the gastro-intestinal tract (Möstl *et al*., 2005). On the other hand, it is parent corticosterone can be detected in the urate sphere because corticosterone binds with proteins in the blood which are involved in the formation of urate sphere (Clapp, 2010).

Due to laboratory constraints which meant that levels of deuterated corticosterone and dexamethasone in the plasma could not be determined, it is not possible to provide a full explanation for the results of the current experiment. However, using a similar method of non-invasive exogenous administration of corticosterone by mealworms, Breuner *et al*. (1998) reported a peak in plasma corticosterone in white crown sparrows some 7 minutes after ingestion of mealworms injected with corticosterone. There is the possibility that a similar event occurred in the current study, namely that the dexamethasone was delivered in the blood within a short period after its administration. It has been reported that the response to dexamethasone suppression depends on the amount of dexamethasone present in the blood circulation. Out of 66 psychiatric human patients administered 1 mg oral dexamethasone the night prior to blood sampling the following morning, 59% of patients had no suppression while 41% showed suppression of serum cortisol levels (Morris *et al*., 1986). In that study, patients that had low levels of serum dexamethasone were reported to demonstrate non-suppression (5.3 vs 10.1 nmol/l, $P<0.001$). In the current study however, there was no suppression of corticosterone level in plasma but there was suppression of corticosterone level in the urate sphere which implies a definite delivery of dexamethasone in the bloodstream of the Cort-Dex birds. Immediately after birds ingested Cort-Dex mealworms, there was an increased level of drinking and diuresis (Personal observation). Clapp (2010) also reported suppression of levels of corticosterone in the urate sphere of Great tits administered dexamethasone (wax moth larvae impregnated with 100µg dexamethasone

was fed to the Great tits one day before the study).Similarly, in snowshoe hares (*Lepus americanus*), 10 hours after injection with dexamethasone, level of faecal cortisol metabolites were suppressed by 61% (Sheriff *et al*., 2010). A lack of suppression of endogenous levels of corticosterone by dexamethasone has been associated with chronic stress observed in humans with psychiatric disorders (Cole *et al*., 2000). In the current study there was no attempt made to create conditions of chronic stress, so that environmental, nutritional, and lighting conditions were kept at normal levels. Nevertheless, future studies could explore the potential for the urate sphere to detect chronic stress in birds. Apart from chronic stress, the HPA axis could be hyperactive if there are tumours either in the adrenal or pituitary glands (Lumeij, 1994a) leading to an increase in the level of corticosterone in circulation. Therefore, with further work to confirm these findings, the urate sphere could be considered as a potential non-invasive measure of chronic stress in broiler chickens.

4.8 Conclusions

This experiment confirms the presence of endogenous corticosterone in the urate sphere of broiler chickens and so suggests that the condition of the HPA axis in broiler chickens could be detected from the urate sphere as previously reported in Great tits. Therefore, the use of urate sphere in estimating levels of endogenous corticosterone appears to have some promise as a non-invasive tool of assessing stress in broiler chickens which warrants further investigation to fully validate this method. Although the processing of the urate spheres takes longer than the plasma nevertheless, the urate sphere can be collected from the bird without any disturbance or infringement.

Chapter 5: Development and validation of an improved cognitive bias task for assessing affective state in broiler chickens

5.1 Introduction

Animal welfare encompasses both the physical and mental conditions of the animal (Dawkins, 2004; Koknaroglu and Akunal, 2013). The approach to animal welfare based on biological functioning focusses on measurement of physiological parameters such as autonomic responses and hormonal changes (Fraser, 2003), especially the stress hormone, corticosterone. With increasing awareness of the sentient nature of animals and their ability to experience positive and negative states (Duncan, 2006), the approach to animal welfare based on subjective feelings is on the increase. Whilst it is relatively easy to measure physiological parameters in animals, subjective feelings cannot currently be reliably inferred from physiological measurements. Some authors have argued that measurements of stress hormones, such as corticosterone, are more likely to give an indication of the degree of arousal (high or low) of an animal, whereas subjective feelings relate to the valence (positive/negative or pleasant/unpleasant) of the animal's response to a stimulus (Mendl *et al*., 1999). Therefore, improved measures of welfare measures that might be more closely related to subjective feelings and hence the valence of an animal's affective state are urgently needed.

Despite the fact that unlike humans, animals cannot report their feelings verbally, several methods have been developed in animal welfare that claim to assess the valence of an animal's state, namely**,** appraisal theory (Désiré *et al*., 2002), anticipatory behaviour (Spruijt *et al*., 2001; Zimmerman *et al*., 2011) and cognitive bias (Harding *et al*., 2004). Of these three methods, cognitive bias has been the most popular method used to assess affective state of animals. The relationship between cognitive bias and welfare has been investigated in a wide range of species including rats (Harding *et al*., 2004; Burman *et al*., 2008; Burman *et al*., 2009 and Richter *et al*., 2012), dogs (Cassey *et al*., 2008), rhesus macaques (Bethell *et al*., 2012), capuchins (Pomerantz *et al*., 2012), pigs (Douglas *et al*., 2012), sheep, (Destrez *et al*., 2013), honey bees (Bateson *et al*., 2011), starlings (Bateson and Matheson, 2007; Brilot *et al*., 2010), chicks (Salmeto *et al*., 2011) and laying hens (Wichman *et al*., 2012).

A cognitive bias task is a test used to assess the affective state of an animal based on its interpretation of ambiguous stimuli after the animal has first learnt to discriminate between positive (rewarded) and negative (non-rewarded/aversive) stimuli (Mendl *et*

al., 2009). In other words, cognitive bias is an evaluation of how the emotional state influences information processing (Mendl *et al*., 2009). The influence of emotion on cognitive processes was derived from human studies in which it has been established that people in a negative affective state, such as depression or anxiety, are found to be more pessimistic in their judgement of ambiguous stimuli (Mogg *et al*., 2006). Optimism and pessimism are defined operationally as an increased expectation of a positive or negative event, respectively (Salmeto *et al*., 2011).

The first attempt to develop a cognitive bias task in a non-human animal was by Harding *et al*. (2004) who used a go/no-go operant task. Rats were trained to press a lever on hearing a positive tone to obtain a food reward (45 mg food pellets). On hearing another, negative, tone the rats were trained to refrain from pressing the lever otherwise they would be punished with a blast of white noise (30 s of 70 dB). Once the discrimination was learnt, the rats were presented with ambiguous tones intermediate between the positive and negative tones and their responses recorded. The results showed that rats subjected to 9 days of stressful environmental conditions took longer to press the lever when presented with positive and near positive ambiguous tones which was interpreted as an indication of a negative affective state akin to depression (Harding *et al*., 2004).

Following this achievement, several improvements and variations in cognitive bias tasks have been developed (see summary in Table 5.1) and this approach has been extended to other animal species with the aim of assessing the affective state of animals under different environmental conditions such as barren/enriched housing (Wichman *et al*., 2012), light intensity (Burman *et al*., 2009), predatory attack (Bateson *et al*., 2011) and isolation (Salmeto *et al*., 2011). For instance, instead of the lever pressing operant task, a touch screen operant task was used in training monkeys (Bethell *et al*., 2012) where the monkeys have to touch a screen to receive a reward (food) or refrain from touching the screen to avoid an aversive noise. Apart from operant tasks, spatial task have been devised in rats (Burman *et al*., 2008; Burman *et al*., 2009) and dogs (Cassey *et al*., 2008),where the animal has to discriminate between a rewarded (food) and unrewarded (no food) locations.

Given the promising nature of cognitive bias tasks for assessing negative affective state in animals highlighted in the brief review above, the aim of the current experiment was to develop and validate an improved cognitive bias task for broiler chickens. In the

sections that follow we discuss first the rationale underlying our design of an improved cognitive bias task, and second, the rationale for our validation of this task using direct manipulation of corticosterone levels.

5.1.1 Task design

A major problem with operant cognitive bias tasks is the length of time taken by the animals to learn the initial discrimination. Starlings required about 26 sessions (a daily session comprising of 54 trials) before learning was established (Matheson *et al*., 2008). A further refinement could be the addition of an aversive food at the unrewarded location in a spatial task. In the studies by Burman *et al*. (2008, 2009), rats subject to a spatial task learnt faster when the unrewarded location had an aversive food compared to when the rats could see the food but could not access it (2 vs 6 days). Aversive food was prepared by soaking feed pellets in 2% quinine solution and allowing it to dry overnight (Burman *et al*., 2009). However, this comparison might be deceptive. In the study of Burman *et al*. (2008), the unrewarded position had food which the rats could visibly see and smell but could not eat. Possibly the rats might have been trying to get access to the food underneath the wire mesh and this might have elongated the time required to learn the task. Hence, the advantages of making the unrewarded location actively aversive have not been thoroughly tested yet.

Table 5.1: Summary of different tasks used in training animals for a cognitive bias test

Animal species	**Reward**	**Punisher**	**Training**	**Manipulation of affective state**	**Reference**
Rats	Food	White noise	Operant task	Unpredictable environmental conditions	Harding *et al*. (2004)
Rats	Accessible food	Inaccessible food	Spatial task	Housing enrichment	Burman *et al*. (2008)
Rats	Food	Unpalatable food	Spatial task	Light intensity	Burman *et al*. (2009)
Starlings	Food	Unpalatable food	Visual discrimination task	Cage enrichment	Bateson and Matheson (2007)
Laying hens	Food	No food	Spatial task	Housing enrichment	Wichman *et al*. (2012)

In laying hens, spatial cognitive bias tasks have been used for assessing the affective state of birds which have undergone transfer from a battery cage to a litter pen (Lindström, 2010) or different levels of environmental enrichment (Wichman *et al*., 2012). In both studies, laying hens required more than 12 training sessions before they successfully reached the learning criterion. The long period required to learn the spatial task in birds could be due to the absence of a 'punisher' at the unrewarded location. Wichman *et al*. (2012) presented birds with an empty bowl in the unrewarded position, and in addition only eight trials were presented per day during the training (four rewarded and four unrewarded) compared to Burman *et al*. (2009) who used 12 trials per day of training (six rewarded and six unrewarded).

In designing an improved spatial cognitive bias task for broilers, we considered the use of unpalatable food at the unrewarded location in order to speed up discrimination learning. However, from our previous training of broilers to discriminate between palatable and unpalatable food produced by spraying feed with 4 % quinine solution (in Chapter 3), we found that broilers of a commercial strain still consumed the unpalatable feed, probably because they have been genetically selected for high feed intake to sustain rapid growth and had undergone a degree of feed withdrawal before the test was conducted. Previous reports in starlings showed that feed-restricted birds consumed more of quinine treated mealworms than the birds without feed restriction (Barnett *et al*., 2007). Hence the use of an aversive stimulus which is not food based might be a more effective punisher for broilers. Air puffs have been reported to be aversive to rats (Moriarty *et al*., 2012) and domestic hens (Edgar *et al*., 2011). In the latter study, after a 30 s air puff mother hens showed both physiological and behavioural changes such as a decrease in eye temperature and level of preening but an increase in standing alert behaviour indicative of vigilance to a threatening situation. We therefore hypothesised that the introduction of an air puff at the unrewarded location might be an appropriate improvement of the spatial cognitive bias task for broilers.

5.1.2 Task validation – manipulation of affective state in broiler chickens

To validate a cognitive bias task, it was necessary to show that the animals' judgment of ambiguous stimuli is sensitive to their affective state. This required us to manipulate affective state. In most previous tests authors have elected to manipulate state indirectly via changing the animals' environment (enrichment, light intensity), but in this study we decided to manipulate state more directly by feeding the stress hormone corticosterone

to mimic a state of chronic stress. Changes in environmental conditions such as unpredictable housing conditions (Harding *et al*., 2004), predatory attack (Bateson *et al*., 2011), barren environment (Douglas *et al*., 2012) have been associated with pessimistic judgement biases and hence by assumption with negative affective states. Recently, Pomerantz *et al*. (2012) measured both cognitive bias and faecal corticosterone level in capuchins exhibiting stereotypic behaviour. They found that capuchins which showed higher level of stereotypic behaviour and had higher faecal corticosterone levels were in a negative affective state. Also, honey bees subjected to predatory attack also showed physiological changes related to stress (Bateson *et al*., 2011). Together, these results suggest that stress has a link with the induction of negative affective state (Paul *et al*., 2005). The secretion of corticosterone during short-term (acute) stress is beneficial because it prepares the animal to cope with the situation. However, long-term (chronic) stress response is detrimental to the birds (Dickens *et al*., 2010) thereby compromising welfare. Some of the negative consequences of chronic stress are breakdown of muscle proteins and fat and suppression of immune function (Hill *et al*., 2008) changes in behaviour, disruption of the HPA (Dickens *et al*., 2010) and impaired cognitive functions (Lupein *et al*., 2007).

Normally, the negative feedback system regulates the concentration of corticosterone in circulation, but at times the negative feedback system is inhibited and this consequently leads to a continuous increase in the concentration of corticosterone (Pariante and Lightman, 2008), indicative of chronic stress (Wolf, 2008). In the wild, birds are faced with series of stressors such as predators, harsh weather and lack of food which cause their levels of corticosterone to increase (Pravosudov, 2005). Broilers could similarly experience multiple stressors including high temperature, high RH, lighting condition, wet litter, increased ammonia level and noise (Rosales, 1994).

Chronic stress can be mimicked by implanting animals with stress hormones (Puvaldopirod and Thaxton, 2000; Olanrewaju *et al*., 2006) or feeding hormones through drinking water (Post *et al*., 2003; Shini *et al*., 2008) to achieve a continuous elevation of corticosterone level for several days (7-10 days). Puvaldopirod and Thaxton (2000) undertook a comprehensive study to investigate changes in broiler chickens associated with chronic stress. Their study showed that as early as 4 days post implant of an ACTH mini-osmotic pump (delivering 8IU/kg BW/day), the performance (depression in body weight), immune system (reduction of relative spleen weight) and metabolism (increased blood glucose and relative liver weight) were affected. Chronic

stress also induces changes in acid-base balance in broilers (Olanrewaju *et al*., 2006). Changes in internal organs and acid-base status could serve as indicators of chronic stress in replacement of levels of plasma corticosterone which is known to be easily affected by procedures involved in blood sampling such as catching and handling, especially if blood is not sampled within 2-3 minutes of catching the bird (Mormede *et al*., 2007). A regulated and non-invasive way of administration of exogenous corticosterone in white crown sparrows was reported by Breuner *et al*. (1998) where birds were offered mealworms injected with either 4µg of corticosterone dissolved in dimethyl sulfoxide (DMSO) or DMSO only. Breuner *et al*. (1998) reported a significant peak of in the levels of corticosterone in the plasma seven minutes after ingestion and this stimulated the activity level of the birds in terms of an increase in perch hopping behaviour.

To obtain further confirmation that we had manipulated the affective state of the birds, we chose to use the novel object test as a behavioural assay of fear. As mentioned earlier, increased standing alert behaviour in mother hens subjected to an aversive stimulus, namely an air puff, was an indication of fear (Edgar *et al*., 2011). Fear is generated when an animal is faced with threatening conditions such that behavioural and physiological responses are triggered (Cockrem, 2007) in preparation for danger (Forkman *et al*., 2007). Fear is considered to be a type of emotion (Forman *et al*., 2007), obviously a negative one because animals cannot be fearful in the absence of a negative stimulus. Fear can be measured through the use of a novel object test (Wichman *et al*., 2012), such that an animal that avoids a novel object is considered fearful. Other tests of measuring fearfulness in animals are open field test and tonic immobility (Cockrem, 2007). The use of an open field test does not however indicate the true level of fear because it is masked by the effect of isolation (Forkman *et al*., 2007). Interestingly, Wichman *et al*. (2012) found a positive correlation ($P<0.05$) between the tonic immobility and novel objects tests of fearfulness. Hence, a novel object test seems to have promise in measuring fear because it is based on how threatening the bird appraises the novel object. Destrez *et al*. (2013) found that sheep which had been chronically stressed sheep for 6 weeks were more fearful in a novel object test as indicated by displaying emotional responses such as increased vocalisation and making less contact with the novel object. In another study of Destrez *et al*. (2013), chronically stressed sheep (6 weeks) judged ambiguous cues (empty buckets placed in three ambiguous positions in between the two trained positions) in a cognitive bias test

pessimistically. In amphibians, a frog exposed to a toad had increased level of corticosterone metabolites in its urine indicative of stress and subsequently displayed a longer duration of tonic immobility which implies a condition of fear (Narayan *et al*., 2013). Hence it seems that animals subjected to stressful conditions tend to be more fearful and this subsequently reduces productivity of the birds in terms of body weight, egg weight and even mortality (de Haas *et al*., 2013).

In summary therefore, the aim of this study was to develop an improved cognitive bias task for broiler chickens similar to that of Wichman *et al*. (2012) but with some modifications. We elected the use of a 'punisher', in this case an air puff, in the unrewarded position instead of an empty food bowl. We hypothesised that the averseness of the air puff would reduce the time required by birds to learn the spatial discrimination necessary for the task. Additionally, birds will be presented with 12 trials per day of training (after Burman *et al*., 2008 and 2009). We hypothesised that by giving the birds more trials per day, we would further reduce the number of days necessary to learn the discrimination. This new task will be validated on broilers whose internal state has been manipulated to mimic chronic stress by direct administration of corticosterone.

We hypothesised that birds whose internal state had been manipulated to mimic chronic stress would be more likely to show a pessimistic interpretation of ambiguous positions. We also predicted that the manipulation would cause changes in internal organs and blood parameters indicative of chronic stress. Finally, we predicted that broilers under a condition of mimicked chronic stress would be more fearful to a novel object and exhibit behaviours different from the control birds.

5.2 Materials and methods

5.2.1 Ethical considerations

This experiment was conducted under Project Licence number PPL 60/4270 of the Animals (Scientific Procedures) Act 1986. Approval for the project was given at both national (Home Office) and local (Ethical Revenue Committee) level, with the number of birds used considered to be the minimum required to obtain a statistically significant difference between treatments.

5.2.2 Experimental design and location

The experiment was a between-subjects design with a single factor which was the treatment at two levels namely: i) Control birds fed mealworms (*Tenebrio* larvae) injected with DMSO solvent and ii) Corticosterone birds fed mealworms injected with corticosterone dissolved in DMSO (a non-polar solvent) for quick delivery of corticosterone into the bloodstream (Breuner *et al*., 1998). Birds were trained in a spatial task to differentiate between a rewarded and unrewarded position after which they were randomly allocated to treatment, either Control or Corticosterone. On the third day of treatment, birds were subjected to a cognitive bias test were three ambiguous locations between the rewarded and unrewarded locations were introduced. The cognitive bias test was undertaken for three consecutive days (Days 3 to 5 of treatment). On Day 6, birds undertook a novel object test in the test arena. On the last day of the study, Day 7, the behaviour of the birds in their home pen was recorded after which they were weighed, blood sampled, euthanized and the internal organs recovered during *post mortem*.

The study was conducted in the controlled environment rooms in the Ridley Building of Newcastle University in two batches, i.e. first and second replicate. The second replicate commenced one week after the end of the first replicate; details of the replicates are as follows:

1st replicate: 15 Ross 308 broiler chickens, 17 days of age (average weight of 1.3 kg) were purchased from a poultry farm (Oakland Farms, Moor Monkton, York, UK). The medical history showed that the birds had been given repeat vaccinations of an infectious bronchitis (IB) vaccine.

2nd replicate: 27 Ross 308 broiler chickens, 13 days of age (average weight 950 g) were purchased from a poultry farm (Oakland Farms, Moor Monkton, York, UK). The medical history showed that the birds had received treatment for Chronic Respiratory Disease (CRD) for 3 days when they were 1-3 days old, and were given an IB vaccine when they were 10 days old.

On arrival at the laboratory in Newcastle, the birds were weighed individually and placed in holding pen, 5 birds were randomly allocated to each plastic pen (diameter of 90 cm and height of 30 cm) containing wood shavings (Goodwill's Wood shavings and Timber Products Ltd, Ponteland, UK) approximately 5cm deep. Commercial specification broiler feed (20%CP, 13.0MJ/kg ME, 4% oil and 6% ash; W.E Jameson &

Son Ltd, Masham, UK) and tap water was provided *ad libitum*. Lighting conditions were 14L:10D whilst temperature and relative humidity were maintained at 20^{o}C and 50% RH respectively. The birds were allowed to settle for three days to adjust to the new environment and to become familiar with the feeding and drinking apparatus. The birds were offered standard mealworms once a day (approximately 6 mealworms per bird) to allow them to become accustomed to feeding on mealworms. On Day 1 of the experiment (three days after arrival), the birds commenced the training (fully described in Section 5.2.5). Learning was confirmed when the birds showed significant difference between the rewarded and unrewarded positions.

After the learning was established (4 days), birds were randomly allocated to one of two treatment groups as described previously, namely Control (fed mealworms injected with DMSO only) or Corticosterone (fed mealworms in which corticosterone dissolved in DMSO solvent had been injected) as described in Section 5.2.3. Because of the diurnal nature of basal corticosterone levels (Hess, 2006), corticosterone was fed indirectly via mealworms twice a day i.e. 10 am and 5 pm. We mimicked chronic stress by feeding corticosterone for 7 days according to Puvaldopirod and Thaxtion (2000). The first two days was referred to as the 'corticosterone build up stage' and on the 3rd day of treatment, the cognitive bias test commenced. Previous study has shown that the use of 4 mg/kg in drinking water elevated plasma levels of corticosterone from the day 1 to 8 of treatment (Post *et al*., 2003), so this level was chosen as an appropriate dose of corticosterone. The initial weights of the birds were similar between treatment groups.

5.2.3 Preparation of test mealworms

To prepare the experimental mealworms, firstly 100 mg of corticosterone (Sigma-Aldrich Ltd, Dorset, UK) was dissolved in 1 ml of DMSO (Sigma-Aldrich Company, Dorset, UK) and vortexed to give a concentration of 100mg/ml. The aim was to provide each Corticosterone bird with approximately 4 mg of corticosterone per kg of body weight, a dosage previously established by Post *et al*. (2003). Injecting mealworms was based on the method of Breuner *et al*. (1998).The mealworms were inactivated by placing them in plastic bags on ice for about 20 minutes. According to treatment, corticosterone mixed in DMSO or DMSO only were injected into the mealworms using a 100µl Hamilton syringe (Hamilton Bonaduz, AG, CH-7402 Bonaduz, Switzerland) and immediately these mealworms were then offered to the birds. Birds were fed two

mealworms (in total giving 30µlof either corticosterone dissolved in DMSO or DMSO only) twice a day.

5.2.4 Test arena for training and cognitive bias task

A separate climate chamber of identical size (l × b × h = 4.1m × 2.4m × 2.2m), temperature and lighting conditions to that used for housing the birds was used as the place to locate the test arena which consisted of a wooden platform measuring 2.2 × 2.35m. At one end of the arena towards the left hand side (known as the unrewarded position, point A in Figure 3,1), a small hole was drilled in the floor to allow for the delivery of a blast of ambient air through a pipe connected to an air compressor. The air compressor (Portable air compressor, KNF Neuberger Ltd, Witney, UK) was fitted with a general purpose air gun (Cromwell Ltd, Leicester, UK) (see Figure 5.3) and was located outside the chamber where the training/testing was undertaken. A connecting pipe transferred air from the compressor to the unrewarded position, so that whenever the bird flipped opens the white cone, the assistant (outside the chamber) pressed a lever on the air gun and deliver a short blast of ambient air at a pressure of 3 bars. The ambient air puff delivered at the unrewarded position (U) might be considered as a 'punisher' because after experiencing a number of air puffs some birds jumped while others froze for a few seconds (personal observation).

In the middle of the short side of the test arena was a start box where the bird under test was placed, (see Figure 5.1). The bird could only gain entry to the test arena through a manually operated sliding door controlled by the operator. At a distance of 100 cm from the start box, a series of five locations (rewarded, P25, P50, P75 and unrewarded) were marked on the platform. The distance between the location furthest to the right (rewarded) and the one furthest to the left (unrewarded) positions was 1.8 m (after Wichman *et al*., 2012). During the early stages of training (acclimatisation and pre training stage, see Section 5.2.5), it was observed that when mealworms were placed 200cm from the start box, the birds did not walk to the end of the test arena, so it was decided to use half the length of the test arena was used. Therefore, a temporary wooden barrier was positioned half-way across the arena to reduce the area of the arena so that it now measured 2.35 m × 1.2m. Figure 5.2 shows a bird in the test arena after opening a cone in the rewarded position.

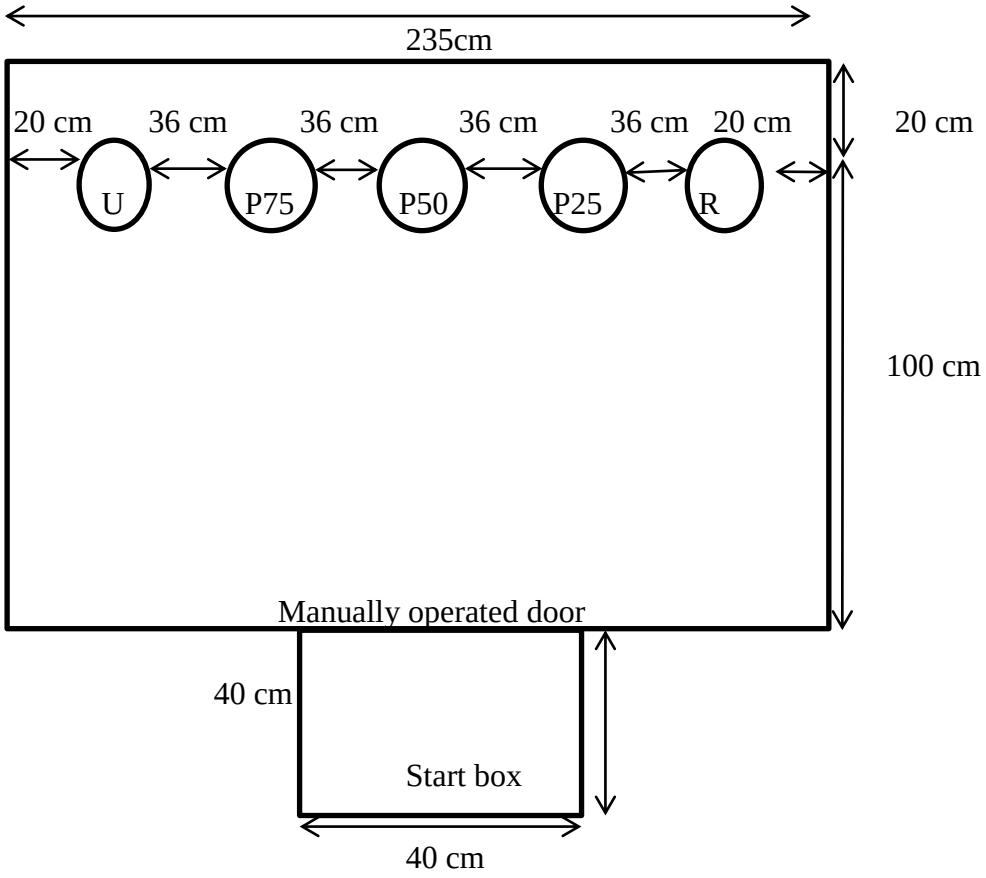

Figure 5.1: Layout of the test arena showing the five positions during a cognitive bias task namely; U (Unrewarded), P75 (ambiguous, close to air blast), P50 (ambiguous, at the middle), P25 (ambiguous, close to reward) and R (reward) positions.

Figure 5.2: A bird standing in the test arena after flipping open the cone placed in the rewarded position to access mealworms underneath the cone.

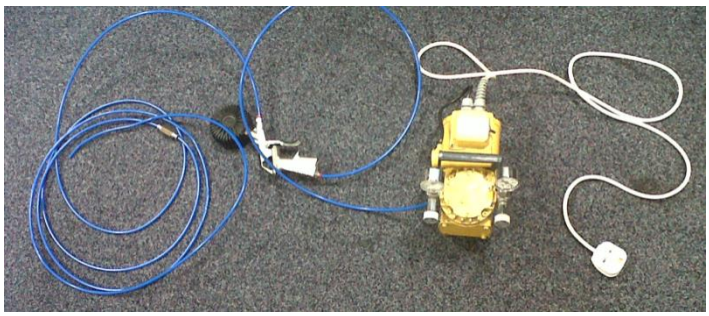

Figure 5.3: Air compressor (in yellow) connected to the air gun (silver) by means of a plastic tube (blue)

5.2.5 Protocol for training birds to distinguish between positive and negative positions in the test arena

Acclimatisation (Day 1): Birds were placed in the start box and offered a mealworm after which the door was opened and the bird given access to the test arena where mealworms had been placed in three petri dishes positioned on the floor. The bird was allowed a period up to two minutes to eat the mealworms before it was returned to the start box. The birds were paired during the first two sessions of acclimatisation to reduce isolation stress and speed up acclimatisation. For the second two sessions, birds were allowed into the test arena individually so that they could become accustomed to feeding in the test arena alone.

Pre-training 1 (Day 2): Each bird was placed in the start box and offered two mealworms (to motivate them to participate in the training) and then the door of the start box was opened to allow access into the test arena. In order to make the mealworms more visible to the bird, two mealworms were placed inside each of the three white paper cones positioned on their side on the floor and inside each cone. Each bird had five sessions of this pre-training on the same day, with each session lasting for 1 minute.

Pre-training 2 (Day 3): Each bird was placed in the start box and offered two mealworms and then the door of the start box was opened to allow access to the test arena. At this stage, mealworms were placed on an open petri dish and then half covered over with a white paper cone in such a way that the birds could see the mealworm but only access it by flipping the cone. The petri dish was placed in the rewarded position in the test arena. The aim of this stage was to teach the bird to flip open the cone positioned in the rewarded position to access the mealworm underneath. Each bird was

given five of these sessions in one day, with each session lasting 1 minute. A bird could only proceed to the training stage when it successfully flipped opened the cone to access the mealworms on three consecutive occasions.

Training (Day 4 - 7): Each day each bird had twelve trials (after Burman *et al*., 2009) in which the petri dish was placed six times in the rewarded position (R; and contained mealworms) and six times in the unrewarded position (U; where instead of having mealworms, the bird was punished by the use of an unpleasant stimulus namely a short puff of air).Birds were not counterbalanced for rewarded and unrewarded positions because of the physical constraints of fixing the air puff to a particular position in the test arena.

To enhance discrimination between the rewarded and unrewarded positions, on the first day of training, for trials 1-6 the petri dish was placed in the same position for two consecutive trials and then in the opposite position for the next two trials (e.g. RRUURR) starting with the rewarded position (following protocol from Burman *et al*., 2009 and Wichman *et al*., 2012). For trials 7-12, the location was alternated, so at the end of the 1st day of training the birds had undergone 7 R and 5U trials. In this study, a period of 2 minutes was given to the birds per trial on the first day so that they would have the opportunity to experience the air puff. However, from Day 5 onwards (until the birds had learnt the task) the order of presenting the rewarded and unrewarded runs was conducted in a pseudo-random order (again, after Burman *et al*., 2009 and Wichman *et al*., 2012) with a maximum of two consecutive trials in the same position hence an equal number of both R's and U's with each trial lasting for one minute. Each trial was presented in a pseudo-random order by writing on a small piece of paper an R or U (six each), pieces which were then folded and mixed together. The experimenter picked one of the folded pieces of paper at random, opens it and whatever is written in the paper is the trial that will be undertaken by the bird, i.e. R or U. Birds had two sessions of training per day (morning and afternoon).

The latency to flip open the cones placed on the petri dish was recorded for each bird. Having established that the bird had learnt the task (i.e. a significant difference between the latency to open the cones in the R and U positions for three consecutive days), then the bird proceeded to the corticosterone build up stage. An inter-trial interval of one minute was maintained in order to clean the test arena and to set up the next trial.

In replicate 1, nine birds learnt the task so five birds were assigned to the Corticosterone group while the other four birds served as Controls. However, in replicate 2, six birds learnt the task so three birds were randomly allocated to each treatment group. Overall, there were eight birds in the Corticosterone group and seven birds in the Control group that underwent the cognitive bias test. In addition to the six birds which learnt the task in replicate 2, a further eight birds were randomly allocated to treatment i.e. four birds/treatment to supplement the number of animals available for other data collection such as blood, behaviour and internal organs.

Corticosterone build up stage (Day 8 - 9): There was a two-day break between the end of training and the start of cognitive bias test in order to build up the corticosterone level of the corticosterone birds. According to treatment, each bird was offered two mealworms injected with corticosterone dissolved in DMSO (Corticosterone birds) or DMSO only (Control bird) twice daily (10 am and 5 pm). At the start of the treatment, birds in replicate 1 had an average bodyweight of 1.5 kg while those in replicate 2 were 1.1 kg. Hence, to take account of differences in body weight, through the mealworms birds in replicate 1 and 2 were fed a total of 6 mg of corticosterone (two mealworms were offered in the morning and evening, each injected with 1.5 mg of corticosterone) and 4 mg of corticosterone (one mealworm was offered in the morning and evening injected with 2 mg of corticosterone) per day respectively from two days preceding the cognitive bias test until five days after the test (so 7 days of treatment).

Cognitive bias test (Day 10 - 12): Two birds (one Corticosterone and the other Control) were moved to the test room and kept in a holding pen identical to their home pen. Commencement of cognitive bias test was staggered by 2 minutes to allow birds to perform the test one at a time. According to treatment, each bird was fed two mealworms previously injected with either corticosterone/DMSO or DMSO. At 7 minutes post-ingestion of the mealworms, when corticosterone delivery was believed to be at its peak in the plasma (Breuner *et al*., 1998), the bird placed in the start box was allowed access into the test area by opening the door. One round of the test was performed by presenting the petri dish three times in the rewarded position, three times in the unrewarded position, and three probe tests. The three test probes (ambiguous cues, P) consisted of petri dishes placed at points P25, P50 and P75 between the rewarded and unrewarded positions (as shown in Figure 5.4). An example of a test series was R, U, P25, R, U, P50, U, R and P75. The test series was divided into three equal portions, such that after three test positions the birds (there were two birds in a

pair being tested together) were swapped until they had completed the test series. The presentation of the three probe tests was randomised within the test series on each day of the cognitive bias test. The latency to reach the rewarded, unrewarded and the test probes (near reward, middle and near air puff) was recorded for each bird. The cognitive bias test was undertaken for three consecutive days, on Day 3 to 5 of treatment.

5.2.6 Novel object test

Since the birds appeared to be comfortable walking from the start box to access mealworms placed underneath a white cone in the rewarded position, therefore the novel object test was undertaken in the test arena on Day 6. The birds were fed mealworms appropriate to Corticosterone or Control treatments and 7 minutes after ingestion of mealworms the bird was placed in the test arena for 2 minutes to undertake a novel object test (see Figure 5.4). The novel object test consisted of a plastic model of a dog, approximately 10cm tall, placed in the rewarded position, in front of which was a standard white cone with a mealworm underneath. The latency of the bird to open the cones in the rewarded positions was recorded and if the bird did not open the cone within 2 minutes, a latency of 120s was recorded. In addition, the behaviour of each bird during this period was recorded by a camera directly onto a laptop computer (see Table 5.2 for description of behaviour).

Figure 5.4: A novel object (a plastic model of a dog; approximately 10cm tall) placed behind the cone in the rewarded position of the test arena (shown in top right corner of the picture).

Table 5.2 Behaviour categories monitored during exposure of broilers to a novel object test

Behaviour category	Definition
Observation of novel object- halfway	Bird walking toward the rewarded position but stops halfway and then changes its movement towards the opposite end
Observation of novel object- close to start box	Bird notices the novel object immediately after stepping out of the start box and then walks away from it
Defecation	Passing of droppings in the test arena
Sitting	Sitting in the test arena
Motion	Walking in the test arena
Escape attempt	Bird tries to jump out of the test arena

Subsequently, the video was set at play back mode and each bird was scored for 2 mins during the novel object test using a 0 and 1 scale, where 1 means the behaviour was displayed and 0 means the behaviour was not displayed, according to Prayitno *et al*. (1997a).

5.2.7 Behavioural observations in the home pen

On Day 7, the general activity of birds in each treatment group (Control or Corticosterone) was recorded by a video camera positioned over the home pen and connected to a PC. Birds were paired in each pen (one from each treatment group) and were fed mealworms based on their treatment. In each pen was a feeder, bell drinker and a wooden perch. Some 7 minutes after ingestion, the behaviour recording started and continued for a period of 10 minutes. Previously, Breuner *et al*. (1998) had observed a relative increase in perch hopping behaviour 15 minutes after birds were offered corticosterone-injected mealworms .The behaviour categories monitored (from preview of video recording) are presented in the ethogram in Table 5.3, categories which were adapted from Wang *et al*. (2013).

Table 5.3: Behavioural ethogram used to describe activity of broilers in the home pen

Behaviour	**Description**
Preening	beak used to ruffle feathers
Feeding	beak placed in the feeder
Drinking	beak placed in the drinker
Foraging	pecking at the litter

Subsequently the video recorded on the laptop was played back and at 1 minute intervals each bird was scored as being engaged in one of four categories of behaviour, namely preening, feeding, drinking or foraging.

5.2.8 Blood sampling

After the behaviour recordings on Day 7, a blood sample was taken from each bird. A total of 0.5 ml of blood was sampled from the intertarsal vein using a 1 ml syringe and needle and immediately 5 drops of blood was put into an i-STAT cartridge ($CG8^+$, Quality Clinical Reagent, Limited, York, UK) and then inserted into a blood gas analyser (Abaxis Vet Scan i-STAT®1 Analyser, Quality Clinical Reagent, Limited, York, UK) for the analysis of blood partial pressure of carbon dioxide (pCO_2), pH, sodium ion (Na^+) and blood glucose.

5.2.9 Body weight and internal organ collection

After the recording of behaviour, birds were weighed, blood samples were taken and then euthanized through cervical dislocation also known as the schedule 1 method of euthanasia (average weight 1.7 kg) and a *post mortem* undertaken to recover internal organs, namely the liver, heart and spleen. Organs were weighed using a sensitive scale and then expressed relative to bodyweight. The weights of the internal organs were expressed relative to the body weight (kg) of the birds. The difference between bodyweight of the bird prior to and after the 7 day experiment was used to derive the mean weight gain.

5.3 Statistical analyses

All data were checked for normality and if normality was satisfied we proceeded with a parametric test otherwise data was transformed or non-parametric tests were undertaken. All statistical tests were undertaken using SPSS statistical package (version 19, IBM).

i) Training

Data of the first day of training was not included because of the imbalance between the rewarded and unrewarded trials and also because birds were given a period of 2 minutes with the aim that bird would flip the cones in the unrewarded position and experience the air puff (Burman *et al*., 2009). The latency to open cone in the rewarded and unrewarded positions by each bird during the subsequent three day training period was subjected to a paired sample t-test. Birds had two sessions of training per day, so the average for each day was calculated and used for subsequent analysis. Each data point is an average of 18 data sets (6 for each position per day for 3 days).

ii) Cognitive bias

The cognitive bias test was carried out over a three day period, with each bird having three rewarded trials, three unrewarded trials and one trial for each ambiguous position per day, so for each bird, the average latency for the nine rewarded, nine unrewarded and three ambiguous positions was calculated.

Latency to open the cone was subjected to a repeated measures analysis of variance with the five positions as the within subject variable and treatment as the between subject variable. In order to account for individual differences in performance during the test (time taken to open the cone indicative of motivation), latency to open cones in the ambiguous position was adjusted using the formula of Burman *et al*. (2011) to derive an adjusted latency as follows:

- latency to open cones in each position– reward latency/ unrewarded latency – reward latency

On each day of the cognitive bias task, the average of the mean latency to approach rewarded and unrewarded positions was calculated and this was used to derive the adjusted latency. After which the mean adjusted latency for the three days was calculated. The mean adjusted latency was inputted into a general linear model (GLM) repeated measures analysis of variance. Multiple comparisons between positions were

adjusted for using Bonferroni correction (SPSS version 19). Between group differences were determined using a LSD post hoc-test.

Because of the difference in the number of data points for each position during the cognitive bias test (there were nine data sets for the rewarded and unrewarded positions per bird whereas there were only three data sets per bird for each of the ambiguous positions), we decided to analyse the mean adjusted latencies for the three ambiguous positions to check for differences between treatments using a univariate analysis.

ii) Novel object test

The latency to flip open the cone placed in the rewarded position after a novel object was placed behind it was recorded. Out of the 15 birds that took part, only one bird opened the cone during the 2 minute period. Data collected was subjected to a chi-square analysis using Cross tab in SPSS statistical package (version 19, SPSS, Rothampstead, UK).

iii) Behaviour in the home pen

Since data on behaviour in the home pen did not satisfy the normality test (Shapiro-Wilks test), data were subjected to a $\log_{10}$transformation before we could proceed with a GLM analysis having treatment as fixed factor.

iv) Body, internal organ weight and blood parameters

The levels of key blood parameters, body weight gain, internal organ weights and relative internal organ weights were analysed using the GLM command in order to analyse for the effect of treatment.

5.4 Results

5.4.1 Training to distinguish rewarded and unrewarded positions

The latency to open cones in the unrewarded position was longer ($P<0.001$) than that of the rewarded position (Figure 5.5).

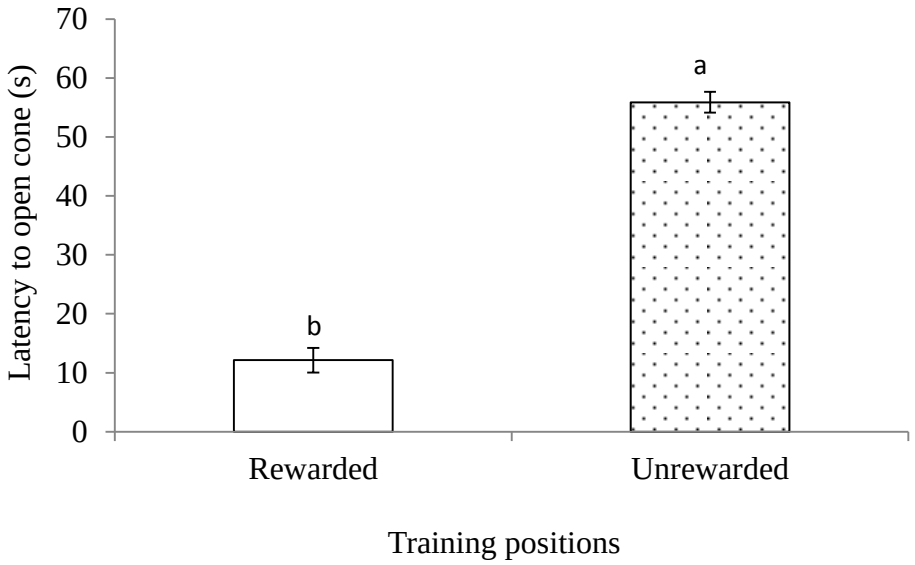

Figure 5.5: Mean latency of broiler chickens to open cone placed in either the rewarded or unrewarded positions. Values are means±1SEM. Average of three days training (6 sessions), each point is a mean of 18 dataset per bird (6 data for each bird per position per day of training), n=15. ab Means with different letter differ significantly between positions at P<0.001.

5.4.2 Effect of corticosterone (mimicking chronic stress) on cognitive bias

Overall, treatment had not significant effect on mean latency to open cones, but there was a tendency for Corticosterone birds to take longer time to open the cone than Control birds ($F_{1, 13} = 4.39$, P= 0.056).The latency to open the cones differed across the five positions in which the cone was located ($F_{2.097, 27.262} = 1.44$, P<0.001). It took the birds a significantly longer time to open a white cone positioned at the unrewarded position than at any of the other four positions (reward, near reward, middle and near unrewarded), see Figure 5.6. However, there was no significant interaction between position and treatment for latency to open the cone at any of the five positions (P>0.05).

With adjusted latency however, the effect of treatment was significant so that Corticosterone birds took longer to open cones than Control birds ($F_{1, 12} = 4.73$, P=0.050). Adjusted latency to open the cones also differed across the five positions ($F_{2.026, 247.309} = 28.022$, P<0.001), such that it took birds a longer time to open a cone placed in the unrewarded position than in the other four positions (reward, near reward, middle and near unrewarded), Figure 5.7. There was no significant interaction between

position and treatment on the adjusted latency to open cone at any of the five positions (P>0.05).

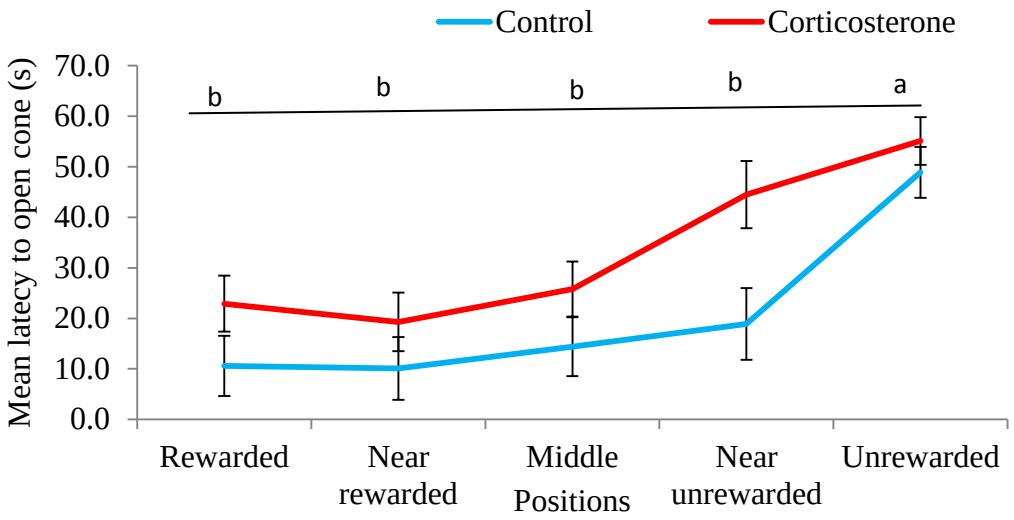

Figure 5.6:Mean latency for Control and Corticosterone birds to open a cone placed in one of five different positions during a cognitive bias task. Data is pooled for the two replicates. Values are means ± 1SEM.Control birds (n= 7), corticosterone birds (n= 8). ab Means with different letter indicate the overall latency (mean value for both treatment at each position) differ significantly between positions at P<0.001.

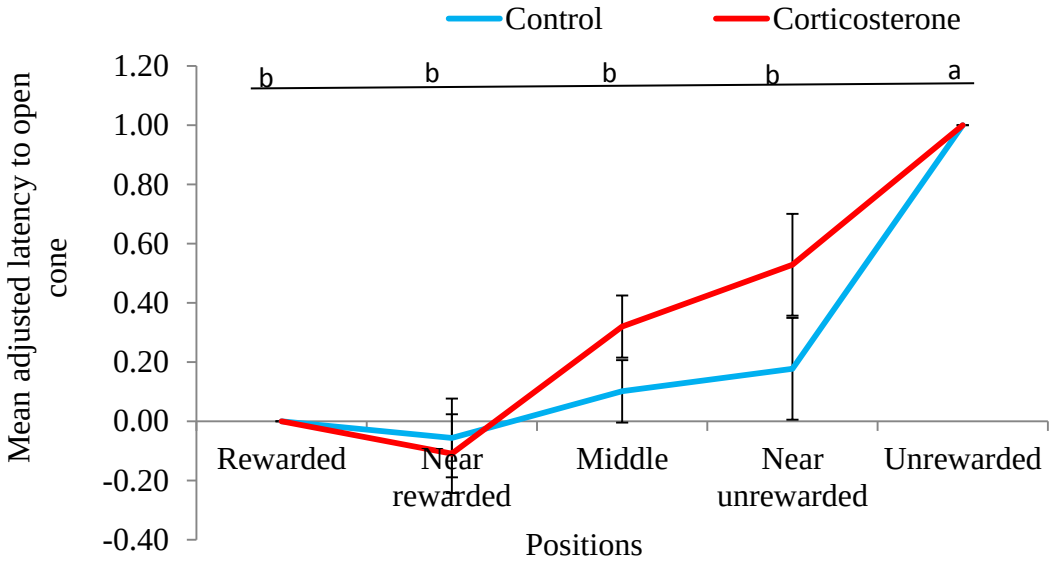

Figure 5.7: Adjusted mean latency for corticosterone and control birds to open cone in five different positions during cognitive bias task. Data is pooled for the two replicates. Values are means ± 1SEM.Control birds (n= 7), corticosterone birds (n= 7). ab Means with different letter indicate the overall adjusted latency (mean value for both treatment at each position) differ significantly between positions at P<0.001

5.4.2.1 Effect of corticosterone (mimicking chronic stress) on the judgement bias of ambiguous positions

After adjusting the latency to correct for the different level of motivation, Corticosterone birds were slower in opening cones in the three ambiguous positions ($F_{1, 12} = 4.731$, $P<0.05$) than the Control birds as can be seen in Figure 5.8.

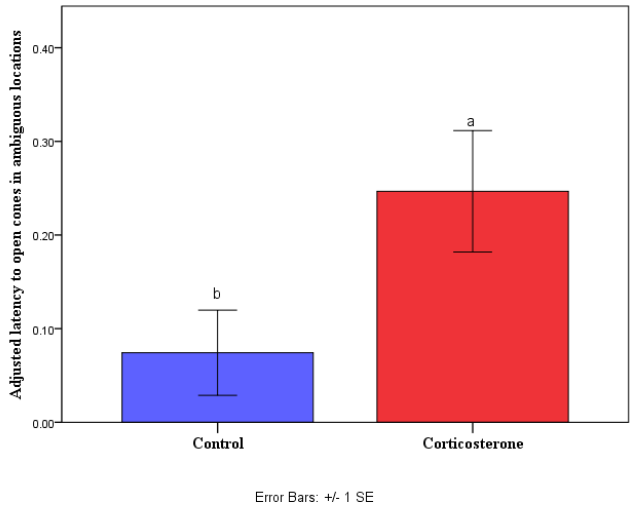

Figure 5.8: Adjusted latency for Control and Corticosterone birds to open a cone in the three ambiguous positions during a cognitive bias task. Data is pooled for two replicates. Values are means ± 1SEM.Control birds (n= 7), Corticosterone birds (n= 7). ab Means with a different letter differ significantly ($P<0.05$).

5.4.3 Effect of corticosterone (mimicking chronic stress) on the response of birds to a novel object

Only one bird (Corticosterone group) opened the cone placed in front of the novel object (within 3 seconds of coming out of the start box) while the other 14 birds did not open the cone within the 2 minute period allowed for this test. The behaviour of the birds during the novel object test (2 minute period) is presented in Figure 5.9, with none of the behaviour categories significantly affected by treatment, probably because of the small sample size. Numerically at least, it appeared that the percentage of birds in motion in the test arena seemed greater in the Corticosterone (87.5%) treatment compared to the Control (57.1%), whereas the percentage of birds which displayed attempted escape was greater in the Control (71.4%) compared to the Corticosterone treatment (37.5%).

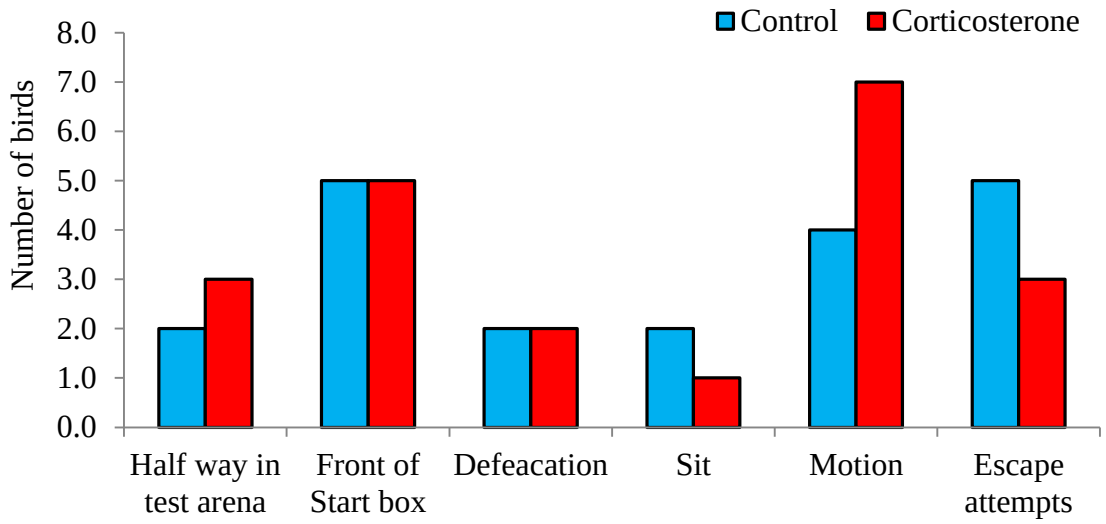

Figure 5.9: Behaviour of Control and Corticosterone birds exhibited during the two minute period of a novel object test. Corticosterone birds (n= 8) and Control birds (n=7). Values are medians.

5.4.4 Effect of corticosterone (mimicking chronic stress) on behaviour of broilers in the home pen

There was no significant effect of treatment in the number of instances of preening, feeding, drinking and foraging behaviour (Figure 5.10), although again the small sample size may have been partly responsible for this.

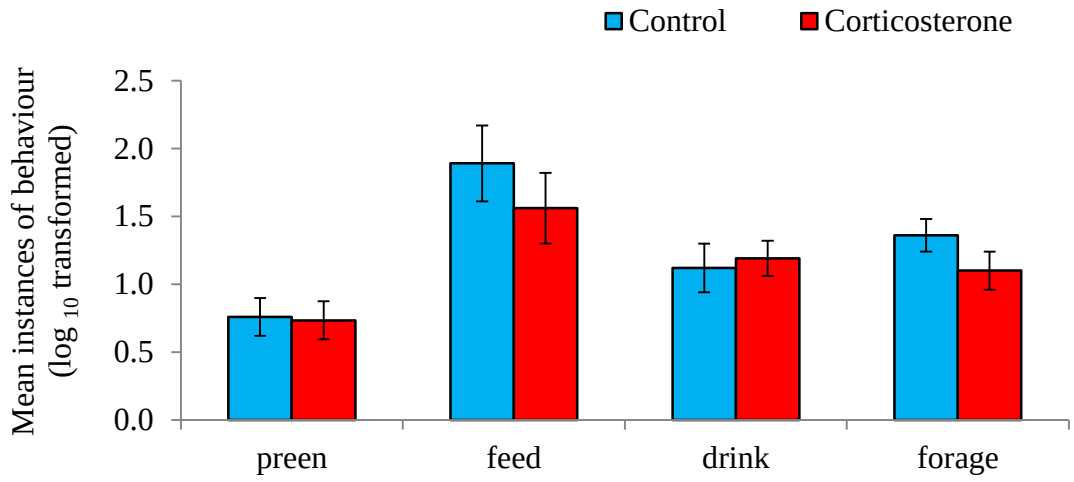

Figure 5.10: Behaviour of Control and Corticosterone birds in their home pen in a 10-minute period of time beginning 7 minutes after ingestion of mealworms injected with DMSO or corticosterone/DMSO respectively. Corticosterone (n= 8) and control (n=7). Values are means±1SEM

5.4.5 Effect of corticosterone (mimicking chronic stress) on blood parameters, internal organs and body weight of broilers

The Control and Corticosterone birds had similar levels of blood pH and pCO_2. However, the Corticosterone birds had a lower level of Na^+ ($F_{1, 14}$= 6.46, P<0.05) compared to the control birds. There was a trend towards significance for Corticosterone birds to have a greater level of blood glucose ($F_{1, 14}$ = 3.65, P = 0.077) than control birds (see Table 5.4).

Table 5.4: Blood parameters of Control and Corticosterone birds offered mealworms injected with either DMSO only corticosterone/DMSO for 7 days. Values are means ± 1 SEM

Blood parameter	**Control (n=7)**	**Corticosterone (n=9)**
pCO_2 (mmHg)	41.1±3.22	39.1±2.84
pH	7.4±0.03	7.4±0.03
Na^+ (mmol/l)	145.4±0.49[a]	143.8±0.43[b]
Glucose (ng/dl)	237.7±11.41	266.8±10.07

[a,b] Means with different superscript across the row differ significantly (P<0.05)

Treatment had no effect on initial weight of the birds before application of treatment, final weight or mean weight gain after 7 days of treatment as presented in Table 5.5. However, Corticosterone birds had a significantly greater liver weight ($F_{1, 23}$ = 12.85, P<0.05) and tended to have a lower spleen weight ($F_{1, 23}$ = 4.08, P=0.056) compared to the control birds. On the other hand, expressing internal organ weight relative to body weight showed that Corticosterone birds had a lower relative spleen weight ($F_{1, 21}$ = 9.64, P<0.05) but a greater relative liver weight ($F_{1, 21}$ = 37.58, P<0.001) than the Control birds. Both absolute and relative heart weight were similar across treatments.

Table 5.5: Body and internal organ weight of Control and Corticosterone birds fed mealworms injected with either DMSO or corticosterone/DMSO for 7 days. Values are means ±1 SEM

Parameter	**Control (n=11)**	**Corticosterone (n=12)**
Initial body weight (g)	1277.3±62.4	1351.8±59.7
Final body weight (g)	1620.1±71.50	1680.3±68.46
Average body weight gain (g)	342.8±20.04	328.4±19.19
Liver weight (g)	46.1±3.23[b]	62.1±3.09[a]
Relative liver weight (g/kg BW)	28.2±1.03[b]	36.9±0.98[a]
Spleen weight (g)	1.9±0.14	1.5±0.14
Relative spleen weight (g/kg BW)	1.2±0.07[a]	0.9±0.06[b]
Heart weight (g)	8.1±0.40	8.3±0.38
Relative heart weight (g/kg BW)	5.0±0.03	5.0±0.22

[ab] Means with a different superscript differ at $P<0.05$

5.5 Correlation between cognitive bias and physiological indicators of chronic stress

Table 5.6 shows the correlation between latency to approach cones in the ambiguous positions and physiological indicators of chronic stress. There was a positive correlation between relative liver weight and latency to approach a cone placed in the ambiguous positions.

Table 5.6: Correlations (R^2 values) between performance of broiler chickens in a cognitive bias task and physiological indicators of chronic stress

	Adjusted latency for ambiguous positions	**Relative spleen weight**	**Relative liver weight**
Adjusted latency for ambiguous positions	1.000		
Relative spleen weight	-0.351	1.000	
Relative liver weight	0.606*	-0.489	1.000

*P<0.05

5.6 Discussion

The aim of this study was to achieve two main objectives namely, i) to develop an improved cognitive bias task for broilers that would reduce training time by introducing a punisher (air puff), and, ii) to validate this task as a non-invasive welfare measure by exploring how broilers under a mimicked chronic stress condition judge ambiguous positions. Physiological and behavioural indicators were adopted as indicators of chronic stress.

5.6.1 Improved cognitive bias task for broilers

i) Training

This section discusses whether the introduction of an air puff improved acquisition of the cognitive bias task. Ad hoc observations suggested that birds found the air puff aversive. Birds jumped, stepped back or froze for some time after receiving an air puff on their face. Freezing is a response to threatening situation (Wang *et al*., 2013). In fact, in an experiment using rats, a single air puff for one second deterred the animals from entering the part of the arena where they had received an air puff the previous day (Moriarty *et al*., 2012). Thus the introduction of an air puff in the current study could have contributed to the rapid discrimination between the rewarded and unrewarded positions.

The results of the current study showed an improvement over that of Wichman *et al*. (2012) who reported that none of the laying birds learnt a spatial discrimination task

after six training sessions. Indeed the laying birds used by Wichman *et al*. (2012) required an average of 12 training sessions before they could discriminate between the rewarded (corn) and unrewarded positions (empty bowl). However, in our study birds discriminated between rewarded (mealworm) and unrewarded (air puff) positions after only 6 training sessions. Similar findings was reported in rats that initially required 6 days to attain the learning criterion in a spatial task involving a visible but inaccessible food in the unrewarded position (Burman *et al*., 2008). Furthermore, the introduction of an aversive stimulus (feed treated with quinine) reduced to training period to 2 days (Burman *et al*., 2009).

Regarding issues relating to the relatively small proportion of birds that learnt the task, although it was our intention to train as many birds as possible for the cognitive bias task, only 60% and 22% of the birds from Replicates 1 and 2 respectively were able to attain learning criterion. A lower number of the younger birds in Replicate 2 learnt the task. In contrast, some other studies have reported success in training 4 day old birds (Salmeto *et al*., 2011; Hymel and Sufka, 2012), albeit using a different task. Therefore the difference may not be related to age per se, but perhaps a lower level of motivation by the younger birds to participate in the training or the possibility that younger birds were more fearful in the test arena. In the study of Salmeto *et al*. (2011), the latency to reach a goal box was longer in the presence of a chick stimulus cue (still picture of a chick) than a mirror cue where the birds could see a moving image. Our observation was that most of the birds that were excluded during the training could be categorised into one of three main groups namely, i) birds that failed to feed on the mealworms during the acclimatisation or pre training period, ii) birds that fed only in the presence of a companion but failed to feed when tested singly in the test arena, and iii) birds that failed to flip open the cones to access the mealworms hidden underneath it. Perhaps if we had persisted with training for a longer time, a higher number of birds would have learnt the task. Previous studies have demonstrated the need to extend the training period to avail animals the opportunity to learn a task. Douglas *et al*. (2012) reported that training of pigs had to continue for ten days to allow all the pigs to reach learning criterion. In another study on rate, after three days of training only 65.2% of animals learnt a discrimination task so an extra training period (4-5 days) was provided for rats that were yet to learn the task (Ritcher *et al*., 2012).There is a possibility that the few birds that learnt the task in our study were the fast learners; hence conclusions from the

cognitive bias task might be restricted to the subset of animals that successfully learnt the cognitive bias task in three days.

The impact of stress on the performance of birds in a cognitive bias task was not evident in the current study despite the report that imposing stress on animals after learning a task could affect their memory in subsequent tests (Mendl, 1999). After 20 minutes of restraint (the stress), zebra finches failed to continue in a visual task but not in a spatial task (Hodgson *et al*., 2007). In the current study, birds which had successfully learnt the task and which were subsequently offered corticosterone-injected mealworms for two days before the cognitive bias test still demonstrated significant discrimination between the two trained positions, except for a single bird which failed to approach the cone in any of the five positions. It seems that the effect of stress on the performance of animals in a cognitive bias task depends on the particular task.

ii) Cognitive bias task

This is the first study to report the direct effect of elevated corticosterone on cognitive bias, and hence by assumption, the affective state of birds. The current study found that corticosterone-treated birds took longer to approach ambiguous positions compared to the control birds, a reaction typical of a pessimistic judgement suggesting therefore that the corticosterone birds may well be in a negative affective state. The overall latency to approach the cones in the five positions was longer in corticosterone birds which could indicate less motivation of corticosterone birds to perform the task. Hence, we decided to adjust the latencies of individual birds to approach the ambiguous cones according to their latencies to approach the rewarded and unrewarded positions. Despite this adjustment, corticosterone birds still took longer to open cones in the ambiguous positions. A similar finding was reported by Enkel *et al*. (2010), where rats subjected to a pharmacological treatment to increase stress (in this case a noradrenergic-glucocorticoid treatment) were pessimistic in their judgement of ambiguous cues.

5.6.2 Validation of the non-invasive method of mimicking chronic stress in broilers

In this section, the effect of elevated corticosterone on physiological changes, level of fear and behaviour of birds will be discussed.

i) Physiological indicators

Chronic stress causes several anatomical changes within the body system of birds. The current study therefore considered the use of other blood parameters apart from corticosterone and changes in internal organs as indicators of chronic stress. The method adopted in this study involved feeding corticosterone-injected mealworms to birds and was first reported by Breuner *et al*. (1998) in white crowned sparrows. The corticosterone dosage (4 mg/kg body weight) was adopted from Post *et al*. (2003) and Shini *et al*. (2008) who offered similar levels of corticosterone to birds through drinking water.

The current study found physiological changes indicative of chronic stress similar to that reported in previous studies such as suppression of the relative spleen weight, increase in relative liver weight and (albeit a tendency for) increase in blood glucose levels (Puvaldopirod and Thaxton, 2000; Post *et al*., 2003; Shini *et al*., 2008; Wang *et al*., 2013). In a different study, significant changes in the internal organs (relative spleen and liver) of broilers were reported as early as 4 days post treatment with ACTH (Puvaldopirod and Thaxton, 2000). The suppression of the relative spleen weight suggests an impaired immune system (Post *et al*., 2003) or an immunosuppressive effect of corticosterone (Wiepkema and Koolhaas, 1993). Furthermore, the suppression of the relative spleen weight in broilers was accompanied by an inhibition of antibody production in response to sheep red blood cell vaccine (Post *et al*., 2003). Since the immune system is responsible for safeguarding the body from infections and foreign material (Hill *et al*., 2008), an impairment of the immune system could easily predispose animals to disease infections which consequently result in mortality.

The increased relative liver weight is caused by the accumulation of lipid in the liver during the process of fat breakdown for the production of glucose (Puvaldopirod and Thaxton, 2000; Wang *et al*., 2013). Gluconeogenesis is the process whereby non-carbohydrate substrates like proteins and fats are converted into glucose to avail birds with the required energy to survive during stress (Ognik and Sembratowicz, 2012) because of the suppression of the digestive processes (Øverli *et al*., 2002). In the current study, there was a tendency for corticosterone birds to have a greater level of blood glucose, however this is in contrast with Olanrewaju *et al*. (2006), Vahdatpour *et al*. (2009) and Lin *et al*. (2004) who all found that corticosterone-treated birds had increased blood glucose levels.

One of the detrimental effects of elevated corticosterone is a depressed body weight. In our study, the body weight gain of the corticosterone birds was 14.4g less than the control birds (P>0.05). It could be that if the study had persisted for longer or if a higher dosage of corticosterone had been used then this reduction in weight gain might have been significant. Previous studies undertaken for a longer duration (either 10 or 49 days) found significant reduction in body weight of chronically stressed birds (Post *et al*., 2003 and Vahdatpour *et al*., 2009 respectively). Decline in body weight was reported in birds treated with a higher dosage of ACTH (8IU/kg BW/day), Puvaldopirod and Thaxton (2000). The reduction in body weight arising from elevated corticosterone could be explained by the inhibitory effect of corticosterone on growth hormone (Hill *et al*., 2008) or the catabolic effect of corticosterone on muscle tissues.

Apart from changes in internal organs and body weight, elevated levels of corticosterone act on the kidney thus impairing the blood acid-base balance. Although we found no effect of corticosterone supplementation on levels of blood pCO_2 or pH, Olanrewaju *et al*. (2006) reported that chronic stress increased the level of arterial pCO_2 but had no effect on pH. The kidney regulates blood pH controlling the levels of bicarbonates (Powell, 2000). In addition, the reduced level of Na^+ in the blood of corticosterone birds could be attributed to increased urinary excretion (Ewing *et al*., 1999). Stress has been reported to increase the water content of the excreta of birds by as much as 187% to that of control birds (Puvadolpirod and Thaxton, 2000). The loss of sodium could be detrimental because of its role in the regulation of blood pressure (Goldstein and Skadhauge, 2000). Probably increased level of corticosterone restricts the production of aldosterone, which is a steroid hormone secreted by the zona glomerulosa of the adrenal cortex that regulates the epithelial cells of the late distal tubule and early collecting duct of the nephron to reabsorb Na^+ into the interstitial fluid and plasma for the conservation of Na^+ (Hill *et al*., 2008).

ii) Response to a novel object test

This section will discuss whether chronic stress increases behavioural indicators of the level of fear in birds. The novel object test was conducted in the same test arena used for the training and cognitive bias task because it was believed that birds were accustomed to being tested alone in the arena, so it was considered an appropriate venue to estimate the level of fear in the birds. Previous studies suing the chronic mild stress paradigm on rats, quails and sheep have shown that simulated chronic stress increases the level of fear (Yang *et al*., 2006; Laurence *et al*., 2012; Destrez *et al*., 2013).

Therefore we expected that in the current study the corticosterone-treated birds would be fearful, however there was no difference in the response of the corticosterone and control birds to the novel object. Corticosterone birds displayed fewer escape attempts during the novel object test than control birds; however this difference did not reach statistical significance perhaps because of the low sample size used so that it might be worth repeating this test in further investigations. During the training and cognitive bias test, the birds were fast in approaching the cone placed in the rewarded position (<15 seconds). With the novel object placed behind the cone in the rewarded position, all the birds refused to open the cone during the two-minute test period. One corticosterone bird opened the cone and accessed the mealworm within 3 seconds of stepping into the test arena, after which the bird raised its head up and discovered the novel object and then it hurriedly moved away suggesting that the bird was afraid of the novel object (personal observation).

iii) Behaviour of birds in their home pen

It is known that there is a bidirectional relationship between hormones and behaviour such that the secretion of certain hormones could trigger the display of specific behaviour or the other way round (Nelson, 2005). In the current study, the levels of preening, feeding, drinking and foraging were similar both treatment groups. Wang *et al*. (2013) found that the percentage of broilers walking increased under chronic stress but stress had no effect on other behaviours such as feeding, drinking, sitting, standing, foraging and dust-bathing. Our study also found an increase in walking behaviour in corticosterone birds (P>0.05) during the novel object test. Probably corticosterone enhances motion/walking and could be a signal of restlessness or anxiety in broilers. In fact, increased locomotion is one of the behavioural responses of the mother hen when they perceive that their chicks were subjected to aversive conditions (Edgar *et al*., 2013). The opposite trend was observed in rainbow trout where locomotor activity was greater in rainbow trout subjected to a single day cortisol treatment than those given a three-day cortisol treatment.

In the current study, behavioural recording started 7 minutes after the birds had ingested mealworms injected with either corticosterone or DMSO with the aim of finding a significant change in behaviour at the point when the plasma levels of corticosterone is at its peak (Breuner *et al*., 1998). After an acute dose of corticosterone, Breuner *et al*.

(1998) reported an increase in perch hopping behaviour which coincided with the period when the plasma corticosterone level was at its peak (7 mins); however, this did not persist throughout the 60 minute period during which plasma corticosterone levels were elevated before returning to baseline values (Breuner *et al*., 1998). Breuner *et al*. (1998) suggested that the initial increase in perch hopping behaviour was needed to rapidly adjust to the stressful conditions. Hence, behavioural changes with respect to increased level of corticosterone may be more of an acute than chronic effect. Such a rapid adjustment in behaviour was not found in this study as behaviour recording was undertaken on the seventh day of treatment so the results cannot be compared to that of a single dose.

A cumulative effect of corticosterone (after the second and fourth dose) on feeding behaviour was reported in red-eyed vireos Vireo (Lõhmus *et al*., 2006) with an increase in the number of visits to the food bowl compared to control birds. The current study did not find a difference in preening behaviour between birds which were in a positive (control birds) or a negative (corticosterone birds) affective state although a behavioural study by Zimmerman *et al*. (2011) reported increased preening in birds expecting a positive stimuli (mealworm) compared to those treated to an unconditioned negative stimuli (water spray).

5.7 Conclusions

The improved spatial task developed by employing the use of an air puff as a punisher in the unrewarded position enhanced the learning process compared to previous reports in laying hens. To validate this task, physiologically stressed birds whose internal state was manipulated through a non-invasive administration of corticosterone (indirectly through mealworms), took longer to open cones placed in the ambiguous positions which is interpreted as a pessimistic judgement indicating a negative affective state. In addition, physiological stress in broilers was associated with reduced relative spleen weight, reduced sodium ion levels but increased plasma glucose levels and relative liver weight. Physiologically-stressed broilers had a similar level of fear to that of control birds. Body weight gain, feeding, preening, drinking and foraging behaviour were not different from that of the control birds. In conclusion, welfare markers of physiological stress in broilers include suppression of relative spleen weight and levels of sodium ion, increase in relative liver weight, blood glucose and the presence of negative affective state. Out of all these physiological indicators of chronic stress, only the relative liver

weight was positively correlated with the latency to open cones in the ambiguous positions.

Chapter 6: Validation of techniques to measure the core body temperature of broilers exposed to simulated episodic heat stress caused by high temperature and relative humidity

6.1 Introduction

Broiler chickens exposed to high temperature and relative humidity (RH) find it difficult to maintain their core body temperature (Borges *et al*., 2007) because hot humid environments reduce the opportunity for heat loss through sensible and evaporative means (Widowski, 2010). Hence, an animal can be said to be under heat stress if the prevailing environmental conditions (high temperature and RH) impairs heat loss from the body thus resulting in an increase in core body temperature (CBT) (Jensen and Toates, 1997). According to (DEFRA, 2005), welfare problems from heat stress arise when the CBT of the bird begins to rise. This implies that a measure of the core body temperature could serve as a useful indicator of welfare during heat stress.

Holistically, the degree of heat stress experienced by a bird is the cumulative effect of the temperature and water vapour density (derived from the RH), known as the apparent equivalent temperature (AET), (Mitchell and Kettlewell, 1998). AET has been adopted by the broiler breeding company Aviagen in their Ross broiler manual (Aviagen, 1999), in order to make producers aware of the appropriate environmental conditions for rearing birds throughout the growth cycle. However, despite information on heat stress such as this being available to producers, it seems that commercially-reared broilers can still endure periods when their environmental conditions are less than ideal. In a survey of commercial poultry farms in the United Kingdom and Denmark, Jones *et al*. (2005) reported that there were times during the growth cycle when temperature and RH in the poultry house were outside recommended levels, particularly when the birds were between 3-6 weeks of age which coincides with the period when birds are most likely to be affected by heat stress. Jones *et al*. (2005) found that factors such as stocking density, drinker type and ventilation system might have contributed to the lack of adequate regulation of temperature and RH in the poultry house.

Acute heat stress could be experienced by commercially-reared broilers in a number of occasions, namely during a diurnal variation in the temperature and/or RH in the poultry shed (Yahav, 2009), catching for transportation (Ritz *et al*., 2005) or during subsequent transportation to the slaughterhouse (Mitchell and Kettlewell, 1998). In fact, one study found that 1.0% of broilers sent for slaughter arrived at the slaughterhouse dead due to

the effects of heat stress during transportation (Ritz *et al*., 2005). The stress experienced by birds during transportation is an aggregate of heat and other stressors, including vibration of the vehicle and high stocking density (Abeyesinghe *et al*., 2001), and this could be more challenging to birds in underdeveloped countries with poor roads and longer journeys. The physiological and behavioural responses of birds under acute heat stress reflect the rapid need to maintain homeothermy (Yahav, 2009) and if such needs are not met, then problems can quickly arise. Broilers could be exposed to an extended (i.e. chronic) period of heat stress (Balnave, 2004) throughout their short growing period of just 6 weeks. Broilers could experience chronic heat stress if the birds are densely stocked in a building with poor environmental control and at a time of the year when the temperature/RH was particularly high.

During summer, birds could experience a heat wave. A period of high temperature and RH which persists for a minimum of five days is regarded as a heat wave in the USA (Robinson *et al*., 2001). In Korea, the common duration of a heat wave is mostly two to three days but not more than five days (Son *et al*., 2012). In Brazil, a heat wave could range from one to five days (Vale *et al*., 2010). These reports clearly show that there is some variation in the duration of a heat wave across the world, and that an average duration of three days could be accepted. However, there are no studies reporting the number of hours in a day during a heat wave episode when the temperature and or RH is high. Heat waves can result in substantial mortality in poultry flocks. With the predicted increase in the frequency, duration and intensity of heat wave due to global warming (Robinson, 2001), then mortality of broiler chickens resulting from heat stress might well increase.

In a simulated heat wave experiment involving broilers exposed to cyclic changes in temperature (21-30-21^{o}C) for 3.5 h/day for three consecutive days, the levels of plasma corticosterone were elevated ($P<0.05$) on the 1st and 3rd day with the 2nd day being intermediate (Mahmoud *et al*., 2004), suggesting that a cyclic high temperature was stressful to the birds. In a study of acute heat stress, Lin *et al*. (2006) found no increase in the levels of plasma corticosterone of five week old broilers exposed to 32^{o}C for 6 h, despite a significant rise in CBT of 0.7^{o}C. Perhaps measuring CBT could be more useful in detecting moderate levels of heat stress in birds.

CBT can be estimated from digital thermometers inserted into the rectum/cloaca of the animal (Quimby *et al*., 2009). Such an application may have limited use in large

populations of broilers and requires a degree of restraint for the bird. So, for continuous measurement of CBT, surgically implanted radio-telemetry data loggers (Dawson and Whittow, 2000) and telemetry devices (Lacey *et al*., 2000) have been developed. Data loggers could be quite expensive to purchase especially for a large number of animals (Chen and White, 2006). Aside from the risk of infection after invasive surgery, there is also the possibility that surgically-implanted devices could impair the welfare of the birds by obstructing the gastro-intestinal tract or respiratory system (Flecknell, 2000), which could have implications for growth performance, well-being and survival. Temperature-ID microchips have the dual purpose of measuring CBT and identification of the individual animal. Temperature-ID microchips work on the principle of passive RFID (radio frequency identification), which respond to a radio signal from the scanner to transmit the bar code (identification number) and CBT of the animal to a receiver device. Provided these microchips deliver an accurate estimate of CBT, they could serve as a replacement for data logger and radio-telemetry devices and because it is a less invasive technique that does not require surgery, so offer a major refinement in the method of measuring CBT in animals. Compared to the data logger, the microchip permits an instant measurement of CBT, thus rapid intervention can be given to an animal showing signs of hyperthermia or other disease conditions associated with a rise in CBT. However, whilst the use of microchips to measure CBT has been validated in goats (Torrao *et al*., 2011), cats (Quimby *et al*., 2009) and rabbits (Chen and White, 2006) against other measures of core and rectal body temperature. However, there are no reports of the use of temperature-ID chips in broiler chickens.

Physiological responses of broilers during heat stress involve vasodilation which enhances blood flow to the body surface causing an increase in skin temperature (Yahav *et al*., 2005). In poultry, the less feathered parts of the body (comb, legs, under the wings and the area around the cloaca) are mainly involved in heat dissipation and have been described as 'thermal windows' (Gerken *et al*., 2006). In fact, the use of thermal imaging on broilers exposed to heat stress (35^{o}C) detected an increase in the comb, face and leg temperatures (Yahav *et al*., 2005). As an alternative to an expensive thermal image camera, an infrared thermometer could be used to monitor the surface body temperature because it has the facility to convert radiation emitted from the body into a temperature reading without requiring contact with the animal (Rextroat *et al*., 1999). During heat stress, both surface body temperature and CBT increase resulting in a drop in the thermal gradient between the body core and the periphery of the animal.

Therefore, it is conceivable that it might be possible to establish a relationship between surface body temperature and CBT that could serve as another non-invasive means of estimating CBT of broilers under heat stress conditions. Facial temperature has been used to predict CBT in broilers under heat stress conditions (Giloh *et al*., 2012).

Behavioural responses of birds to heat stress can include increased levels of panting (respiration rate, RR) and drinking and reduced levels of feeding (Etches *et al*., 2008) which are directed at either increasing heat loss or reducing heat production. However, there is some disagreement about how heat stress affects levels of preening behaviour. Gerken *et al*. (2006) proposed that the levels of preening behaviour during heat stress would increase, so as to maximise heat loss by ruffling the feathers to release heat trapped in the feathers. (Lolli *et al*., 2010) on the other hand considered preening to be a comfort behaviour. Broilers under heat stress (33^{o}C) displayed less preening behaviour as the stocking density increased from 16.6 to 32.5 kg/m^{2} (Lolli *et al*., 2010). Collectively, physiological parameters of increased respiratory rate, a rise in CBT and a decrease in levels of preening behaviour could be considered as an indication of poor welfare of broilers exposed to heat stress.

Therefore, the aim of this experiment was to develop a heat stress protocol of diurnal changes in temperature/RH lasting for a duration of three hours for three consecutive days to simulate conditions in a moderate heat wave to address four objectives:

1. To validate three different techniques used to estimate core body temperature in broiler chickens exposed to heat stress, namely a data logger, a temperature-ID chip and an infra-red thermometer.
2. To develop a model that would relate welfare indicators such as CBT and RR to AET that could avail farmers a better understanding of the consequences of moderate heat stress.
3. To investigate the consequences of episodic heat stress on the growth performance, physiology and behaviour of broilers.
4. To investigate the impact of an implanted data logger on the welfare of broiler chickens under different levels of AET.

6.2 Materials and methods

6.2.1 Ethical considerations

This experiment was conducted under Project Licence number PPL 60/4270 of the Animals (Scientific Procedures) Act 1986. Approval for the project was given at both national (Home Office) and local (Ethical Revenue Committee) level, and it was considered that overall the severity of this work was mild.

Since this was to be the first in a series of experiments to investigate the effects and subsequent alleviation of heat stress, close consultation took place with staff from the University's Comparative Biology Centre (CBC) including the Superintendent, Named Veterinarian and Named Animal Care and Welfare Officer (NACWO).

A template of humane end points was agreed in advance, with the understanding that the details of this matrix would be refined and improved upon in subsequent experiments. Parameters that were considered appropriate for this matrix included mortality, as an extreme example of reduced welfare under heat stress as well as aspects of behaviour (incidence of panting), physiology (blood pH, changes in CBT) and other welfare indicators relevant to broiler chickens (e.g. incidence of breast blisters and contact dermatitis; litter quality; weight loss).

6.2.2 Experimental Design

This pilot study was designed to develop methods of simulating moderate episodic heat stress in the laboratory using climate-controlled chambers, so that a controlled and modest rise in CBT might be induced without resulting in mortality through hyperthermia. The study was conducted at the Ridley Building using four specialised rooms (l × b × h = 4.1m × 2.4m × 2.2m) equipped with computerized temperature and humidity controllers. The rooms were identical in size, construction materials, climatization equipment, feeders and drinkers etc. The experiment was arranged in a 2 × 2 factorial design, with two levels of temperature (normal = 20^{o}C and high = 30^{o}C) and two levels of relative humidity (RH, dry = 40% and humid = 70%) as shown in Table 6.1. Thus, each of the two temperature levels was paired with one of the two RH levels so that each chamber represented a particular temperature-RH combination i.e. one treatment per chamber.

The design of the experiment was to subject birds to heat stress for a period of three hours each day for three consecutive days. Vale *et al*. (2010) reported that the average

duration of a heat wave is approximately three days, although they failed to report the number of hours within each day in which temperature is very high. Therefore we adopted a time period of three hours which is the maximum duration of transportation of birds to the slaughterhouse in the UK (Nicol and Scott, 1990). Similarly, commercially-grown broilers in the UK are said to experience heat stress when ambient temperature reaches 30^{o}C (personal communication, Peter Morgan of Moy Park Ltd, Craigavon, UK). Thus, depending on the particular treatment, the level of temperature-RH set on the control panel of the climate chamber was gradually increased from 'control' levels (i.e. 20^{o}C and 40% RH) over a period of 1 h (step up in temperature by 2^{o}C every 12 mins; while the RH was increased from 40 % to 70 %) and then held constant for a period of three hours (see Table 6.1). Once the three hours had elapsed, temperature and RH were returned to control levels again over a period of 1 h (step down in temperature by 2^{o}C every 12 mins; and RH decreased from 70% to 40%). The pre heat stress phase is the period prior to the step up of temperature/RH in the chamber while the post heat stress period (PHS) is a period of 1 h after the temperature/RH in the chamber has returned to baseline values. Chamber temperature and RH were automatically logged on a PC which confirmed that the temperature/RH created in each chamber was according to the experimental design (Appendix 2). During the experimental period, the broiler chickens had free access to water and feed. The apparent equivalent temperature was derived from the formula: $AET = T + e/\gamma^*$ where T is temperature, e is water vapour pressure, γ^* is corrected psychometric constant (derived from the ratio of the resistances to heat and water vapour transfer) according to Mitchell and Kettlewell (1998).

6.2.3 History of birds

A total of 65, female Ross broiler chickens (age 26 days, weight range of 950-1050 g) were obtained from a commercial poultry farm in Yorkshire (Oakland Farms, Moor Monkton, York, UK) and transported in poultry crates from Yorkshire to Newcastle, a journey of approximately 1.5 h. The birds had previously been vaccinated against infectious bronchitis and infectious bursal disease (Gumboro) when they were aged 7 and 18 days respectively. This study was not designed to measure the effect of sex, so the decision was made to source only female birds to reduce overall variation in bodyweight.

Table 6.1: Experimental design showing conditions of temperature and relative humidity for each of the four treatments

	Temperature and RH at the different phases of the heat stress experiment					
Simulated environmental conditions during heat stress	Pre heat stress (PrHS)	Step up (ST)	Heat stress (3HS)	Step down (SD)	Post heat stress (PHS)	AET (^{o}C) during 3HS
Normal Dry (ND)	20^{o}C, 40%		20^{o}C, 40%		20^{o}C, 40%	35.3
Normal Humid (NH)	20^{o}C, 40%		20^{o}C, 70%		20^{o}C, 40%	46.7
Hot dry (HD)	20^{o}C, 40%		30^{o}C, 40%		20^{o}C, 40%	57.4
Hot Humid (HH)	20^{o}C, 40%		30^{o}C, 70%		20^{o}C, 40%	78.0

6.2.3.1 Housing prior to experimental period

Upon arrival, the broiler chickens were housed in circular pens (90 cm in diameter, 30 cm high) already placed in the four chambers (three pens per room). The temperature and RH of all four rooms was set at 20^{o}C and 40% RH respectively as recommended by the Ross broiler manual for birds of this age (Aviagen, 1999). Commercial pelleted feed (20% crude protein, 4% oil, 6% ash and 13.0 MJ/kg ME; Poultry Pro Finisher Pellets, W.E. Jameson & Son Ltd, Masham, UK) and water were provided *ad libitum* from hoppers placed on the floor of each pen. Five birds were randomly chosen and placed in each pen, giving feeder and drinker spaces of 5.00 and 8.75 cm per bird respectively. Lighting conditions were sixteen hours light and eight hours dark (16L:8D) with an intensity of 30 lux during the light period.

Under veterinary advice, eleven days after arrival, the lighting regimen was changed to 14L:10D and the diet diluted with whole wheat (in ratio 50:50 wheat to commercial feed) in an attempt to reduce feeding behaviour/ nutrient intake and so to slow down growth rate of the birds. Although some UK broiler companies provide a proportion of whole wheat in the diet of broilers as part of a package of environmental enrichment, the standard illumination programme for broilers is 18L:6D. In addition, five days prior to the start of the experiment, the number of birds in each pen was reduced to four to

reduce the stocking density of the remaining birds. Thus, for this experiment, 48 broiler chickens were used giving 12 birds per treatment and 4 birds per replicate pen. However, only 12 birds (one in each pen) had a data logger and temperature-ID chip implanted.

6.2.3.2 Temperature sensing equipment to be fitted during surgery

Within each pen, one bird was subject to surgery to implant a data logger device and temperature-ID chip (thus twelve 'logger' birds in total). Prior to implantation, a tiny talk data logger (Tiny tag, Gemini Talk 2 data logger, Omni Instruments, UK) was calibrated in a water bath and programmed to record core body temperature at three minute intervals. The data logger is battery-powered and logs data at intervals into its memory. The loggers all conformed to the specification provided by the manufacturer, the absolute accuracy was 0.5°C or better in the specified range (-40 to 85^{o}C) and the resolution was 0.05°C or better. Each logger (as shown in Figure 6.1a) was fitted with a new battery (3V Lithium battery), placed inside a black film cassette (shown in Figure 6.1b) and the lid secured to the main body of the canister by means of PFTE tape. The logger was then implanted into the abdominal cavity as described in detail below. After the experiment, birds were euthanized through cervical dislocation and following *post mortem* the data logger was retrieved and data of CBT downloaded onto a PC.

In the same bird, a new, sterile identification chip (identichip® with Bio-Thermo®, Animalcare Limited, York, UK; see Figures 6.1c) was injected 3 cm deep (maximum depth to avoid the problems of chip migration and environmental influence) into the left breast muscle to allow for subsequent temperature readings by scanning with a hand-held reader® (418-S53-B003-ENG Bio thermo reader, serial number 072942, Digital Angel Corp, MN, USA, Figure 6.1d). The temperature-ID chips used in this experiment could estimate temperature range between 25-50^{o}C and gave a reading to the nearest 0.1^{o}C. The temperature-ID chips work based on passive RFID (radio frequency identification) by responding to a radio signal from the scanner to transmit the bar code (identification number) and CBT.

Implantation of the data logger and temperature-ID chip on the same bird was undertaken for the validation of the core body temperature measured using the data logger and temperature-ID chip. The length and breadth of the data logger were 4.0 cm and 2.7 cm respectively while that of the temperature-ID chip were 1.5 cm long and

diameter 0.1 cm. The weights of the data logger and temperature-ID chip were 20.8 g and 0.1 g respectively.

Figure 6.1a: Back view of data logger

Figure 6.1b: Data logger fitted in film canister

Figure 6.1c: Temperature-ID chip

Figure 6.1d: Temperature-ID chip scanner

6.2.3.3 Surgical procedure for fitting body temperature sensing equipment

On the day of surgery, feed was withdrawn from the birds 2 h beforehand. All the birds were weighed individually and legs rings fitted to allow for individual identification. The 12 birds selected (one per pen) had a mean bodyweight of 1445.8 ± 14.3 g (they were chosen as being close to the average bodyweight for each pen) and were marked on the neck region with a nontoxic spray (East Riding Farm Services Ltd, UK) for easy identification. These 12 selected birds were then transported to the theatre (a 10 min drive from the Ridley Building) for implantation of the data logger and the temperature-ID chip. Surgery was performed by the Veterinary staff of the Comparative Biology Centre, Newcastle University.

In the theatre, each bird received 2 mg/kg i/m of butorphanol (Pfizer Ltd, Kent, UK) and 1mg/kg i/m of midazolam (Hameln Pharmaceuticals Ltd, Gloucester, UK) to provide pre-emptive analgesia and sedation. General anaesthesia was subsequently induced by administration of 8% sevoflurane in oxygen at a flow rate of 1.5 litre /min through a small, open flow face mask. Satisfactory induction of anaesthesia was confirmed by the abolition of withdrawal reflexes to comb and toe pinches, attenuation of corneal and nictitating membrane reflexes and reduction in the pupillary response to light. In

addition, 2 mg/kg s/c of Carprofen (Pfizer Ltd, Kent, UK) was administered to provide post-operative analgesia, and 10 mg/kg i/m of enrofloxacin (Animal Health Division, Berks, UK) given as a precautionary measure to prevent wound infection. Feathers were removed from the surgical site around the sterna carina and the bird was then placed in dorsal recumbence on a heated table and covered with an insulating blanket. An area of skin in the sternal mid-line was cleaned and disinfected with hibiscrub (chlorexhidine). All subsequent procedures were undertaken under aseptic conditions.

A 50 mm midline incision was made starting just below the sternal carina (keel), then the duodenum and pancreas were gently lifted out of the abdomen and carefully placed on a piece of cotton wool which had been soaked with saline. The data logger (previously assembled following sterilization of components with ethylene oxide) was inserted into the abdomen and gently manipulated so that it was positioned behind (dorsal to) the abdominal viscera. The duodenum and pancreas were repositioned, and the peritoneum and abdominal muscle closed with PDS (3-0) interrupted sutures (i.e. a mono-filament synthetic absorbable suture Polydioxalone of diameter 0.3 mm and tensile strength 12.3N; Ethicon Division of Johnson & Johnson Medical Limited, Scotland, UK). The skin was closed using PDS (4-0) using a subcuticular continuous suture (i.e. a mono-filament synthetic absorbable suture Polydioxalone of diameter 0.2 mm and tensile strength 7.5N; Ethicon Division of Johnson & Johnson Medical Limited, Scotland, UK). Finally, tissue glue was applied to the skin margins.

Next, the bird was placed in an incubator set at 20^{o}C and the temperature-ID chip was injected 3 cm deep into the left breast muscle using a needle applicator provided by the manufacturer. The bird remained in the incubator under the observation of a theatre nurse until recovery (typically 20-30 mins later). Some four hours after surgery, each bird was given a second intramuscular injection of enrofloxacin (0.6 ml; Animal Health Division, Berkshire, UK) as a further precaution against infection.

The 12 birds that underwent surgery were then returned to the Ridley Building and to the same climate chamber from which they originated. The three birds from each treatment were temporarily housed together (3 logger birds/ pen) in a newly made pen placed in the chamber overnight in order to avoid pecking from other birds. The following morning the logger birds were checked for health before returning them back

to their home pens. Recovery of birds that underwent surgery was monitored by weighing them at intervals after surgery (3rd and 10th day post-surgery).

6.2.4 Data collection

6.2.4.1 Core and surface body temperature

Core body temperature (CBT) was automatically measured by the data logger device every three minutes. However, the mean of five data points closest to when the microchip and under-wing temperatures were estimated within each treatment was used to derive the mean core body temperature from the data logger. The change in CBT measured from the baseline was calculated by subtracting the CBT at pre heat stress from that of subsequent phases. To measure CBT from the temperature-ID chip and surface body temperatures, the bird was picked up from its pen and the temperature-ID chip scanned by passing the hand-held scanner device across the breast of the bird. Next surface body temperatures were estimated with the infra-red thermometer. Scanning the chip and measuring surface body temperature took less than two minutes per bird, therefore the procedure should have had little effect on temperature estimates.

Surface body temperatures from four different body parts identified as thermal windows namely under the wing (henceforth referred to as wing temperature), feet, cloaca and comb, were taken using a surface laser temperature probe (ThermoWorks TW2 mini pocket infrared thermometer, Lindon, USA). This infra-red thermometer has a distance: spot ratio of 6:1, emissivity of 0.95, response time of 1 second, operating range of 0-50^{o}C, sensitivity to wavelength of 5-14µm and dimensions of 23×50×103 mm (H×W×D). The thermometer was calibrated according to the guidelines of the manufacturer. The mean of the four body surface temperatures was subsequently calculated for each phase of the heat stress protocol as being representative of overall surface body temperature.

CBT from the temperature-ID chip and surface body temperatures were measured on the 1st and 3rd days of heat stress at the end of each of the five phases. A single measurement for each of the four body surfaces was taken at the PrHS, ST, 3HS, SD and PHS. No measures of surface body temperature were taken on the second day because of time constraints in taking blood samples that day.

6.2.4.2 Haematological and hormonal parameters

Due to ethical limitations on the amount of blood that could be sampled from a single bird, blood sampling was only undertaken on the 2nd day of exposure to heat stress. A blood sample was taken from each of six birds on each treatment (three 'logger' and three other birds) on three specific occasions during the day. Stress level of birds was found to be similar in each of the three days of exposure to cyclic heat stress (Mahmoud *et al*., 2004). The 1st blood sample was taken at the PrHS phase, the 2nd at the end of 3HS phase and the 3rd at the end of the PHS phase.

For easy repeated blood sampling within the shortest possible time (<1 minute), to reduce handling stress and to prevent the development of haemorrhage which is common from samples taken from the wing, birds selected for blood sampling were fitted with an IV catheter. A 22 gauge over the needle IV catheter (Abbott laboratories, Ltd, Berkshire, UK) was inserted into the intertarsal vein and an intermittent injection cap inserted onto the hub of the catheter. The catheter was firmly secured onto the leg of the bird by bandage and tape. Whilst the bird was held securely, a 0.5 ml sample of blood was drawn into a syringe via a 22 gauge syringe through the injection cap. When there was difficulty in obtaining a sample through the catheter, a sample was taken from the vein in the other leg of the bird using a needle and syringe (this occurred in 4 cases).

The 1st blood sample was taken at the point when the IV catheter was fitted, performed in the corridor outside the climate chamber, but subsequent samples were taken inside the climate chambers. At the end of PrHS, 3HS and PHS phases, a 0.5 ml sample of blood from the intertarsal vein was collected directly into a syringe. A few drops of whole blood were immediately put into an i-STAT cartridge ($CG8^+$, Quality Clinical Reagent, Limited, York, UK) and then inserted into a blood gas analyser (Abaxis Vet Scan i-STAT®1 Analyser, Quality Clinical Reagent, Limited, York, UK) to give instantaneous analysis of the partial pressure of carbon dioxide (pCO_2) and oxygen (pO_2) along with levels of pH, glucose and bicarbonate (HCO_3^-).

6.2.4.3 Respiratory rate (RR)

The respiratory rate (RR) of two birds per pen (a logger and a control bird) was estimated on the 1st and 3rd day of heat stress, to correspond with days when core and surface body temperatures were taken. RR was counted by direct observation, so that 10 minutes prior to the end of four phases of the heat stress protocol (ST, 3HS, SD and

PHS phases), the number of breaths a bird took in 30 seconds was recorded and then converted to the number of breaths per minute.

6.2.4.4 Behaviour recording

In each climate chamber, two digital cameras (DCS-900, Ethernet camera, BT plc, London, UK) were fitted on a wooden beam suspended from the ceiling to capture activity in two out of the three pens in that particular chamber. The cameras recorded activity continuously onto a laptop computer on the 1st and 3rd day of heat stress. In each pen, two focal birds (the logger bird and a control bird similar in weight to the logger bird) identified by marking their tail feathers with a nontoxic spray (East Riding Farm Services Ltd, Driffield, UK) were monitored. Upon playback of the video, the observer recorded the number of bouts of each of four behaviour categories within a 10-minute period prior to the end of the ST, 3HS, SD and PHS phases. It was anticipated that any effects of temperature/RH would be seen most clearly during this time. The four behaviour categories measured are defined in the ethogram presented in Table 6.2. These behaviours were chosen as they were previously shown to change during heat stress (Etches *et al*., 2008 and Bozakova *et al*., 2009).

Table 6.2 Behavioural ethogram of the four categories used to classify behaviour

Behaviour category	**Definition of behaviour**
Feeding	Beak in the feeder
Drinking	Beak in the drinker
Preening	Beak used to ruffle feathers
Wing drooping	Separation of wings from the body

6.2.4.5 Growth performance and feed and water intake

On each day of the heat stress experiment, feed intake (during and after heat stress), and water intake (only during heat stress) were measured. The amount of feed in the feed hopper was measured before and after exposure to heat stress and the difference was recorded as feed intake. For water intake, three litres of water was placed in a bell drinker in each pen at the end of PrHS, and at the end of PHS the volume of water remaining was measured using a calibrated jug. The difference between these two

parameters gave an estimate of the amount of water consumed per pen during heat stress. No incidence of spillage was observed in the food and water during the experiment.

For body weight gain, birds were weighted individually on the morning prior to the start of the heat stress experiment (starting weight) and then 72 hrs. later at the same time of day when it was the end of the experiment (finishing weight). To weigh the birds, a bucket was placed on a scale and tarred before the bird was placed in the bucket and its weight recorded.

Subsequently, the following production indices were determined:

a) Mean feed intake during heat stress = amount of feed provided at the end of PrHS - feed left over at the end of PHS (a period of 6 hours).
b) Mean feed intake after heat stress = amount of feed provided at the end of PHS - feed left over the following morning (a period of 18 hours).
c) Mean daily feed intake = amount of feed provided at the end of PrHS - feed left over the following morning (a period of 24 hours).
d) Water intake = water supplied at the end of PrHS - water remaining at the end of PHS
e) Estimated daily weight gain = finishing weight – starting weight / 3.

6.2.5 Statistical Analyses

All data collected were checked for normality using the Shapiro Wilk's test before statistical analyses were performed using the statistical package SPSS (version 19, SPSS, Rothampstead, UK). Repeated measures analysis was used because data were collected from the same bird over five phases of the heat stress protocol. Core body temperature and change in core body temperature measured by the data logger and temperature-ID chip, individual and mean surface body temperature, respiratory rate, blood parameters and behaviour were analysed using a repeated measures (phases of the heat tress protocol) General Linear Model (GLM) fitted in a full factorial model reflecting the fact that we predicted interactions between our independent variables (temperature and RH). If the test assumption of sphericity was violated (Mauchly's test, $P<0.05$), then the degrees of freedom were corrected using Greenhouse - Geisser estimates of sphericity.

The relationships between CBT-chip, CBT-logger and SBTs, as well as those between change in CBT-chip, change in CBT-logger and change in SBTs were analysed using Pearson correlation and simple linear regression analyses.

A simple linear regression analysis was undertaken to develop an equation for predicting the change in CBT from the chip and logger and RR of birds exposed to different AET conditions. Since a Pearson's correlation demonstrates the degree of relationship between temperatures measured from each device, then for further validation of CBT measured from the temperature-ID chip and that measured from the data logger, the data were subjected to a Bland-Altman plot to determine the limit of agreement (Bland and Altman, 1986). A Bland Altman plot shows the mean of the differences in CBT between the temperature-ID chip and the logger and the limit of agreement between them (mean difference ± 1.96 SD). The Bland-Altman plot (shown subsequently in Figure 6.4) is a graph showing the difference between the change in CBT-chip and CBT-logger on the y-axis, and mean change in CBT-chip and CBT-logger was plotted on the x-axis. Therefore, validation of core body temperature estimated by the temperature-ID chip and logger was based on the Bland- Altman plot.

Finally, to determine whether the welfare of birds were impaired arising from the implantation of data logger in the abdominal cavity, a GLM analysis was undertaken to investigate the differences between logger and control birds in terms of RR, behaviour and blood parameters having bird type (logger or control) and AET as factors.

6.3 Results

6.3.1 Validation of techniques to estimate core body temperature in broiler chickens exposed to heat stress – use of a data logger, temperature-ID chip and infra-red thermometer

6.3.1.1 Core body temperature from chip (CBT-chip) and logger (CBT-logger)

Although all 12 temperature-ID chips functioned appropriately to produce barcode and temperature readings, two of the data loggers malfunctioned so that data could not be retrieved from them (one from the 20^{o}C, 40% RH treatment, and the other from the 30^{o}C, 40% RH treatment). The mean core body temperature estimated from the data logger at the end of three hours of heat stress for each day is presented in Table 6.3. Since there was no significant interaction between day × temperature × RH on core

body temperature estimated at the end of 3 h of heat stress, subsequent analysis will be presented as the mean for the three days.

Phase of the heat stress protocol had a significant effect on CBT-logger ($F_{4, 24} = 14.69$, $P<0.001$) and CBT-chip ($F_{4, 32} = 5.82$, $P<0.05$). The mean CBT estimated from the data logger showed that CBT was significantly greater at the end of the 3HS & SD phases than in the PrHS, ST & PHS phases (Figure 6.2a). CBT from the temperature-ID chip was significantly greater at the end of the 3HS than in the PrHS, ST and PHS phases with CBT at the end of SD being intermediate (Figure 6.2a). There was a significant phase × temperature interaction for CBT-logger ($F_{4, 24} = 5.35$, $P<0.05$), specifically at 3HS where birds kept at high temperature (30^{o}C) had a higher CBT than those kept in the normal temperature. There were no significant interactions of phase × RH or phase × temperature × RH for CBT-logger or CBT-chip

Table 6.3: Mean core body temperature of broiler chickens estimated from an implanted data logger at the end of 3 hours of exposure to one of four different environmental conditions for three consecutive days.

Environmental conditions	**Day 1 (^{o}C)**	**Day 2 (^{o}C)**	**Day 3 (^{o}C)**	**Day × temperature × RH**
20^{o}C, 40% RH	41.4±0.33	41.3±0.26	41.3±0.29	
20^{o}C, 70% RH	41.4±0.27	41.6±0.21	41.3±0.24	
30^{o}C, 40% RH	41.8±0.33	41.8±0.26	41.7±0.29	
30^{o}C, 70% RH	42.0±0.28	42.0±0.21	41.8±0.24	NS

NS = non-significant

Due to the variation observed in the baseline CBT (a range of 1.2 ^{o}C) measured by the temperature-ID chip (see PrHS values in Figure 6.2a), temperature estimates were standardized by subtracting the values of the CBT in the PrHS phase from subsequent values obtained in the other four phases. Such standardization provides the change in core body temperature (ΔCBT-chip, ΔCBT-logger respectively) of the broiler chicken at different phases of the heat stress protocol.

Phase of the heat stress protocol had a significant effect on ΔCBT-logger ($F_{3, 18} = 18.35$, $P <0.001$) and ΔCBT-chip ($F_{3, 24} = 5.82$, $P<0.05$). The ΔCBT was significantly greater at the 3HS and SD phases than at the PrHS, end of ST or end of PHS phases (Figure 6.2b). There was a significant phase × temperature interaction for ΔCBT-logger

($F_{3, 18} = 5.63$, $P<0.05$), specifically at the end of 3HS ($P<0.05$) where birds exposed to high temperature had a greater ΔCBT than birds kept at normal temperature. For instance, at the end of the 3HS phase, in birds exposed to high temperature and humid conditions the ΔCBT-logger was 0.7°C while the ΔCBT-chip was 0.5°C. There were no significant phase × RH or phase × temperature × RH interactions for ΔCBT-chip or ΔCBT-logger.

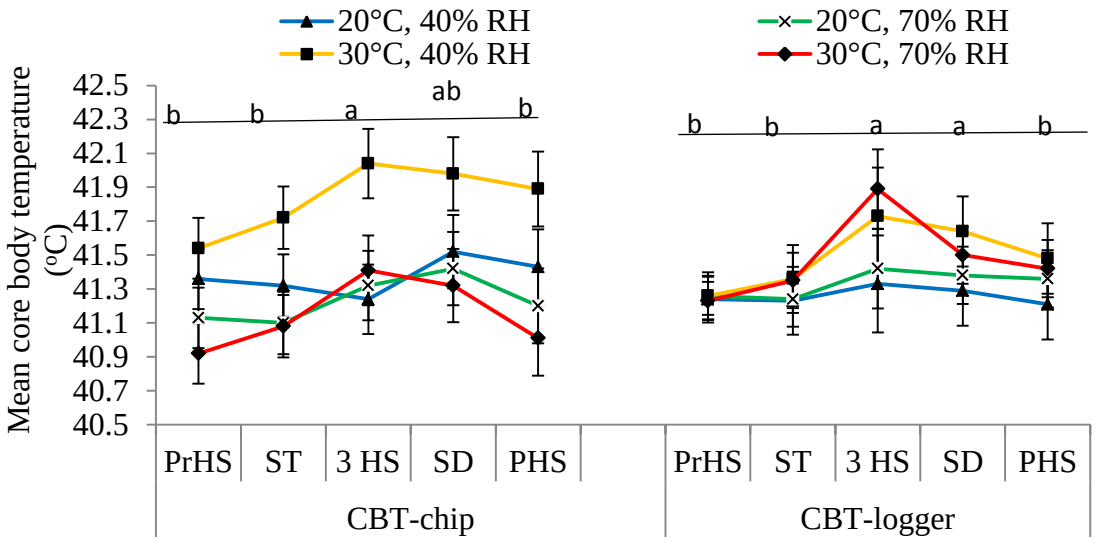

Figure 6.2a: Core body temperature at different phases of the heat stress protocol measured by a temperature-ID chip (n=12 birds) and a data logger (n=10 birds). Values are mean for 2 days ±1 SEM. ab Means with different letters indicate the overall CBT value (single mean for all treatments during that phase) differs between phases ($P<0.05$).

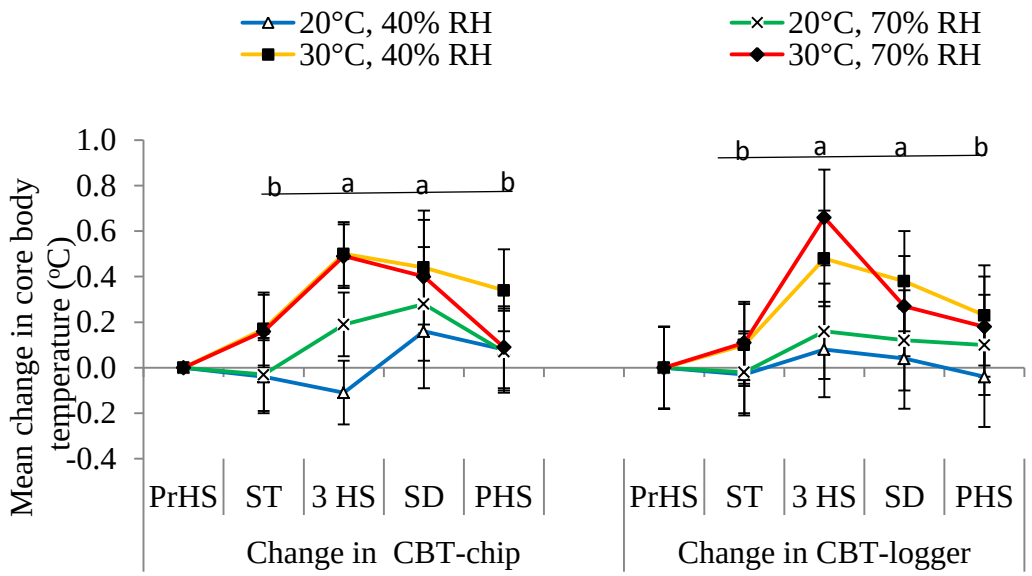

Figure 6.2b: Change in core body temperature at different phases of the heat stress protocol of measured by a temperature-ID chip (n=12 birds) and a data logger (n=10 birds). Values are mean for 2 days ±1 SEM. ab Means with different letters indicate the overall ΔCBT value (single mean for all treatments during that phase) differs between phases ($P<0.05$)

6.3.1.2 Relationship between the temperature-ID chip and the data logger in measuring either CBT or change in CBT

There was no significant correlation between CBT-chip and CBT-logger ($R^2 = 0.358$, $P>0.05$) at the end of 3HS. However, there was a significant correlation between the ΔCBT-chip and ΔCBT-logger ($R^2 = 0.714$, $P<0.05$) at the end of 3HS. The regression equation which could reliably predict ΔCBT-logger from ΔCBT-chip is ΔCBT-logger = 0.171 + 0.749 ΔCBT-chip ($P<0.05$). The scatter plot of ΔCBT-logger against ΔCBT-chip ($R^2 = 0.51$) is presented in Figure 6.3.

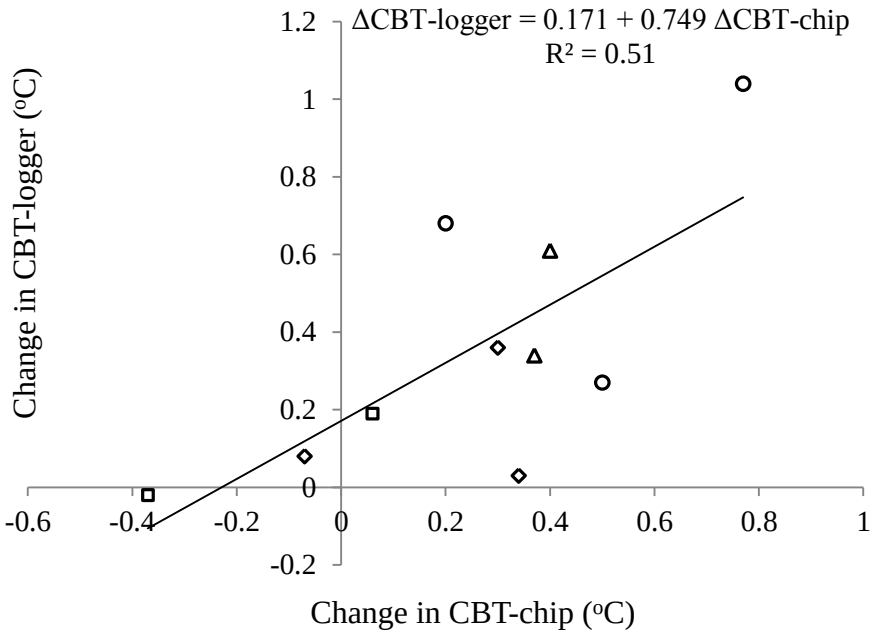

Figure 6.3: A scatter plot showing the relationship between change in change in core body temperature (CBT) measured from a temperature-ID chip and from a data logger in birds exposed to one of four different environmental conditions ($R^2 = 0.51$). □ (20°C, 40% RH); ◊ (20°C, 70% RH); Δ (30°C, 40% RH) and ○ (30°C, 70% RH)

6.3.1.3 Agreement between the temperature-ID chip and the data logger in measuring the change in CBT

Data for the ΔCBT-chip and ΔCBT-logger were used to construct a Bland-Altman plot which had on the y-axis the mean difference between ΔCBT-chip and ΔCBT-logger and on the x-axis the mean of ΔCBT-chip and ΔCBT-logger, as shown in Figure 6.4. The mean difference between ΔCBT-chip and ΔCBT-logger (known as the bias) was -0.1 ± 0.25 (mean ± SD) which means that the mean ΔCBT-chip was 0.1°C less than ΔCBT-logger (presented as the solid horizontal line in Figure 6.4). The limit of agreement between the chip and data logger devices lies within the mean ± 1.96 SD i.e. 0.4°C and -0.6°C (represented by the two dotted lines).

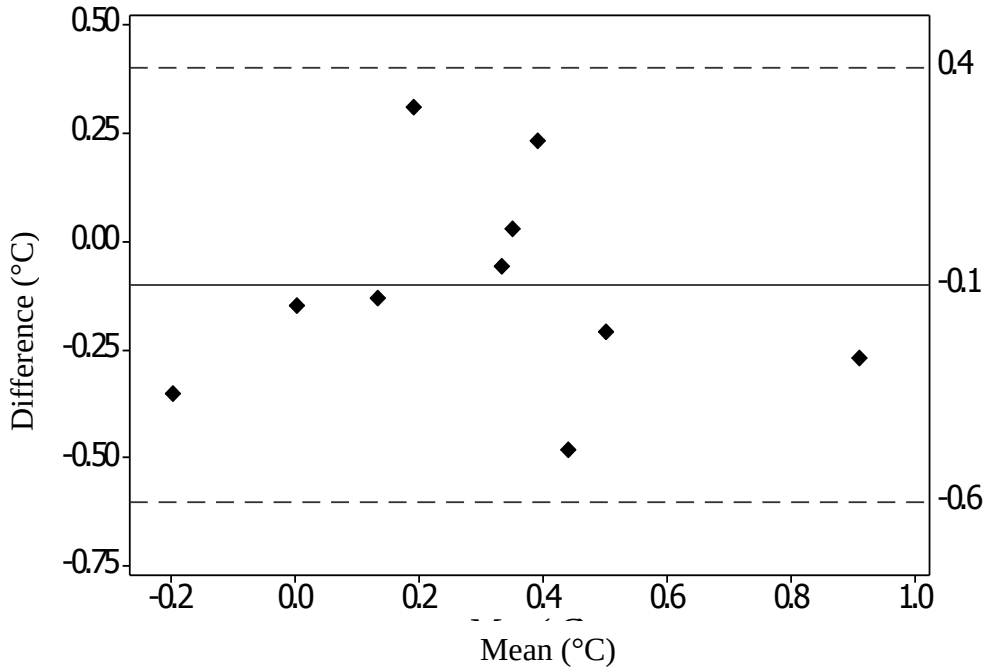

Figure 6.4: Bland-Altman plot showing the difference between the change in core body temperature estimated by a data logger and that from a temperature-ID chip (n=10). The difference in temperature estimation methods are plotted against the pairwise means

6.3.1.4 Relationship between core and surface body temperatures

From Table 6.4, showing data at the end of 3HS, it can be seen there was a significant correlation between CBT-logger and wing SBT ($P<0.05$). There were also significant correlations between wing and comb SBT ($P<0.001$), between wing and feet SBTs ($P<0.05$) and between comb and feet SBTs ($P<0.001$).

Table 6.4: Correlation values (R^2) between core body temperature measured from a data logger (CBT-logger) or a temperature-ID chip (CBT-chip) and surface body temperatures measured from an infra-red thermometer at the end of 3 h of heat stress.

	CBT-logger	**CBT-chip**	**Wing**	**Comb**	**Feet**	**Cloaca**
CBT-logger	1.000					
CBT-chip	0.358	1.000				
Wing	0.713*	0.190	1.000			
Comb	0.536	0.339	0.882**	1.000		
Feet	0.592	0.540	0.764*	0.802**	1.000	
Cloaca	0.192	0.619	0.342	0.331	0.537	1.000

*P<0.05, **P<0.001.

Table 6.5 presents the correlation between the changes in core and surface body temperatures between the pre heat stress phase and the end of 3 h of heat stress. Change in CBT estimated by the data logger was significantly correlated with that estimated by the temperature-ID chip (P<0.05) and with wing (P<0.001) and feet (P<0.05) SBTs. In addition, the change in CBT-chip was significantly correlated with change in wing SBT (P<0.001). Finally, there was a significant correlation between change in wing and change in feet SBTs (P<0.05).

Table 6.5: Correlation values (R^2) between changes in core body temperature measured from a data logger (ΔCBT-logger) or a temperature-ID chip (ΔCBT-chip) and four surface body temperatures measured from an infra-red thermometer between the pre heat stress and end of 3 h of heat stress phases.

	ΔCBT-logger	**ΔCBT-chip**	**ΔWing**	**ΔComb**	**ΔFeet**	**ΔCloaca**
Δ CBT-logger	1.000					
Δ CBT-chip	0.714*	1.000				
Δ Wing	0.865**	0.819**	1.000			
Δ Comb	0.308	0.411	0.318	1.000		
Δ Feet	0.756*	0.416	0.672*	0.359	1.000	
Δ Cloaca	0.168	0.205	0.071	0.480	0.553	1.000

*P<0.05, **P<0.001.

There were significant regression equations relating CBT-logger and wing SBT (WT) (equation 1), ΔCBT-logger and ΔWT (equation 2) and ΔCBT-chip and ΔWT (equation 3) as follows:

CBT-logger = 36.625 + 0.141 WT ($P < 0.05$) ……Equation 1

ΔCBT-logger = 0.079 + 0.174 ΔWT ($P < 0.05$)…….Equation 2

ΔCBT-chip = - 0.002 + 0.157 ΔWT ($P < 0.05$)……..Equation 3

The scatter plot of CBT-logger against WT (Figure 6.5a), ΔCBT-logger against ΔWT (Figure 6.5b) and ΔCBT-chip against ΔWT (Figure 6.5c) gave R^2 values of 0.51, 0.75 and 0.67 respectively.

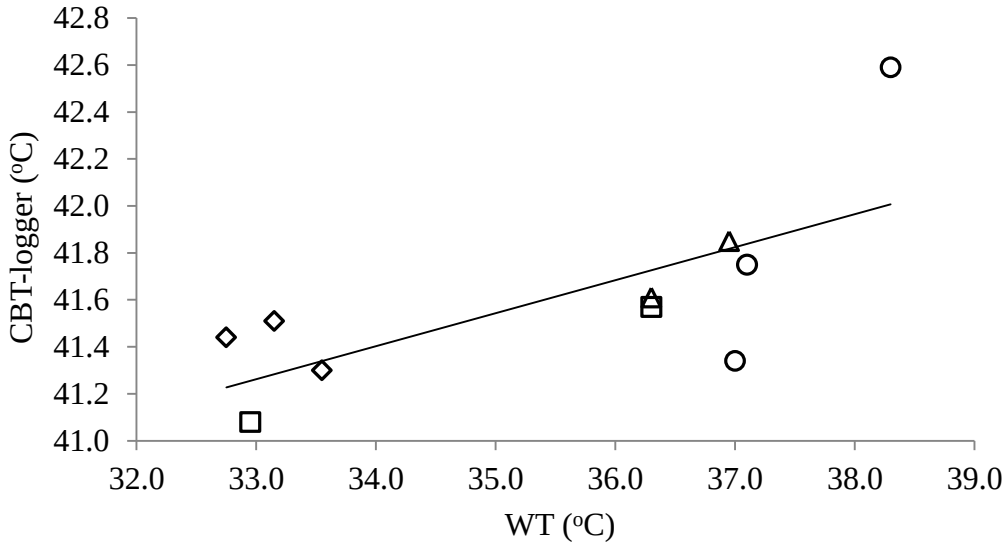

Figure 6.5a: A scatter plot showing the core body temperature (CBT) estimated from a data logger and wing temperature (WT) from an infra-red thermometer ($R^2 = 0.51$) in broilers exposed to one of four different environmental conditions. □ (20°C, 40% RH); ◊ (20°C, 70% RH); Δ (30°C, 40% RH) and ○ (30°C, 70% RH)

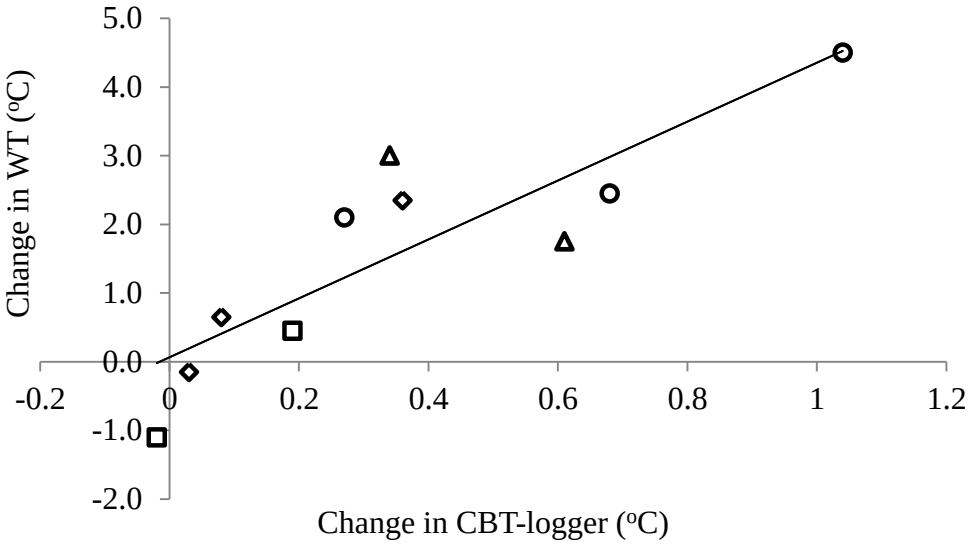

Figure 6.5b: A scatter plot showing the change in core body temperature estimated from a data logger and change in wing temperature from an infra-red thermometer ($R^2 = 0.75$) in broilers exposed to one of four different environmental conditions. □ (20°C, 40% RH); ◊ (20°C, 70% RH); Δ (30°C, 40% RH) and ○ (30°C, 70% RH)

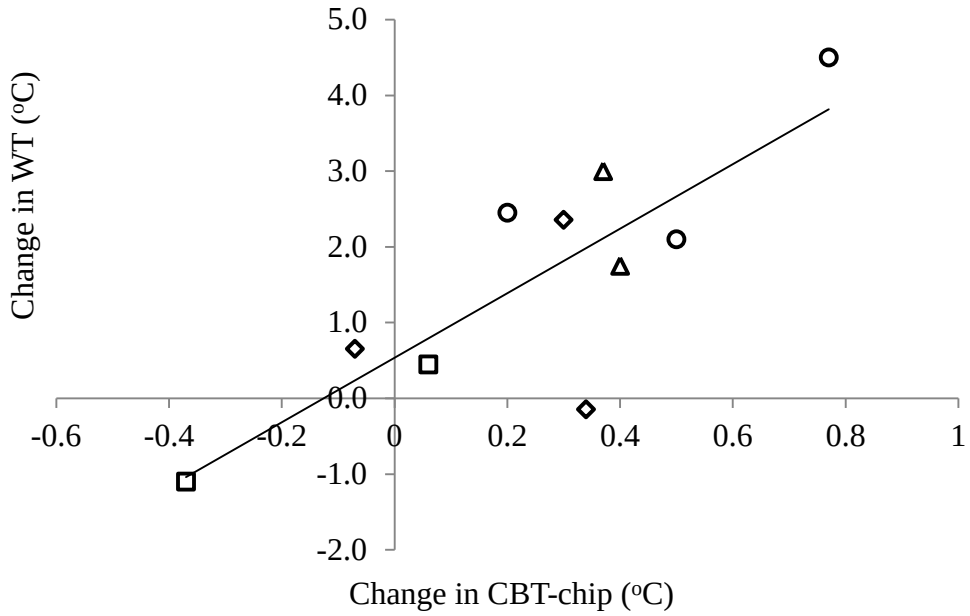

Figure 6.5c: A scatter plot showing the change in core body temperature estimated from the temperature-ID chip and change in wing temperature from an infra-red thermometer ($R^2 = 0.67$) in broilers exposed to one of four different environmental conditions. □ (20°C, 40% RH); ◊ (20°C, 70% RH); Δ (30°C, 40% RH) and ○ (30°C, 70% RH)

6.3.2 Prediction of change in core body temperature and respiratory rate of broiler chickens exposed to different apparent equivalent temperatures

It was established in Section 6.3 that the estimate of changes in core body temperature from the temperature-ID chip was positively correlated with that of the data logger. The previous section has demonstrated the consequences of short-term episodic heat stress on physiology, behaviour and growth performance of broilers of market age. In this section, the interest is in predicting the changes in core body temperature and respiratory rate of broilers kept under different levels of apparent equivalent temperature (AET). Regression plots were used to establish a relationship between change in core body temperature and respiratory rate against different AETs. Arising from these regression plots were significant equations that could reliably predict ΔCBT-chip and ΔCBT-logger and RR from AET, namely:

ΔCBT-chip = -0.5 + 0.01 × AET (R^2 = 51.6 %, P<0.05)

ΔCBT-logger = -0.46 + 0.01 × AET (R^2 = 54.9%, P<0.05)

RR=-21.65 + 1.58 × AET (R^2 = 82.0%, P<0.001).

From these three regression plots, it was observed that the ΔCBT and RR increased linearly when AET was above 47.6^{o}C. Thus, AETs between 35.3^{o}C and 46.7^{o}C fall within the range of temperature which is within the thermoneutral zone of the birds. However, with an increase in AET above 46.7^{o}C, then the birds could begin to experience a certain degree of heat stress due to the increase in ΔCBT and RR. Full results are presented in Appendix 3.

6.3.3 Consequences of simulated episodic heat stress for physiology, behaviour and growth performance of broiler chickens

This section presents result of the impact of episodic heat stress on other welfare indicators such as physiology and behaviour as well as feed intake and growth performance. Results on the effect of episodic heat stress on absolute and change in core body temperature have been presented in the previous section.

6.3.3. 1 Surface body temperatures

i) Wing temperature (WT)

There was a significant effect of phase on WT ($F_{4, 80} = 4.92$, $P<0.05$). The mean WT was greater at the end of ST and 3HS compared to in the PrHS, SD and PHS phases (Figure 6.6). Overall, temperature had a significant effect on WT ($F_{1, 20} = 21.43$, $P<0.001$) with birds exposed to high temperature having a greater WT than those at normal temperature. Although RH had no effect on WT, there was a significant interaction between temperature and RH ($F_{1, 20} = 7.89$, $P<0.05$), so that broilers kept in normal dry conditions (20°C, 40% RH) had a significantly greater WT ($P<0.05$) than those kept in normal humid conditions (20°C, 70% RH). There was a significant phase × temperature ($F_{4, 80} = 7.06$, $P<0.001$) interaction on WT, with a greater WT in birds exposed to 30°C at the ST ($P<0.05$) and 3HS ($P<0.001$) phases. There were no phase × RH or phase × temperature × RH interactions for WT.

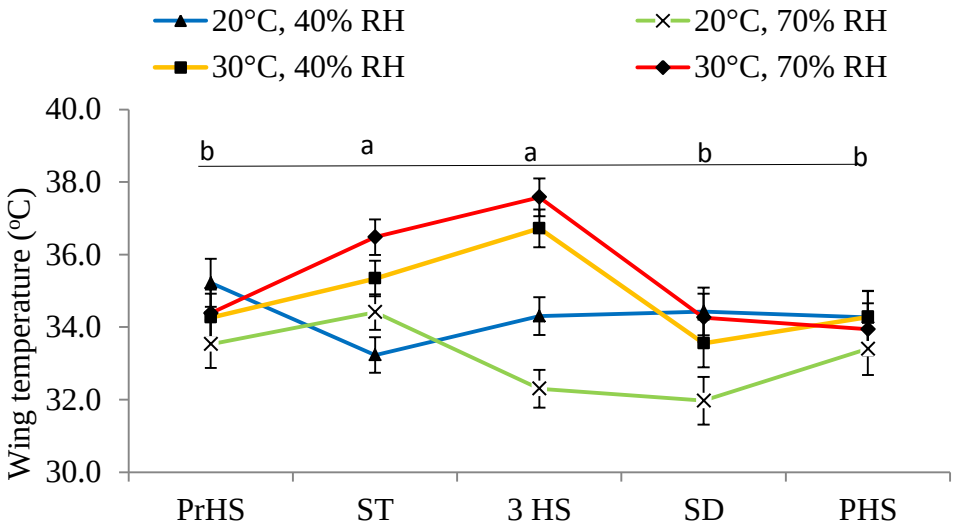

Figure 6.6: Wing temperature of broiler chickens in different phases of the heat stress protocol. Values are means ±1 SEM. n=12 birds/treatment. ab Means with different letters indicate that overall the WT (mean value for all treatments during that phase) differs significantly between phases ($P<0.05$)

ii) Cloaca temperature (CLT)

Figure 6.7 shows that phase had a significant effect on surface body temperature of the cloaca (CLT) ($F_{4, 80} = 5.11$, $P<0.05$). CLT was greater at the end of ST and 3HS

compared to at the end of the PrHS, SD and PHS phases. Overall, there was a marginally significant effect of temperature on CLT ($F_{1, 20} = 4.29$, $P = 0.051$), with CLT being greater in birds exposed to high temperature than those exposed to normal temperature. RH had no effect on CLT and nor was there any interaction between temperature and RH, phase and temperature or phase × temperature × RH for CLT.

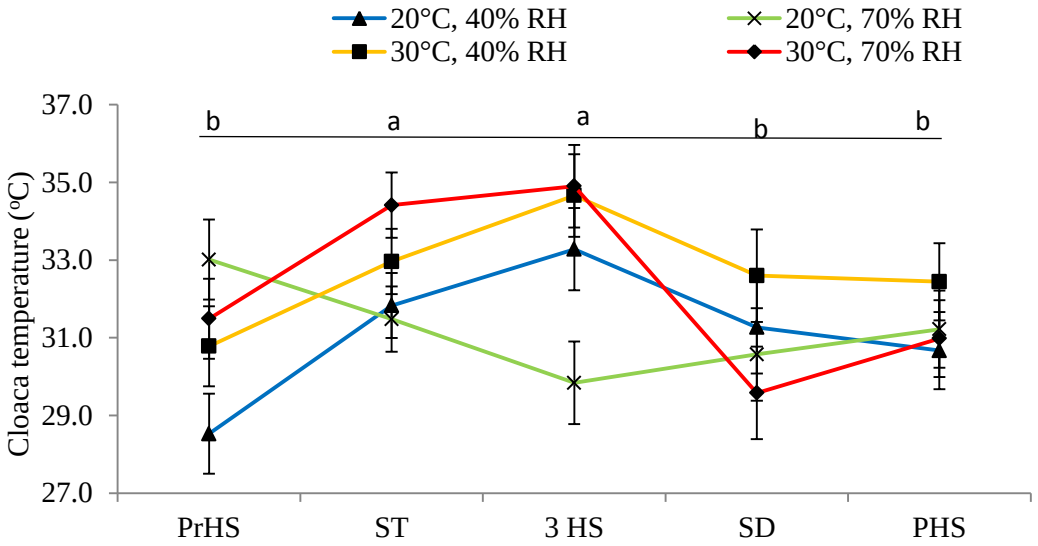

Figure 6.7: Cloaca temperature of broiler chickens in different phases of the heat stress protocol. Values are means ±1 SEM. n=12 birds/treatment. ab Means with different letters indicate that overall CLT (mean value for all treatments during that phase) differ significantly between phases (P<0.05)

ii) Comb temperature (CT)

There was a significant effect of phase on mean surface body temperature of the comb (CT) ($F_{2.473, 49.460} = 14.07$, $P<0.001$). CT was greater at ST and 3HS compared to at the PrHS, SD and PHS phases. Overall, temperature had a significant effect on CT ($F_{1, 20} = 39.01$, $P<0.001$) with CT being greater in birds exposed to 30°C than those kept at 20°C (Figure 6.8). There was a significant effect of RH on CT ($F_{1, 20} = 5.23$, $P<0.05$) with birds kept at a constant RH of 40% having a greater CT than those kept at 70% RH. There was a significant phase × temperature interaction for CT ($F_{2.473, 49.460} = 20.72$, $P< 0.001$), due to birds exposed to 30°C at the ST and 3HS phases having a higher CT than those kept at a constant 20°C.There were no significant temperature × RH, phase × RH or phase × temperature × RH interactions for CT.

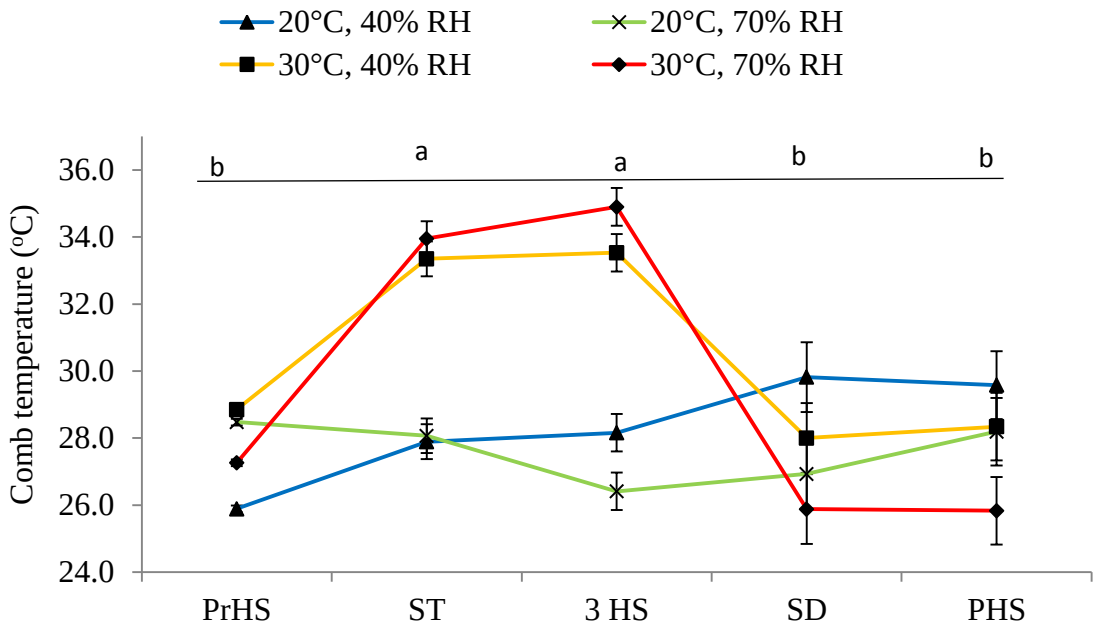

Figure 6.8: Comb temperature of broiler chickens in different phases of the heat stress protocol. Values are means ±1 SEM. n=12 birds/treatment. ab Means with different letters indicate that overall CT (mean value for all treatments during that phase) differ significantly between phases (P<0.001)

iii) *Feet temperature (FT)*

There was a significant effect of phase on mean surface body temperature of the feet (FT) ($F_{4, 80} = 12.45$, $P<0.001$). FT was greater at the end of ST and 3HS than at the end of the PrHS, SD and PHS phases. There was a significant effect of temperature on FT ($F_{1, 20} = 46.68$, $P<0.001$), with birds exposed to 30°C having a greater FT than those kept at constant 20°C. Although RH had no effect on FT, there was a significant temperature × RH interaction for FT ($F_{1, 20} = 4.61$, $P<0.05$), so that FT was greater in birds exposed to 30°C, 40% RH than those exposed to 30°C,70% RH which in turn was greater than birds exposed to 20°C with either 40 or 70% RH. There was also a significant interaction of phase × temperature for FT ($F_{4, 80} = 10.47$, $P<0.001$) with broilers exposed to 30°C having a higher FT in the ST ($F_{1, 20} = 61.48$, $P<0.001$) and 3HS ($F_{1, 20} = 44.71$, $P<0.001$) phases. There was no significant phase × RH or phase × temperature × RH interaction for FT (Figure 6.9).

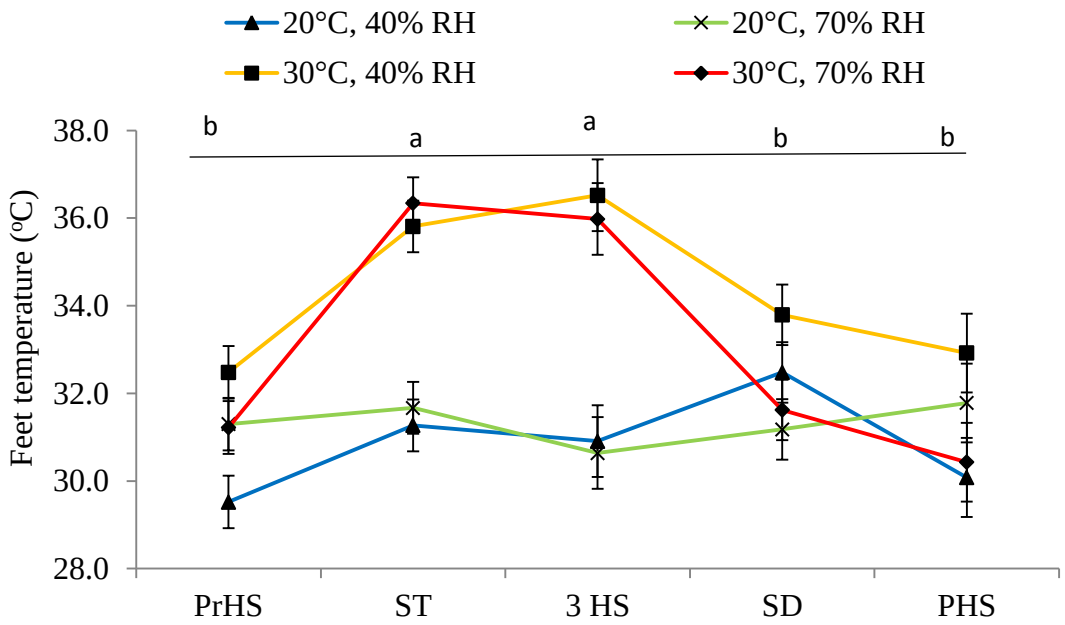

Figure 6.9: Feet temperature of broiler chickens in different phases of the heat stress protocol. Values are means ±1 SEM. n=12 birds/treatment. ab Means with different letters indicate that overall FT (mean value for all treatments during that phase) differ significantly between phases (P<0.001)

iv) Mean surface body temperatures (MSBT)

There was a significant effect of phase on the mean surface body temperature (MSBT) ($F_{4, 80}$ = 30. 60, P<0.001). The MSBT was greater at the end of the ST and 3HS phases compared to that at the end of the PrHS, SD and PHS phases (see Figure 6.10). Overall, MSBT was greater in birds exposed to 30°C than those kept at 20°C ($F_{1, 20}$ = 55.18, P<0.001). RH had no effect on MSBT and not was there a temperature × RH interaction for MSBT. There was a significant interaction of phase × temperature for MSBT ($F_{4, 80}$ = 31.85, P<0.001) with broilers exposed to 30°C having a greater MSBT than birds kept at 20°C at the end of ST ($F_{1, 22}$ = 116.82, P<0.001) and 3HS ($F_{1, 22}$ = 112.64, P<0.001) phases. A significant interaction of phase × RH for MSBT ($F_{4, 80}$ = 6.74, P<0.001) was observed for the SD phase ($F_{1, 20}$ = 15.50, P<0.05), where the MSBT was greater in birds exposed to 70% RH than those exposed to 40% RH. There was also a significant phase × temperature × RH interaction for MSBT ($F_{4, 80}$ = 5.49, P<0.05), which occurred at the 3HS phase (P<0.05, Figure 6.6e) in which birds exposed to 30°C, 40% RH and 30°C, 70% RH had greater MSBT than those kept at 20°C, 40% RH, which in turn was greater than birds exposed to 20°C, 70% RH.

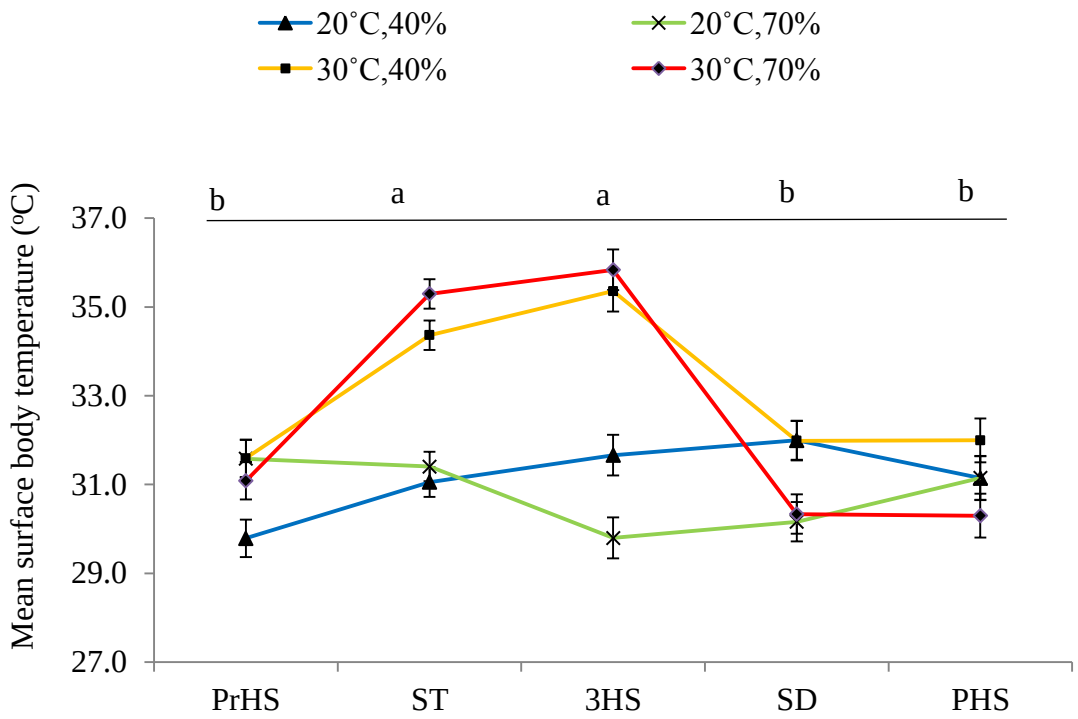

Figure 6.10: Mean surface temperature of broiler chickens in different phases of the heat stress protocol. Values are means ±1 SEM. ab Means with different letters indicate that overall MSBT (mean value for all treatments during that phase) differ significantly between phases (P<0.001).

6.3.3.2 Respiratory rate (RR)

There was a significant effect of phase on respiratory rate (RR) ($F_{3, 60}$ = 22.71, P<0.001). Mean RR was greater at the end of 3HS compared to at the end of ST or PHS phases, with the level for SD being intermediate. Overall, RR was greater ($F_{1, 20}$ = 107.27, P<0.001) in birds exposed to 30°C than those kept at 20°C, and in birds kept at 70% RH than those kept at 40% RH ($F_{1, 20}$ = 10.04, P<0.05) (see Figure 6.11). There was a significant interaction between temperature and relative humidity for RR ($F_{1, 20}$ = 30.70, P<0.001),whereby the RR of birds exposed to 30°C, 70% RH was greater than those exposed to 30°C, 40% RH which in turn was greater than those kept at 20°C, 40% RH or 20°C, 70% RH. There was a significant phase × temperature interaction for RR ($F_{3, 60}$ = 29.21, P<0.001), where broilers exposed to 30°C were breathing faster than those kept at 20°C from the ST phase until the end of PHS. There were no significant interactions of phase × RH or phase × temperature × RH for RR.

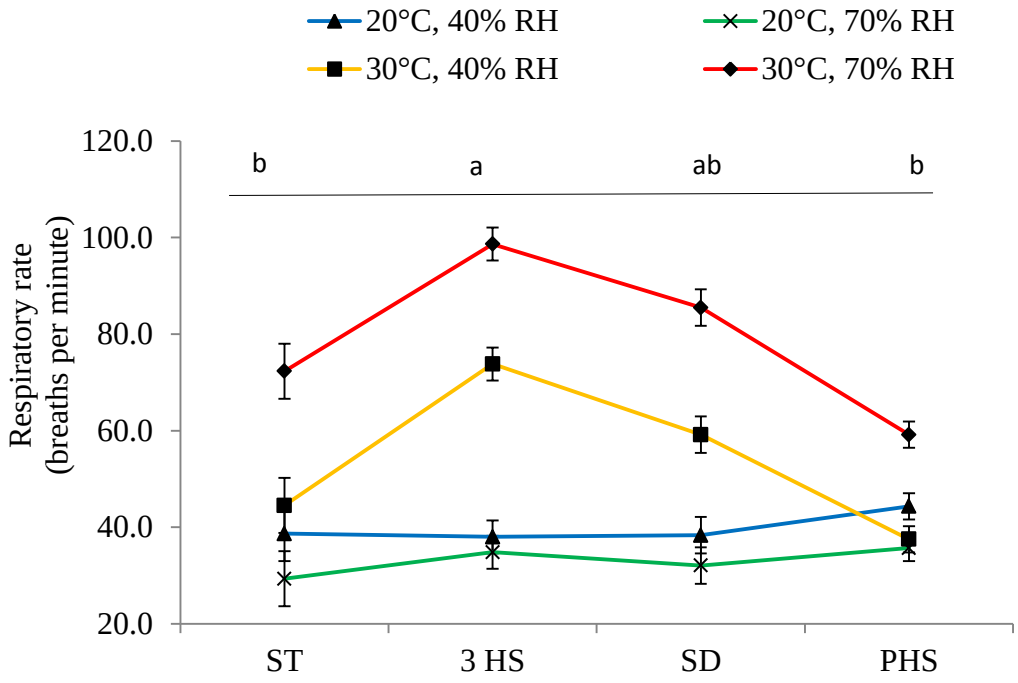

Figure 6.11: Respiratory rate of broiler chickens in different phases of the heat stress protocol. Values are means ±1 SEM. ab Means with different letters indicate that overall RR (mean value for all treatments during that phase) differ significantly between phases (P<0.001)

6.3.3.3 Number of behaviour bouts

Table 6.6 shows the effect of environmental temperature and RH on the number of bouts of each of four different behaviour categories. Phase of heat stress had a significant effect on the number of preening bouts ($F_{3, 36} = 3.76$, P<0.05; see Table 6.6). The number of preening bouts was greater at the end of ST compared to after the PHS phase, with an intermediate number in the other phases (ST and PHS). There was a significant interaction of phase × RH for the number of preening bouts ($F_{3, 36} = 4.15$, P<0.05), especially at the end of 3HS ($F_{1, 12} = 5.88$, P<0.05) where the number of preening bouts was lower in birds exposed to 70% RH compared to those exposed to 40% RH. There was also a significant phase × temperature × RH interaction for the number of preening bouts ($F_{3, 36} = 4.01$, P<0.05) specifically at the 3HS phase ($F_{1, 12} = 5.88$, P<0.05) where birds exposed to 30°C and 70% RH exhibited a lower level of preening bouts than those exposed to 30°C and 40% RH, with levels in the other treatment being intermediate (Table 6.6).

The number of wing drooping bouts was greater (P<0.05) in birds exposed to 20°C than those exposed to 30°C, but RH had no effect on the number of wing drooping bouts. Equally, the number of drinking or feeding bouts was not affected by phase, temperature or RH. There were no first or second order interactions for the number of bouts of wing drooping, drinking or feeding (Table 6.6).

6.3.3.4 Blood parameters

Table 6.7 presents the blood parameters taken on the second day of the heat stress experiment at three different phases, namely pre heat stress (PrHS), end of 3 h of heat stress (3HS) and post heat stress (PHS). Blood pH ($F_{2, 26}$ = 10.76, P<0.001) and bicarbonate levels, HCO_3^- ($F_{2, 26}$ = 9.49, P<0.05) were greater at the end of 3HS and PHS than at the PrHS phase. The blood glucose ($F_{2, 26}$ = 5.57, P<0.05) level was greater at the end of 3HS than the PrHS phase. Blood pO_2 level was greater ($F_{1, 13}$= 5.73, P<0.05) in birds exposed to 30°C than those kept at 20°C, and there was a tendency for pO_2 to be enhanced in birds exposed to 70% RH compared to those kept at 40% RH.

There were significant interactions between phase and RH for pCO_2 ($F_{2, 26}$ = 5.28, P<0.05), blood pH ($F_{2, 26}$ = 4.03, P<0.05) and pO_2 ($F_{2, 26}$ = 3.50, P<0.05) at the end of 3HS, where blood pH and pO_2 levels were enhanced whereas pCO_2 levels were reduced in birds exposed to 70% RH compared to those exposed to 40% RH. There was. There was also a significant phase × temperature × RH interaction for pO_2 ($F_{2, 26}$ = 4.05, P<0.05), whereby birds exposed to 20°C, 40% RH had a lower pO_2 than birds in the other three treatments at the end of 3HS.

6.3.3.5 Feed intake, water intake and growth performance

Table 6.8 shows the feed intake, water intake and weight gain of broiler chickens subjected to 3 h of heat stress per day for three consecutive days. There was a significant effect of temperature on mean feed intake during the six hour period, from the end of PrHS to the end of PHS ($F_{1, 8}$ = 5.58, P<0.05), with lower feed intake in broilers exposed to 30°C compared to those exposed to 20°C. RH had no effect on feed intake during this period, nor was there any interaction between temperature and RH. Feed intake in the 18 h period after heat stress, from the end of PHS to the end of PRHS the following day, was affected by temperature ($F_{1, 8}$ = 9.80, P<0.05), with broilers exposed to 30°C eating more than birds that had been exposed to just 20°C. There was

no effect of RH on feed intake after heat stress. However, there was a significant temperature × RH interaction ($F_{1, 8}$ = 15.03, P<0.05), whereby broilers which were exposed to 30^{o}C, 70% RH consumed more feed than broilers kept in 30^{o}C, 40% RH. Neither temperature nor RH had any effect on mean overall daily feed intake, but there was a significant temperature by RH interaction ($F_{1, 8}$ = 8.76, P<0.05) whereby birds kept at 30°C & 70% RH ate more than those exposed to 30^{o}C & 40% RH.

Birds exposed to 30^{o}C drank more water than those kept at 20^{o}C ($F_{1, 8}$ = 5.30, P = 0.05). However, there was no effect of RH on water intake nor was there a temperature by RH interaction. For body weight gain, neither temperature nor RH had any effect and nor was there a temperature by RH interaction.

Table 6.6 The number of behaviour bouts displayed by birds in a 10 minute period prior to the end of each phase of the heat stress protocol. Values are means

	20°C		30°C								
	40% RH	**70% RH**	**40% RH**	**70% RH**	**SEM**	**P**	**T**	**RH**	**P×T**	**P× RH**	**P×T×RH**
Preen											
ST	2.4	2.1	1.8	1.5	0.42						
3HS	1.9	1.9	3.0	0.4	0.54						
SD	0.9	1.8	2.0	1.5	0.35						
PHS	1.0	0.9	1.5	1.5	0.43	*	NS	NS	NS	*	*
Drink											
ST	1.3	0.9	1.4	0.8	0.28						
3HS	0.9	1.1	1.0	1.3	0.41						
SD	1.4	1.8	0.6	0.6	0.45						
PHS	0.6	0.6	1.0	0.8	0.29	NS	NS	NS	NS	NS	NS
Wing droop											
ST	1.6	1.4	0.6	0.4	0.42						
3HS	1.1	2.1	1.1	1.3	0.42						
SD	1.1	1.0	1.1	0.5	0.30						
PHS	0.6	1.0	0.9	0.6	0.31	NS	*	NS	NS	NS	NS
Feed											
ST	0.9	0.5	0.5	0.4	0.17						
3HS	0.4	0.9	0.3	0.3	0.25						
SD	0.5	0.8	0.5	0.8	0.25						
PHS	0.5	0.9	0.5	0.8	0.23	NS	NS	NS	NS	NS	NS

P= phase of heat stress, T=temperature, RH=relative humidity. ST= step up, 3HS= end of 3 hours of heat stress, SD=step down and PHS= post heat stress. *P<0.05, **P<0.001, NS= not significant

Table 6.7: The effect of episodic heat stress on blood parameters taken on the second day of the heat stress experiment across the different phases of the heat stress protocol. Values are mean ±SEM.

	20°C		**30°C**							
	40% RH	**70% RH**	**40% RH**	**70% RH**	**P**	**T**	**RH**	**P×T**	**P× RH**	**P×T×RH**
pCO_2 (mmHg)										
PrHS	42.5±2.47	42.5±1.43	37.9±1.43	37.0±2.02						
3HS	46.6±4.02	36.9±2.32	37.5±2.32	33.9±3.28						
PHS	38.8±2.80	38.1±1.62	36.9±1.62	35.5±2.29	0.058	0.054	NS	NS	*	NS
pH										
PrHS	7.38±0.02	7.39±0.01	7.42±0.01	7.44±0.02	**	NS	NS	NS	*	NS
3HS	7.37±0.04	7.46±0.03	7.45±0.03	7.50±0.04						
PHS	7.44±0.03	7.46±0.02	7.46±0.02	7.47±0.02						
HCO_3^- (mmHg)										
PrHS	25.0±0.91	25.7±0.52	24.4±0.52	25.2±0.74						
3HS	26.1±1.00	26.3±0.58	26.1±0.58	26.3±0.82						
PHS	26.1±0.76	26.7±0.44	26.4±0.44	26.1±0.62	*	NS	NS	NS	NS	NS
pO_2 (mmHg)										
PrHS	50.5±3.38	48.2±1.95	51.7±1.95	52.0±2.76						
3HS	37.5±3.32	55.0±1.91	53.7±1.91	53.0±2.71						
PHS	39.0±4.77	48.2±2.75	47.8±2.75	50.0±3.90	0.058	*	0.056	NS	*	*
Glucose (ng/dl)										
PrHS	235.5±5.89	222.8±3.40	229.7±3.40	227.7±4.81						
3HS	246.5±9.858	233.0±5.69	239.5±5.69	239.7±8.05						
PHS	228.0±8.09	225.8±4.67	234.5±4.67	239.3±6.61	*	NS	NS	NS	NS	NS

P= phase of heat stress, T=temperature, RH=relative humidity. PrHS= pre heat stress, 3HS= end of 3 hours of heat stress and PHS= post heat stress.

*P<0.05, **P<0.001, NS= not significant

Table 6.8: The effects of episodic heat stress on feed intake, water intake and daily weight gain of broiler chickens over a 3-day period. Values are means

Parameter	**20°C**		**30°C**			**P value**		
	40%RH	**70%RH**	**40%RH**	**70%RH**	**SEM**	**T**	**RH**	**T×RH**
[1]Feed intake (g/bird)	213.1[ab]	184.3[b]	182.4[b]	233.6[a]	13.52	NS	NS	*
[2]Feed intake (g/bird)	120.9	136.3	97.7	114.8	9.47	*	NS	NS
[3]Feed intake (g/bird)	92.2[b]	47.1[c]	84.7[b]	118.8[a]	10.11	*	NS	*
[1]Mean daily weight gain (g/bird)	81.8	81.3	78.1	83.3	7.10	NS	NS	NS
[2]Water intake (ml/bird)	277.2	279.7	310.6	325.0	17.07	*	NS	NS

[ab] means followed by a different superscript across each row differ at P≤0.05. [1] Daily=24 h, [2] during heat stress (6 h, PrHS to PHS), [3] after heat stress (18 h, PHS to PrHS the following day).

6.3.4 Effects of implanting a temperature data logger on the welfare of broiler chickens exposed to different apparent equivalent temperatures (AET)

The objective of this section was to investigate whether the implantation of a data logger caused discomfort to the birds, especially when subjected to different apparent equivalent temperatures. The hypothesis tested was that there would be no difference between birds subjected to surgery to implant a data logger and a temperature-ID chip, (logger birds) and control birds in their physiological, behavioural and growth responses under different levels of AET.

6.3.4.1 Weight gain of birds after surgery

Three days after surgery, logger birds had sustained a lower weight gain compared to control birds ($F_{1, 46}$=72.928, P<0.001) (See Figure 6.12). However, ten days post-surgery weight gain was not significantly different between logger and control birds.

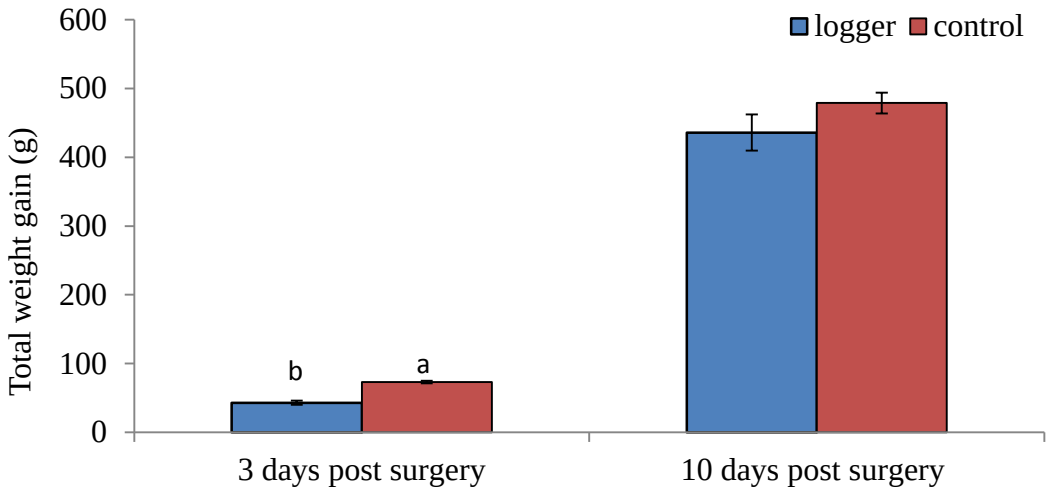

Figure 6.12: Weight gain of logger and birds some three and ten days after surgery compared to weight before surgery. Logger birds=12, Control birds=36, values are means ±1SEM. ab Means with different letters differ at P<0.001.

6.3.4.2 Behaviour, physiology and blood profile of logger and control birds exposed to different apparent equivalent temperatures

Table 6.9 summarises the effect of surgery to fit a logger and apparent equivalent temperature on the weight gain, behaviour, physiology and blood profile of broilers. There was no effect of bird type (logger or control) or AET on weight gain. There were no significant differences in behaviour of the logger and control birds monitored 10 minutes prior to the end of exposure to different AET. However, there was a significant

effect of AET on the number of instances of preening ($F_{3, 8} = 4.71$, $P<0.05$) and feeding ($F_{3, 8} = 6.30$, $P<0.05$). The number of instances that birds preened was greater in broilers exposed to AET of 35.3^{o}C and 57.4^{o}C compared to those exposed to 78.0^{o}C but intermediate in birds exposed to AET of 46.7^{o}C. On the other hand, the number of instances of feeding was greater in broilers exposed to an AET of 46.7^{o}C compared to birds exposed to the other three AETs. There was a significant effect of bird type on pCO_2 ($F_{1, 17} = 7.20$, $P<0.05$), so that logger birds had a greater pCO_2 level compared to control birds. There was no significant effect of bird type on blood pH, pO_2 level or respiratory rate. AET had a significant effect on pO_2 ($F_{3, 17} = 7.20$, $P <0.05$), so that pO_2 was greater in birds exposed to AET levels of 46.7^{o}C, 57.4^{o}C and 78.0^{o}C compared to those kept at 35.3^{o}C. AET also had a significant effect on respiratory rate ($F_{3, 24} = 65.52$, $P<0.001$), whereby it was greater in birds exposed to AETs of 57.4 and 78.0^{o}C compared to those exposed to 35.3 and 46.7^{o}C. There was no significant interaction between bird type and AET for any of the parameters listed in Table 6.9.

Table 6.9: The performance, number of behaviour bouts and blood parameters of broiler chickens implanted with a data logger and a temperature-ID chip compared to control birds when exposed to different apparent equivalent temperatures. Values are means.

	AET = 35.3°C		**AET = 46.7°C**		**AET = 57.4°C**		**AET = 78.0°C**					
	Logger	**Control**	**Logger**	**Control**	**Logger**	**Control**	**Logger**	**Control**	**SEM**	**BT**	**AET**	**BT×AET**
Mean weight gain	70.1	64.4	57.2	77.9	70.8	51.6	67.8	78.9	5.50	NS	NS	NS
Number of behaviour bouts												
Preening	7.3	5.8	6.8	9.5	5.5	0.0	1.5	0.0	2.07	NS	*	NS
Wing drooping	1.3	1.0	1.3	1.0	3.0	1.3	1.3	1.3	0.57	NS	NS	NS
Feeding	1.8	2.0	1.3	0.5	9.3	5.8	1.3	0.3	1.81	NS	*	NS
Drinking	6.0	3.3	4.3	1.8	5.5	7.3	5.3	3.8	3.07	NS	NS	NS
Respiratory rate	37.7	38.3	33.7	36.0	76.0	71.7	98.7	98.7	5.34	NS	**	NS
Blood parameters												
pCO_2 (mmHg)	56.1	37.1	37.9	35.8	38.7	36.3	35.5	30.7	2.77	*	NS	NS
pH	7.3	7.5	7.4	7.5	7.5	7.5	7.5	7.5	0.32	NS	NS	NS
pO_2 (mmHg)	42.0	33.0	54.0	56.0	51.3	56.0	53.5	52.0	2.76	NS	*	NS

BT=bird type (control or logger), AET= apparent equivalent temperature, BT×AET=interaction, NS=not significant, *P<0.05 and **P<0.001

6.4 Discussion

6.4.1 Validation of less-invasive means of estimating CBT

The aim of this study was to simulate a heat wave condition during which validation of measuring core body temperature using two less invasive devices (namely an injectable temperature-ID chip or a surface infra-red thermometer was undertaken.

The simulated episodic heat wave protocol used in this experiment to mimic moderate heat stress showed that core body temperature estimated from an implanted data logger at the end of 3 h of exposure to heat stress was similar on each of the three consecutive days of heat stress. If the birds had become acclimatised to either of the heat stress conditions (30^{o}C, 40% RH or 30^{o}C, 70% RH) then a decline in CBT would have been observed from the first to the third day. Since that was not seen, birds could be said to have experienced a similar level of heat stress on each of the three days. Although our focus in this study was to use CBT as an indication of the level of heat stress experienced by birds, the actual stress level could be determined by measuring the level of corticosterone in the plasma. (Mahmoud *et al.*, 2004) found a similar rise in the level of plasma corticosterone in broilers on each of the three days of exposure to cyclic high temperature. This also agrees with the report of Yahav *et al*. (1996), namely that broilers require 4-7 days to become acclimatised to high temperature. A more recent report also confirms that acclimatisation of broilers can be achieved in 7 days when birds are exposed to intermittent repeated heat stress (38^{o}C, 62% RH for 2, 3 or 4 h/day), so that on subsequent exposure to acute heat stress (43^{o}C, 55% RH, 4 h), acclimatised birds had a lower rectal temperature, rate of increase in rectal temperature, rate of evaporative water loss and mortality compared to non-acclimatised birds (Abdelqader and Al-Fataftah, 2014). Hence, high mortality of birds recorded during a heat wave could be attributed to insufficient time for birds to become acclimated to the high temperature with or without high RH.

Change in core body temperature (ΔCBT) measured by the temperature-ID chip (ΔCBT-chip) and the data logger (ΔCBT-logger) were greater in birds exposed to heat stress (30^{o}C, 40% RH or 30^{o}C, 70% RH) at the end of 3HS and the step down phases. Hence, it can be inferred that change in core body temperature measured from the data logger and temperature-ID were actually related to the change in environmental conditions and not diurnal variation in CBT. Three hours exposure to heat stress resulted in a ΔCBT-

chip and ΔCBT-logger of 0.5°C and 0.7°C respectively. The combination of high temperature and high RH (AET of 78.0°C) did not result in a further incremental increase in CBT, which might possibly be due to the relatively short period of heat stress (three hours) and also the relatively low number of birds involved in the current study. Another reason could be attributed to the greater thermoregulatory effort (respiratory rate) of birds exposed to an AET of 78.0°C compared to those exposed to an AET of 57.4°C (see respiratory rate results in Section 6.3.3.2). Results of the ΔCBT and Δwing temperature were also highest at the end of 3HS.

The use of a temperature-ID chip to estimate CBT in chickens has not been previously validated against other, robust measurements of CBT such as that provided by a data logger implanted deep inside the body cavity. However CBT estimated from a thermometer inserted into the terminal colon was positively correlated (R = 0.824) with an implanted telemetry device in the abdominal cavity of chickens, with the thermometer underestimating the CBT by 0.57°C under heat stress conditions (De Basilio *et al*., 2003). The large difference between CBT estimated by the thermometer and that from the telemetry device reported by De Basilio *et al*. (2003) emphasises the need for a better means of estimating CBT in broilers under heat stress conditions. In the current study, there was no relationship established between CBT-logger and CBT-chip and this could be attributed to the high baseline variation in muscle temperature between birds, despite efforts of the veterinary surgeon to position the temperature-ID chip at the same depth in each bird. CBT estimated from the temperature-ID chip had a large variation even at the pre heat stress phase when all birds were kept in the same thermoneutral conditions (20°C, 40% RH). Torrao *et al*. (2011) also reported a large difference between CBT measured from an implanted logger and that from microchips implanted in the muscle, flank and between the shoulder blades of goats exposed to thermoneutral conditions (24°C, 24-26% RH).

Thus, to overcome the initial variability of the CBT-chip, a form of standardization was undertaken whereby baseline CBT was deducted from subsequent CBT estimates to give a new parameter of change in CBT (ΔCBT). A similar change was calculated for CBT-logger. It was found that ΔCBT-chip had a reliable relationship with ΔCBT-logger, as confirmed by a Bland-Altman plot, and so could be used to predict the ΔCBT measured by the data logger. These promising results demonstrate that a temperature-ID chip implanted subcutaneously could be used to monitor the ΔCBT of broiler chickens.

Actually, ΔCBT temperature is a major parameter widely used as a clinical sign of the immediate condition of an animal (Kort *et al*., 1998), to classify the degree of heat stress as moderate or severe in broilers (Mitchell and Kettlewell, 1998) and layers (Wolfenson *et al*., 1981). Indeed, a ΔCBT of 4^{o}C is known to be lethal to birds (DEFRA, 2005). Therefore ΔCBT estimated from a temperature-ID chip could indicate the degree of heat stress to which the bird is exposed, and serve as a cue for the provision of alleviation measures to avert CBT from reaching a lethal point.

The Bland–Altman plot used in the current study showed that ΔCBT-chip was only 0.1^{o}C less than ΔCBT-logger, which has little biological significance but confirms that the relationship is a sound one given that the threshold for limit is 1^{o}C. This could suggest that the site chosen for the implantation of the temperature-ID chip in the breast muscle of birds gave a reading which was extremely close to the true CBT values. Torrao *et al*. (2011) reported that microchips implanted in the retro peritoneum of goats gave the best agreement (0.2^{o}C lower) with CBT measured from a data logger or rectal temperature estimated from a digital thermometer, with little variation within and between goats. However, in the same study microchips implanted in the groin, semimembranosus muscle, subcutaneously in the flank or subcutaneously between the shoulder blades had a low agreement (differed by 3.5^{o}C) with the CBT measured from a data logger, due to the high variability between goats.

It is worth noting however, that the results of (Torrao *et al*., 2011) were based on absolute core body temperatures measured from the data logger and microchips, whereas in the current study absolute CBT failed to give a significant correlation between temperature-ID chip and data logger as the ΔCBT. Equally, results from the current study cannot be compared with those of (Chen and White, 2006 and Quimby *et al*., 2009) who validated rectal temperatures from a thermometer against microchips implanted subcutaneously between the shoulder blades of rabbits and cats respectively, and a limit of agreement of 1.5^{o}C between thermometer and microchips was reported. Therefore differences in results between studies could be due to the different species used and use of various sites referred to as 'core' (abdominal cavity vs rectal temperature). In fact, rectal temperature has previously been reported to underestimate CBT (De Basilio *et al*., 2003 and Torrao *et al*., 2011) temperature readings from subcutaneously implanted microchips could be influenced by environmental conditions.

The current study demonstrated that 51% of the variation in CBT measured by an implanted data logger could be explained by surface wing temperature (WT) measured by an infra-red thermometer. Moreover, ΔWT explained about 67% or 75 % of the variation of ΔCBT-chip or ΔCBT-logger respectively. This relationship between ΔWT and ΔCBT may be due to increased peripheral blood flow arising from vasodilation of blood vessels when an animal is under heat stress (Al-Tamimi, 2007). However, the use of under wing temperatures as an indicator of imminent hyperthermia may be limited because surface body temperatures rapidly changes in environmental conditions (discussed in Section 6.3.3.1).

6.4.2 Effect of heat stress on physiology, behaviour and growth performance

The aim of this study was to develop a model to simulate episodic moderate heat stress by creating diurnal changes in temperature/RH for three consecutive days and then to investigate the consequences of this heat stress on the welfare of broiler chickens through indicators such as physiology, behaviour and growth performance. The discussion will be sectioned into two main parts, namely i) to validate the simulated episodic heat stress model based on changes in welfare indicators, and ii) to investigate the consequences of simulated episodic heat stress based on the environmental parameters used, i.e. temperature, relative humidity and their interaction.

Validation of the simulated episodic heat stress on changes in welfare indicators

Heat stress in poultry is a potential welfare problem because it poses thermal discomfort to birds (Widowski, 2010) by preventing heat dissipation. The inability of birds to dissipate heat during heat stress results in the accumulation of heat within the body that can eventually lead to death. DEFRA (2005) reported when the CBT of birds begins to rise then welfare problems exist because within a short period of time the bird could die. In order to achieve sufficient heat loss during periods of heat stress, birds resort to panting which is seen as an increased respiratory rate (Etches *et al*., 2008).

Both ΔCBT and respiratory rate of birds were elevated at the end of 3HS, an effect which persisted until the end of the step down phase (the gradual decrease in temperature and RH, SD phase). This suggests a positive relationship between ΔCBT and respiratory rate. Zhou *et al*. (1997) found that the respiratory rate of male broilers began to increase when CBT rose above 41.5^{o}C, peaking when CBT was 42.5^{o}C after which RR began to decline. At the point when RR begins to decline, the levels of heat

production and heart rate have increased significantly - increased heart rate was required to satisfy the need for increased blood flow toward surface body parts to assist with heat dissipation. However, this could lead to the accumulation of blood in the heart and consequently heart failure (Aengwanich and Simaraks, 2004). The simulated heat wave used in the current study caused a moderate ΔCBT of 0.7^{o}C over a 3 h period compared to the control birds and could be considered as a moderate level of heat stress (Mitchell and Kettlewell, 1998). Heat wave conditions were simulated by increased temperature and RH for three hours repeated for three consecutive days. The three days used in the current study was based on the typical duration of heat waves reported in the literature by Vale *et al*. (2010) and Son *et al*. (2012). If the heat stress period was extended for more than 3 h then the ΔCBT would have increased further and this may have resulted in mortality (Quinteiro-Filho *et al*., 2010) which was not desired in the current study.

As it was, one hour after the environmental conditions had been returned to normal, ΔCBT and RR still remained elevated, but after a further hour (2 h post heat stress), both CBT and RR had returned to baseline levels. Nascimento *et al*. (2012) reported that the respiratory rate of broiler chickens exposed to heat stress at the 4th (37^{o}C, 80%RH), 5th (36^{o}C, 80% RH) and 6th (35^{o}C, 80% RH) week of age for 90 minutes returned to baseline levels within 30 minutes after the withdrawal of the heat stress condition. Nascimento *et al*. (2012) also reported that after a heat stress exposure, a period of 30 mins was enough for the birds' cloaca temperature and RR to return to baseline levels. Differences between the current study and that of Nascimento *et al*. (2012) could be attributed to their use of a short period of heat stress (35^{o}C, 80% RH for 90 mins) resulting in a smaller rise in CBT (0.45^{o}C) whereas in the current study, a rise in CBT of 0.7^{o}C was recorded in birds exposed to hot humid conditions (30^{o}C, 70% RH) for 3 hours. Since the duration of heat stress has an influence on the degree of rise in CBT, then the greater the rise in CBT the more time is needed for the accumulated heat stored in the body to be dissipated and the temperature to return to baseline.

In the current study, increased RR was required to dissipate the stored heat causing the rise in CBT but at the end of 2 h PHS the ΔCBT and RR had both returned to baseline values. Most studies on heat stress have concentrated on the welfare of birds during the heat stress period which is very important, but our results have shown that the welfare of broilers were still poor even after the heat stress conditions had been removed. This emphasises the fact that the birds need time for their welfare to be restored, even after the environmental conditions have apparently returned to normal.

The response of MSBT was quite different from that of ΔCBT and RR in that the MSBT reached its peak at the end of ST and remained elevated till the end of 3HS. This is in line with Nascimento *et al*. (2011) who, using a more sophisticated thermal image camera, reported an increase in mean surface body temperature with increase in ambient temperature. In the current study, the rapid increase in MSBT at the end of ST phase could be due to the presence of peripheral thermoreceptors scattered in the body surfaces that detect changes in environmental conditions (Rastogi, 2007). Signals from the peripheral thermoreceptors in the skin or changes in blood temperature are sent to the anterior hypothalamus to initiate heat loss by triggering vasodilation and panting (Rastogi, 2007). Nääs *et al*. (2010) found a positive correlation between the temperature of the featherless body parts and ambient temperature (R^2= 0.8) indicating a rapid response to change in ambient temperature. Similarly, in the current study, one hour after the gradual decrease in environmental conditions (i.e. return to control temperature and RH) the MSBT had dropped back to the baseline value. This indicates that a measure of the surface body temperature could detect changes in environmental condition.

Although blood pH and levels of bicarbonates and glucose were greater at the end of 3HS, and this effect persisted until the end of the PHS, the lack of a significant effect of temperature and RH on these parameters could be associated with the establishment of merely moderate heat stress. This finding agrees with Lin *et al*. (2006) where an increase in CBT of 0.7^{o}C in birds exposed to acute heat stress (32^{o}C) was not associated with changes in blood pH, pO_2, and pCO_2.

Considering all these welfare indicators estimated across the heat stress phases, increased CBT and RR suggests that broiler chickens still suffered the aftereffects of moderate heat stress even after the heat stress had been removed. Thus extra steps may be necessary to promote recovery once the immediate stressor has been removed. In fact it is already known that slaughtering birds immediately after they have been exposed to heat stress can cause changes to breast muscle pH which is associated with changes in core body temperature and acid/base status (Sandercock *et al*., 2001). Hence it is recommended that birds exposed to heat stress during transportation to the slaughterhouse should be allowed a ‘resting period’ before slaughtering in order to maintain meat quality.

Consequences of episodic heat stress for animal welfare arising from high temperature

Exposure of birds to 30^{o}C for 3 h for 3 consecutive days produced a significant rise in CBT of 0.7^{o}C which was accompanied by an increase in MSBT and RR to assist heat loss through sensible and evaporative means respectively (Teeter and Belay, 1996). Broiler chickens with ΔCBT of 0.4-1^{o}C are classified as experiencing moderate heat stress while a ΔCBT of more than 1^{o}C is indicative of severe heat stress (Mitchell and Kettlewell, 1998). Using an egg laying strain of chicken, Wolfenson *et al*. (1981) classified birds having a ΔCBT of 1^{o}C and 2^{o}C as experiencing moderate and severe heat stress respectively. The reason for this difference between egg layers and meat birds could be attributed to the lower tolerability of broilers than layers to heat stress even when subjected to similar level of heat stress at the same age or body weight (Sandercock *et al*., 2006).

The current study was conducted with broiler chickens stocked at only 15 kg/m^2, which is approximately half the level of stocking density specified by the European Union as the minimum space required for commercial broiler chickens. If the experiment was repeated on broiler chickens stocked at the EU minimum stocking density of 33 kg/m^2, then a much greater ΔCBT could be envisaged because reduced space would impede heat loss and this could be exacerbated by the release of additional metabolic heat from the bird to the microclimate (typically.10-15 W/bird) (Mitchell and Kettlewell, 1998). Higher stocking density could lead to an increased litter temperature (Lolli *et al*., 2010) as heat is trapped within the birds' microclimate and reduced space between birds constrains them to sitting a position which facilitates heat transfer from the body to the litter (Gerken *et al*., 2006). For example, 6 week old broilers kept at 20^{o}C and 78.3% RH had an increased level of panting when stocked at 34 and 40 kg/m^2 compared to those stocked at 28 kg/m^2 (8.1 vs 8.8 vs 5.6%), McLean *et al*. (2002). Hence, high stocking density could aggravate the intensity of heat stress experienced by birds, especially when farmers want to maximise occupancy of the space. Countries in tropical or subtropical regions of the world might consider developing an appropriate stocking density that would enhance the welfare of the birds during heat stress, since stocking density has been identified as one of many other factors that affect the birds' effective environmental temperature (Widowski, 2010). Increased stocking density of 43 day old broiler chickens (for five days) from 26.5 kg/m^2 to 45.0 kg/m^2 elevated the core, head, body and shank temperatures by 0.5, 1.7, 2.3 and 1.2^{o}C respectively (Abudabos *et al*., 2013), probably arising from a reduced space between the birds.

Overall, broiler chickens in the current study exposed to 30^{o}C had a lower blood pCO_2 level but greater pH and pO_2 levels than birds exposed to 20^{o}C. This result agrees with those of Hocking *et al*. (1994) that the thermoregulatory effort of birds exposed to heat stress can be determined by estimating venous pCO_2 and pH levels. Contrary to expectations, there was no significant effect of temperature on blood parameters at the end of 3HS, presumably due to the short duration of the heat stress period coupled with the level of temperature used to induce heat stress or the age/size of the birds. Other studies have reported changes in blood parameters within a shorter period of heat stress than was used in the current study. For example, subjecting 35 and 63 day old broilers to acute heat stress (32^{o}C, 75% RH) for 2 h suppressed pCO_2 levels and this was accompanied by an increase in blood pH levels, and the changes in acid-base status were found to be more pronounced in older birds (Sandercock *et al*., 2001). The birds in the current study were 46 days old and had an average weight of 2.4 kg which falls within the age range of birds used in the Sandercock *et al*. (2001) study but the environmental temperature used in the current study to create heat stress was less (30 versus 32^{o}C).

Birds exposed to 30^{o}C had a lower feed intake during the period of heat stress but this was compensated for after the heat stress conditions were removed. The suppression of feed intake during heat stress could be explained by a decrease in digestive activity explained by reduced blood flow (44-48%) to the digestive tract (Wolfenson *et al*., 1981), which is a physiological response aimed at reducing metabolic heat production (Ferket and Gernat, 2006). Level of heat production is associated with increased feed intake (Zhou and Yamamoto, 1997). Increased core and surface body temperature which occurs during heat stress has been found to be negatively correlated with metabolic rate (measured from triiodothyronine level, T_3), Giloh *et al*. (2012). Birds exposed to heat stress (32^{o}C) showed a significant correlation between body temperature and feed consumption (Cooper and Washburn, 1998). Since in the current study birds were reared under an illumination programme of 14L:10 D, and the heat stress occurred during 8 h of the light period, this implies that the birds had the opportunity (a further 6 hours of light) to compensate for their poor feed intake during heat stress. Hence, at the end of the three days experimental period, the weight gain of birds exposed to heat stress was similar to those reared in control conditions.

As expected, in the current study birds exposed to 30^{o}C drank more water than those exposed to 20^{o}C, although this was not reflected in the number of drinking bouts.

Therefore it could be argued that birds exposed to episodic heat stress simply consumed more water at each visit. The number of preening bouts was not affected by high temperature. However, in a study by Lolli *et al*. (2010) increase in stocking density of broiler chickens from 16.5 kg/m^2 to 32.5 kg/m^2 and exposed to heat stress (33^{o}C) resulted in an increase in litter temperature of approximately 5^{o}C which subsequently suppressed the levels of preening behaviour. The suppression of preening behaviour could be a cumulative effect of heat stress and reduced space in birds kept at high stocking density, whereas in the current study birds were kept at a relatively modest stocking density of only 15 kg/m^2 and so results of the current study might be more associated with the effects of heat stress.

Consequences of episodic heat stress for animal welfare arising from high RH

RH had no impact on the ΔCBT and MSBT. It was expected that high RH would exacerbate the degree of heat stress leading to a greater ΔCBT and perhaps greater MSBT if heat loss through sensible means was required. Lin *et al*. (2005) exposed 4-week old broiler chickens to either 35^{o}C or 30^{o}C and RH of either 35, 60 or 85% for 24 h, and reported that RH above 60% elevated the rectal temperature of birds exposed to 35^{o}C but suppressed surface body temperature. On the other hand, Lin *et al*. (2005) found that RH had no effect on rectal temperature of birds exposed to 30^{o}C but surface body temperature increased with increase in RH. This suggests that at high environmental temperature RH suppressed sensible heat loss, thereby resulting in an elevated rectal temperature, but at a mild environmental temperature (30^{o}C), the rectal temperature could be maintained. However, the surface body considered by Lin *et al*. (2005) was quite different from that measured in the present experiment, since they measured temperature in the plumage and breast surface, which could be affected by the level of feathering on the birds, whereas the current study concentrated on SBTs from less feathered body surfaces.

Since the current study found that RH elevated RR but not surface body temperature, it implies the regulation of CBT was mainly through evaporative cooling than sensible heat loss means. This could be the reason for the reduction in the level of preening behaviour. The increased respiratory rate of birds under high RH caused the levels of pO_2 and pH to rise but the level of pCO_2 to decrease. A similar trend of a lack of an effect of RH on CBT and MSBT was reported by Yahav *et al*. (1995) in a study of longer duration (3 weeks) where there was no effect of RH (40-45% or 70-75%) on rectal temperature, skin temperature, body weight gain or feed intake of broilers kept at

35°C. However, in this study by Yahav *et al*. (1995), there is the possibility that the birds had become fully adapted to the conditions because of the extended period of exposure to heat stress.

Consequences of episodic heat stress on welfare arising from high temperature and high RH

Overall, the results of the respiratory rate recorded from the ST to the PHS phases showed that birds exposed to 30°C, 70% RH had a greater respiratory rate than those exposed to 30°C, 40% RH. This greater respiratory rate of birds in the 30°C, 70% RH suggests the need for a greater thermoregulatory effort required to regulate CBT. High humidity (70%) under normal temperature (20°C) suppressed MSBT and pCO_2 but enhanced the levels of pO_2 and pH. The reason for this is not clear, however it has been reported that the lack of control of RH in commercial poultry houses was associated with an increase in the levels of faecal corticosterone and mortality of 5-week old broilers (Dawkins *et al*., 2004). It could be that at high RH, heat stress can be experienced by birds even when the temperature is within the normal range (Mitchell and Kettlewell, 1998), thereby triggering the secretion of corticosterone (Cockrem, 2007). In one particular study, mortality of birds during the growth period especially between 3-5 weeks of age was found to be positively correlated with RH levels and temperature. Unfortunately none of the ten commercial poultry companies who took part in the study routinely measured or controlled RH in their poultry houses (Dawkins *et al*., 2004), probably because relative humidity is affected by several factors such as litter moisture, drinker types etc. Relative humidity has been implicated as a major environmental factor associated (R^2 = 0.77) with the incidence of hock lesions in broilers (Mcllroy *et al*., 1987), presumably because increased RH enhances deterioration of the litter. Birds kept under conditions of moist litter were found to be more fearful evident from an increase in the duration of tonic immobility compared to birds on dry litter (263s vs 184s, $P<0.05$; Campo and Prieto, 2009). This suggests that apart from RH exacerbating the degree of heat stress experienced by birds, the indirect effect of RH on litter could also trigger poor welfare in broilers.

In this study, preening behaviour of birds exposed to heat stress was suppressed in humid conditions. Panting (increased respiration) and preening involves the use of the beak, and since the birds find the combination of hot and humid conditions more challenging and need to increase their respiratory rate to regulate CBT, so they reduce time spent preening. Preening behaviour in birds has been reported to be reduced under

poor welfare conditions such as reduced outdoor access, increased levels of fear, continuous lighting conditions and high stocking density (Zhao *et al*., 2014; Wang *et al*., 2013 and Lolli *et al*., 2010), but not thermoregulatory behaviour as proposed by Gerken *et al*. (2006).

Combining the results of the interaction of temperature and humidity on respiration rate and level of preening, a behavioural matrix was proposed that could be used to classify the degree of heat stress experienced by broilers, namely: 1) normal RR plus high frequency of preening bouts is suggestive of freedom from no heat stress, 2) moderate RR plus high frequency of preening bouts equates is indicative of moderate heat stress, and finally 3) high RR plus low frequency of preening bouts suggests high heat stress.

The increase in feed intake after the heat stress period was greater in birds that were exposed to hot and humid conditions compared to those exposed to hot and dry conditions, thus suggesting that the birds could have found the hot humid environment more energy demanding in terms of increased muscle activity which results in an increased energy requirement (Tao and Xin, 2003a) so need they need to eat more in order to compensate for the energy loss.

6.4.3 Prediction of changes in CBT and RR under different AET and the effect of implanting data loggers in the abdominal cavity of birds

The aims of this section were firstly to predict the change in core body temperature and respiratory rate of broiler chickens exposed to different apparent equivalent temperatures, and secondly to investigate the impact of implantation of data logger and injection of the temperature-ID chip on the welfare of broiler chickens under different apparent equivalent temperatures.

Above an AET of 46.7^{o}C, a significant regression equation was developed (R^2 = 89.6) that could be used to predict the respiratory rate of birds. The high R^2 value suggests that the increase in respiratory rate is proportional to the increment in thermal load (AET) experienced by birds. An increase in respiratory frequency has been correlated to an increase in blood flow (Wolfenson *et al*., 1981). Also, thermoregulatory efforts of broiler chickens are proportional to the degree of thermal load experienced (Mitchell and Kettlewell, 1998). Therefore, respiratory rate can be used to predict the degree of heat stress experienced by broiler chickens, as has been developed for cattle (Gaughan *et al*., 2000). Since respiratory rate is a measure that can be easily obtained and does not

involve disturbing or restraining the animals, then it might be an index of heat stress that could be employed by poultry farmers as a non-invasive method of predicting the thermal load experienced by birds and so a welfare indicator.

Surgery for the implantation of the data logger was undertaken under general anaesthesia and necessary pain relief was given to reduce the pain experienced by the birds. Nevertheless birds which underwent surgery still experienced some distress, evident from the poor weight gain during the three days post-surgery. Some of the known consequences of implanting data logging devices are a reduction in feed and water intake, restricted breathing or hyperventilation and restricted movement (Flecknell and Waterman-Pearson, 2000). In this study, respiratory rate in logger and control birds was similar, but logger birds had greater levels of blood pCO_2. Since the attachment of radio telemetry transmitters in wildlife studies have been reported to have no effect on animal behaviour, Small *et al*. (2005) proposed that blood parameters should be assessed, probably because haematological parameters could reflect mild stressful conditions which may not be observed from the behaviour of the birds alone. Small *et al*. (2005) failed to detect changes in heterophil/lymphocyte ratio (an indicator of chronic stress) in wild birds fitted with loggers either externally or by surgery. In the current study, there were changes in blood parameters but not in the behaviour or performance of the birds.

When the birds in this study were exposed to different AETs, the frequency of preening, wing drooping, feeding and drinking behaviour were similar in logger and control birds. Having established preening as comfort behaviour, our conclusion that the implantation of data loggers did not cause discomfort for the birds is based on the lack of difference in the level of preening behaviour between logger and control birds. Moreso, obstruction of the gastro-intestinal tract or respiratory tract was not found in the current study, as there were no differences between logger and control birds in feeding and drinking behaviour or respiratory rates. Indeed, after the 3-day recovery period post-surgery, the logger and control birds gained similar weight under each of the AET conditions.

6.5 Summary and conclusions

Although validation study was based on a small number of chickens (n=10), the results nevertheless suggest that ΔCBT could be reliably predicted from an injectable temperature-ID chip. This is a less invasive procedure than the fitment of a data logger.

Another option is the use of an infra-red thermometer to measure temperature under the wing however, although this requires handling the bird before this measure can be taken.

The heat stress protocol used in this study was successful in simulating moderate episodic heat stress as evident in the physiological responses of the birds. Surface body temperature and preening behaviour proved to be the most sensitive parameters to gradual change in temperature and relative humidity conditions.

During this short-term episodic heat stress, high temperature resulted in an increased change in core body temperature, MSBT and respiratory rate. On the other hand, RH had less impact than temperature on body temperatures. High RH caused an increase in respiratory rate and blood pH, but suppressed blood pCO_2 and pO_2 levels. High environmental temperature coupled with high RH exacerbated respiratory rate, further suppressed preening bouts and increased the feed intake of the birds after the heat stress conditions had been removed. Blood parameters were not affected, though these effects could be exacerbated in commercial broilers stocked at high density. Birds subjected to heat stress should be availed some period of time (2 h) to recover from the after-effect of the heat stress.

Broiler chickens subjected to surgery for the implantation of a data logger and a temperature-ID chip in their abdominal cavity recovered after three days and began to gain weight similar to the control birds. The implantation of data loggers/temperature-Id chips in broiler chickens did not appear to affect their performance, respiratory rate, behaviour or blood parameters except that it elevated blood pCO_2 level. With an increase in AET above 46.7^{o}C, the ΔCBT and RR increased linearly.

Chapter 7: Effect of intensity and duration of episodic heat stress on the growth performance, physiology and welfare of broiler chickens

7.1 Introduction

Substantial economic losses occur every year in poultry production because of mortality and low growth performance arising from heat stress (Toyomizu *et al*., 2005). In the USA alone, it has been estimated that heat stress costs the poultry industry between $128-$165 million every year (St-Pierre *et al*., 2003). Although this estimate covers losses from broilers, layers and turkeys, broilers are more commonly affected by heat stress than layers. In one particular study, when broilers and layers were subjected to similar heat stress conditions (32^{o}C for 2 h) at the same age or body weight, the impact of heat stress on broilers in terms of changes in acid-base balance and impaired muscle function was greater than on layers (Sandercock *et al*., 2006). The difference between these two types of chicken was associated with the rapid growth rates of the broiler line (Sandercock *et al*., 2006; Gous and Morris, 2005). Nevertheless, heat stress can still impact upon layers. A study of laying birds at the peak stage of production (31 weeks of age) exposed to chronic heat stress for 5 weeks showed that egg production was suppressed compared to control groups, with effects on daily egg production (56.2% versus 87.4%), egg weight (46.9g versus 56.4g), shell thickness (0.028 versus 0.034 mm), antibody production (5.3 versus 7.1) and mortality (31.7% versus 5%) (Mashaly *et al*., 2004). The reduced performance of the birds subsequently leads to reduced income for both laying hen and broiler chicken farmers.

A considerable amount of literature has been published on the effect of heat stress in broilers using different temperatures, levels of relative humidity and duration. Most acute heat stress experiments are undertaken with a duration of heat stress of between 1.5 to 24 hours, with temperature ranging between 30 to 38^{o}C (Nascimento *et al*., 2011; Sandercock *et al*., 2001; Sandercock *et al*., 2006; Soleimani *et al*., 2011; Quinteiro-Filho *et al*., 2012; Lin *et al*., 2005).

During thermoregulation, the temperature gradient between the body surface and the environment regulates the rate of heat loss through conduction, convection and radiation (Cangar *et al*., 2008). Hence, surface body temperature is a reflection of heat flow from the core to the periphery (Lin *et al*., 2005). In broilers kept under ideal thermoneutral conditions, surface body temperature at the first and sixth week of age has been reported

to be 36 and 28^{o}C respectively (Cangar *et al*., 2008), which implies a greater thermal gradient for heat loss as the birds increase in age. Hence, heat loss through conduction, convection and radiation are the main means of heat loss in birds reared under thermoneutral conditions (Nicol and Scott, 1990).

The cardiovascular system is very important in the regulation of both heat dissipation to organs actively involved in heat loss, and also oxygen levels which has implications for metabolism in the animal (Olanrewaju *et al*., 2010). However, with an increase in core body temperature, heat loss through sensible means is suppressed and then respiratory rate increases rapidly. This is accompanied by suppression of levels of CO_2 present in the blood, causing the partial pressure of CO_2 (pCO_2) level to drop, which in turn leads to an increase in excretion of bicarbonate (HCO_3^-) from the kidney and in turn an increase in blood pH (Borges *et al*., 2007), a condition called respiratory alkalosis. Change in acid-base balance during heat stress is greater in older birds than in young birds (Sandercock *et al*., 2001) due to the greater metabolic rate associated with the larger size which makes them more susceptible to heat stress. Furthermore, another physiologically controlled means of heat loss in birds is through increased level of excretion during heat stress although this has consequences for the loss of serum electrolytes such as sodium ion (Borges *et al*., 2007). Exposure to stress often triggers an increase in corticosterone release and concomitant rise in blood glucose levels which are needed for survival (Ognik and Sembratowicz, 2012). Whilst Lin *et al*. (2006) failed to detect any change in levels of blood glucose or corticosterone in birds exposed to heat stress (32^{o}C) for 6 h compared to control birds, this could be related to the fact that only a moderate change in core body temperature (CBT) of 0.7^{o}C was seen in the heat stressed birds.

In commercial broiler rearing, birds can experience heat waves lasting continuously for between one to five days, with mortality due to hyperthermia reported even after a single day of a heat wave (Vale *et al*., 2010). Due to global warming, heat waves have been predicted to increase in frequency, duration and intensity (Robinson, 2001) and hence poultry production could be affected. Indeed, despite the installation of fans and foggers to assist with temperature regulation inside commercial broiler houses, mortality of birds can still occur during a heat wave (Vale *et al*., 2010). Furthermore, in many regions of the world broilers are still kept indoors in systems which do not have anything but basic environmental control. In Brazil for example, approximately 60% of the poultry produced are from non-climate controlled housing systems (Nääs *et al*.,

2010). Hence it might be expected that losses of birds during heat waves could be even higher under these conditions, especially in tropical countries.

An increase in CBT of 1^{o}C in a 3.6 kg broiler is equivalent to increased heat storage in the body tissues of 12.5 kJ (Maloney, 1998). If the increase in CBT is not curtailed somehow then the bird could die when CBT increases by 4^{o}C (DEFRA, 2005). Mortality of broilers exposed to a heat stress of 36^{o}C between day 35 and 42 of age was 43.3% greater than exposed to 31^{o}C or control birds kept at 21^{o}C (Quinteiro-Filho *et al*., 2010). Comparing birds in these two levels of heat stress, Quinteiro-Filho *et al*. (2010) reported that birds exposed to 36^{o}C had a greater increase in corticosterone level than control birds (147% versus 110%), and considered that this subsequently accounted for a decrease in relative spleen and thymus weight (lymphoid organs). However, in a follow-up study, using similar temperature levels for just one day, Quinteiro-Filho *et al*. (2012) found that heat stress lead to increased serum corticosterone levels but there was no effect on the lymphoid organs.

From the evidence provided above, it is clear that acute heat stress causes an increase in core and surface body temperature, respiratory rate, damage to skeletal muscle resulting in poor meat quality, corticosterone concentration and mortality accompanied with suppressed feed intake and body weight gain. Clearly heat stress is an issue for animal welfare as well as animal production. The effect of heat stress on broiler chickens is a function of the intensity and duration (Widowski, 2010) and, given that heat waves could become more common in the future (Robinson, 2001), this implies that broilers could experience more than one episode of heat stress during their growth cycle.

The aim of this chapter then was to investigate the impact of intensity and duration of episodic heat stress on the welfare of broiler chickens. To achieve this, two objectives were involved:

1) To investigate the stress responses of birds exposed to either a high intensity of heat stress for 3 hours or a moderate intensity of heat stress for 6 hours
2) To investigate whether repeat exposure to heat stress will help birds to cope with subsequent exposure to moderate heat stress.

7.2 Materials and methods

7.2.1 Ethical considerations

This experiment was conducted under Project Licence number PPL 60/4270 of the Animals (Scientific Procedures) Act 1986. Approval for the project was given at both national (Home Office) and local (Ethical Revenue Committee) level, and it was considered that overall the severity of this work was mild.

7.2.2 Experimental design

In this experiment, groups of broiler chickens were subjected to either high heat stress (HHS; 32^{o}C, 70% RH) for 3 hours, moderate heat stress (MHS; 30^{o}C, 70% RH) for 6 hours, or control conditions (20^{o}C, 50% RH) for 6 hours, each day for two consecutive days. In addition, a fourth treatment (namely MHS-2) investigated the effects of conditioning to MHS, so that birds were exposed to MHS (30^{o}C, 70% RH) for 6 hours for three consecutive days, 5 days beforehand (during that initial exposure, birds in the other three treatments were maintained at control levels of temperature and RH). Thus there were four treatments – control, HHS and MHS and MHS-2 (see Table 7.1 for summary). All birds arrived at the same age and were tested at same age, except that MHS-2 birds had already had some MHS.

In this experiment, MHS was extended for 6 h based on the previous experiment were birds exposed to MHS for 3 h had a change in CBT of 0.7^{o}C. Naturally, high temperatures could last for more than 3 h per day.

According to treatment, a controlled gradual increase and subsequent decrease in temperature/RH was achieved using a similar pattern of heat stress and the same climate chambers (one treatment per chamber) as that used in the previous experiment. Thus, on each day of heat stress, and according to treatment, the temperature and RH was gradually increased during a 1 hour period (‘step up’, ST), then held constant for a period of time three or six hours (‘heat stress’) and then gradually reduced over a period of 1 hour (‘step down’, SD) as shown in Table 7.1. Additional measures were taken pre heat stress (PrHS) and in the 1-hour post heat stress period (PHS).

Some 16 birds were allocated to each treatment, with four birds in each of four replicate pens in each climate chamber. Of these, 8 birds per treatment were subjected to brief anaesthesia when a temperature-ID chip was injected 3cm deep into the left breast muscle, as described in Section 7.2.3 below.

To achieve the first objective of this experiment data from control, MHS and HHS were involved, whereas for the second objective the treatments considered were control, MHS and MHS-2.

7.2.3 Description and history of the birds

A total of 64, male Ross 308 broiler chickens (age 21 days, liveweight of approx. 950-1000 g) were obtained from a commercial poultry farm (Oakland Farms Ltd, York, UK) and transported to Newcastle University. The birds had previously been given vaccinated against infectious bursal disease and infectious bronchitis at 7 and 14 days of age, respectively. On arrival to the laboratory, the birds were weighed individually and placed in a holding room littered with wood shavings (Goodwill's Wood Shavings and Timber Products Ltd, Ponteland, UK) 5 cm deep. On the next day, the birds were randomly selected and placed in groups of four in one of four pens in each of the four treatment rooms. Commercial feed (20% crude protein, 4% oil, 6% ash and 13MJ/kg ME from W.E. Jameson & Son Ltd, Masham, UK) and tap water was provided *ad libitum* from hoppers placed on the floor of each pen. Lighting conditions were 14L:10D with an intensity of 30 lux- during the light period.

7.2.4 Procedure for surgery

Seven days after arrival (28 days old, live weight of approx. 1.64 kg), 32 birds were randomly selected for implantation with a temperature-ID chip (identical to those used in the previous experiment). This procedure was undertaken under brief anaesthesia by mask induction of 8% sevoflurane vaporised in oxygen (1.5 litres per minute). The skin on the left breast muscle was disinfected with hibitone, and using the sterile applicator provided the chip was injected 3 cm deep into the left breast muscle. After this, 0.2 mg/kg of meloxicam was administered to the bird as a means of pain relief. Each 'chip' bird, as it was known, was then placed in a box to allow recovery, which was confirmed when they exhibited the righting reflex. The bird was then returned to its home pen. The experiment commenced 12 days after implantation of the temperature-ID chips, at a point when the birds were 40 days old and weighed approximately 2.8 kg.

Table 7.1: Experimental design showing the environmental conditions across the heat stress protocol

Treatment	Pre heat stress (PrHS)	Step up (ST), 1 h	Heat stress	Step down (SD), 1 h	Post heat stress (PHS), 1 h
Control	20^{o}C, 50% RH		20^{o}C, 50% RH (6 h)		20^{o}C, 50% RH
MHS	20^{o}C, 50% RH		30^{o}C, 70% RH (6 h)		20^{o}C, 50% RH
HHS	20^{o}C, 50% RH		32^{o}C, 70% RH (3 h)		20^{o}C, 50% RH
MHS-2	20^{o}C, 50% RH		30^{o}C, 70% RH (6 h)		20^{o}C, 50% RH

Control: no heat stress, standard environmental conditions for housing broilers at this age of 20^{o}C and 50% RH; MHS: moderate heat stress for 6 hours on Day 1 and 2; HHS: high heat stress for 3 hours on Day 1 and 2; MHS-2: moderate heat stress for 6 hours on Day 1 and 2, with the first exposure occurring 5 days beforehand, on Day -5.

Figure 7.1: Position of a temperature-ID chip in the breast muscle of a broiler bird after the experiment.

7.2.5 Data collection for measures of welfare

Table 7.2 presents a summary of timepoints showing when data were collected on Day 1 and Day 2.

7.2.5.1 Core body temperature (CBT) and surface body temperatures

On the two experimental days, the temperature–ID chips on all chip birds were scanned during PrHS, at the end of 3 and 6 h of heat stress and then during the PHS phase using a pocket reader (418-S53-B003-ENG Bio thermo reader, Digital Angel Corp, MN, USA). In addition, in half of the chip birds (birds in pens 1 and 4), core body temperature (CBT) was measured at the end of each hour of heat stress. The change in CBT (ΔCBT) was subsequently calculated by subtracting the CBT during the pre heat stress period (PrHS) from that recorded in subsequent phases. Surface body temperatures (SBT) from four different body parts, namely under the wing, the feet, cloaca and comb, were estimated using a surface laser temperature probe (Raytek 3V gun infra-red thermometer model- RAYMX2G, Berlin), according to Chen and White (2006).Surface body temperatures were measured only on the 1st day of heat stress at the same time when the temperature-ID chips were scanned (i.e. at PrHS, 3HS, 6HS and PHS) and not on the second day because of the time constraints of blood sampling on Day 2. Mean surface body temperature was subsequently derived by taking the average of the four body surface measures.

Table 7.2: Summary of data collected on body temperature and feed intake during the 2 day episodic heat stress experiment.

	Sampling point			
Day of experiment	**Age of bird (days)**	**Pre heat stress (PrHS)**	**Heat stress 3 or 6 hrs**	**Post heat stress (PHS)**
1	40	CBT, SBT, weight of feed provided	CBT, SBT, weight of feed leftover	CBT, SBT, weight of feed leftover
2	41	Blood, CBT, weight of feed provided	Blood, CBT, weight of feed leftover	Blood, CBT, weight of feed leftover

CBT = core body temperature, SBT = surface body temperature

7.2.5.2 Haematological parameters

Blood sampling was undertaken on Day 2, the 2nd day of exposure to heat stress. Blood was sampled from 4 birds per treatment (i.e. 1 bird from each replicate) on 4 different occasions as follows: the 1st blood sample was taken pre heat stress to serve as basal levels (PrHS), the 2nd at the end of 3HS, the 3rd at the end of 6HS and the 4th at the end of PHS. Thus, birds in the HHS treatment had blood sampled three times, namely PrHS, 3HS and PHS, while birds in the other three treatments were sampled four times, namely PrHS, 3HS, 6HS and PHS.

Veterinary advice cautioned against repeat blood sampling from the wing vein, due to the significant risk of haemorrhage. Hence a 22 gauge over needle IV catheter (Abbott Laboratories, Berkshire, UK) was inserted into the leg vein and fixed to the leg of the bird. At each sampling time, the injection cap of the catheter was removed and then blood was drawn into a syringe via the catheter. A 0.5 ml of blood sample was immediately transferred to an i- STAT CG8^{+}cartridge and then inserted into a Vet Scan i-STAT®1 blood analyser. Analysis was made of levels of blood partial pressure of CO_2 (pCO_2), partial pressure of oxygen (pO_2), pH, bicarbonates (HCO_3^-), ionized calcium (iCa), total carbon dioxide (TCO_2), sodium ion (Na^+), glucose and haematocrit.

7.2.5.3 Growth performance, feed intake and mortality

The production indices that were monitored were feed intake (during and after heat stress) and weight gain (see Table 7.2), along with any mortality. Water intake was also monitored during heat stress period. No incidence of feed or water spillage was observed during the experiment. Determination of all production indices was as follows:

1. Feed intake during heat stress = amount of feed provided prior to heat stress minus feed left in the tough at the end of heat stress.
2. Feed intake after heat stress = amount of feed consumed between the end of heat stress and the post heat stress phases.
3. Water intake = water supplied prior to heat stress minus water remaining at the end of heat stress.
4. Daily weight gain = (bodyweight on Day 2 minus bodyweight on Day 1) / 2
5. Percentage mortality during the 2 days heat stress experiment = (number of dead birds/treatment) / (total number of birds/treatment) × 100

7.3 Statistical analyses

All data collected were tested for normality using a Shapiro-Wilks test before the appropriate parametric analysis was undertaken. Analysis was undertaken using the statistical software from SPSS (version 19, Rothampstead, UK) and multiple comparisons between treatments were adjusted for using the Bonferroni correction.

To analyse for the effect of different intensities of heat stress for 3 hours, data on core and change in core body temperature (CBT and ΔCBT respectively), surface body temperatures (SBT) and blood parameters at the end of the 3 h period were subjected to a general linear model (GLM) analysis with treatment (control, MHS or HHS) as a fixed factor. Based on the hourly CBT obtained from the birds from the first to the third hour of heat stress, a simple linear regression of ΔCBT and hour of heat stress was undertaken.

For the relative comparison of high heat stress for 3h (HHS) and moderate heat stress for 6 h (MHS), data on CBT and ΔCBT, SBTs and blood parameters (HHS for 3 h with those of control and MHS for 6 h) were subjected to a GLM analysis with treatment (control, HHS or MHS) as a fixed factor. To achieve the second objective, for comparison between birds exposed to moderate heat stress for the second time and another group of birds exposed to moderate heat stress for the first time (thus MHS-2 versus MHS, respectively), data on CBT, ΔCBT, SBTs and blood parameters at the end of 6 h of heat stress were subjected to a GLM analysis with treatment (control, MHS or MHS-2) as a fixed factor.

Any significant differences between treatments were separated using Least Significant difference (LSD).

7.4 Effect of intensity of heat stress on welfare and productivity of broiler chickens over a 3 h period

7.4.1 Core body temperature

The intensity of heat stress had a significant effect on CBT ($F_{2, 21}= 41.38$, $P<0.001$), so that CBT was greater in birds exposed to HHS than to MHS, which in turn was significantly greater than control birds (Figure 7.2). The intensity of heat stress also had a significant effect on ΔCBT ($F_{2, 21}= 46.15$, $P<0.001$), with ΔCBT greater in birds

exposed to HHS than those exposed to MHS which in turn was greater than control birds (ΔCBT values are given in each corresponding absolute CBT bar in Figure 7.2).

For birds exposed to 32°C and 70% RH, the equation predicting the change in CBT over time is ΔCBT= 0.917 + 0.663 × hours (P<0.05), whereas for birds exposed to 30°C and 70% RH, the equation predicting the change in CBT over time is ΔCBT= 0.371 + 0.338 × hours (P<0.05). Using the ΔCBT of 4°C as the end point according to DEFRA (2005), it implies that birds exposed to heat stress of 32°C and 70% RH or 30°C and 70% RH would reach their end point after approximately 5 or 11 h respectively.

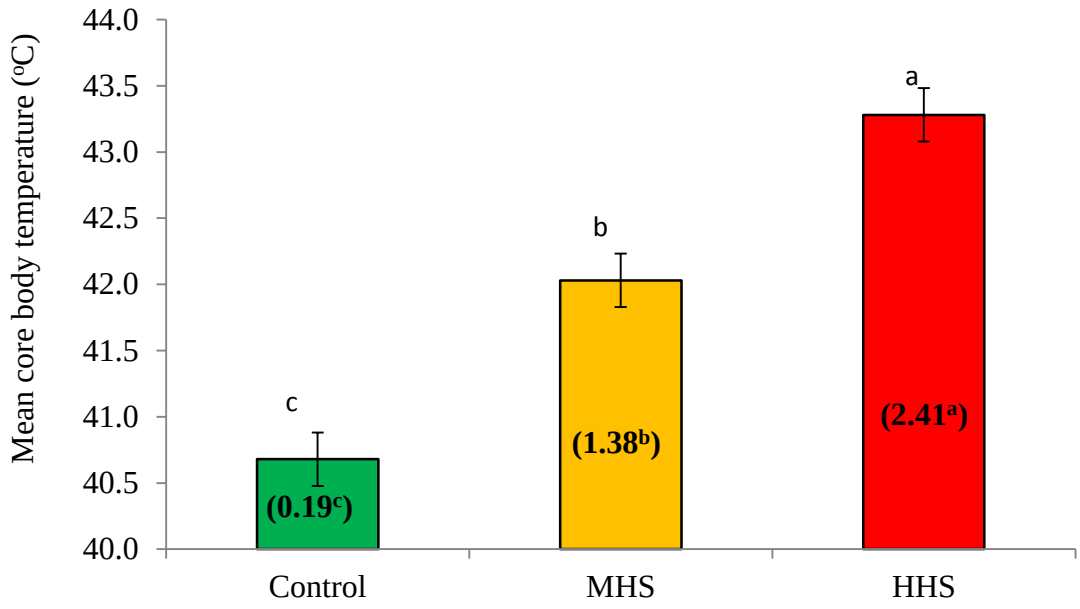

Figure 7.2: Effect of intensity of heat stress on core body temperature and change in core body temperature (values shown in bold, in brackets) of broiler chickens after 3 hours exposure to one of three environmental conditions. Values are Means ±1 SEM, n=8 birds/treatment. [abc] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress (30°C, 70% RH), HHS = high heat stress (32°C, 70% RH).

7.4.2 Surface body temperature at the end of 3 hours of exposure

The intensity of heat stress had a significant effect on wing ($F_{2, 21}$= 46.77, P < 0.001), feet ($F_{2, 21}$= 371.10, P <0.001), comb ($F_{2, 21}$= 206.27, P < 0.001) and cloaca ($F_{2, 21}$= 72.95, P < 0.001) SBT, as shown in Figure 7.2a. All four surface body temperatures were greater in birds exposed to HHS than in birds in MHS, which in turn were greater than in control birds. Similarly, intensity of heat stress had a significant effect on the

mean of the four body surfaces ($F_{2, 21}$= 214.014, P<0.001), so that MSBT was greater in birds exposed to HHS than those exposed to MHS, which in turn was greater than control birds (Figure 7.3 b).

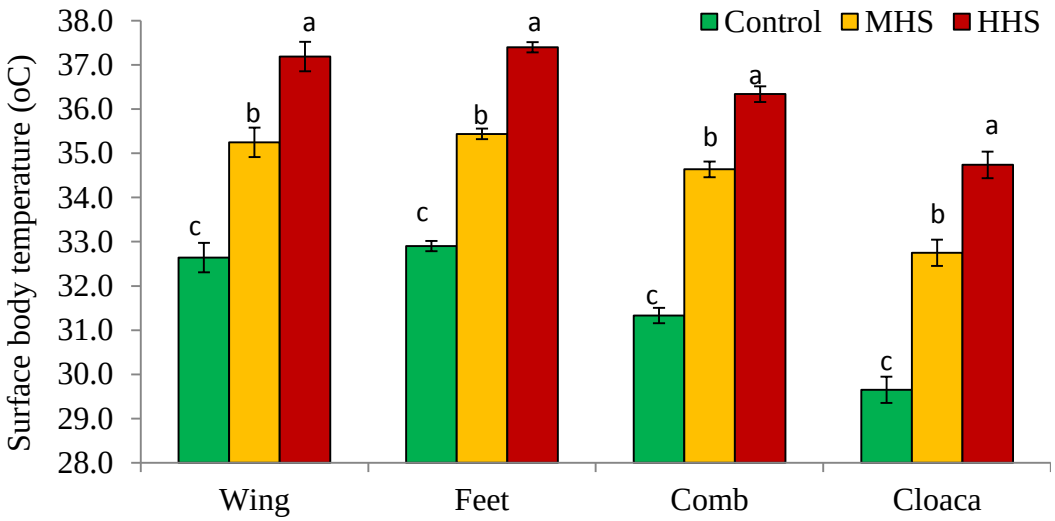

Figure 7.3a: Effect of intensity of heat stress on surface body temperature of broiler chickens at the end of 3 hours exposure to one of three environmental conditions. Values are means ±1 SEM, n=8 birds/treatment. [abc] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress (30°C, 70% RH), HHS = high heat stress (32°C, 70% RH).

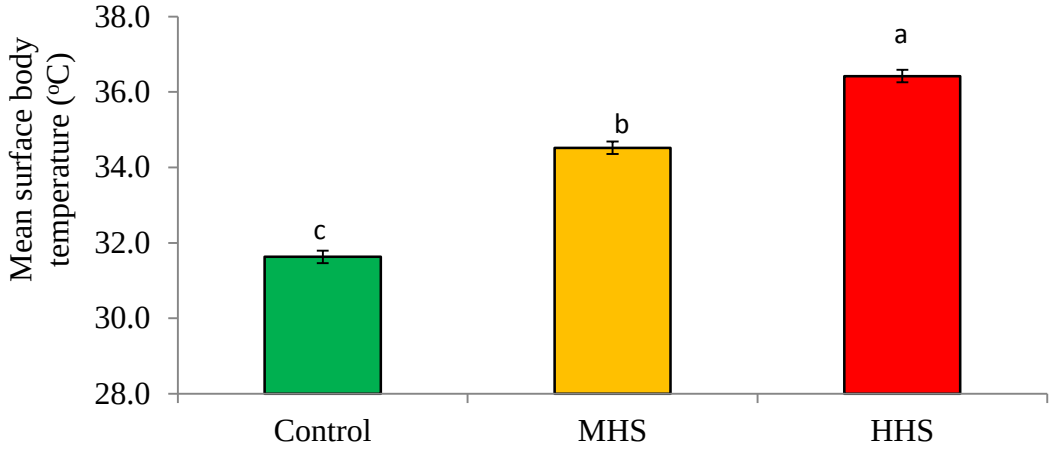

Figure 7.3b: Effect of intensity of heat stress on mean surface body temperature of broiler chickens at the end of 3 hours exposure to one of three environmental conditions. Values are means ±1 SEM. MHS - moderate heat stress and HHS- high heat stress. n= 8 birds/treatment. [abc] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress (30°C, 70% RH), HHS= high heat stress (32°C, 70% RH).

7.4.3 Blood parameters at the end of 3 hours of exposure

Although four birds per treatment were sampled for blood, limitations of the blood gas analyser meant that results are only available for three samples per treatment as shown in Table 7.3. Intensity of heat stress had a significant effect on blood pH ($F_{2, 6} = 10.23$, $P<0.05$), pCO_2 ($F_{2, 6} = 16.62$, $P < 0.05$), iCa ($F_{2, 6} = 12.32$, $P<0.05$), HCO_3^- ($F_{2, 6} = 6.43$, $P<0.05$) and total carbon dioxide ($F_{2, 6} = 6.20$, $P<0.05$). However, intensity of heat stress had no effect on levels of blood glucose or haematocrit. In the six blood parameters which were affected by the intensity of heat stress, there was no significant difference between the HHS and MHS.

Table 7.3 Effects of intensity of heat stress on blood parameters of broiler chickens at the end of 3 hours exposure to one of three environmental conditions. Values are means ± SEM (n=3 birds per treatment)

Blood parameter	Control	MHS	HHS	P value
Blood pH	7.45±0.02^b	7.53±0.02^a	7.53±0.02^a	*
pCO_2 (mmHg)	39.23±1.54^a	29.03±1.54^b	27.83±1.54^b	*
Ionized calcium, iCa (mmol/l)	1.43±0.02^a	1.31±0.02^b	1.37±0.02^{ab}	*
Bicarbonates, HCO_3^- (mmol/l)	27.00±0.74^a	24.07±0.74^b	23.50±0.74^b	*
Total CO_2, TCO_2 (mmol/l)	28.33±0.86^a	25.00±0.86^{ab}	24.33±0.86^b	*
Sodium ion, Na^+ (mmol/l)	141.33±0.82	142.33±0.82	144.33±0.82	NS
Blood glucose (ng/dl)	254.00±13.05	250.33±13.05	263.67±13.05	NS
Haematocrit (% PCV)	20.67±1.75	21.00±1.75	20.68±1.75	NS

*Significant treatments effect at $P<0.05$. ^{ab} Means with different superscript across each row differ at $P<0.05$. MHS = moderate heat stress (30^{o}C, 70% RH), HHS= high heat stress (32^{o}C, 70% RH).

7.4.4 Feed intake at the end of 3 hours of heat stress

Feed intake was significantly suppressed in birds exposed to MHS compared to control birds ($F_{2, 9} = 4.41$, $P<0.05$), whilst the feed intake of birds in HHS was intermediate, as shown in Figure 7.4.

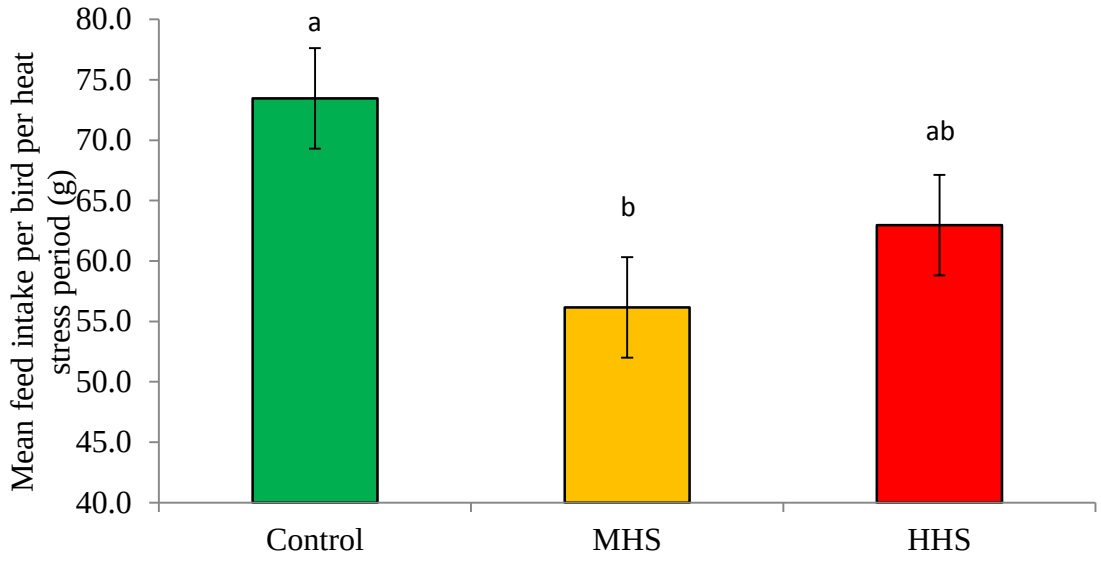

Figure 7.4: Effect of intensity of heat stress on feed intake of broiler chickens during the 3 hours of exposure to one of three environmental conditions. Values are means ±1 SEM, n= 4 pens/treatment. [ab] Means with different letters are significantly different at P<0.05. MHS = moderate heat stress (30^{o}C, 70% RH), HHS= high heat stress (32^{o}C, 70% RH).

7.5 Comparison of the stress response of broiler chickens exposed to high heat stress for 3 h (HHS) or moderate heat stress for 6 h (MHS)

7.5.1 Core and change in core body temperature

The CBT of broiler chickens in HHS and MHS was greater than in control birds ($F_{2,21}$= 31.81, P<0.001), whilst intensity of heat stress had no effect on CBT, see Figure 7.5. Similarly, ΔCBT of broilers subject to HHS and MHS was greater than in control birds ($F_{2,21}$= 34.79, P<0.001), whilst intensity of heat stress had no effect on ΔCBT (ΔCBT values are inserted in each bar in Figure 7.5).

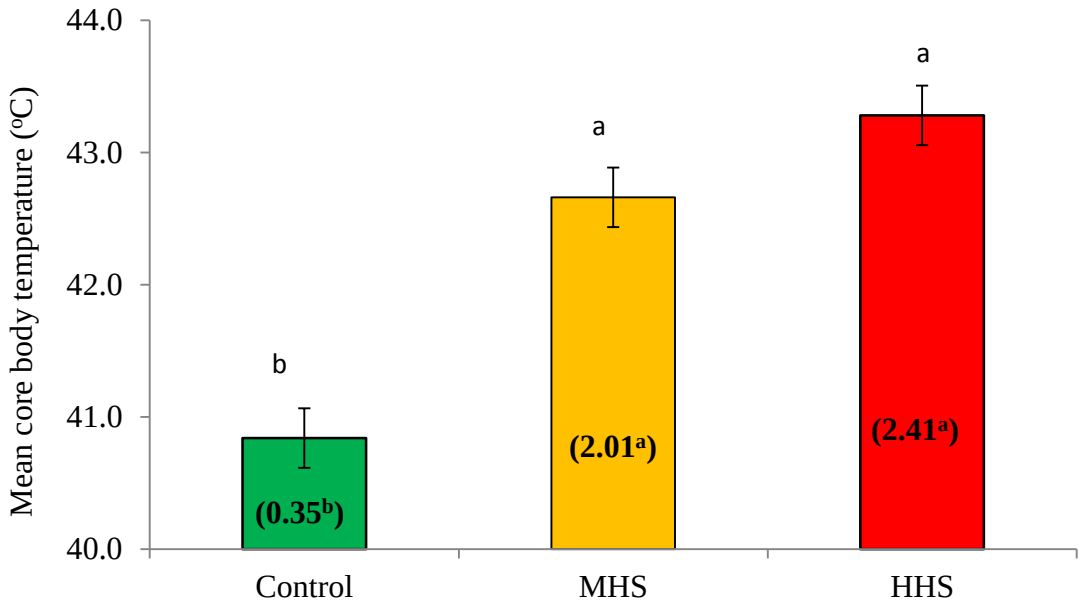

Figure 7.5: Effect of high heat stress for 3 hours and moderate heat stress for 6 hours on core and change in core body temperature (change in core body temperature between the pre heat stress and the end of 3 hours for HHS and 6 hours for MHS and control are presented inside each bar for the respective treatment, shown in bold in brackets). Values are means ±1 SEM, n= 8 birds/treatment. [ab] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress (30°C, 70% RH), HHS = high heat stress (32°C, 70% RH).

7.5.2 Surface body temperature

The wing ($F_{2, 21}$= 14.44, P<0.001) and cloaca ($F_{2, 21}$= 26.07, P<0.001) temperatures were greater in birds exposed to MHS and HHS than in the control birds. However, feet ($F_{2, 21}$= 117.17, P<0.001), comb ($F_{2, 21}$= 52.40, P<0.001) and mean surface body temperature ($F_{2, 21}$= 90.45, P<0.001) were greater in birds exposed to HHS than to MHS, which in turn were greater than the control birds (Figure 7.6a and 7.6b).

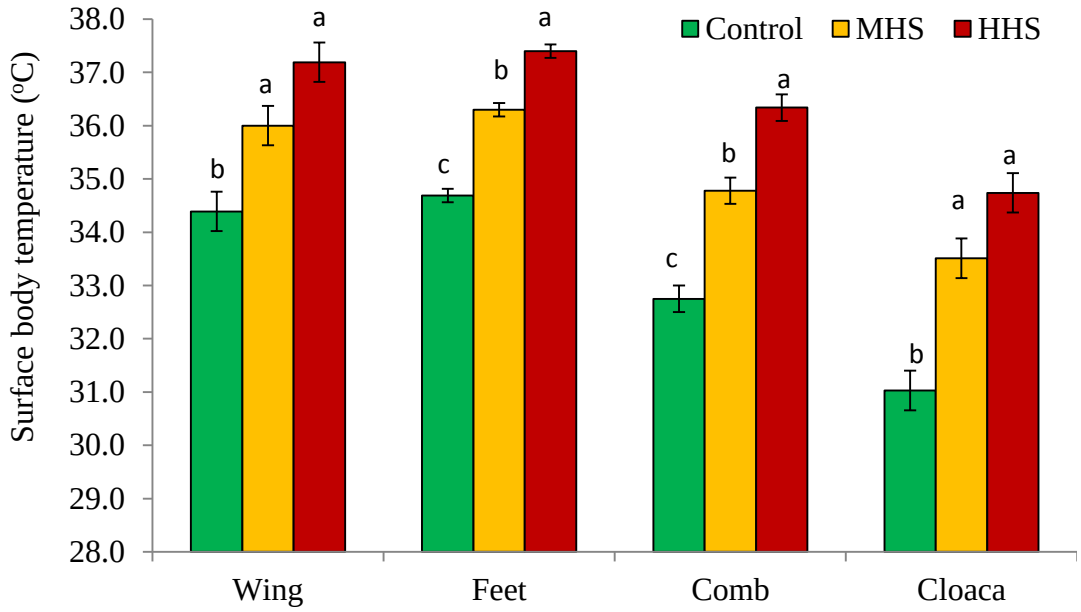

Figure 7.6a: Effect of high heat stress for 3 hours and moderate heat stress for 6 hours on the surface body temperature of broiler chickens. Values are means ±1 SEM, n = 8 birds/treatment. [abc] Means with different letters for each body surface are significantly different at P<0.001. MHS=moderate heat stress (30°C, 70% RH), HHS= high heat stress (32°C, 70% RH).

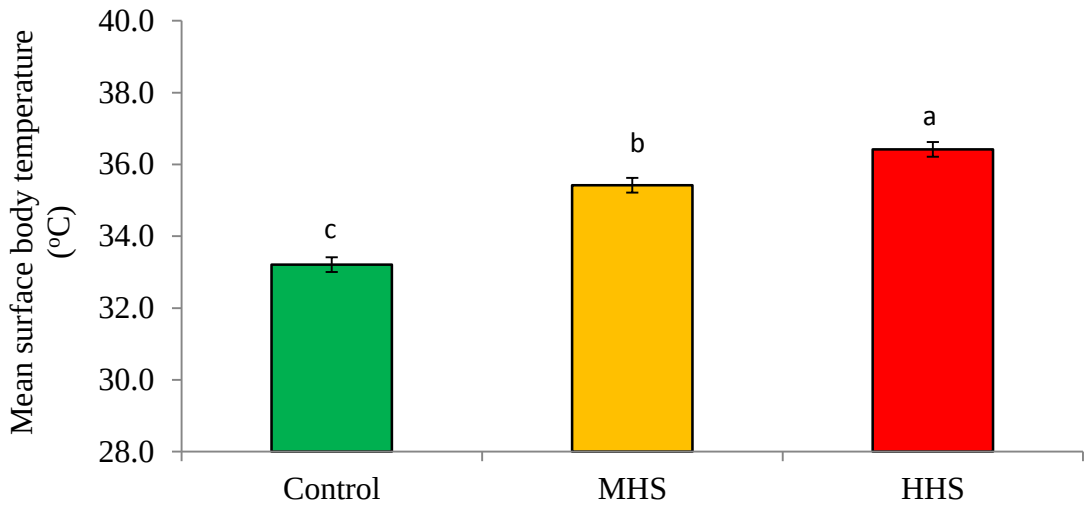

Figure 7.6b: Effect of high heat stress for 3 hours and moderate heat stress for 6 hours on the mean surface body temperature of broiler chickens. Values are means ±1 SEM, n=8 birds/treatment. [abc]Means with a different letters are significantly different at P<0.001. MHS = moderate heat stress (30°C, 70% RH), HHS = high heat stress (32°C, 70% RH).

7.5.3 Blood parameters

The level of pCO_2 was greater in HHS birds than in control birds; however there was no difference in the levels of pCO_2 between HHS and MHS birds ($F_{2, 7} = 7.65$, $P<0.05$). There was no significant effect of treatment on the other blood parameters (Table 7.4).

Table 7.4: Effect of high heat stress for 3hours and moderate heat stress for 6 hours on blood parameters. Blood samples from the control birds were taken at the end of the 6 hour period. Values are means ± SEM; n: control = 4 birds, MHS = 3 birds and HHS = 3 birds.

Blood parameter	**Control (6 h)**	**MHS (6 h)**	**HHS (3 h)**	**P value**
Blood pH	7.5±0.02	7.5±0.02	7.5±0.02	NS
pCO_2 (mmHg)	38.7±1.83 [b]	32.8±2.18[ab]	27.8±2.18 [a]	*
Ionized calcium, iCa (mmol/l)	1.4±0.02	1.4±0.03	1.4±0.03	NS
Bicarbonates, HCO_3^- (mmol/l)	27.3±0.88	25.6±1.02	23.5±1.02	NS
Total CO_2, TCO_2 (mmol/l)	28.5±0.97	26.7±1.12	24.3±1.12	NS
Sodium ion, Na^+ (mmol/l)	143.3±0.66	142.3±0.76	144.3±0.76	NS
Blood glucose (ng/dl)	236.3±14.44	283.3±16.67	263.7±16.67	NS
Haematocrit (% PCV)	23.0±1.54	22.0±1.78	20.7±1.78	NS

[ab] Means with different superscript across a row are significantly different at $P<0.05$, NS= not significant.

7.5.4 Growth performance, feed intake and mortality

Both feed intake and daily weight gain were reduced in birds exposed to heat stress (($F_{2, 9} = 37.121$, $P<0.001$and $F_{2, 43} = 19.38$, $P<0.001$, respectively), as shown in Figure 7.7a and 7.7 b respectively. There was no difference in feed intake or daily weight gain between birds in MHS or HHS treatments. On the 2nd hour of heat stress exposure on the 2nd day of the heat stress, 2 birds from the HHS treatment died, which amounts to 12.5% mortality.

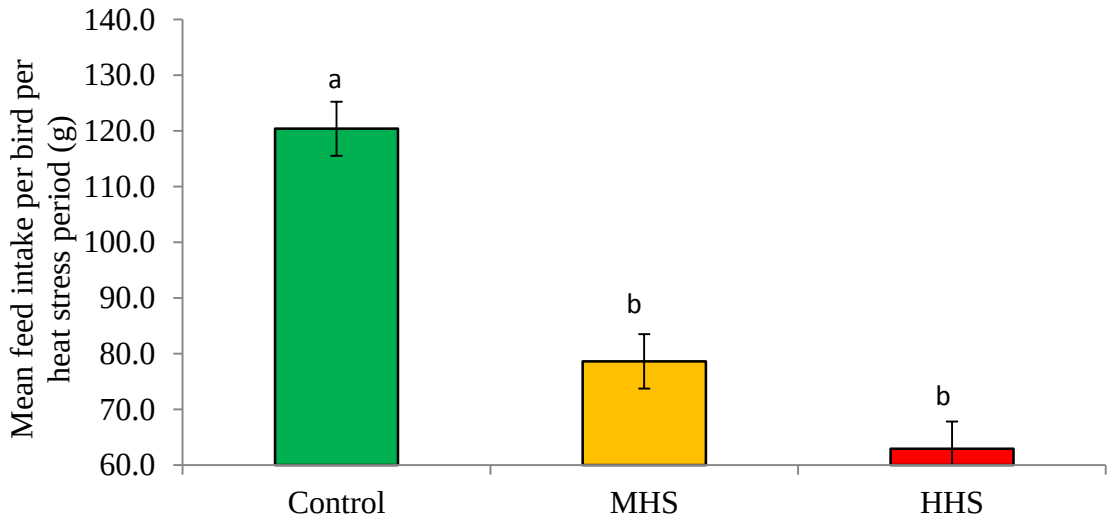

Figure 7.7a: Effect of high heat stress for 3h and moderate heat stress for 6h on mean feed intake of broiler chickens during heat stress period. Data for control birds was after 6 h. Values are means ±1 SEM, n = 4 pens per treatment. [ab] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress (30^{o}C, 70% RH), HHS = high heat stress (32^{o}C, 70% RH).

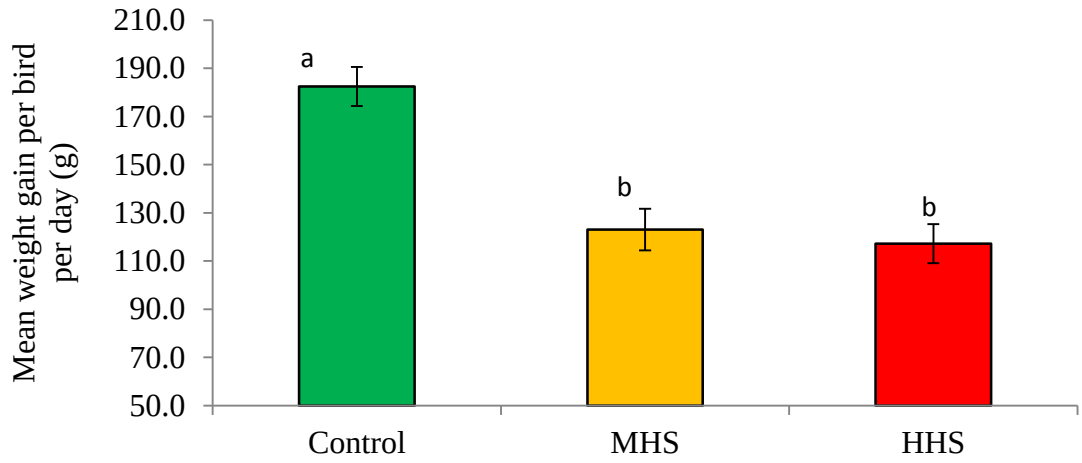

Figure 7.7b: Effect of high heat stress for 3h and moderate heat stress for 6h on mean daily weight gain of broiler chickens. Control (n=16), MHS (n=16) and HHS (n= 14). Values are means ±1 SEM. [ab] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress (30^{o}C, 70% RH), HHS = high heat stress (32^{o}C, 70% RH).

7.6 Comparison of stress responses of birds exposed to moderate heat stress for the second or first time

7.6.1 Core and surface body temperature at the end of 6 hours of moderate heat stress

Both CBT and ΔCBT were greater in birds exposed to MHS and MHS-2 than in control birds ($F_{2, 21}$= 52.99, P<0.001 and $F_{2, 21}$= 47.393, P<0.001, respectively), as shown in Figure 7.8a and 7.8 b respectively. CBT and ΔCBT were similar between birds exposed to MHS or MHS-2 (ΔCBT values are in bold and bracketed in each bar, Figure 7.8a).

The number of exposures to MHS had a significant effect on mean wing ($F_{2, 21}$= 4.69, P<0.05), feet ($F_{2, 21}$= 25.49, P<0.001), comb ($F_{2, 21}$= 12.08, P<0.001) and cloaca ($F_{2, 21}$= 5.51, P<0.05) SBT, as shown in Figure 7.8b. Feet and comb temperatures were greater in birds in MHS than those in MHS-2, which in turn were greater than for the control birds. Wing and cloaca temperatures however were greater in MHS birds than in control birds, but were similar between MHS and MHS-2 birds. The number of exposures to moderate heat stress also had an effect on MSBT ($F_{2, 21}$= 22.48, P<0.001), whereby MSBT was greater in birds exposed to MHS than those exposed to MHS-2, which was in turn higher than control birds (Figure 7.8c).

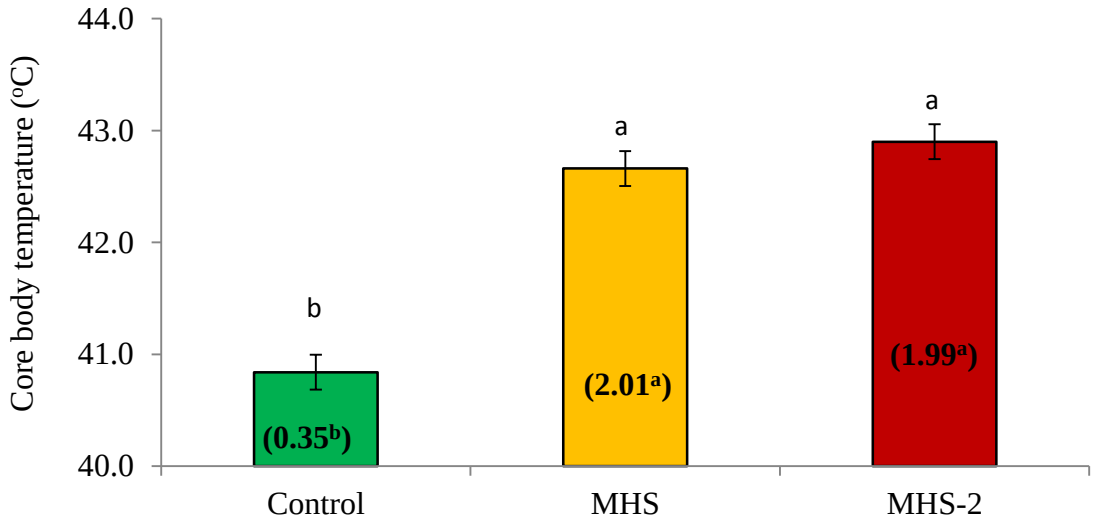

Figure 7.8a: Effect of moderate heat stress on core and change in core body temperature of broiler chickens (change in core body temperature between the pre heat stress and the end of 6 h are presented inside each bar for the respective treatment in brackets). Values are means ±1 SEM, n = 8 birds/treatment. [ab] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress for the first time, MHS-2 = moderate heat stress for the second time, some 5 days after first exposure to MHS.

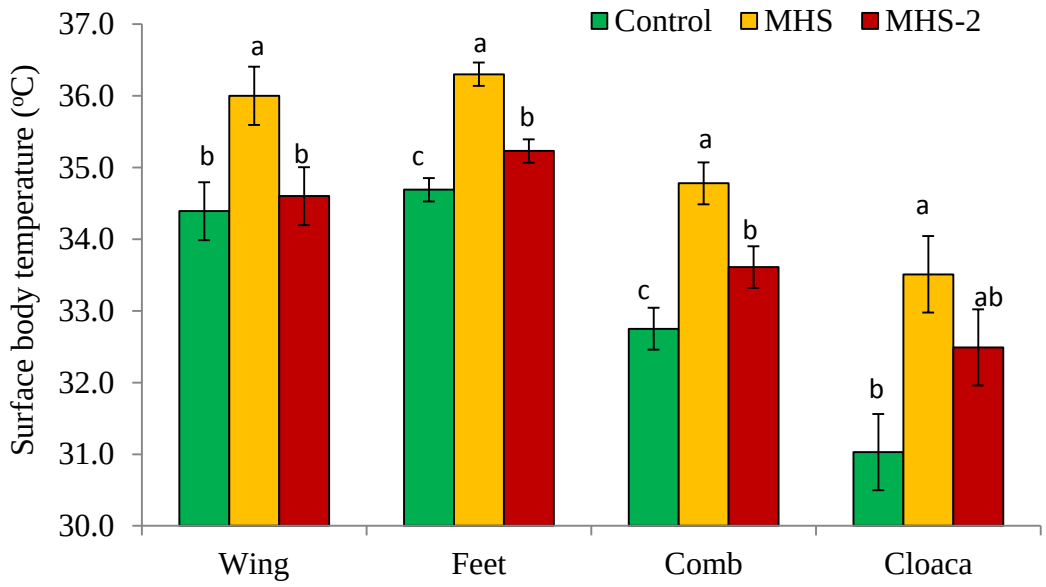

Figure 7.8b: Effect of moderate heat stress surface body temperature of broiler chickens at the end of 6 h. Values are means ±1 SEM, n = 8 birds/treatment. [abc] Within each surface body temperature, means with a different letter are significantly different at P<0.05. MHS = moderate heat stress for the first time, MHS-2 = moderate heat stress for the second time, some 5 days after first exposure to MHS.

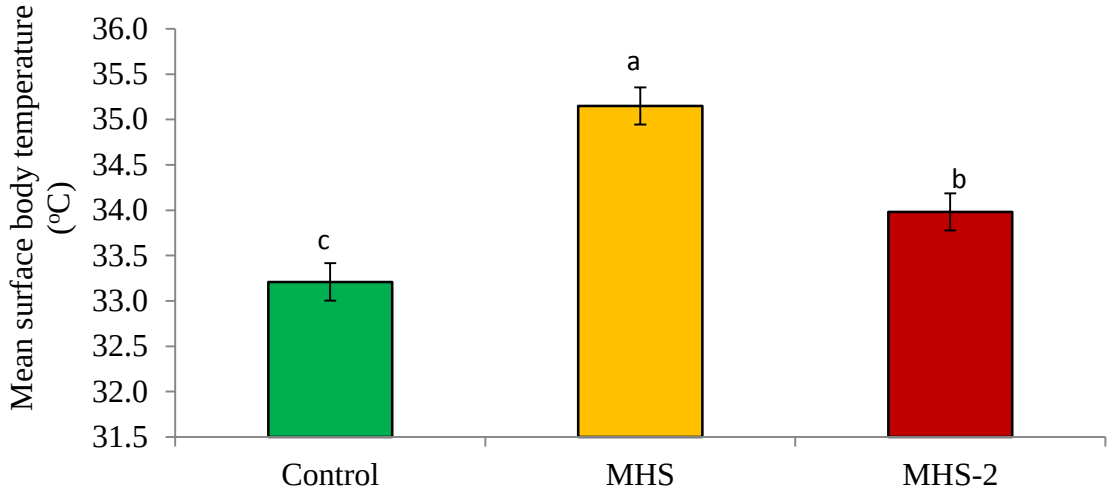

Figure 7.8c: Effect of moderate heat stress on mean surface body temperature of broiler chickens at the end of 6 h. Values are means ±1 SEM, n = 8 birds/treatment. [abc] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress for the first time, MHS-2 = moderate heat stress for the second time, some 5 days after first exposure to MHS.

7.6.2 Blood parameters at the end of 6 hours of moderate heat stress

None of the blood parameters were significantly affected by repeated exposure to MHS, as shown in Table 7.5.

7.6.3 Growth performance and feed intake

Feed intake was higher in control birds than in MHS-2 birds, which in turn was higher than in MHS birds ($F_{2, 9} = 35.54$, $P<0.001$), as shown in Figure 7.9a. Daily weight gain was significantly greater in control birds compared to MHS birds, with an intermediate value for MHS-2 birds ($F_{2, 45} = 15.30$, $P<0.001$; see Figure 7.9b.

Table 7.5: Effect of repeat exposure to moderate heat stress on blood parameters of broiler chickens at the end of 6 h. Values are means ±1 SEM; n: control = 4 birds, MHS = 3 birds and MHS-2 = 4 birds

Parameters	**Control**	**MHS**	**MHS-2**
Blood pH	7.46±0.03	7.50±0.03	7.46±0.03
pCO_2 (mmHg)	38.70±3.06	32.77±3.53	36.10±2.74
Ionized Calcium, iCa (mmol/l)	1.43±0.03	1.39±0.03	1.38±0.03
Bicarbonate, HCO_3^- (mmol/l)	27.33±1.18	25.57±1.36	25.00±1.05
Total CO_2, TCO_2 (mmol/l)	28.50±1.33	26.67±1.54	26.00±1.19
Sodium ion, Na^+ (mmol/l)	143.25±0.99	142.33±1.15	142.00±0.89
Blood glucose (ng/dl)	236.25±19.68	283.33±22.72	273.20±17.60
Haematocrit (% PCV)	23.00±1.60	22.00±1.85	22.00±1.43

MHS = moderate heat stress for the first time, MHS-2 = moderate heat stress for the second time, some 5 days after first exposure to MHS.

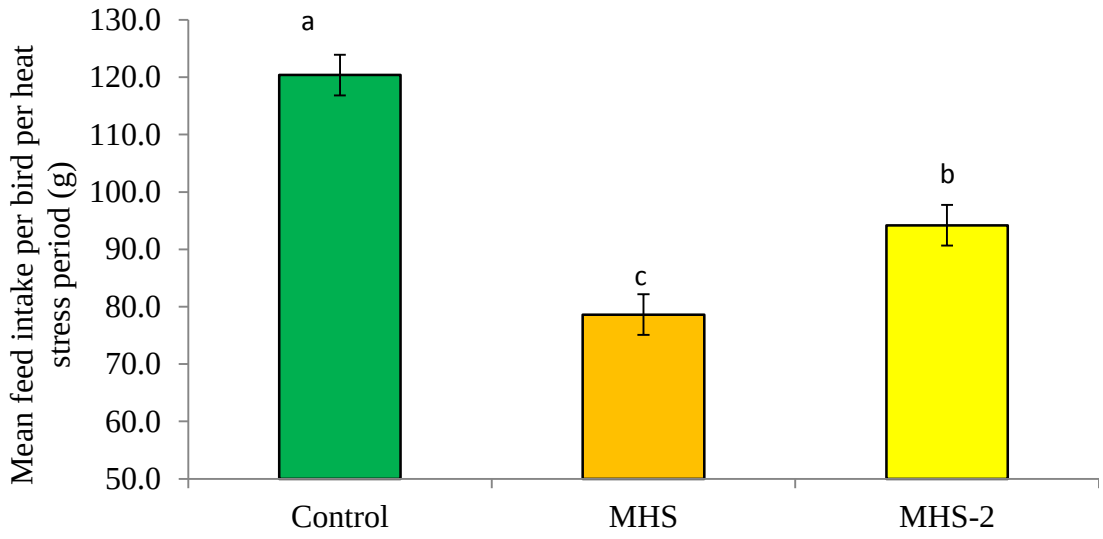

Figure 7.9a: Effect of repeat exposure to moderate heat stress on the feed intake of broiler chickens during the 6 h period of exposure to moderate heat stress. Values are mean ± 1 SEM, n=4 pens/treatment. [abc] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress for the first time, MHS-2 = moderate heat stress for the second time, some 5 days after first exposure to MHS.

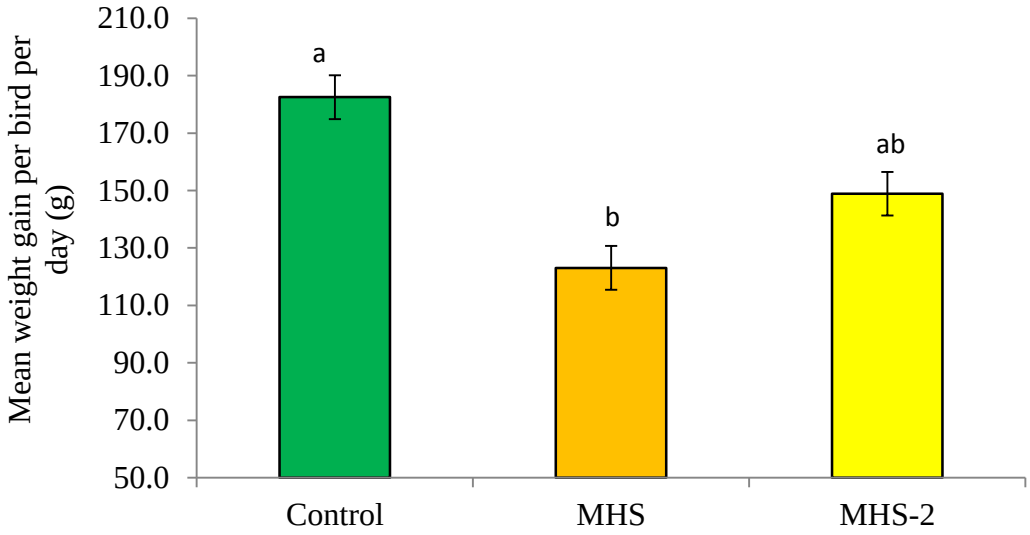

Figure 7.9b: Effect of moderate heat stress on weight gain of broiler chickens. Values are mean ± 1 SEM, n=16 birds/treatment. [ab] Means with different letters are significantly different at P<0.001. MHS = moderate heat stress for the first time, MHS-2 = moderate heat stress for the second time, some 5 days after first exposure to MHS.

7.7 Discussion

The aim of this study was to investigate the impact of intensity and duration of episodic heat stress on the welfare of broiler chickens. To achieve this, two objectives were involved:

1) To investigate the stress responses of birds exposed to either a high intensity of heat stress for 3 hours or a moderate intensity of heat stress for 6 hours
2) To investigate whether repeat exposure to heat stress will help birds to cope with subsequent exposure to moderate heat stress.

7.7.1 Effect of intensity and duration of heat stress on broiler chickens

The two heat stress intensities used in this experiment both caused a significant increase in the CBT of broiler chickens, indicating a condition of thermal discomfort i.e. increased heat storage within the body, which can lead to hyperthermia and death. Thermal imbalance in these birds meant that heat production was greater than heat loss, and so this excess heat stored in the body causes the CBT to rise (Lin *et al*., 2004). A greater intensity of heat stress (HHS) caused a greater change in CBT (1^{o}C more than moderate intensity of heat stress, MHS), therefore indicative of greater heat storage in birds exposed to HHS. In this study, broilers subjected to HHS or MHS for 3 h experienced a rise in CBT greater than 1^{o}C, a threshold which was considered to be indicative of severe heat stress in broilers (Mitchell and Kettlewell, 1998). In laying hens, an increase in CBT of 1^{o}C and 2^{o}C was considered to be moderate and severe heat stress respectively (Wolfenson *et al*., 1981). Compared to birds having a 1^{o}C rise in CBT, birds enduring a 2^{o}C rise in CBT would not have experienced a further increase in capillary blood flow to the digestive organs, but rather a further increase in capillary blood flow to the wattle and trachea (Wolfenson *et al*., 1981). Such changes in the relative capillary flow to different parts are to aid dissipation of heat from the respiratory tract. Therefore it might have been expected in the current study that feed intake in birds exposed to high heat stress would be lower than those exposed to moderate heat stress at the end of 3h, as the birds sought to minimise metabolic heat output, but that this was not seen.

In the current study, broilers exposed to HHS treatment for 3 h had their CBT elevated by 2.4^{o}C, which is similar to that obtained by Sandercock *et al*. (2006) who found a ΔCBT of 2.3^{o}C after exposing 35 day old (2.27 kg) broilers to a single day of acute heat

stress (32°C, 75% RH, for 2 h). However, Sandercock *et al*. (2006) withdrew feed from the birds during the heat stress period, whereas in the present study birds had continuous access to feed. Withdrawal of feed from birds during a period of heat stress has been reported to reduce core body temperature (Ahmad *et al*., 2006), probably because of the reduced amount of heat that must be dissipated. Increase in core body temperature is associated with a significant increase in plasma creatinine kinase levels, indicative of skeletal damage which could subsequently affect meat quality (Sandercock *et al*., 2001).

With further exposure to MHS for 6 h, the ΔCBT of the birds in MHS treatment was comparable to that of birds exposed to HHS for 3 h. This suggests that the greater the intensity of heat stress the shorter the duration necessary for it to reach a lethal point. Thus birds subjected to sudden high heat stress rather than moderate heat stress may find it difficult to cope thus leading to mortality. On the second day of heat stress, two birds from the HHS treatment died at the end of the second hour of exposure to high heat stress. The two birds which died each had experienced a substantial rise on CBT (individual ΔCBT was 3.4 and 4.7°C just immediately after death), averaging 4.1°C which is in keeping with the rise in CBT reported to be lethal to broilers (DEFRA, 2005). Hence, it seems that the temperature-ID chips could give a reliable measure of ΔCBT under heat stress conditions. Building on the linear regression of change in core body temperature against duration of heat stress reported in Section 7.4.1 to detect the point at which a 4°C increase in core body temperature would be seen (the lethal ΔCBT of birds exposed to heat stress, DEFRA, 2005), then death from hyperthermia could be expected after 5 hours exposure to moderate heat stress or 11 hours exposure to high heat stress. Information such as this relating the intensity and duration of degree of heat stress to rise in core body temperature and the likely time when there is a risk of mortality could enable poultry farmers to better understand the need for alleviation to be provided. Hegeman (2011) reported that during a heat wave in the USA, a power shortage for just 45 minutes resulted in the death of 50,000 broilers in a single farm, although there was no report on the total number of birds on the farm. The failure of important organs such as the heart, lungs, liver and kidney to cope with the challenge of maintaining homeostasis results in heat stroke (Aengwanich and Simaraks, 2004), since in broilers typically consideration was not given to the capacity of these organs during the process of genetic selection for improved growth performance (Havenstein *et al*., 2003).

In the UK, broilers may experience acute heat stress during the 3 h of transportation to the slaughterhouse (Nicol and Scott, 1990). If acute heat stress is experienced during transportation, then closer proximity of the rearing sites to the slaughterhouse could save birds from prolonged exposure to stress and resulting mortality. Alternatively, air-conditioned vehicles may be necessary to transport birds from sites positioned some distance from the slaughterhouse, perhaps for reasons of disease control. There could be variations in the distance through which birds have to be transported in different countries and some countries may not even have regulations relating to the maximum transportation time for birds. In one study, mortality of broilers during transportation to the slaughterhouse increased in longer journeys such that compared to a standard journey of 50 km, transport of distances over 300 km resulted in a four-fold increase in mortality rate (0.15 versus 0.86% respectively; Vecerek *et al*., 2006). Assuming the transport truck travels at 50 km/h, then a distance of 300 km will be covered in approximately 6 h. Hence, transportation of broilers for 6 h under conditions of high heat stress as used in the current study, along with the increased stocking density of birds in the transport crate thereby limiting the opportunity for dissipation by conduction and convection would further increase the risk of mortality that could be experienced in broilers reared in tropical regions of the world.

Regarding the surface body temperature at the end of 3 hours of exposure to two intensities of heat stress, all four surface body temperatures (wing, feet, cloaca and comb) were greater in birds exposed to high heat stress than those exposed to moderate heat stress. This result corresponds to the greater ΔCBT of birds in these conditions. Vasodilation occurs mainly in the unfeathered parts of the body, such as the feet, legs, comb and wattles, in order to assist heat loss. The feet have specialised features called arteriovenous anastomoses which help in heat dissipation to the environment (Etches *et al*., 2008). To achieve heat loss through this means, the arteriovenous anastomoses and core vein become constricted to prevent heat transfer from the incoming arterial blood to the core vein, so that heat carried by the arterial blood gets to the body surface through the already dilated peripheral veins (Willmer *et al*., 2005). Heat transferred to the feet can easily be lost through conduction to the litter in a deep litter system, whereas this avenue of heat loss is limited in a cage system where no litter is provided as occurs in many housing systems for laying birds.

The rate of heat transfer from the core to the surface is affected by the flow of blood to the body surface (Cangar *et al*., 2008). The regulation of blood flow to the skin is

controlled by the sympathetic nervous system and heat loss through the skin is dependent on increased blood flow to the skin and feather covering (Rastogi, 2007). Another heat exchange system is called the reteopthamicum which is situated between the optic cavity and the brain and helps to dissipate heat through the eye, mouth, beak and nose (Etches *et al*., 2008) and so to help control the amount of heat reaching the brain. Giloh *et al*. (2012) found a positive correlation between facial temperatures and CBT of birds under heat stress. The surface body temperatures estimated by a thermal image camera showed that the temperature of the comb, eye, ear, wattle and leg were greater than other body parts which are covered with feathers (Naas *et al*., 2010). Of the four surface body temperatures measured in the present study, the comb is the closest one to the brain and has a larger surface area for heat loss. At the end of 6 h of exposure to MHS, the wing and cloaca temperatures were comparable to that of birds exposed to HHS for 3h while the feet and comb temperatures were lower than those of HHS after 3 h. This implies a differential response of surface body temperature to an extended period of exposure to moderate heat stress, hence differences in the rate of heat loss through the different thermal windows.

Birds exposed to MHS for 6 h and HHS for 3 h had similar levels of ΔCBT and absolute wing and cloaca temperature, but absolute feet and comb temperatures were lower in birds exposed to MHS than those subject to HHS. This result suggests a reduction in heat transfer from the core to the feet and comb under conditions of prolonged moderate heat stress compared to the wing and cloaca areas (which became just as hot as they did under HHS). This finding is in agreement with Wolfenson *et al*. (1981), who reported that blood distribution to the comb reduced as the change in CBT increased from 1 to 2^{o}C, indicating the need to protect the brain from increased heat load. The rapid increase in comb temperature of birds in the HHS group suggests a lack of regulation of brain temperature and this might have contributed to mortality of the two birds in this treatment. Tolerance to increased CBT is related to the ability to keep the brain cool (Baker, 1982).

The mean surface body temperature of birds exposed to HHS for 3 hours was greater than that in birds exposed to MHS for 6 h, suggesting a rapid response needed for heat dissipation. A rise in surface body temperature has a great role to play in controlling the thermal status of an animal by assisting in sensible heat loss (conduction, convection and radiation), especially when there is a considerable thermal gradient between the animals' surface temperature and the environment (Cangar *et al*., 2008). This need for

greater heat loss was observed more in the HHS birds than those of extended periods of MHS. The elevated surface body temperature observed during heat stress is a cumulative effect of the high environmental conditions and increased blood flow to the body surface (Giloh *et al*., 2012). During heat stress, birds are faced with the situation of an increase in both core and surface body temperature, causing a reduction in the temperature gradient between the core and body surface and also between the body surface and the environment. Collectively, these changes reduce the opportunity for sensible heat loss through conduction, convection and radiation (Lin *et al*., 2005).

Although in this study the number of replicate blood samples was relatively low, nevertheless the results suggest that exposure to heat stress for 3 hours (either HHS or MHS) suppressed levels of pCO_2, iCa and HCO_3^- but increased blood pH. Hence both intensities of heat stress used in this study impaired the acid-base status of the blood compared with control birds. Changes in acid-base balance are indications of increased heat loss through evaporative means (Gous and Morris, 2005) arising from the increased respiratory rate required to achieve cooling. Although respiratory rate was not determined in the current study, the reduction in pCO_2 level and subsequent increase in pH could be indicative of increased respiratory rate (Sandercock *et al*., 2001) required for thermoregulation (Hocking *et al*., 1994). The reduction in levels of ionised calcium during heat stress has implications for the productivity of egg laying birds. A reduced level of blood ionised calcium in laying hens exposed to heat stress (35^{o}C, 50% RH) was attributed to reduced calcium uptake in the duodenal epithelial cells, which consequently resulted in the production of eggs with thin shells (Mahmoud and Yaseen, 2005). Such effects could be extended to broiler breeders, where the integrity of the shell is an important factor to safeguard the development of a healthy broiler chick. In the current study, changes in blood parameters were not further aggravated in birds exposed to the higher intensity of heat stress (HHS), despite the 1^{o}C increase in CBT. In comparison, a greater increase in serum corticosterone was observed in birds subjected to greater levels of heat stress (36^{o}C) compared to moderate level (31^{o}C), a 147% versus 110% increase respectively compared to the control birds (Quinteiro-Filho *et al*., 2012).

In the present study blood pH and pCO_2 were similar in birds exposed to HHS for 3 h and MHS for 6 h, thus suggesting that with a further exposure period of three hours to MHS, blood pH and pCO_2 levels were not further affected. Changes in the blood profile of birds in the present study could also indicate that the HHS treatment had a more pronounced effect on the birds than the MHS treatment. Both feed intake and weight

gain were reduced in birds exposed to either MHS or HHS conditions. Although the birds had opportunity to compensate for the reduced feed intake during the heat stress period, and they could have consumed more feed during the hours when temperature and RH were returned to normal, control levels, this did not happen. This implies that in commercially reared broilers, short term (3 to 6 h) heat stress for two days could slow down the rate of growth, hence extending the period required to reach market weight. If such a two day simulated episode of heat stress as used in the current study could suppress weight gain of 40 day old broilers, then broilers reared in countries with longer growing periods such as the USA and France (typically having a 70 day rearing period and to attain a heavier weight) (Piestun *et al*., 2013) are likely be even more affected by episodic heat stress.

In summary, broilers exposed to a period of high heat stress had difficulty coping with this stressful condition and regulating their core body temperature. This resulted in the death of two birds, reflecting that the impact of thermal stress on an animal is a combination of the intensity and duration of heat stress (Widowski, 2010).

7.7.2 Comparison of stress responses of birds exposed to MHS for the first or second time

At the end of 6 h of exposure to MHS, the stress responses of birds exposed to MHS for the second time (MHS-2) were similar to those exposed to MHS for the first time, in terms of the ΔCBT, cloaca surface body temperature and weight gain. However, wing, feet, comb and mean surface body temperature were lower at the end of 6 h for birds which had a prior exposure to moderate heat stress. Surface body temperature is dependent on ambient temperature and surface blood circulation (Abudabos *et al*., 2013). The current study shows that broilers, especially those that have reached market weight, did not respond differently to increased frequency of a heat wave which could amount into a further increase mortality of birds hence the need for an effective means of heat alleviation. However, as a strategy to, perhaps the three days of exposure to moderate heat stress, some five days before testing, used here was not sufficient to promote adaptation to high heat load. These findings confirm that acclimatisation requires a longer period of time, typically a period of seven days as reported elsewhere in the literature (Abdelqader and Al-Fataftah, 2014). Similarly, there is interest in increasing the thermotolerance levels of broilers to prepare them for future occurrences of heat waves by exposure to high temperature (and RH perhaps) between the period of

embryo genesis and 10 days of age because at this point brain development is still incomplete (Collin *et al*., 2007; Piestun *et al*., 2013).

7.8 Summary and conclusions

The results of this experiment showed that broiler chickens were more tolerant of moderate heat stress (30^{o}C, 70% RH) than high heat stress (32^{o}C, 70% RH), the latter which resulted in some mortality. However, moderate heat stress for 6 h or high heat stress for 3 h had a similar impact on both broiler welfare, in terms of a number of welfare indicators including change in core body temperature and selected blood parameters, as well as broiler performance, based on estimates of feed intake and weight gain. Mean surface body temperature however was elevated in birds kept under high heat stress birds at the end of 3 h. The impact of heat stress is highly dependent on its intensity and/or duration. Prior exposure of broilers aged approximately 40 days to a three-day episode of moderate heat stress did not change their stress response in a subsequent exposure to moderate heat stress, suggesting that there was no adaptation induced.

Chapter 8: Attempts to alleviate episodic moderate heat stress in broiler chickens through provision of additional cup drinkers

8.1 Introduction

The last two chapters have described the substantial impact that episodic heat stress can have on the behaviour, physiology and growth performance of broiler chickens. Therefore, it is appropriate to investigate measures that could be used to alleviate the effects of heat stress in broilers and so safeguard their welfare and growth performance. Elucidating means to alleviate heat stress is an important goal so that modern commercial broiler strains of temperate origin which have been genetically selected for high growth rate can cope with the challenges of heat stress and so remain in full health and remain productive, even under tropical conditions. Management strategies that have been used to alleviate heat stress in indoor-housed broilers include installation of fans in the building, as well as sophisticated climatic control and ventilation systems. These strategies are quite expensive (Kutlu and Forbes, 1993) and depend upon a constant power supply, reasons which limit their potential uptake in many tropical countries. There are a number of other possible strategies, however, which could be considered.

The provision of water-cooled perches to broiler chickens exposed to heat stress (32.6^{o}C) for 4 weeks enhanced heat loss through conduction so that both final bodyweight and total weight gain were greater than control birds which had access to ambient temperature perches (Reilly *et al*., 1991). Another natural way through which birds might cool themselves providing that a source of open water is readily available includes splashing water on their combs and wattles to enhance the rate of evaporative cooling from these surfaces (Dawson and Whittow, 2000). For every 1 g of water evaporated, 2.4 kJ of heat is absorbed from the respiratory surface which aids in heat dissipation from the body (Renaudeau *et al*., 2012). Other water-related behavioural responses to heat stress include increased water intake (May and Lott, 1992). In one particular study, the survival of birds exposed to a high heat stress of 40^{o}C and 30% RH for 3 hours was attributed to their increased water consumption (Bogin *et al*., 1996).

Comparing two commercially used drinker types, namely nipple and bell drinkers, Bruno *et al*. (2011) reported that total water intake was greater in broilers which had bell drinkers compared to those with nipple drinkers (28.1 ml vs 3.7 ml, $P<0.001$), within a 10-minute observation period because mean water intake per visit to the bell drinker was greater than that of the nipple drinker (0.86 ml versus 0.02 ml, $P<0.01$).

Since birds increase their water intake during heat stress, Pereira and Nääs (2008) undertook a behavioural study to determine the thermoneutral zone (TNZ) of broilers according to the frequency of drinking. Their results gave a significant regression equation (R=51.4%) that could be used to predict the frequency of the use of the drinker according to the prevailing environmental conditions of temperature and RH, such that an increase in the frequency of drinker use indicates environmental conditions above the TNZ. In another study, Pereira *et al*. (2007) found a negative correlation between increasing temperature/RH and the frequency of other behaviours, so that an increase in the frequency of feather ruffling, preening or wing opening behaviour could be indicative of being in thermally comfortable environmental conditions.

Collectively these studies emphasise the critical role of water in helping to maintain thermal balance (Bruno *et al*., 2011). Therefore measures should be put in place to ensure that broilers raised under conditions of heat stress have ready access and opportunity to consume a sufficient quantity of water to meet their thermoregulatory needs. However, commercial housing systems for broiler chickens favour drinkers that reduce water spillage, since this can lead to wet litter which in turn can compromise the welfare of the birds. However, with increasing poultry production across the world, especially of faster growing genotypes in tropical and subtropical regions, then development of an improved drinker that does not compromise litter quality might be a useful means of helping to alleviate heat stress.

Physiological parameters such as an increase in respiratory rate (Nascimento *et al*., 2012) and skin temperature (Yahav *et al*., 1995) are important indicators of thermal comfort levels in animals, presumably because of the role of these parameters in heat transfer (Cangar *et al*., 2008). However, evaporative heat loss through increased respiratory rate is associated with water loss, and consequently can lead to dehydration (Yahav *et al*., 2005) if the body's water reserves are not replaced. Since dissipation of heat from the body surface of broiler chickens is limited due to feather coverage, body surface heat loss is mainly achieved from the less feathered parts such as wattles, combs and legs (Lolli *et al*., 2010). Heat loss from these parts can be explained by the increased capillary blood flow to the comb and wattles under conditions of heat stress, typically of about 427 and 195% respectively of the levels of blood flow in thermoneutral conditions (Wolfenson *et al*., 1981). To enhance the rate of evaporative heat loss from other body surfaces, the idea of intermittent sprinkling of water onto the head and appendages of laying hens subjected to heat stress was explored by Chepete

and Xin (2000). This intervention reduced the rise in core body temperature by 1.4^{o}C thus extending the survival time of the birds. Although such an intervention might be difficult to realise in commercial litter floor broiler rearing systems broilers, nevertheless the possibility of developing a drinker which could avail the birds the opportunity to splash water on their combs under conditions of heat stress warrants some investigation.

This study was undertaken to investigate whether the provision of additional cup drinkers in addition to standard bell drinkers would afford broilers the opportunity for greater water intake and hence enhanced cooling under conditions of moderate heat stress. The aim was to investigate the extent to which the behavioural, physiological and growth performance of broiler chickens exposed to heat stress could be alleviated through the provision of additional cup drinkers.

8.2 Materials and methods

8.2.1 Ethical considerations

This experiment was conducted under Project Licence number PPL 60/4270 of the Animals (Scientific Procedures) Act 1986. Approval for the project was given at both national (Home Office) and local (Ethical Revenue Committee) level, and it was considered that overall the severity of this work was mild.

8.2.2 Experimental design and treatments

The experiment was conducted at Newcastle University in the same climatic chambers used in the last two heat stress experiments. The experimental design was a 2 × 2 factorial, with two levels of environmental conditions, namely normal (N; 20^{o}C and 50% RH) or moderate heat stress (MHS; 30^{o}C and 70% RH) and two levels of drinker treatment namely standard (S; one standard poultry bell drinker for 4 birds, giving a drinker space of 6.25 cm/bird; Wells Poultry Equipment, Ebbw Vale, UK; shown in Figure 8.1a) or additional cup drinkers (A; same as standard, but with two additional cup drinkers per pen, giving a total drinker space of 17.65 cm/bird). The cup drinkers (depth = 7 cm and width = 8.5cm, BEC, Broiler Equipment Company, Craven Arms, UK; shown in Figure 8.1b) each have a capacity of 300 ml and were fastened to the sides of the pen housing the birds at a height of 10 cm above the floor by cable ties.

During the heat stress protocol the cup drinkers were replenished at the end of each hour of heat stress.

The experiment was designed to induce moderate heat stress on the birds for a period of six hours each day for three consecutive days, simulating episodic moderate heat stress. On each day of heat stress, the temperature and RH were gradually increased during a 1 hour period (ST: step up), then held constant for six hours (HS: heat stress) and then gradually reduced over a period of 1 hour (SD: step down), as shown in Table 8.1. Together with a pre heat stress phase (PrHS) and a post heat stress phase (PHS), this gave a total of five phases. Sixteen birds were randomly assigned to each treatment and housed in floor pens (4 birds per pen) in one climate chamber.

Figure 8.1a. Standard bell drinker (side view)

Figure 8.1b. Cup drinker (view from above)

Table 8.1: Experimental design showing how environmental conditions of temperature and relative humidity varied across the four treatments during each of three days of simulated moderate heat stress

	Temperature and RH at the different phases of the heat stress experiment				
Treatment	**Pre heat stress (PrHS)**	**Step up (ST) 1 hr**	**Heat stress (HS)***	**Step down (SD) 1 hr**	**Post heat stress (PHS) 1 hr**
N-S	20°C, 50%	No change	20°C, 50%	No change	20°C, 50%
N-A	20°C, 50%	No change	20°C, 50%	No change	20°C, 50%
MHS-S	20°C, 50%	Gradual increase	30°C, 70%	Gradual decrease	20°C, 50%
MHS-A	20°C, 50%	Gradual increase	30°C, 70%	Gradual decrease	20°C, 50%

N-S: Normal conditions + standard bell drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers. *HS was split into 3HS and 6HS, after 3 and 6 hours respectively.

8.2.3 Description of birds and housing

On Day -10, a total of 64, male Ross 308 broiler chickens (age 21 days, and weight approximately 1.0 kg) were obtained from a commercial poultry farm (Oakland Farms Ltd, York, UK) and transported to Newcastle University in poultry crates. On the poultry farm from which the birds were sourced, water was provided through nipple drinkers. On arrival at Newcastle University, the birds were weighed and tagged with leg rings for individual identification, and then placed in a holding pen the floor of which was covered 5cm deep with wood shavings (Goodwill's Wood shavings & Timber products Ltd, Ponteland, UK). Commercial feed (20% crude protein, 4% oil, 6% ash and 13.00 MJ/kg ME from W.E Jameson & Son Ltd, Masham, UK) and water were provided *ad libitum* from feed hoppers and a bell drinker on the floor of each pen.

On Day –9, the birds were randomly allocated to one of the four treatments (one treatment per climate chamber) and placed in floor pens (4 birds per pen, 4 pens per chamber). These pens were identical in layout to pens used in previous two heat stress experiments, namely circular, bounded by a plastic perimeter and bedded with wood shavings. The diameter of the pen was 90 cm, allowing a space allowance per bird of 15kg/m^2. For the climate chambers utilising the additional drinkers, on experimental days (Day 1, 2 and 3) the cup drinkers were placed in the pen for an 8 h period, from PrHS until the PHS phase. From the time the birds arrived at the laboratory, provision of feed and water and health checks were undertaken daily. Estimates of feed and water intake were made at intervals during the heat stress protocol, as described in Section 8.2.5.5 below.

8.2.4 Implantation of temperature-ID chips

On Day - 5 (when the birds were 28 days old and weighted approximately 1.64 kg), 32 birds (two birds per pen, hereafter called 'chip birds') were selected for surgical implantation of a temperature-ID chip. This procedure was undertaken under brief anaesthesia by mask induction of 8% sevoflurane vaporised in oxygen (1.5 litres / minute). The skin on the left breast muscle was disinfected with hibitone and the chip injected 3cm deep into the left breast muscle using the sterile applicator provided. After this, 0.2 mg/kg of meloxicam was administered to the birds as a means of pain relief. The birds were then placed in a box to allow recovery, confirmed when they exhibited the righting reflex. The heat stress experiment commenced five days later (Day 1), at which point the birds were 32 days old and weighed approximately 2.0 kg.

8.2.5 Collection of welfare indicator data

8.2.5.1 Core and surface body temperature

Core body temperature (CBT-chip) was estimated from each of the 32 birds that were implanted with the temperature-ID chip by passing a pocket reader across the breast of the bird whilst the bird was restrained by a handler. CBT was taken on the 1st and 3rd day of the heat stress experiment on four occasions, namely PrHS, 3HS, 6HS and PHS. The change in CBT-chip reading (ΔCBT-chip) from the pre-heat stress phase to 3 hours of heat stress, 6 hours of heat stress and post heat stress phases was subsequently

derived by subtracting the CBT measured at PrHS from the CBT at the end of each of 3HS, 6HS and PHS phases respectively.

Estimates of surface body temperature (SBT) were taken from four different body parts, namely on the comb, under the wings, under the feet and on the cloaca. SBT was estimated using a surface laser temperature probe (Raytek 3V gun thermometer, RAYMX2G, Berlin, Germany) immediately after the CBT was estimated at the end of each phase, namely PrHS, 3HS, 6HS and PHS. The surface laser thermometer was calibrated according to the manufacturer's guidelines before use.

8.2.5.2 Respiratory rate

The respiratory rate of two randomly selected birds per pen was measured for 30 seconds at the end of 3HS and 6HS, and then converted to the mean number of breaths per bird per minute.

8.2.5.3 Haematological parameters

Blood sampling was undertaken on Day 2, the 2nd day of the heat stress experiment using the same procedure as described in Chapter 5 and 6. Blood was sampled from one chip bird per pen (i.e. four birds per treatment) on four different occasions during the day. The 1st blood sample was taken at the pre heat stress (PrHS) phase to serve as basal levels, the 2nd at the end of 3HS, the 3rd at the end of 6HS and the 4th at the end of the PHS phase. Blood sampled at each of these phases was used for instantaneous analysis of the levels of partial pressure of carbon dioxide (pCO_2), pH, haematocrit (Hct), haemoglobin (Hb) and bicarbonate (HCO_3^-) by a portable blood analyser (Vet Scan i-STAT®1 blood analyser, Quality Clinical Reagent, Limited, York, UK).

8.2.5.4 Behaviour sampling

In each climate chamber, two digital cameras (DCS-900 and DCS- 910, Ethernet camera supplied by BT, UK) were fitted to a wooden beam suspended above the pens and connected to a PC to record the behaviour of the birds. Scan sampling of behaviour was subsequently undertaken on two pens per treatment for two days (Day 1 and 3, the 1st and 3rd day of heat stress). Behaviour of all four birds in each pen was scored for one hour during the period of heat stress (specifically the 3rd and 6th hour of HS) at 1-minute

intervals. At each minute, the four birds in each pen were classed as exhibiting one of 11 mutually exclusive behaviour categories: feeding, drinking, standing, sitting, lying, foraging, preening, wing drooping, splash water or panting as defined in Table 8.2. The total number of birds observed in each behaviour category during a 60-minute session was expressed as a proportion of the total number of bird-minutes (i.e. number of birds × number of observations, typically 4 × 60 = 240) and referred to as the proportion of total observation time. Drinking behaviour is the sum of drinking from both cups and standard drinker.

Table 8.2: Behavioural ethogram for the 11 behaviour categories monitored during the 3rd and 6th hour of heat stress.

Behaviour category	**Description of behaviour**
Feeding	Beak in the feeder
Drinking-cup †	Beak in the cup drinker
Drinking-bell †	Beak in the bell drinker
Foraging	Pecking of litter
Preening	Beak touches the plumage of the bird itself
Wing drooping	Separation of wings from body
Panting	Fast breath with mouth slightly or wide open
Splash water	Splashing of water on comb/wattles
Standing inactive	Bird is standing on two feet
Sitting inactive	Resting the abdomen on the litter with raised head
Lying inactive	Head resting on litter while sitting or resting on one side of its body

† 'Drinking' behaviour was the sum of 'drinking-cup' and 'drinking-bell'

8.2.5.5 Production parameters and litter quality

Feed intake and growth rate

Estimates of feed intake on Day 1, 2 and 3 at various points during the heat stress protocol were made as follows: average daily feed intake (over a 24 hour period), feed intake during heat stress (specifically the period of 8 hours from PrHS to 6HS), after heat stress (a 2 hour period between 6HS and the end of PHS) and the intervening

period of 14 h (from PHS to PrHS the following day). Water intake from the standard bell drinker during heat stress (an 8 hour period, from PrHS to 6HS) was also estimated, whilst the amount of water consumed from the cups was not measured. The weight of each bird was measured prior to and after the 3 day heat stress experiment (birds were placed in a bucket and weighed on a weighing scale) and the average daily weight gain per bird subsequently estimated.

Litter moisture content

On Day -1, the day prior to the start of the heat stress experiment, the litter in each pen was removed and replaced with fresh litter. At the end of the experiment (on Day 4) litter samples were taken from three different positions in each pen, namely close to the drinkers, the middle of the pen and close to the feeder. These three samples were mixed together thoroughly and a 20 g subsample taken, placed in an aluminium dish in an oven set at 100^{o}C until the weight of the tray was constant. Percentage moisture content was then calculated for each of 4 pens per treatment.

8.2.6 Statistical Analyses

Statistical tests were performed using repeated measures analysis of variance in the statistical package SPSS (version 19, SPSS, Rottampstead, UK) and fitting environmental conditions and drinker treatment as factors in the model. Data were tested for normality using a Shapiro-Wilks test, before undertaking the appropriate parametric test. In all cases, the GLMs fitted were full factorial models to allow for interactions between the independent variables (environmental condition and drinker treatment). The CBT, ΔCBT and SBTs were analysed using a repeated measures GLM with phase of the heat stress protocol fitted as the within-subjects factor (PrHS, 3HS, 6HS and PHS) in a full factorial model. Post hoc tests for multiple comparisons were adjusted for using the Bonferroni correction. If the test assumption of sphericity was violated, (Mauchly's test, $P<0.05$), then the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity.

The results of the blood analysis, respiratory rate and behaviour at the end of 3HS and 6HS and percentage litter moisture content were subjected to a GLM analysis, with environmental conditions and drinker treatment as fixed factors. Post hoc tests were carried out using the least significant difference (LSD). Percentage moisture content of

the litter was subjected to GLM analysis. Finally, for treatments with additional cup drinkers, the proportion of observation time that the birds drank from each of the two different types of drinker at the end of 3HS and 6HS were subjected to a GLM analysis with environmental conditions (N-A and MHS-A only) as a fixed factor.

8.3 Results

All temperature-ID chips functioned properly giving both individual animal identification and estimates of core body temperature. Although the temperature–ID chips were scanned on four different occasions on the 1st and 3rd day, values for 6HS and PHS for birds in the MHS-S treatment were only available for the e3rd day because on the 1st day temperature in the climate chamber started to decline at the end of just five hours of heat stress.

8.3.1 Core and change in CBT-chip in response to heat stress

There was a significant effect of phase on CBT ($F_{3, 84} = 75.45$, $P<0.001$) whereby the CBT in birds at the end of 6HS was greater than at the end of 3HS, which in turn was greater than at PrHS or PHS. Overall, environmental condition had a significant effect on core T-chip ($F_{1, 28} = 38.034$, $P < 0.001$) with birds exposed to 30°C, 70% RH having a higher core T-chip than those at 20°C, 50% RH. There was neither a significant effect of drinker treatment nor environmental condition × drinker treatment on CBT.

There was a significant phase by environmental conditions interaction for CBT ($F_{3, 84} = 108.20$, $P<0.001$), specifically at 3HS ($P<0.001$), 6HS ($P<0.001$) and PHS ($P<0.05$), where birds exposed to MHS had a greater CBT than birds kept in the normal (control) conditions. There was no significant effect of drinker treatment, nor was there an interaction between phase and drinker treatment on CBT. Finally, there was a significant interaction of phase × environmental condition × drinker treatment on CBT ($F_{3, 84} = 3.44$, $P <0.05$), specifically at the end of 3HS where the CBT was greater for birds in the MHS-S and MHS-A treatments than those in N-A and N-S treatments (Figure 8.2a).

The change in core body temperature with respect to the PrHS phase is presented in Figure 8.2b. There was a significant effect of phase on ΔCBT ($F_{2, 56} = 78.85$, $P<0.001$) whereby the ΔCBT for birds at the end of 6HS was greater than at 3HS, which in turn was greater than the end of PHS. Overall, environmental conditions had a significant

effect on ΔCBT ($F_{1, 28}$ = 139.698, P < 0.001) with a greater ΔCBT in birds exposed to 30°C, 70% RH than their counterpart at 20°C, 50% RH. Drinker treatment was significant on ΔCBT ($F_{1, 28}$ = 8.633, P <0.05) with a smaller ΔCBT in birds which had additional cup drinkers compared to those that had standard bell drinkers. There was no significant interaction of environmental condition and drinker treatment on ΔCBT. There was a significant phase × environmental conditions interaction for ΔCBT-chip ($F_{2, 56}$ = 89.92, P<0.001) specifically at 3HS (P<0.001) and 6HS (P<0.001), where birds exposed to MHS had a greater ΔCBT than birds kept in the normal conditions. There was also a significant interaction between phase and drinker treatment for ΔCBT ($F_{2, 56}$ = 4.59, P <0.05) which occurred at both 3HS (P<0.05) and 6HS (P<0.05). Thus, at the end of 3HS and 6HS, the ΔCBT was reduced when the birds had access to additional cup drinkers. Finally, there was a significant interaction of phase × environmental conditions × drinker treatment for ΔCBT ($F_{2, 56}$ = 5.65, P <0.05) at the end of PHS, where the ΔCBT was lower in birds kept in the N-A treatment than those in the other three treatments.

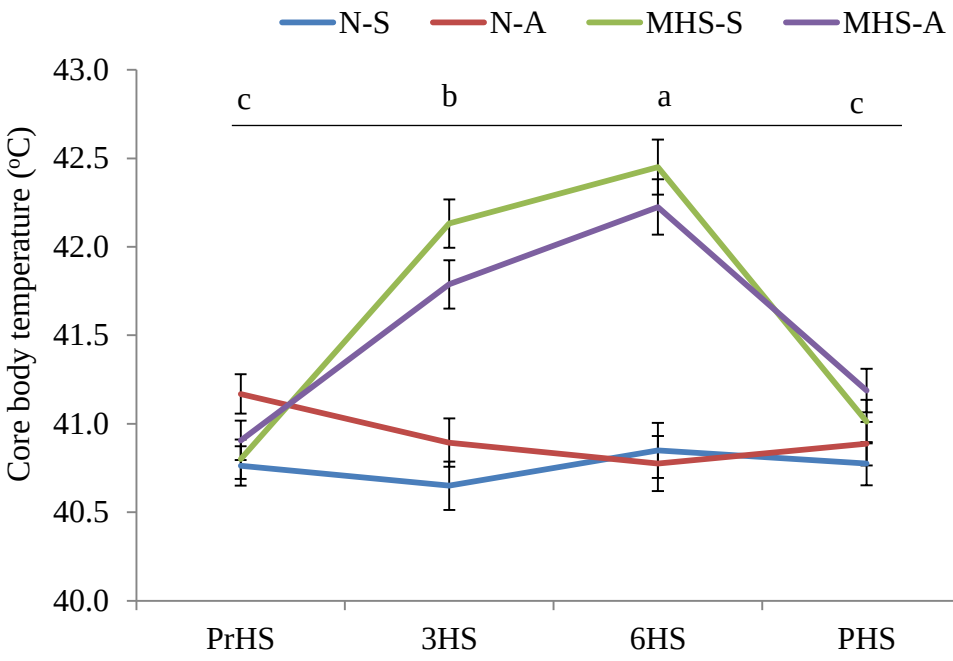

Figure 8.2a: Interaction of environmental conditions and drinker treatment on core body temperature of broiler chickens estimated from a temperature-ID chip at different phases of a moderate heat stress protocol. Values are means ±1 SEM. [abc] Means with different letters indicate the overall CBT value (single mean for all treatments during that phase) differs between phases (P<0.001). N-S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

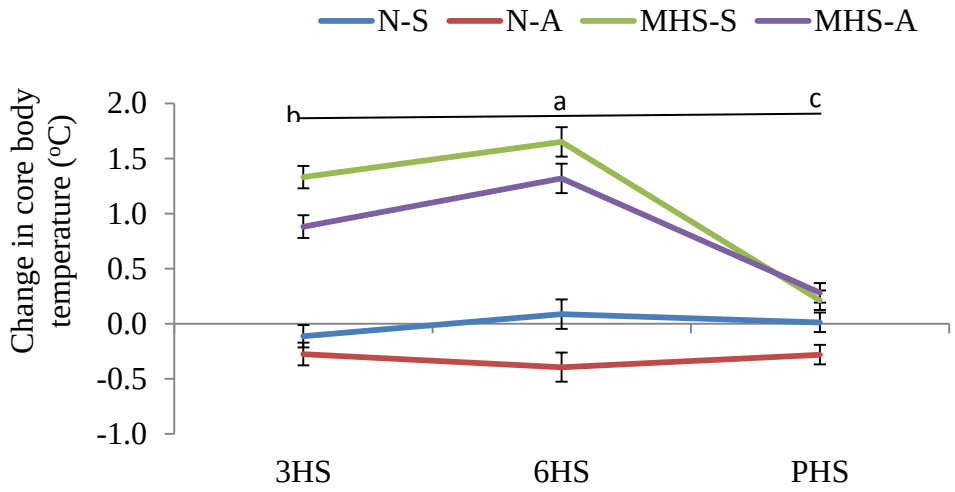

Figure 8.2b: Interaction of environmental conditions and drinker treatment on change in core body temperature of broiler chickens estimated from a temperature-ID chip at different phases of a moderate heat stress protocol. Values are means ±1 SEM. [abc] Means with different letters indicate the overall change in CBT value (single mean for all treatments during that phase) differs between phases (P< 0.001). N-S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

8.3.2 Surface body temperatures

8.3.2.1 Comb temperature (CT)

There was a significant effect of phase on CT ($F_{3, 84} = 28.12$, $P<0.001$), where CT at the end of 3HS was greater than at the end of 6HS which in turn was greater than at the end of PrHS and PHS phases. Overall, environmental conditions had a significant effect on CT ($F_{1, 28} = 32.444$, $P < 0.001$) with greater CT in birds exposed to 30°C, 70% RH than 20°C, 50% RH. Drinker treatment had a significant effect on CT ($F_{1, 28} = 4.919$, $P<0.05$) with greater CT in birds having additional cup drinkers than standard bell drinkers. There was a significant interaction of environmental conditions and water provision on CT ($F_{1, 28} = 6.240$, $P <0.05$) such that birds exposed to 30°C, 70% RH had a greater CT when provided with additional cup drinkers than standard bell drinkers. However, water provision did not have a significant effect on CT of birds at 20°C, 50% RH.

There was a significant effect of phase × environmental conditions interaction for CT ($F_{3, 84} = 52.46$, $P<0.001$) specifically at 3HS & 6HS ($P<0.001$) phases where CT was greater in birds exposed to MHS than to the normal conditions. There was a significant

interaction between phase × drinker treatment interaction on CT ($F_{3, 84}$ = 10.38, P<0.001) at the end of 3HS (P<0.05) and PHS (P<0.001). At these two phases CT was greater in birds which had additional cup drinkers than those which had only standard drinkers. Lastly, there was a significant interaction between phase × environmental conditions × drinker treatment for CT ($F_{3, 84}$ = 11.17, P<0.001) with an environmental conditions × drinker treatment interaction at both 3HS (P<0.001) and PHS (P<0.05) phases. At the end of 3HS phase, CT was greater (P<0.05) for birds in the N-A treatment than those in the N-S treatment. Similarly, at the PHS phase, CT was greater in birds in the MHS-A treatment than those in MHS-S (Figure 8.3a).

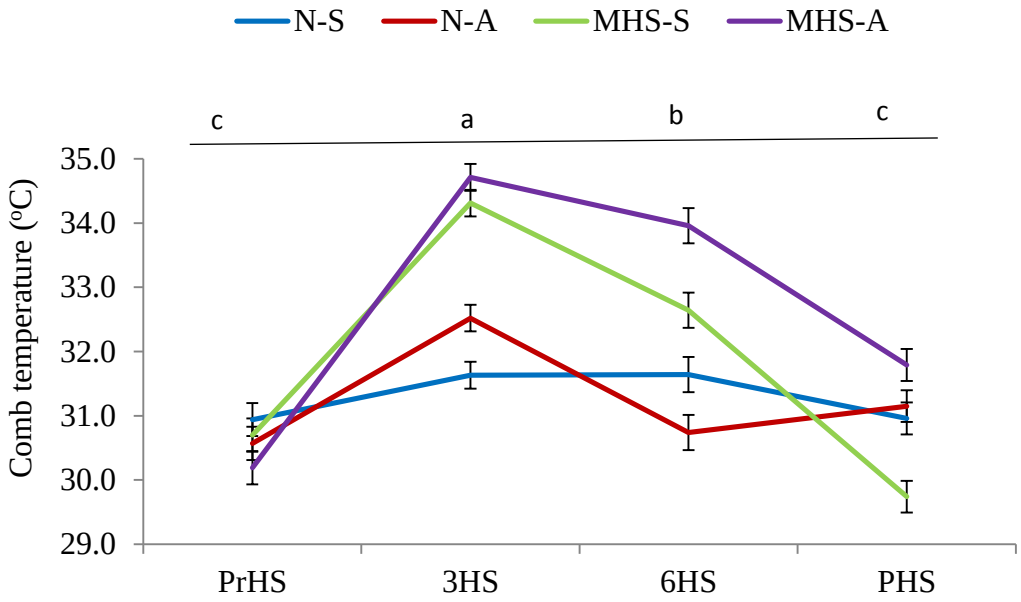

Figure 8.3a: Interaction of environmental conditions and drinker treatment on comb temperature of broiler chickens estimated by surface temperature probe at different phases of a moderate heat stress protocol. Values are means ± 1 SEM. [abc] Means with different letters indicate the overall CT value (single mean for all treatments during that phase) differs between phases (P< 0.001). Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

8.3.2.2 Cloaca temperature (CLT)

There was a significant effect of phase on CLT ($F_{3, 84}$ = 25.55, P<0.001), so that CLT of birds at the end of 3HS and 6HS was greater than at PrHS or PHS. Overall, environmental conditions had a significant effect on CLT ($F_{1, 28}$ = 6.978, P <0.05), CLT was greater in birds exposed to 30°C, 70% RH than 20°C, 50% RH. Drinker treatment

had a significant effect on CLT ($F_{1, 28} = 6.620$, $P < 0.05$), with a greater CLT in birds which had additional cup drinkers in their pen than those that had standard bell drinkers. There was a significant interaction of environmental conditions and drinker treatment on CLT ($F_{1, 28} = 6.978$, $P < 0.05$), such that birds exposed to 30^{o}C, 70% RH, had a greater CLT when provided with additional cup drinkers than standard bell drinkers. There was a significant interaction of phase × environmental conditions for CLT ($F_{3, 84} = 18.44$, $P<0.001$), specifically at 3HS ($P< 0.001$) and 6HS ($P<0.05$) where birds exposed to MHS had a greater CLT than those in the normal conditions. There was no significant interaction of phase × drinker treatment on CLT (Figure 8.3b).

8.3.2.3 Wing temperature (WT)

There was a significant effect of phase on WT ($F_{3, 84} = 28.12$, $P<0.001$) with WT at the end of 3HS was greater than at the end of 6HS which in turn was greater than at the end of PrHS and PHS phases. Overall, environmental conditions had a significant effect on WT ($F_{1, 28} = 8.920$, $P < 0.05$) with a greater WT in birds exposed to 30^{o}C, 70% RH than 20^{o}C, 50% RH. Neither drinker treatment nor interaction of environmental conditions and drinker treatment had a significant effect on WT.

There was a significant interaction of phase × environmental conditions for WT ($F_{3, 84} = 9.53$, $P<0.001$) at the end of both 3HS and 6HS, where the WT of birds exposed to MHS was greater than those kept in the normal conditions. There was no significant phase × drinker treatment interaction on WT. Finally, there was a significant phase × environmental conditions × drinker treatment interaction for WT ($F_{3, 84} = 5.78$, $P<0.001$), specifically at PHS ($P<0.05$), where WT was greater for birds exposed to MHS-A than those exposed to MHS-S (Figure 8.3c).

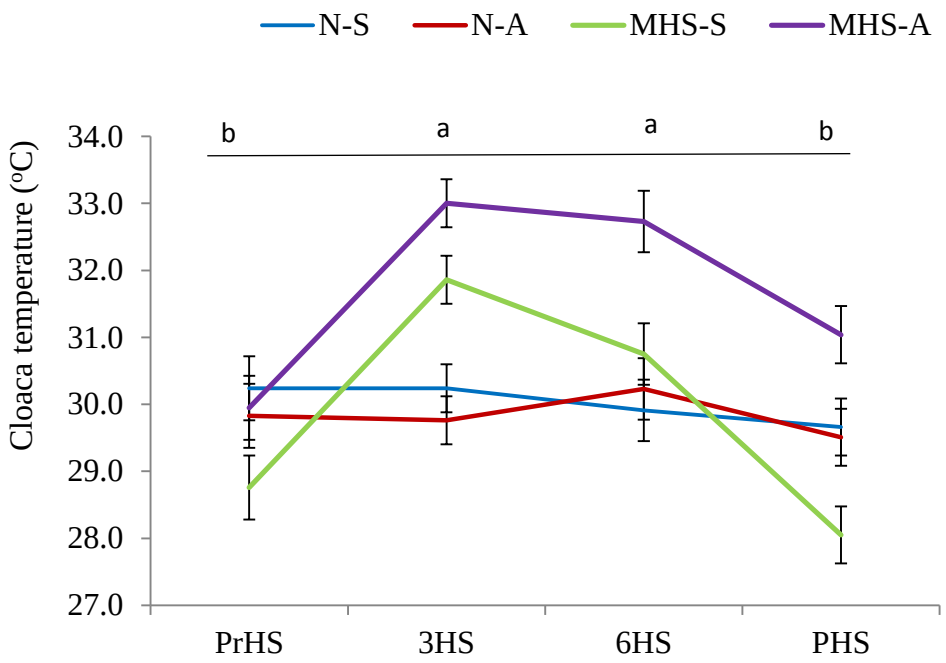

Figure 8.3b: Interaction of environmental conditions and drinker treatment on cloaca temperature of broiler chickens estimated by a surface temperature probe at different phases of a moderate heat stress protocol temperature. Values are means ± 1 SEM. [ab] Means with different letters indicate the overall CLT value (single mean for all treatments during that phase) differs between phases ($P<0.001$). N-S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

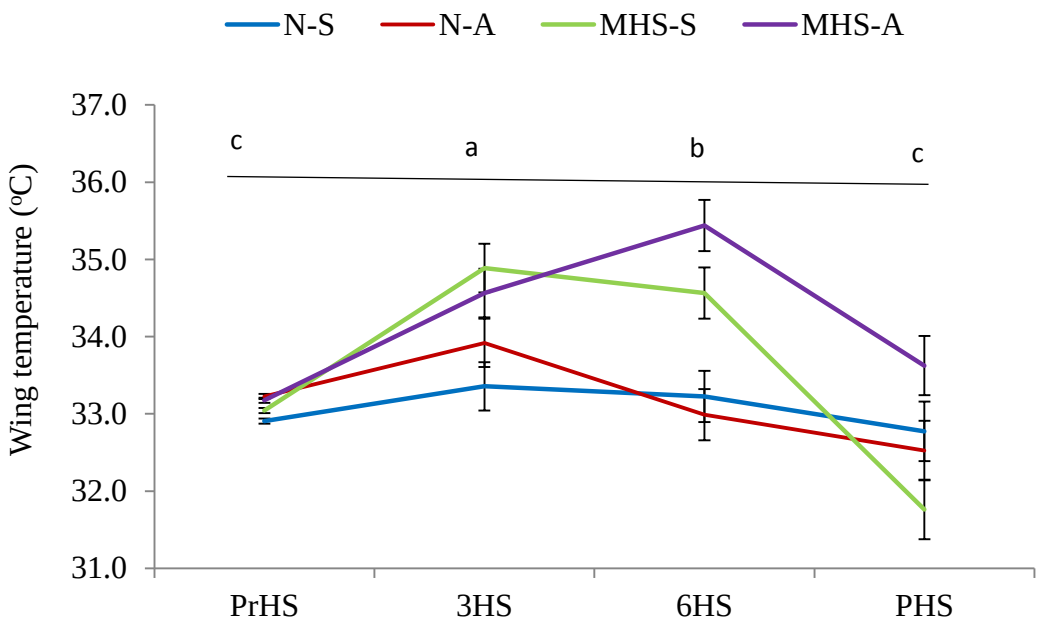

Figure 8.3c: Interaction of environmental conditions and drinker treatment on wing temperature estimated by surface temperature probe at different phases of a moderate heat stress protocol. Values are means ± 1 SEM. [abc] Means with different letters indicate the overall WT value (single mean for all treatments during that phase) differs between phases (P<0.001). N=S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

8.3.2.4 Feet temperature (FT)

There was a significant effect of phase on FT ($F_{2.010, 56.273} = 33.94$, P<0.001) with the FT of birds at 3HS and 6HS greater than at PrHS or PHS. Overall, environmental conditions had a significant effect on FT ($F_{1, 28} = 74.187$, P< 0.001) with birds exposed to 30°C, 70% RH having a greater FT than those at 20°C, 50% RH. Drinker treatment had a significant effect on FT ($F_{1, 28} = 4.702$, P <0.05) with a greater FT in birds which had additional cup drinkers than those with standard bell drinkers. There was no significant environmental condition × drinker treatment interaction on FT.

There was a significant interaction of phase × environmental conditions for FT ($F_{2.010, 56.273} = 13.60$, P<0.001) at both 3HS and 6HS, where FT was greater in birds exposed to MHS than those kept in normal conditions. There was also a significant interaction of phase × drinker treatment for FT ($F_{2.010, 56.273} = 4.55$, P<0.05) at both 3HS and PHS,

where FT was greater in birds provided with additional cups than those with just a standard drinker. There was a significant interaction of environmental conditions × drinker treatment at PHS ($P<0.05$), where FT was greater in birds in MHS-A than MHS-S birds. Finally, there was a significant interaction of phase × environmental conditions × drinker treatment for FT ($F_{2.010, 56.273} = 4.46$, $P<0.05$), Figure 8.3d.

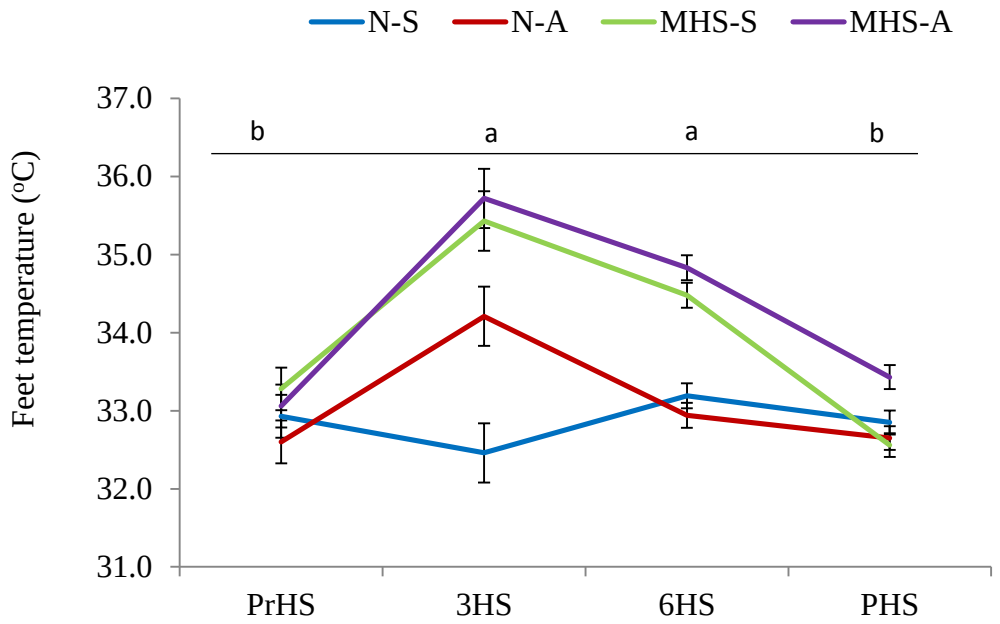

Figure 8.3d: Interaction of environmental conditions and drinker treatment on feet temperature of broiler chickens measured at different phase s of a moderate heat stress protocol. Values are means ± 1 SEM. [ab] Means with different letters indicate the overall FT value (single mean for all treatments during that phase) differs between phases ($P<0.001$). N-S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

8.3.3 Respiratory rate at the end of 3HS and 6HS

The environmental conditions in which broiler chickens were kept had a significant influence on the respiratory rate at the end of 3HS ($F_{1, 12} = 104.25$, $P<0.001$) and 6HS ($F_{1, 12} = 56.554$, $P<0.001$), so that broiler chickens exposed to MHS had a greater respiratory rate compared to birds in the normal conditions. There was no significant

effect of drinker treatment on respiratory rate at 3HS and 6HS nor was there an environmental conditions × drinker provision interaction (see Figure 8.4).

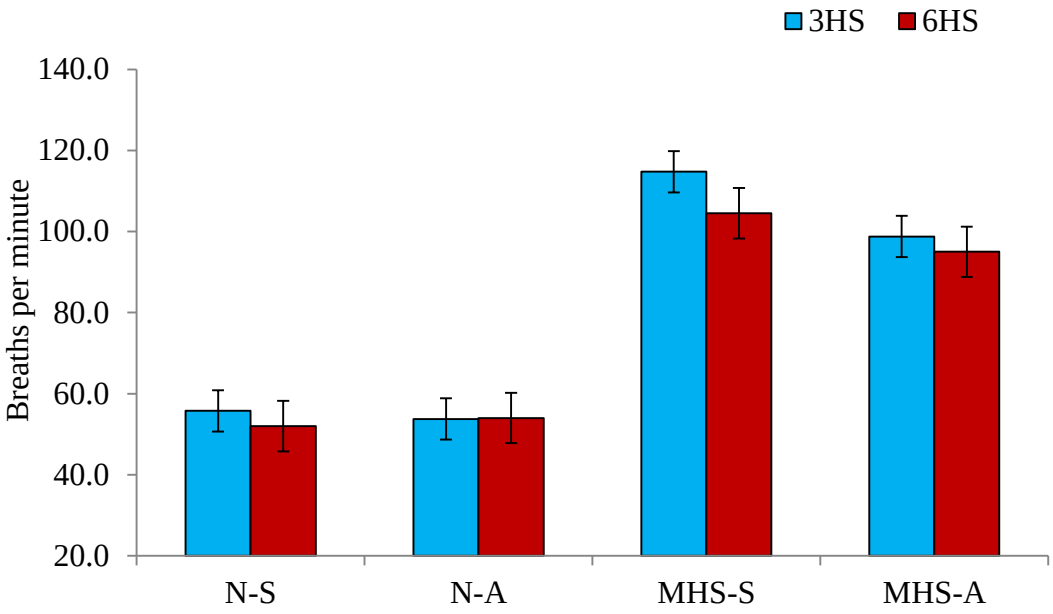

Figure 8.4: Interaction of environmental conditions and drinker treatment on respiratory rate of broiler chickens at the end of 3 and 6HS (n = 16) of exposure to moderate heat stress. Values are means ± 1 SEM. N-S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

8.3.4 Blood parameters

At the end of 3HS, environmental conditions had a significant effect on levels of haematocrit, Hct ($F_{1, 7} = 5.73$, $P<0.05$) and haemoglobin, Hb ($F_{1, 7} = 16.54$, $P<0.05$) such that birds exposed to MHS had lower levels of Hct and Hb (see Table 8.3). There was no significant effect of environmental conditions on pCO_2, pH and HCO_3^- levels. Drinker treatment had no significant effect on the levels of blood parameters at any point. The only significant interaction of environmental conditions × drinker provision on blood parameters was for HCO_3^- ($F_{1, 7} = 10.045$, $P<0.05$), where birds exposed to MHS-A had a greater HCO_3^- level than those in MHS-S. .

At the end of 6 hours exposure to heat stress, there was no effect of environmental conditions or drinker treatment on any of the blood parameters, and nor were there any interactions between these factors. There was a tendency for environmental conditions

to affect levels of haematocrit ($F_{1, 7} = 3.94$, $P = 0.088$) and haemoglobin ($F_{1, 7} = 3.97$, $P = 0.087$), but differences were not significant.

8.3.5 Behaviour sampling

At the end of 3HS, environmental conditions had a significant effect on all behaviour categories except standing behaviour and splash water (see Table 8.4). Birds exposed to MHS spent a greater proportion of total observation time on feeding, wing drooping, panting and drinking. On the other hand, birds in MHS spent less time on preening, lying, foraging and sitting. The provision of additional cup drinkers enhanced the proportion of total observation time broiler chickens spent feeding, but tended to suppress levels of foraging behaviour. There was a significant interaction between environmental conditions and drinker treatment for the proportion of total observation time the birds spent wing drooping, so that whilst overall birds exposed to heat stress showed greater levels of wing drooping, this was significant only for standard pens with standard drinkers (Table 8.4).

The mean incidence of birds splashing water on their combs was zero, in both 3HS and 6HS

At the end of 6HS, birds exposed to MHS spent a greater proportion of total observation time standing and panting, and less time preening, lying, sitting and foraging than birds in normal conditions. There was no effect of drinker treatment nor was there any interaction of environmental conditions and drinker treatment on any behaviour categories (Table 8.4).

Table 8.3: Interaction of environmental conditions and drinker treatment on blood parameters of broiler chickens at the end of 3 and 6HS of exposure to moderate heat stress. Values are means ± 1 SEM. N-S: Normal condition + standard drinker (n=2); N-A: Normal conditions + additional cup drinkers (n=3); MHS-S: Moderate heat stress + standard bell drinker (n=3); MHS-A: Moderate heat stress + additional cup drinkers (n=3).

Parameter					P value		
3HS	N-S	N-A	MHS-S	MHS-A	EC	DT	EC × DT
pH	7.4±0.04	7.4±0.03	7.4±0.03	7.5±0.03	NS	NS	NS
Hb (g/dl)	7.3±0.39	7.3±0.32	5.4±0.32	6.4±0.32	*	NS	NS
Hct (% PCV)	21.5±3.20	21.3±2.61	10.9±2.61	18. 7±2.61	*	NS	NS
HCO_3^- (mmol/l)	27.2±1.12^{ab}	26.3±0.92^{ab}	23.5±0.92^{b}	28.8±0.92^{a}	NS	NS	*
pCO_2 (mmHg)	40.5±3.65	45.1±2.98	35.2±2.98	41.4±2.98	NS	NS	NS
6HS							
pH	7.4±0.04	7.4±0.03	7.5±0.03	7. 5±0.03	NS	NS	NS
Hb (g/dl)	7.0±0.55	6.7±0.45	5. 7±0.45	6.1±0.45	NS	NS	NS
Hct (% PCV)	20.5±1.60	19.7±1.31	16.7±1.31	18.0±1.31	NS	NS	NS
HCO_3^- (mmol/l)	23.8±2.08	27.3±1.70	27.0±1.70	28.8±1.70	NS	NS	NS
pCO_2 (mmHg)	37.4±3.88	42.7±3.17	36.9±3.17	41.2±3.17	NS	NS	NS

*P<0.05; NS-non significant; Note: EC = environmental conditions, DT = drinker treatment and EC×DT= interaction

Table 8.4: Effect of environmental conditions and drinker treatment on mean proportion of total observation time spent on 10 behaviour categories at the end of 3 and 6 hours exposure to moderate heat stress. Values are the means, n= 2 pens/ treatment. N-S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

Behaviour	**Treatment**					**P value**		
3HS	N-S	N-A	MHS-S	MHS-A	SEM	EC	DT	EC×DT
Feeding	0.06	0.07	0.07	0.10	0.01	*	*	NS
Drinking	0.07	0.07	0.13	0.14	0.01	*	NS	NS
Standing	0.04	0.04	0.07	0.07	0.01	NS	NS	NS
Sitting	0.55	0.54	0.00	0.00	0.01	**	NS	NS
Preening	0.11	0.10	0.03	0.04	0.01	*	NS	NS
Wing droop	0.00^{b}	0.01^{ab}	0.03^{a}	0.02^{ab}	0.04	*	NS	*
Panting	0.00	0.00	0.67	0.66	0.02	**	NS	NS
Lying	0.06	0.09	0.00	0.00	0.01	*	NS	NS
Foraging	0.08	0.06	0.01	0.00	0.01	**	NS	NS
Splash water	0.00	0.00	0.00	0.00	0.00	NS	NS	NS
6HS								
Feeding	0.08	0.07	0.07	0.06	0.01	NS	NS	NS
Drinking	0.07	0.06	0.10	0.12	0.02	NS	NS	NS
Standing	0.04	0.04	0.08	0.04	0.01	*	NS	NS
Sitting	0.58	0.62	0.00	0.10	0.17	*	NS	NS
Preening	0.10	0.10	0.03	0.03	0.01	*	NS	NS
Wing droop	0.01	0.00	0.01	0.00	0.01	NS	NS	NS
Panting	0.00	0.00	0.71	0.73	0.01	**	NS	NS
Lying	0.08	0.08	0.00	0.00	0.02	*	NS	NS
Foraging	0.05	0.05	0.02	0.00	0.01	*	NS	NS
Splash water	0.00	0.00	0.00	0.00	0.00	NS	NS	NS

*P<0.05; **P<0.001; NS- non-significant; Note: EC = environmental conditions, DT = drinker treatment and EC×DT = interaction

8.3.5.1 Drinking from either standard bell drinker or additional cup drinkers

At the end of 3HS, the proportion of observation time drinking from the bell drinker was greater ($F_{1, 2} = 33.80$, $P < 0.05$) in MHS-A birds than in N-A birds (Figure 8.5). At the end of 6HS, although drinker treatment had no significant effect on which drinker type was used, there was a tendency ($F_{1, 2} = 9.80$, $P = 0.089$) for the proportion of observation time of drinking from the cup drinkers to be greater in birds exposed to MHS than those exposed to normal conditions.

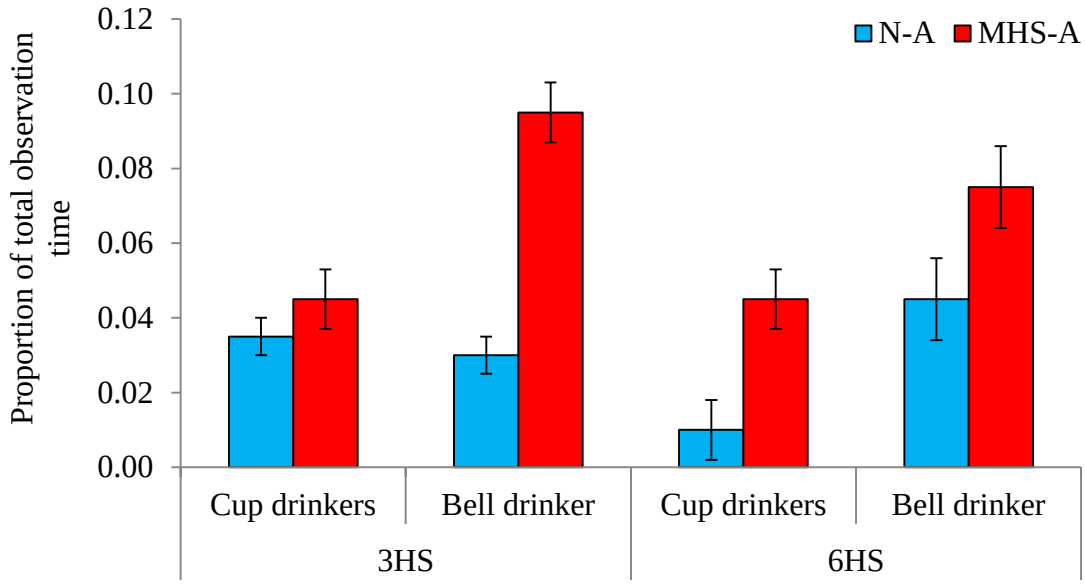

Figure 8.5: Proportion of total observation time spent drinking from the cup and bell drinkers by birds in the N-A and MHS-A groups at the end of 3 and 6 hours of moderate heat stress. Values are means ± 1 SEM. N-A: Normal conditions + additional cup drinkers; MHS-A: Moderate heat stress + additional cup drinkers.

8.3.6 Litter moisture content (%)

The moisture content of the litter at the end of the 3-day heat stress experiment was greater ($F_{1, 12} = 15.12$, $P<0.05$) in pens in the MHS conditions than those in normal conditions as shown in Figure 8.6. There was no effect of drinker treatment nor were there any environmental conditions by drinker treatment interaction for litter moisture content.

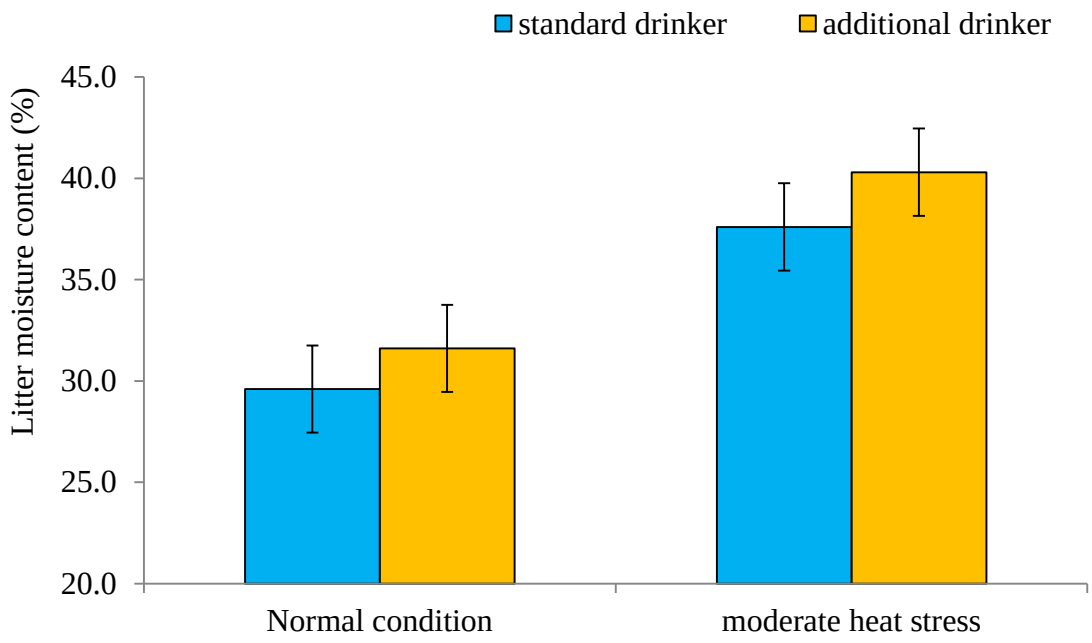

Figure 8.6: Effect of environmental conditions and drinker treatment on moisture content of litter at the end of a three days moderate heat stress experiment (n = 4 pens / treatment).Values are means ± 1 SEM.

8.3.7 Feed intake, bodyweight, weight gain and water intake

Overall, the mean daily feed intake of broilers exposed to MHS was less ($F_{1, 12} = 20.71$, $P<0.05$) than those kept under normal conditions as seen in Table 8.5. Birds exposed to MHS consumed less feed in the 8-hour period when heat stress was imposed ($F_{1, 12} = 38.46$, $P<0.001$) than those in normal conditions. On the other hand, feed intake between the end of 6HS and PHS (a 2 hour period) was greater ($F_{1, 12} = 39.64$, $P<0.001$) in birds which were previously exposed to MHS than those in the normal conditions. Environmental conditions had a significant effect on feed intake in the intervening 14 hour period ($F_{1, 12} = 24.27$, $P<0.001$), so that birds exposed to normal conditions consumed more feed during this period than those which were exposed to MHS. Broiler chickens which had standard drinkers in their pens ate more feed in the 8 h period when heat stress was imposed ($F_{1, 12} = 26.35$, $P<0.001$) than their counterparts which had additional cup drinkers, but ate less feed in the intervening period ($F_{1, 12} = 59.14$, $P<0.001$). Birds which had additional cup drinkers ate more feed during the compensatory period than those which had standard drinkers. Neither environmental conditions nor drinker treatment had any effect on weight gain.

Water consumption during heat stress from the standard drinker (8 h) was greater ($F_{1, 10} = 5.88$, $P<0.05$) in birds exposed to MHS than those kept in normal conditions. Water intake was greater ($F_{1, 10} = 32.34$, $P<0.001$) in birds which had standard drinkers than those which had additional cup drinkers.

There were no environmental conditions by drinker treatment interactions for daily feed intake, feed intake during and after heat stress, compensatory feed intake, weight gain or water intake.

Table 8.5: Effects of environmental conditions and drinker treatment on feed intake, water intake, weight gain of broiler chickens during a 3-day heat stress experiment. Values are means ± 1SEM. N-S: Normal condition + standard drinker; N-A: Normal conditions + additional cup drinkers; MHS-S: Moderate heat stress + standard bell drinker; MHS-A: Moderate heat stress + additional cup drinkers.

					P value		
	N-S	N-A	MHS-S	MHS-A	EC	DT	EC×DT
Daily feed intake (g/bird)	226.4±4.79	230.6±4.79	204.6±4.79	208.9±4.79	*	NS	NS
Feed intake (PrHS - 6HS, 8hrs) g/bird	63.5±1.44	56.9±1.44	55.3±1.44	47.1±1.44	**	**	NS
Feed intake (6HS-PHS, 2hrs) g/bird	21.9±1.51	22.7±1.51	34.1±1.51	29.6±1.51	**	NS	NS
Feed intake (PHS-PrHS, 14hrs) g/bird	77.5±2.72	94.2±2.72	59.8±2.72	85.0±2.72	**	**	NS
Water intake from bell drinker							
(PrHS-6HS, 8hrs) ml/bird	316.7±27.21	148.1±31.42	386.5±27.2	220.8±31.42	*	*	NS
Initial weight (g)	2092.0±44.62	2068.7±44.62	1960.7±46.09	1926.9±44.62	*		
Final weight (g)	2502.9±52.77	2467.4±52.77	2359.7±54.50	2290.6±52.77	*	NS	NS
Weight gain/bird/day (g)	137.0±7.97	134.2±7.97	121.2±7.97	124.7±7.97	NS	NS	NS

*P<0.05; **P<0.001; NS- non-significant; Note: EC = environmental conditions, DT = drinker treatment and EC×DT = interaction

8.4 Discussion

The aim of this experiment was to investigate the extent to which the behavioural, physiological and growth performance of broiler chickens exposed to heat stress could be alleviated through the provision of additional cup drinkers. The discussion will be sectioned into three parts, namely i) the effect of using additional cup drinkers in general, ii) alleviation of moderate heat stress by provision of additional cup drinkers, and iii) changes in welfare indicators with changes in environmental conditions.

8.4.1 General benefits of the provision of additional cup drinkers

The provision of additional cup drinkers enhanced comb, cloaca and feet temperatures (but had no effect on mean wing temperature). These three body surfaces could easily assist in heat loss compared because they are unfeathered and require little effort in order to support heat loss mechanisms compared to wing droop or wing flap behaviour. Wing drooping is one of the thermoregulatory behaviours displayed whereby the birds spread out their wings to expose the less-feathered parts under the wings, thus increasing the surface area to allow for increased heat loss (Gerken *et al*., 2006). The level of hydration (water status) affects evaporative heat loss from the skin (Dawson and Whittow, 2000) as dehydration reduces the comb temperature during heat stress suggesting a reduction in the transfer of heat from the core to the surface (Zhou *et al*., 1999).

Provision of additional cup drinkers enhanced feeding behaviour but this did not correspond to a higher feed intake and so it could be argued that the increase in feeding behaviour could be associated to a reduced feed intake per visit. The provision of additional cup drinkers did not result in an increase in litter moisture content, suggesting that water spillage was not a problem. Although birds kept in the normal environmental conditions were not exposed to heat stress, the provision of additional cup drinkers nevertheless appeared to help birds in reducing their core body temperature. This suggests that the birds must have been able to consume more water from the cup drinkers which allowed the birds to dissipate heat generated from the high metabolic heat production associated with the high potential for feed intake and growth of the modern broiler, and the physical activities afforded by a more generous space allowance than might be expected in commercial rearing systems.

The lack of previous experience of drinking from the cup drinkers could have affected our results, so that perhaps the birds were in some ways reluctant to use the novel cup drinkers because of fear, or indeed they were motivated to use them out of inquisitiveness. Equally, it should be borne in mind that commercially-reared broilers most commonly are provided with nipple drinkers. Hence, further study would be required to determine the likely benefit of providing additional cup drinkers to birds in commercial systems, whether or not they are subject to a period of heat stress.

8.4.2 Alleviation of moderate heat stress through the provision of additional cup drinkers

The provision of additional cup drinkers to birds exposed to MHS suppressed the rise in core body temperature (i.e. ΔCBT) that was seen in contemporaries subjected to heat stress but with just one standard bell drinker per pen. It seems that birds given additional cup drinkers had elevated heat loss from body surfaces, as evidenced by the increase in surface body temperatures, respiratory rate remained high even in birds provided with additional cup drinkers. Due to the increased muscular activity and changes in acid-base balance arising from increased respiratory rate, increasing heat loss through sensible means may be more beneficial to birds (Nascimento *et al*., 2011) than resorting to evaporative means. Yahav *et al*. (2005) showed that the rate of sensible heat loss from broilers was increased by increasing air velocity to 3m/s.

The blood circulates to every part of the body and so helps in the regulation of body temperature by distribution of heat to the different body parts relevant for heat loss (Powell, 2008). Dehydration causes a rise in core body temperature because of the reduction in water available for evaporative cooling (Dawson and Whittow, 2000). In the current study, birds did not appear to be dehydrated, because there was no change in blood haematocrit levels during the first half of heat stress (3HS), and indeed birds exposed to MHS spent a greater proportion of total observation time drinking from the bell drinkers than those in the normal conditions. With further exposure to heat stress during the next three hours (by 6HS), there was a tendency for a greater proportion of total observation time to be spent drinking from the cup than those in the normal conditions indicative of the need for more greater access to water required to meet their thermoregulatory need. This shift in preference of drinking from the bell drinker to the cup drinker with the increase in duration of heat stress corresponded to when the ΔCBT

was at its peak. This suggests a need for greater access to water intake was required to achieve evaporative cooling (Chaiyabutr, 2004) to assist regulation of core body temperature. It can be argued that the cup drinker provided greater access to water, in the sense that the diameter of the space where the birds could pop their head to drink was 8.5 cm and 2.8 cm for the cup and bell drinkers respectively. Bogin *et al*. (1996) showed that birds subjected to heat stress increased their water intake thereby limiting the increase in core body temperature to just 42.7^{o}C aid and assisting with survival (mean water intake of surviving birds was estimated to be 8% greater than those which did not survive which had a core body temperature of 43.2^{o}C). In the current study, the provision of additional cup drinkers suppressed the rise in core body temperature by 0.3^{o}C. However, the amount of water consumed from the cup drinkers was not measured, so that complete assurance about the effect of additional cup drinkers on water intake cannot be proven. In a recent study by Zhao *et al*. (2012) it was shown that broiler chickens made more use of a water-cooled perch than a normal perch to achieve thermoregulation when exposed to heat stress (31.1-34.1^{o}C) from four to six weeks of age. Furthermore, the water-cooled perches were reported to enhance bodyweight gain and carcass quality and decrease rectal temperature and the incidence of panting in birds exposed to heat stress.

The provision of additional cup drinkers under conditions of moderate heat stress enhanced blood bicarbonate levels. The bicarbonate buffer system is the most important regulator of plasma pH and this is achieved through the kidney (excretion of bicarbonates) or ventilation (to remove CO_2) in order to compensate for changes in the acid base system (Frandson *et al*., 2003). Apart from maintaining blood bicarbonate levels similar to non heat-stressed control birds, provision of additional cup drinkers meant that time spent by birds exposed to heat stress in wing drooping behaviour and standing behaviour of was similar to that of those in normal conditions. There is an increased surface area of the body available for heat loss during standing. In observation of Rock Doves in a desert environment, Ferns (1992) reported that 50% of Rock Doves were found to be standing to enhance convective cooling from the legs while those sitting had their feathers erected during the hottest period (35^{o}C, 14:00-17:00) of the day.

8.4.3 Changes in welfare indicators across the different environmental conditions

The mean change in core body temperature at the end of 3HS (+0.9^{o}C) and 6HS (+1.3^{o}C) was greater in birds exposed to MHS than those in the normal conditions. The ΔCBT was greater at the end of 6HS than 3HS indicating that the amount of heat stored (i.e. in this case heat gain was greater than heat loss) within the body kept increasing as the heat stress conditions persisted. However, the increase in core body temperature did not reach the critical point of 4^{o}C which is lethal (DEFRA, 2005). For birds exposed to MHS, respiratory rate was enhanced at the end of 3HS and 6HS, suggesting an increased reliance on evaporative cooling to assist regulation of core body temperature. Increased respiratory rate of broilers exposed to high ambient temperature (26.7^{o}C) indicates effort required to dissipate heat through evaporative means (Olanrewaju *et al*., 2010).

In contrast, Nascimento *et al*. (2011) reported a linear relationship between mean surface body temperature of broilers and ambient temperature within the range 18 to 32^{o}C. In the current study, the mean surface body temperature (MSBT) was greater at the end of 3HS than 6HS, indicating that heat loss through sensible means has a threshold after which it declines so that the reliance on sensible heat loss diminishes with an extended period of heat stress. The comb, cloaca, wing and feet temperatures were greater in birds exposed to MHS than those in the normal conditions, but while the wing and comb temperatures of birds exposed to MHS were greater at the end of 3HS than at the end of 6HS, the cloaca and feet temperatures were greater at the end of the 3HS and this was maintained until the end of 6HS. This suggests that during the early phase of the heat stress (in the first three hours, 3HS) heat was transferred from the core to all four body surfaces but as the heat stress period continued in the next three hours (to 6HS), heat loss through the cloaca and feet became more important than heat loss through the wing and comb surfaces. Elevated cloaca and feet temperatures could suggest that birds made use of the opportunity to transfer heat to the litter, as they attained a sitting or standing position (for cloaca and feet, respectively). Since the surface body temperature is under the influence of both the environmental temperature and the core body temperature, then the reduction in wing and comb temperatures at the end of 6 hours of MHS could infer a reduction in heat transfer from the core to the periphery. Changes in the four surface body temperatures coincides with the greatest

increase in core body temperature and helps understand the dynamics of heat transfer from the body core to the periphery as the duration of heat stress continues.

Broiler chickens exposed to MHS had lower blood haematocrit and haemoglobin levels after three hours of moderate heat stress. With a further three hours exposure to heat stress, levels of these two blood parameters were not dissimilar to birds kept under standard environmental conditions. This suggests a quick response of the blood system to heat stress, after which mechanisms to restore the blood parameters back to normal. Powell (2000) reported a direct relationship between haemoglobin concentration and oxygen concentration in the blood. This is in agreement with Donkoh (1989), who reported on broilers subjected to chronic heat stress (30 and 35^{o}C from 3 to 7 weeks of age). In the current study, the reduction in haemoglobin level suggests changes in energy required (Gous and Morris2005), through suppression of the metabolic rate of the birds to enable them to cope under heat stress (Donkoh, 1989) evident from the reduction in feed intake. Menten *et al*. (2006) subjected broiler chickens to simulated pre slaughter heat stress conditions (35^{o}C, 85% RH) for 2 hours and took samples of physiological parameters at 30 minute intervals. The haematocrit level of the birds was reduced as early as 30 minutes after exposure to heat stress, an effect which persisted until the end of the two hours of heat stress. In another study, birds deprived of water for 2 days had greater haematocrit levels both before and after exposure to heat stress (30^{o}C for 3 hours; Zhou *et al*. (1999). A long-term heat stress study (26.7^{o}C from day 21 to 56 of age) also confirmed a reduction in haematocrit and haemoglobin levels, especially at the 42nd and 56th day (Olanrewaju *et al*., 2010). The reduction in haematocrit observed in birds exposed to heat stress has been associated with an increase in plasma volume which could assist in reducing the rise in core body temperature (Chaiyabutr, 2004). Yahav *et al*. (1997) discovered a linear relationship between haematocrit and relative heart weight which implies that, in order to meet the metabolic rate, the heart mass needs to regulate both the cardiac output and haematocrit concentration.

The combination of the results of respiratory rate and blood gases could give an insight into the pattern of breathing in birds exposed to heat stress. In the current study, birds exposed to MHS had a greater respiratory rate than control birds. However, the levels of pCO_2, pH and bicarbonates were similar in heat stressed and control birds. This suggests that the increased respiratory rate did not result in the development of

respiratory alkalosis (increase in pH), presumably because the birds were respiring at a shallower but higher frequency rate which helps in the ventilation of the upper dead space of the lungs so that the CO_2 exchange in the alveoli is not affected (Willmer *et al*., 2005).

At the end of 3HS, birds exposed to MHS spent a greater proportion of total observation time of wing drooping, panting, drinking and feeding and less time in preening, lying, sitting and foraging. However, with a further increase in the duration of heat stress (up to 6HS) birds exposed to MHS were not observed to display the same high levels of wing drooping and feeding as they had done in the early phase of heat stress. Suppression in time spent preening, lying, sitting and foraging persisted until the end of 6HS. Broiler chickens subjected to a heat challenge of either 28^{o}C or 32^{o}C were observed to be panting, however the latency to start panting was faster in birds exposed to 32^{o}C than to 28^{o}C (Gerken *et al*., 2006). Bozakova *et al*. (2009) showed that the number of turkeys drinking when exposed to a hot summer period (32^{o}C, 52% RH) was greater than those in the control conditions (16^{o}C, 63% RH). Hence, behaviours which are enhanced under conditions of MHS could be regarded as thermoregulatory behaviour (panting and drinking), while those that are suppressed under MHS are comfort behaviours (sitting, preening and foraging). Birds under heat stress increase panting and drinking behaviours, as increased water consumption is required for efficient evaporative cooling from the respiratory tract (Etches *et al*., 2008). Although panting helps in achieving evaporative cooling, it requires increased muscular activity which in turn adds to the heat load (Willmer *et al*., 2005). The intervention of additional cup drinkers used in this study did not cause a reduction in panting behaviour in heat stressed broilers, however, other interventions reported in the literature have had some success. The use of cool perches (flow of cool water, 10^{o}C through galvanised pipes) under heat stress conditions (maximum temperature between 30.1 and 34.1^{o}C) reduced panting in broilers (Zhao *et al*., 2012), probably because the perches provided a wide surface for the bird, thus enhancing the level of heat loss because of the cool water involved.

The occurrence of preening behaviour was reported to be enhanced under conditions in which birds are thermally comfortable (Lolli *et al*., 2010). This agrees with the reduction in preening seen during heat stress in the current study. The reduction in foraging activities seen under moderate heat stress conditions in the current study might

be a means of birds seeking to reduce their total heat production which could arise from the extra activity incurred during foraging (Andersson *et al*., 2001). On the contrary, Syafwan *et al*. (2011) was of the opinion that that birds under heat stress shift from consuming feed to foraging in the litter which does not add to the heat load.

The litter moisture content was greater in the pens of birds exposed to MHS than in pens of birds in normal conditions, suggesting an increase in water loss from the body through the urine. It has been shown that chicks exposed to 35^{o}C for 4 hours had a 64% increase in water loss compare to those at 24^{o}C (Belay and Teeter, 1993). The current study showed that birds exposed to MHS consumed more water than those in the normal conditions, and since there was no evidence that they splashed water from the cups onto themselves or the bedding, it implies that the increase in moisture content of the litter was from the increased water content of the droppings. Increase urine output during heat stress has been reported to be another means of increasing non-evaporative heat loss (Borges *et al*., 2007).

Feed intake during heat stress was suppressed under conditions of MHS. However, as soon as the heat stress conditions were over, the birds increased their feed intake in order to compensate for their low feed intake during the immediate heat stress period. Reduction in feed intake during heat stress may be a direct effect of high temperature (Ferket and Gernat, 2006) or an indirect effect caused by the elevated body temperature which in turn regulates the pattern of feed intake (Lin *et al*., 2004) so that under high body temperature birds eat less in order to avoid excess heat load. The current study has shown the pattern of feed intake both during and after periods of heat stress.

8.5 Summary and conclusions

The provision of additional cup drinkers to birds was associated with limiting the rise in core body temperature of broiler chickens and enhanced heat loss from body surfaces. Broiler chickens showed a preference towards drinking from the cup drinkers as the duration of heat stress increased. Moderate heat stress supressed time spent by broilers on preening, lying, sitting and foraging but enhanced time spent panting and drinking. The intervention of providing additional cup drinkers did not compromise litter moisture content. Hence this intervention seems promising but would require further investigation into the exact mechanism by which the alleviation of heat stress was

achieved, and in particular how this strategy could be used to combat welfare problems associated with heat stress under commercial-scale broiler rearing operations.

Chapter 9: General Discussion

9.1 Introduction

The aims of this thesis were namely, i) to develop and validate non-invasive means of assessing the welfare of broilers under episodic heat and physiological stress conditions, ii) to investigate the impact of episodic heat stress, physiological stress and changes in light wavelength on the welfare of broiler chickens and iii) to investigate a novel means of alleviating heat stress in broiler chickens.

To achieve these aims, six experiments were conducted. The first experiment investigated the effect of light wavelength on the cognitive ability, growth performance and welfare of broilers. The second experiment demonstrated the potential use of the avian urate sphere as a non-invasive measure of stress in birds. The third experiment involved the development of an improved cognitive bias task for broilers and this task was validated on birds under conditions which mimic chronic stress. The fourth experiment involved the validation of three methods of measuring core body temperature under a simulated episodic heat stress condition. The fifth experiment demonstrated the relative comparison between the stress responses of birds subjected to high heat stress for 3 hours versus moderate heat stress for 6 hours. Finally, the sixth experiment explored the potential of alleviating heat stress conditions by providing birds with additional cup drinkers. In this final chapter, a critical discussion of the main findings from each experimental chapter will be undertaken to evaluate the extent to which the aims of the thesis have been achieved, implications for commercial poultry production and potential areas for future research.

The main findings from each experiment are as follows:

- Broilers reared in blue light had greater daily feed intake and were less active compared to their counterparts in the red light. Broilers from both light wavelengths learnt to discriminate cone colours in a visual task after 3 days of training, and had similar performance in subsequent cognitive tests.
- The basal levels of endogenous corticosterone measured in the plasma and the urate sphere demonstrated individual differences between birds. Dexamethasone suppressed the levels of endogenous corticosterone in the urate sphere but not in the plasma.

- The introduction of a punisher (air puff) enhanced learning in a spatial task by halving the number of training sessions previously reported for laying hens.
- In the cognitive bias task, corticosterone-treated broilers were pessimistic in their judgement of ambiguous positions of the cone. A positive correlation was established between the relative liver weight of the bird and the mean adjusted latency to approach ambiguous positions.
- The change in CBT measured from the temperature-ID chip (ΔCBT-chip) and the data logger (ΔCBT-logger) were positively correlated, with the ΔCBT-chip being less than the ΔCBT-logger by 0.1^{o}C.
- High temperature coupled with high RH aggravated the respiratory rate (RR) of broilers and this was accompanied by suppression of levels of preening behaviour.
- Significant positive correlations were found between the change in surface body temperature measured under the wing (ΔWT) and the ΔCBT-chip, and between ΔWT and ΔCBT-logger. Significant positive regression equations relating change in CBT and RR with apparent equivalent temperature (a factor which combines environmental temperature and RH) were also developed.
- High heat stress for 3 h and moderate heat stress for 6 h had similar effects on broilers, but mortality was only recorded in the high heat stress for 3 h treatment.
- For broilers exposed to moderate heat stress, the provision of additional cup drinkers reduced the rise in CBT and the proportion of time spent in wing drooping behaviour, but enhanced surface body temperatures. Provision of additional cup drinkers also enhanced proportion of time that broilers spent feeding, although this was not reflected in the amount of feed consumed.

9.2 Development and validation of non-invasive methods of assessing the welfare of broiler chickens

9.2.1 Core body temperature of broilers during episodic heat stress

Animal welfare is uncompromised when there is early detection and treatment of poor conditions such as disease (FAWC, 1992). The concept of poor conditions could be extended to those of heat stress, So that the development of an appropriate and less

invasive method of monitoring CBT could help animal welfare. This is the first study to report the use of temperature-ID chips to monitor CBT in broilers. In this thesis we established a relationship between change in core body temperature measured from a temperature-ID chip, a data logger and a laser probe placed under the wing during an episode of heat stress (diurnal changes in environmental temperature/RH for 2-3 days consecutively). In Brazil, the average daily diurnal temperature in a broiler house could rise from 23.2^{o}C to 32.2^{o}C (an increase of 9^{o}C; Nääs *et al*., 2010). Developing a less invasive measure of core body temperature is necessary because previous reports in broilers have shown that thermometer readings from the colon underestimated CBT by 0.57^{o}C compared to an implanted telemetry device (De Basilio *et al*., 2003). A change in CBT of 0.57^{o}C cannot be overlooked because this value corresponds to that recorded during moderate heat stress (Mitchell and Kettlewell, 1998) and could be accompanied with significant physiological and behavioural changes in the bird (see Section 9.2.3).

In the process of validating different methods to estimate CBT, it is not enough just to establish a relationship between different methods, so Bland and Altman (1986) suggested the use of a plot which confirms the agreement between the two methods. In the current study, the temperature-ID chip and data logger showed promising agreement in the measure of change in CBT with a mean difference of 0.1^{o}C. However, absolute CBT measured by the two devices did not show a significant correlation, which could probably be due to the wide variation in CBT measured by the temperature-ID chip at the pre heat stress period.

Manufacturers of temperature-ID chips claim that the temperature measured by the chips are a true representation of the CBT, however it seems that such a claim was applicable to heat stress and not thermoneutral conditions. Under thermoneutral conditions, temperature-ID chip readings were highly variable (Figure 6.2a) probably due to differences in muscle depth between birds. For instance, under thermoneutral conditions, the range of CBT measured by the temperature-ID chips in the 1st, 2nd and 3rd heat stress experiments was 1.20, 1.60 and 1.60^{o}C respectively. In goats, a similar finding was reported by Torrao *et al*. (2011) who compared CBT measured from microchips implanted subcutaneously and intramuscularly, rectal temperature and implanted data loggers. They found that the difference in CBT between microchips (implanted subcutaneously or intramuscularly) and an implanted data logger was lowest and least variable in the hot environment which could possibly be due to the reduced

temperature gradient between body surfaces. Since heat stress is associated with a rise in CBT (Jensen and Toates, 1997), then ΔCBT could potentially be an indicator of the actual heat load on the bird.

In the current study, whilst two out of the 12 data loggers used developed faults after implantation, the entire set of temperature-ID chips used in the three heat stress experiments functioned properly giving both identification and CBT readings with no cases of migration around the body. Other studies have reported problems of migration using chips implanted subcutaneously (Chen and White, 2006 and Lohse *et al*., 2010). Although it may be more invasive, intramuscular implantation of chips as demonstrated in our experiments probably helped to avert the problem of chip migration. With these promising result, a temperature-ID chip implanted 3 cm deep into the breast muscle could replace a surgically implanted data logger to reliably estimate the ΔCBT of broilers under conditions of heat stress. In the three heat stress experiments where the protocol involved changes in temperature/RH, the CBT measured by the temperature-ID chips were consistent in detecting changes in environmental conditions. Once implanted, microchips could give readings for several years, although this has less relevance for a broiler chicken reared typically to 42 days of age. However, Elcock *et al*. (2001) did report the development of tumours in rats two years after implantation with microchips.

Several studies have demonstrated the usefulness of selected surface body temperatures to estimate the thermal status of birds under heat stress. Giloh *et al*. (2012) reported a positive correlation between facial temperature and rectal temperature of broilers exposed to heat stress in an open (R^2=0.81) or a climate controlled (R^2=0.91) poultry shed. Nascimento *et al*. (2014) found a clear distinction between feathered and featherless body surface temperatures and their contribution towards sensible heat loss. The current study focused on the featherless body parts identified as thermal windows by Gerken *et al*. (2006). Out of all the four surface body temperatures (SBTs) measured, only the under wing temperature (WT) was positively correlated with CBT measured from a temperature-ID chip and a data logger. WT predicted 51% of the CBT estimated by an implanted data logger (Section 6.4). In addition, the ΔWT predicted 67% or 75 % of the ΔCBT-chip or ΔCBT-logger respectively. However, measurement of WT requires the bird to be restrained. It has been reported that the relationship between the surface body temperature and CBT is a cumulative effect of the high environmental

temperature and peripheral blood flow during vasodilation of blood vessels (Al-Tamimi, 2007). Hence, surface body temperatures are more rapidly influenced by environmental temperature than intramuscular estimates of CBT. Featherless body surfaces have shown positive correlations (R^2=0.8) with ambient air temperature (Nascimento *et al*., 2014; Nääs *et al*., 2010). In the current study, there was a rapid response of SBTs to environmental changes (elevated at the end of step up phase), whereas CBTs took longer to rise. Therefore, measurements from a temperature-ID chip implanted intramuscularly are less influenced by environmental temperatures and thus still provide a better estimate of CBT than surface measurements.

9.2.2 Non-invasive assessment of physiological stress

Physiological stress can be mimicked by subjecting animals to either a chronic mild stress paradigm (a series of acute stressors) or exogenous administration of ACTH or corticosterone hormones to increase the level of corticosterone in the blood (Cyr *et al*., 2007; Grippo *et al*., 2003; Olanrewaju *et al*., 2006; Puvaldopirod and Thaxton, 2000). However, in this thesis, non-invasive method of administering corticosterone to birds through mealworms as developed by Breuner *et al*. (1998) using a dose of 4 mg/kg BW (Post *et al*., 2003) was successfully employed in Chapters 4 and 5.

i) Corticosterone measurement

In Chapter 4, our interest was to measure endogenous levels of corticosterone in the urate sphere and plasma, and then to determine the effect of dexamethasone on the suppression of levels of corticosterone in the urate sphere and plasma. There were variations in the levels of basal endogenous corticosterone in the plasma and urate sphere which could be related to individual differences (Cockrem, 2007). The lack of significant correlation between basal levels of endogenous corticosterone in the urate sphere and plasma could be attributed to the difference in the samples; the urate sphere is an integrated sample (Clapp, 2010) while plasma is a single time point sample (Nelson, 2005). Despite this variation, the mean basal level of corticosterone in the plasma assayed with LC-MS/MS in the current study (3.25 ng/ml) agrees with published values in previous studies (Shini *et al*., 2008; Soleiman *et al*., 2011 and Wang *et al*., 2013) but is less than that reported by Olanrewaju *et al*. (2006) (11.10 ng/ml) and

higher than that of Dehnhard *et al*. (2003) (1.3mg/ml). Differences in corticosterone levels between studies could be related to a number of factors including differences in the method of hormone assay, age of the birds or sampling time.

One major limitation in this study was the inability to determine the levels of deuterated corticosterone and dexamethasone in the plasma and urate sphere due to laboratory errors. Nevertheless, Breuner *et al*. (1998) reported a peak in the plasma corticosterone within 7 mins after white crown sparrows ingested mealworms previously injected with corticosterone. This finding could provide an explanation for the observed suppression of the levels of endogenous corticosterone in the urate sphere after 20-30 mins and 60-90 mins post ingestion of dexamethasone. Personal observation showed that almost immediately after the birds ingested mealworms injected with deuterated corticosterone and dexamethasone there was an increase in water intake and diuresis, suggesting that the treatment had an effect on the physiology of the birds. Rettenbacher *et al*. (2004) detected a peak of radiolabelled corticosterone in the white uric acid component of chicken droppings just 36 mins after the birds had been injected. This finding of Rettenbacher *et al*. (2004) was based on data from a single bird which is unlikely to be truly representative of a population. In combination, it is intuitive to speculate that the transit time between a peak in plasma corticosterone and its detection in the urate sphere is within the range of 20-40 minutes which is a short period compared to that reported for urine and faeces where the average transition time in domestic chickens is 97 and 190 mins respectively (Hirschenhauser *et al*., 2012). However, the transition period of peak levels of corticosterone from plasma to the urate sphere requires further investigation.

There seems to be some consistencies in the suppression of levels of endogenous corticosterone measured in the urate sphere, hence this could serve as a reliable method for testing the sensitivity of the HPA axis in birds. Suppression of endogenous corticosterone in the urate sphere demonstrated in the current study agrees with previous reports by Clapp (2010) who offered Great tits dexamethasone one day prior to sample collection. Dexamethasone has been found to suppress levels of corticosterone in plasma, feathers and faeces (Etches, 1976; Westerhof *et al*., 1994 and Sheriff *et al*., 2010). Absence of a dexamethasone effect in previous experiments reported in the literature was attributed to the use of low dose of dexamethasone (Rettenbacher *et al*., 2004) or an incomplete suppression at the time the samples were collected (Dehnhard *et*

al., 2003). The lack of suppression of endogenous corticosterone by dexamethasone is an indication of the hyperactivity of the adrenal gland (van Praag, 2004) which is common under conditions of chronic stress. Therefore the urate sphere could be considered as a potential biomarker of the stress status of the birds in comparison to the invasive procedure of blood sampling.

ii) Assessing affective states

Whilst measuring corticosterone from the plasma or the urate sphere indicates the level of arousal caused by a stimulus, the degree of positivity/negativity (valence) of that stimulus can be assessed from the affective state of the animal (Mendl *et al*., 2009). Therefore, in Chapter 5, we developed an improved cognitive bias task as a non-invasive measure of welfare (affective state) in broilers under conditions that mimicked exposure to chronic stress for 7 days.

Previous spatial cognitive bias tasks used in laying hens required birds to undergo 12 training sessions before they could discriminate between a rewarded and an unrewarded location (Wichman *et al*., 2012). In rats, it was shown that the introduction of an aversive stimulus (quinine-soaked food) instead of inaccessible food (food covered with wire mesh) reduced the period of training required from 6 to 2 days (Burman *et al*., 2008 and 2009). The results in Chapter 3 of this thesis and Barnett *et al*. (2007) demonstrate that quinine-soaked feed was consumed by birds especially after a period without access to feed. Hence, the improved task developed in this thesis introduced an aversive stimuli, namely an air puff (Edgar *et al*., 2011) such that birds in the current study were puffed with ambient air if they flipped open a cone in the unrewarded position. This task enhanced the learning process so that after only 6 sessions (3 days of training) the birds had successfully learnt the task. In the current study, few younger birds (4 days younger) attained the learning criterion compared to older birds. Further investigation on the use of this improved task in younger birds should be considered.

To validate this improved cognitive bias task, we adopted a novel method which entails manipulating the internal state of birds by offering them mealworms injected with corticosterone for 7 days to mimic chronic stress. Corticosterone-treated birds took longer to open cones in the ambiguous positions, which imply a pessimistic judgement of ambiguous positions. Previous reports showed that rats treated with noradrenaline-

corticosterone to mimic acute stress responded negatively to ambiguous cues which were interpreted as an indication of a negative affective state (Enkel *et al*., 2010) see link A in Figure 9.1. An increased faecal level of corticosterone has also been associated with a pessimistic judgement of ambiguous cues in in capuchins (Pomerantz *et al*., 2012). The current study is the first to report the direct effect of corticosterone on affective state of birds (link A in Figure 9.1). Poor welfare conditions such as unenriched housing, unpredictable housing or other stressful conditions such as predatory attack and isolation have been shown to be associated with a pessimistic cognitive bias in animals (Bateson and Matheson, 2007; Harding *et al*., 2004; Bateson *et al*., 2011), see links B and C in Figure 9.1. Therefore, an improved cognitive bias task with an aversive stimulus to enhance learning could be adopted as a non-invasive measure of welfare (valence) in broilers subjected to a range of environmental stressors including heat stress and lighting conditions. It should be noted however, that not all manipulations have resulted in changes in affective state of animals. Neither increased stocking density in pigs (Scollo *et al*., 2014) or environmental enrichment in laying hens (Wichman *et al*., 2012) affected the animals' judgement for ambiguous cues.

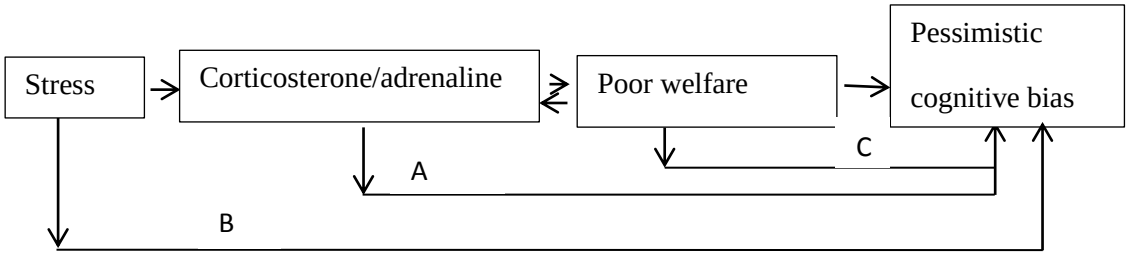

Figure 9.1: Existing links between stress, corticosterone/adrenalin secretion, poor welfare and pessimistic cognitive bias (see text above for explanation of Capital letters)

In support of the pessimistic judgement of ambiguous position by corticosterone-treated birds in the current study, a positive correlation between relative liver weight and adjusted latency to approach ambiguous positions was established. This suggests a relationship between chronic stress and cognitive bias that warrants examination in further research.

9.3 Impact of heat stress on growth performance, physiology and welfare of broiler chickens

Three heat stress experiments were performed to simulate episodic moderate heat stress (MHS) conditions using a protocol that mimics the natural changes in environmental temperature and RH that could occur during a heat wave. A good relationship between the temperature-ID chip and the data logger in measuring change in CBT was established in Chapter 6, so temperature-ID chips were adopted in subsequent heat stress experiments (Chapters 7 and 8). The ΔCBT of broilers exposed to MHS (30^{o}C, 70% RH) for 3 h in the 1st, 2nd and 3rd heat stress experiments were +0.7^{o}C, +1.3^{o}C and +1.4^{o}C respectively. The differences in the magnitude of the ΔCBT between experiments could be attributed to the sex or bodyweight of the birds used in the different studies. For instance, the 1st study involved the use of 46 day-old female broilers (approx. 2.4kg), the 2nd involved 32 day-old male broilers (approx. 2.0kg) and the 3rd involved 40 day-old male broilers (approx. 2.8kg). The weight of birds used in the three experiments ranged from 2.0-2.8kg, hence results obtained in this thesis could be applicable to birds of market age which are more prone to heat stress than younger birds (Sandercock *et al*., 2001) because of their increased level of heat production (Gous and Morris, 2005).

Broiler chickens exposed to a high heat stress (HHS, 32^{o}C, 70% RH, AET= 85.0^{o}C) at the end of 3 h had a ΔCBT of +2.4^{o}C, whereas at the end of 6 h of exposure to MHS (30^{o}C, 70% RH, AET = 78.0^{o}C) in the 2nd and 3rd heat stress experiments the ΔCBT was +1.7^{o}C and +2.0^{o}C respectively. This demonstrates that the greater the intensity of heat stress the greater the rise in CBT and the faster to reach a lethal point, defined as a 4^{o}C rise in CBT (DEFRA, 2005) if adequate means of alleviation are not implemented. On the second day of exposure to HHS, the mean ΔCBT of the two birds that died from heat stroke was 4.1^{o}C which supports the usefulness of the temperature-ID chip in reliably measuring the ΔCBT of broiler chickens.

Whether the ΔCBT is considered as moderate or severe depends on the strain of birds. Mitchell and Kettlewell (1998) believed that a ΔCBT of 0.4-1^{o}C or > 1^{o}C in broilers constitutes a moderate or severe heat experience respectively whereas Wolfenson *et al*. (1981) were of the opinion that a ΔCBT of 1 and 2^{o}C in layers represents moderate and severe heat stress respectively. This emphasises that broilers are more likely to be affected by heat stress conditions than layers (Sandercock *et al*., 2006), although growth performance, egg production and reproductive behaviour in laying birds are

nevertheless suppressed by heat stress (Mashaly *et al*., 2004 and Bozakova *et al*., 2009). Sandercock *et al*. (2001) had reported that older birds were more affected by heat stress than younger birds (63 vs 35 days). In the three heat stress experiments conducted, older and heavier birds had a greater rise in core body temperature than the younger ones when exposed to moderate heat stress conditions. At the end of 3 h of exposure to MHS in the 1st and 2nd heat stress experiments, the levels of pCO_2, pO_2 and pH were not affected suggesting that the birds did not develop respiratory alkalosis. Respiratory alkalosis is caused when excess CO_2 is blown off during panting (also called hyperventilation) leading to a drop in pCO_2 level and subsequently a rise in pH (Borges *et al*., 2003). It could therefore be speculated that the breathing pattern of birds in the MHS treatment was shallow breaths superimposed or alternated with deep breaths thus allowing for the simultaneous ventilation of the upper airways and lungs (Hill *et al*., 2008). However, in the 3rd heat stress experiment, the levels of pCO_2, iCa and bicarbonate in broilers were suppressed at the end of 3 h of exposure to either HHS (32^{o}C, 70% RH) or MHS (30^{o}C, 70% RH), suggesting some degree of respiratory alkalosis.

In the three heat stress experiments, birds exposed to MHS had a lower feed intake during the immediate heat stress period which agrees with other reports in the literature (Cooper and Washburn, 1998; Quinteiro-Filho *et al*., 2010 and Quinteiro-Filho *et al*., 2012) This does not suggest that during heat stress birds stop feeding completely; however, the meal size per feeding bout could be reduced (Savory, 1986). The temporary withdrawal of feed from birds during heat stress has proved to be effective in reducing the CBT (Francis *et al*., 1991; Yalcin *et al*., 2001; Ahmad *et al*., 2006). The short duration (3 or 6 h/day) of heat stress used in the three experiments availed the birds the opportunity to make up for their poor feed intake during the heat stress period, hence weight gain of birds in two out of the three heat stress experiments was not compromised. This may not be the case if the heat stress period was prolonged (e.g. 10 hours) such that birds could not compensate for their poor feed intake, in which case weight gain is reduced (Quinteiro-Filho *et al*., 2012). Increased body weight gain and feed intake corresponds to the period of minimal increase in core and surface body temperature (Yahav *et al*., 1995), in order words maximum growth is attained under thermoneutral conditions. This emphasises the negative correlation between core body temperature and productivity (Cooper and Washburn, 1998).

The predicted increase in intensity, duration and frequency of heat waves due to global warming (Robinson, 2001), implies that in the future broilers could experience heat stress of a greater duration or intensity and may experience more than one heat wave during their growth cycle. Such possibilities were investigated in Chapter 7, firstly, through a relative comparison of broilers subjected to high heat stress for 3 hours (HHS, 3h) or moderate heat stress for 6 hours (MHS, 6h). Mortality of birds was recorded in HHS treatment (no birds in MHS, 6h died) thus suggesting that the birds could not afford the rapid and high thermoregulatory effort required to adjust to such conditions (Yahav *et al*., 1997). However, the feet and comb temperatures of birds in MHS, 6h were lower than those in HHS after 3 h, with a similar trend seen in the 2nd heat stress experiment with a decline in comb temperature at the end of 6 h compared to 3 h (Chapter 8). The comb assists in heat loss (Mukhtar and Khan, 2012) and the reduction of comb temperature as the CBT increases suggests a reduction of heat flow to the brain which is very sensitive to high temperature (Dawson and Whittow, 2000). The maximum duration of transporting birds to the slaughter house in the UK is 3h (Nicol and Scott, 1990), but this could still result in mortality as seen in the current study if the environmental conditions imposed a thermal imbalance in the bird. In commercial broiler production, consideration of how best to transport birds under high environmental temperature and RH is a priority, including consideration or shorter travelling times where possible.

The possibility of repeated exposure to a heat wave was also investigated in Chapter 7. Birds exposed to MHS for the first or second time (MHS and MHS-2 respectively) had a similar ΔCBT, cloaca temperature and weight gain but MHS-2 birds had a lower wing, feet and comb temperatures. The feed intake of MHS-2 birds which had previous exposure to MHS (five days previously) was greater during the second heat stress period and this might have contributed to the high ΔCBT. Since the typical duration of a heat wave has been reported to be 2.7 days (Vale *et al*., 2010) and birds can supposedly achieve acclimatisation after 4-7 days (Yahav *et al*., 1996), this suggests that broilers in the current experiment may not be availed the opportunity to adapt. However, broilers could be prepared for future exposure to heat waves by exposure to heat stress at a much younger age (even in late embryogenesis or up to 10 days post hatch) to increase their thermotolerance level since the brain is yet to be completely developed (Arad and Itsaki-Glucklish, 1991; Zhou *et al*., 1997 and De Basilio *et al*., 2003). However, thermal manipulation needs to be undertaken with caution since this could inadvertently serve as

a source of early life stress to the developing embryo/chick. In the study in this thesis, broilers had their first exposure to MHS at 32 days of age when they had already attained a degree of maturity.

Broiler chickens exposed to MHS for 6 h spent a greater proportion of total observation time standing, panting and drinking but less time preening, lying, sitting and foraging. Drinking behaviour was increased by 52.8% when temperature was changed from a thermoneutral (16^{o}C, 63%RH) to hot conditions (32.2^{o}C, 51.9%RH) in turkey breeders (Bozakova *et al*., 2009). Hence, it could be argued that the behaviours in which birds engaged a greater proportion of time in during heat stress could be referred to as thermoregulatory behaviour, while those which are suppressed by heat stress could be classed as comfort behaviour.

9.4 Impact of mimicked chronic stress on growth performance, physiology and welfare of broilers

Physiological stress is associated with changes in internal organs, body weight and physiology of the birds. The current study did not measure the levels of plasma corticosterone in the plasma but relied on changes in internal organs reported in previous studies as indicators of chronic stress. In the current study, the corticosterone-treated birds had a suppressed relative spleen and an increased relative liver weights which are consistent with previous literature (Puvaldopirod and Thaxton, 2000; Post *et al*., 2003 and Shini *et al*., 2008). Based on these changes, it could be argued that by offering them mealworms injected with corticosterone the current study was able to mimic chronic stress in broilers.

Offering birds' mealworms injected with corticosterone to mimic chronic stress did not affect blood pCO_2 or pH levels, but did reduce sodium ion levels. Olanrewaju *et al*. (2006) reported an increase in levels of pCO_2 in arterial blood of birds implanted with an ACTH mini-osmotic pump for 7 days, but no effect on blood pH. The difference between these studies could be related to the degree of elevation in corticosterone induced by the different methods and the type of blood sample used (venous versus arterial). Venous blood samples are easier to collect and are useful in detecting thermoregulatory changes (Hocking *et al*., 1994). It is known that the kidney regulates the blood-acid base status (Ewing *et al*., 1999) and is one of the target organs of

corticosterone (Hess, 2006) and so the effect of corticosterone on blood acid-base was expected. Blood sodium levels were reduced through increased urinary excretion (Ewing *et al*., 1999) and electrolytes such as sodium, potassium and chloride assist in the maintenance of osmoregulation and acid-base status (Borges *et al*., 2003).

9.5 Impact of light wavelengths on growth performance and welfare of broilers

Research on the impact of light wavelength on broiler chickens has concentrated on behaviour, growth performance, immune system and meat quality (Cao *et al*., 2008; Xie *et al*., 2008 and Ke *et al*., 2011). In the current study, along with behaviour and growth performance, we investigated the effect of light wavelength (red or blue light) on cognitive ability of broilers in a visual discrimination task (Chapter 3). In this study, light wavelength was inadvertently confounded with light intensity, thus definitive conclusions cannot be made. We found no difference between birds from the two light wavelengths during the training and cognitive tests. Previous studies have failed to detect an effect of low light intensity on the growth performance of broilers (Blatchford *et al*., 2009 and Deep *et al*., 2010) but birds reared under low light intensity (1 lux) had pathological changes in their eyes, namely an increase in eye weight (Deep *et al*. 2010), therefore rearing birds under low light intensity should be discouraged. However, in the current study, birds in the low light intensity in chambers fitted with blue light filters were able to differentiate between cone colours in a visual task (white, black and grey coloured coned), which suggests no impairment of the eye. Another reason could be that the high light intensity in the test room (white light of intensity 300 lux) might have made it possible for clear vision during the test. Further studies however are required to clarify the effect of light wavelength on the cognitive ability of birds. The suppressed feed intake in broilers reared under red light in the current study, could be attributed to a diversion from feeding to foraging and walking. Prayitno *et al*. (1997a) also reported an increased level of activity in birds reared under red or white light compared to those in blue or green light, arising from increased stimulation of the hypothalamus (Lewis and Morris, 2000).

9.6 Alleviation of heat stress in broilers by the provision of additional cup drinkers

The provision of additional cup drinkers to birds exposed to MHS suppressed the ΔCBT by 0.3^{o}C, and this was accompanied with an increase in surface body temperatures,

which could perhaps serve as a means of achieving heat transfer from the body core to the periphery. The provision of additional cup drinkers reduced heat stress-related behaviour such as wing drooping. Although water consumed from the cup drinkers was not measured in our study, a previous study has shown that an 8% increase in water intake suppressed the change in CBT by 0.4^{o}C (Bogin *et al*., 1996). Nascimento *et al*. (2011) proposed that alleviation strategies that increase heat loss through sensible means (i.e. conduction, convection and radiation) are more beneficial to birds than evaporative means due to the increased muscular activity requirement for panting and the subsequent changes in acid-base balance. Sensible heat loss can be increased by increasing air velocity (Yahav *et al*., 2005) to assist in convective heat loss. Intermittent sprinkling of water every 15 minutes on the comb and wattles of broilers alongside an air velocity of 0.15-0.20 m/s during acute heat stress (40^{o}C, 45% RH) reduced the ΔCBT by 1.4^{o}C (Chepete and Xin, 2000). The short-term cooling achieved by Chepete and Xin (2000) could be a combined effect of sprinkling (to provide water for evaporation) and air velocity (to increase sensible heat losses). In the current study, based on the lack of a significant change in blood haematocrit levels, we suggest that birds were not dehydrated.

The lack of habituation of birds to the cup drinkers prior to the heat stress period could have caused bias in the use of the cup drinkers during the experiment, namely that they used the drinker during MHS simply out of curiosity. Nevertheless there was a tendency for a shift in preference of drinking from the bell drinker to the cup drinker as heat stress progressed, and this could suggest the need for greater opportunity for water intake needed for thermoregulation (Chaiyabutr, 2004). The wider dimension of the cup drinkers might have provided birds with greater access to water. Several previous studies highlight the limitations of nipple drinkers to supply birds with the required water necessary for thermoregulation during exposure to heat stress. May *et al*. (1997) found that bell drinkers enhanced the daily water consumption of broilers compared to nipple drinkers especially at the peak (28.1-32.2^{o}C) of a cyclic heat stress protocol. Bruno *et al*. (2011) also demonstrated that nipple drinkers do not provide birds with a sufficient quantity of water during heat stress compared to bell drinkers. Equally, Wabeck *et al*. (1994) in the USA showed that provision of bell and cup drinkers enhanced the liveweight gain of broilers during the spring, fall and winter season compared to birds provided with nipple drinkers. Furthermore, during summer the provision of bell and cup drinkers enhanced the live weight gain of broilers more than

the use of nipple drinkers (Wabeck *et al*., 1994). The mechanism behind the results obtained in the current study is yet to be fully understood and this calls for further investigation into the provision of additional cup drinkers to alleviate MHS in broilers.

9.7 Implications for commercial broiler chicken production

Increase in world poultrymeat production between the years 2013-2022 is projected to be 2.2%, with particular expansion forecast for Asia (AVEC, 2013). The environmental conditions in Asia are not optimum for growth of commercial strains of broiler chickens due to the occurrence of heat waves. The intensity, duration and frequency of heat waves could become more pronounced because of global warming (Robinson, 2001) and so the projected increase in poultrymeat production might not be realised unless our understanding of heat stress in broilers and its alleviation is improved.

Management strategies which can help broilers survive and better cope during heat stress conditions are required. Economic losses could be incurred (Toyomizu *et al*., 2005) by poultry farmers when broiler chickens of marketable age experience a short period of episodic high intensity of heat stress. Whilst the simplest option is to build environmentally controlled houses with sufficient capability to deal with periods of extended high temperature and RH and supported by a reliable electricity supply, this is difficult in countries with limited financial resources. Thus a well-designed drinker system may be a less expensive option than a sophisticated environmental control system. However, this will depend on the availability of sufficient water and careful management of to ensure that any spillage from the drinker system does not lead to conditions of wet litter and subsequent health problems.

Traditionally, poultry farmers rely on the behaviour of the chicks during the brooding period to detect whether the extra heat provided is too much or too low. For instance, crowding of birds at the heater indicates low heat supply (Aviagen, 1999). A behavioural matrix proposed in this study could be further developed for the prediction of the degree of heat stress experienced by birds (Table 9. 1). This matrix is based on behavioural findings in the 1st and 3rd heat stress experiments which indicated that during moderate heat stress birds exhibit increased respiratory rate and decreased levels of preening behaviour. The current study measured respiratory rate of bird through visual counting however, it might be difficult to keep track of the respiratory rate as it becomes too rapid under intense heat stress (Menten *et al*., 2006). This proposed matrix

calls for further development since other management factors such as stocking density have been reported to affect the level of preening and respiratory rate (McLean *et al.*, 2002) and (Lolli *et al.*, 2010)). Also visual counting of respiratory rate could be difficult under high degree of heat stress (Menten *et al.*, 2006), probably a better means of measuring respiratory rate could assist in developing this matrix.

Table 9.1: Proposed behavioural matrix for prediction of the degree of heat stress experienced by broiler chickens

Respiratory rate (*)	Level of preening (*)	Likely degree of heat stress
Low	**High**	**No heat stress**
Moderate	**High**	**Moderate heat stress**
High	**Low**	**High heat stress**

* Baseline levels will need to be established for specific farm conditions (i.e. stocking density, age of bird, genotype etc.)

9.8 General conclusions

This thesis established the use of non-invasive measures of welfare during heat stress (temperature-ID chip) and physiological stress (the urate sphere and cognitive bias task). This work has demonstrated for the first time the validation of temperature-ID chips in measuring core body temperature of broilers during heat stress, and the direct effect of corticosterone on the affective state of birds. Moderate levels of heat stress caused an increase in core body temperature, reduced feed intake and body weight gain. High heat stress for a period similar to the maximum transportation time of broilers from farm to the slaughterhouse resulted in some mortality. In line with previous reports, provision of readily accessible water could enhance the welfare of birds during heat stress.

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Appendix

Procedures used for preparation of water and quinine treated feed crumb

1. The feed crumbs was sieved to remove ‘dust’ and large crumbs
2. Feed crumbs was weighed out for each treatment, e.g. 300g for quinine and 300g for water
3. Feed crumbs were then poured into two trays; one for the crumbs to be treated with water and one for crumbs to be treated with quinine
4. Quinine solution was prepared as follows: 100ml water per 150g crumbs, so for 300g crumbs i used 200ml water.

   For 200ml of a 4% quinine solution:

   a. 8g quinine powder was weighed and poured into a glass cylinder / jug
   b. 200ml of hot water was then added
   c. This was stirred well for proper mixing of the quinine powder.
5. A syringe was used to spray the quinine solution over the crumbs; coated well and stirred the crumbs now and again
6. For the water-treated crumbs, a syringe was used to spray 200ml of hot water over the crumbs.
7. The crumbs were well spread out in an even layer and left to dry for 24 hours, ideally somewhere warm and dry.
8. The crumbs were sieved again to ensure they are of a similar size.

TABLE 2: Actual temperature and relative humidity conditions in the climate chamber at each phase of the heat stress protocol

	Temperature and RH at the different phases of the heat stress experiment				
	Pre heat stress (PrHS)	Step up (ST) 1hour	3 hours of heat stress (3HS)	Step down (SD) 1 hour	Post heat stress (PHS) 1 hour
Normal-Dry	20.6^{o}C, 48.6% RH	20.7^{o}C, 47.0% RH	20.9^{o}C, 53.4% RH	20.4^{o}C, 51.5% RH	20.6^{o}C, 48.9% RH
Normal-Humid	20.8^{o}C, 53.3% RH	20.5^{o}C, 76.1% RH	20.4^{o}C, 76.2% RH	20.4^{o}C, 68.6% RH	20.3^{o}C, 60.5% RH
Hot-Dry	20.6^{o}C, 47.4% RH	26.6^{o}C, 35.6% RH	30.5^{o}C, 37.7% RH	26.5^{o}C, 41.1% RH	20.6^{o}C, 57.9% RH
Hot-Humid	20.9^{o}C, 53.5% RH	28.7^{o}C, 69.2% RH	30.6^{o}C, 69.8% RH	22.4^{o}C, 70.6% RH	20.8^{o}C, 72.5% RH

Relationship between Change in core body temperature, respiratory rate and apparent equivalent temperature in broiler chickens

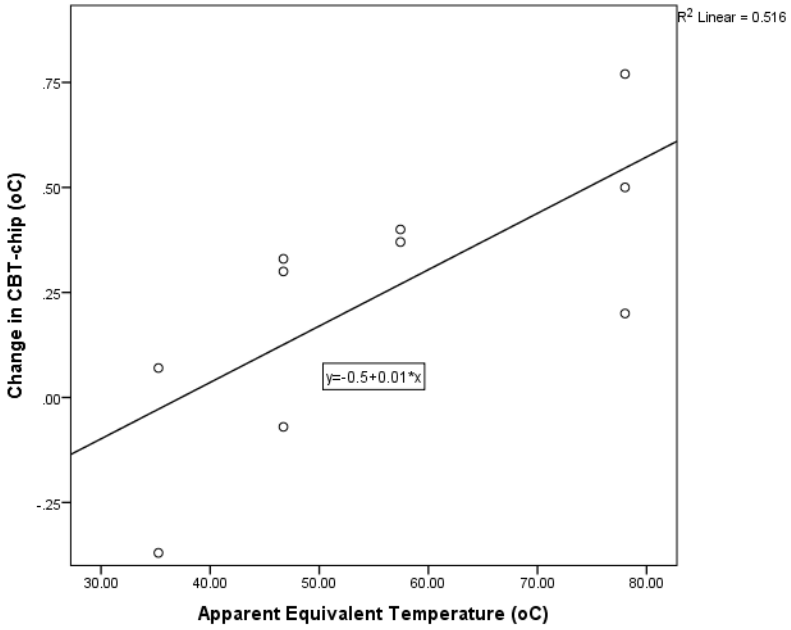

Figure 1: Regression plot showing the change in CBT-chip and apparent equivalent temperature. Each point is a mean of measurements taken over 2 days for each bird.

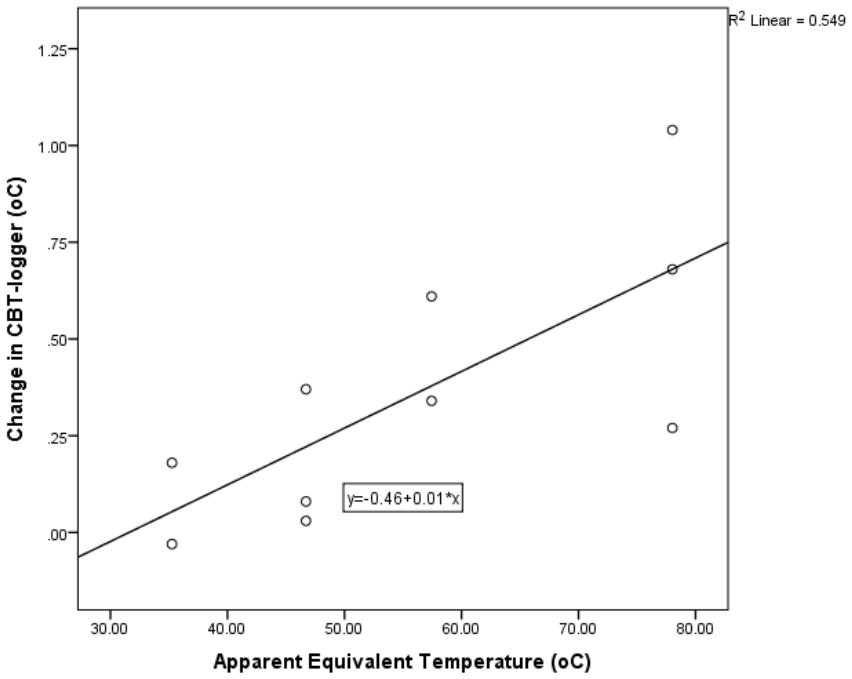

Figure 2: Regression plot showing the change in CBT-logger and apparent equivalent temperature. Each point is a mean of measurements taken over 2 days for each bird.

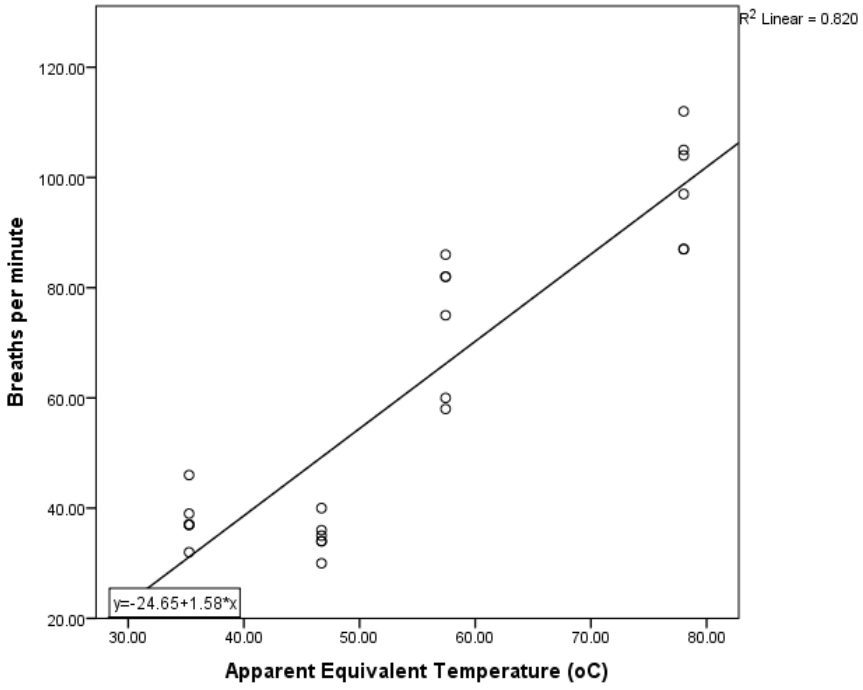

Figure 3: Regression plot showing the respiratory rate and apparent equivalent temperature. Each point is a mean of measurements taken over 2 days for each bird.