EFFECTS OF MELATONIN ON PHYSIOLOGICAL RESPONSES OF BROILER CHICKENS TO CONTINUOUS LIGHTING, HEAT AND ROAD TRANSPORTATION STRESSES DURING THE HOT-DRY SEASON

BY

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NOVEMBER, 2012

DECLARATION

I hereby declare that the work reported in this dissertation was carried out by me, Victor Olusegun SINKALU in the Department of Veterinary Physiology, Ahmadu Bello University, Zaria under the supervision of Prof. J. O. Ayo, Dr. A. B. Adelaiye and Prof. J. O. Hambolu. The work of other investigators referred to is duly acknowledged. No part of this dissertation has been previously submitted for a degree.

Victor Olusegun SINKALU	Signature	Date

CERTIFICATION

The dissertation titled effects of melatonin on behavioural and physiological responses of broiler chickens to heat and road transportation stresses by Victor Olusegun SINKALU meets the regulations governing the award of the degree of Doctor of Philosophy, Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to my Lord Jesus Christ, in whom I live, move and have my being (Acts 17:28).

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ABSTRACT

Experiments were performed with the aim of determining the impact of heat and transportation stresses on the behavioural, physiologic responses and performance of broiler chickens, administered with melatonin during the hot-dry season in the Northern Guinea Savannah zone of Nigeria. Broiler chicks were assigned randomly into three groups, comprising 100 chicks each. Group I was raised under natural photoperiod of 12 h light and 12 h darkness, without melatonin supplementation. Group II was kept under 24-h continuous lighting, without melatonin administration. Group III was raised under 24-h continuous lighting, with melatonin supplementation at 0.5 mg/kg *per os*, via drinking water. Melatonin was administered once daily for 56 consecutive days at 17:00 h from 2nd April to 29th May, 2010. The weekly mean temperature-humidity index recorded inside the poultry house was lowest at week 4 of the study, with the value of 48.60 ± 0.08 ^{o}C, but highest at week 1, with the value of 53.48 ± 0.97 ^{o}C ($P < 0.0001$). On day 42, the mean value of body weight of 2420 ± 50 g/bird was obtained in group III birds, while values recorded in groups I and II broilers were 1470 ± 30 g/bird and 1907 ± 38 g/bird, respectively. Dry-bulb temperature was negatively, but significantly ($P < 0.01$) correlated with feed consumption in groups I (-0.633), II (-0.597) and III (-0.615) broiler chickens. The Giemsa's, Leishman's and Wright's staining methods, successfully used in the laboratory for the first time in the present study, showed broilers' heterophils, lymphocytes, eosinophils, monocytes and basophils. The least erythrocyte, haematocrit, haemoglobin and mean corpuscular volume, but highest values of red cell distribution width were obtained in group I birds. The highest heterophil:lymphocyte ratio was obtained in group II birds. The significantly ($P < 0.05$) highest value of mean corpuscular

haemoglobin was obtained in group III. The overall mean hourly rectal temperature value of 41.50 ± 0.02 ^{o}C, obtained in the group I birds was significantly higher ($P < 0.001$) than the value of 41.14 ± 0.04 ^{o}C recorded in group III, but lower than that of 41.65 ± 0.02 ^{o}C obtained in group II birds. The diurnal variation in osmotic fragility of erythrocytes from the birds was highest in group II broilers, followed by those in group I and lowest in the group III broiler chickens. The overall value of 21.83 ± 1.01 % for panting obtained in group III birds was significantly (Kruskal Wallis Test = 17.45; $P < 0.0001$) lower than the values of 69.72 ± 6.77 % and 67.87 ± 7.19 %, recorded in birds from groups I and II, respectively. The post-transportation malondialdehyde concentration of 2.99 ± 0.20 µmol/ml and 2.57 ± 0.21 µmol/ml recorded in groups II and III, respectively were significantly ($P < 0.05$) higher than the corresponding pre-transportation values. Aspartate aminotransferase, alanine aminotransferase, creatine kinase and alkaline phosphatase activities rose significantly ($P < 0.05$) after transportation in all the groups. However, the activities of the endogenous enzymes, with the exception of superoxide dismutase in group III birds, were not significantly ($P > 0.05$) altered by road transportation. The trend of results obtained demonstrated that melatonin administration to broiler chickens enhanced their growth and productivity. It is concluded that melatonin administration alleviated the deleterious effects of continuous lighting and heat stress on broilers. Its oral administration prior to road transportation may enhance the survival and health status of broiler chickens in the tropical zones of the world.

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LIST OF ABBREVIATIONS

ACTH	-	Adrenocorticotropic Hormone
AFMK	-	Acetyl-formyl-5-methoxykynuramine
ALP	-	Alkaline Phosphatase
ALT	-	Alanine Aminotransferase
AMK	-	N-acetyl-5-methoxykynuramine
AOAC	-	Analysis of the Association of Official Analytical Chemists
AST	-	Aspartate Aminotransferase
AT	-	Ambient Temperature
ATP	-	Adenosine Triphosphate
BSA	-	Bovine Serum Albumin
BW	-	Body Weight
BUN	-	Blood Urea Nitrogen
Ca	-	Calcium
CAT	-	Catalase
CK	-	Creatine Kinase
CL	-	Continuous Lighting
CNS	-	Central Nervous System
CRF	-	Corticotrophin-releasing Factor
CS	-	Corticosterone
DBT	-	Dry-bulb Temperature
EDTA	-	Ethylene Diamine Tetracetic Acid

ELISA	-	Enzyme-linked Immunosorbent Assay
EOF	-	Erythrocyte Osmotic Fragility
ERK	-	Extracellular Signal-regulated Kinases
FCR	-	Feed Conversion Ratio
GC	-	Glucocorticoids
GMT	-	Greenwich Meridian Time
GPx	-	Glutathione Peroxidase
GR	-	Glucocorticoids Receptor
GRd	-	Reduced Glutathione
GSSG	-	Oxidized Glutathione
GSH	-	Glutathione
Hb	-	Haemoglobin
Hct	-	Haematocrit
HDL	-	High-density Lipoproteins
H:L	-	Heterophil:Lymphocyte
HPA	-	Hypothalamic-pituitary-adrenal
HPG	-	Hypothalamic-pituitary-gonadal
HPLC	-	High Performance Liquid Chromatography
H_2O_2	-	Hydrogen Peroxide
IL	-	Intermittent Lighting
IPEMA	-	Indian Poultry Equipment Manufacturer's Association
5-HT	-	5-Hydroxytryptamine (Serotonin)
K	-	Potassium

LDL	-	Low-density Lipoproteins
LDH	-	Lactate Dehydrogenase
MAPK	-	Mitogen-activated protein kinases
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration
MCV	-	Mean Corpuscular Volume
MDA	-	Malondialdehyde
MR	-	Mineralocorticoid Receptor
NaCl	-	Sodium Chloride
NW	-	North-west
NWLSS	-	Northwest Life Science Specialties
OSB	-	Oxidative Stress Biomarker
PCV	-	Packed Cell Volume
PUFA	-	Polyunsaturated Fatty Acids
RBC	-	Erythrocytes
RH	-	Relative Humidity
RT	-	Rectal Temperature
RCS	-	Reactive Chlorine Species
RDW	-	Red Cell Distribution Width
RNS	-	Reactive Nitrogen Species
ROS	-	Reactive Oxygen Species
SCN	-	Suprachiasmatic Nucleus
SD	-	Standard Deviation

SE	-	South-east
SEM	-	Standard Error of the Mean
SOD	-	Superoxide Dismutase
SW	-	South-west
TBA	-	Thiobarbituric Acid
TBARS	-	Thiobarbituric Acid-reactive Substances
THI	-	Temperature-Humidity Index
TI	-	Tonic Immobility
TP	-	Total Protein
TSH	-	Thyroid Stimulating Hormone
T_3	-	Triiodothyronine
T_4	-	Thyroxine
WBC	-	White Blood Cell

LIST OF UNITS AND SYMBOLS

d	-	Day(s)
dl	-	Decilitre(s)
fl	-	Femtolitre(s)
g	-	Gram(s)
h	-	Hour(s)
IU	-	International Units
kg	-	Kilogram(s)
km	-	Kilometre(s)
m	-	Metre(s)
L	-	Litre(s)
mg	-	Milligram(s)
min	-	Minute(s)
mm	-	Millimetre(s)
mmol	-	Millimole(s)
ml	-	Millilitre(s)
n	-	Number of animals
P	-	Probability
pg	-	Picogram(s)
r	-	Correlation coefficient
s	-	Second(s)
^{o}C	-	Degree Celsius

%	-	Percent
µg	-	Microgram (s)
µm	-	Micrometer(s)
µmol	-	Micromole(s)

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

It has been established that the hot-dry season in the Northern Guinea Savannah zone of Nigeria exerts adverse effects on poultry production (Ayo *et al*., 2005, 2011; Sinkalu and Ayo, 2008). The season, which is characterized by high ambient temperature (AT) and high relative humidity (RH) (Ayo *et al.,* 2005), induces heat stress in livestock, including poultry species. Many environmental factors, including high ambient temperature, induce oxidative stress (Sahin *et al.*, 2004; Azad *et al*., 2010) and deplete antioxidants in the body (Feenster, 1985; McDowell, 1989; Bhimaraj *et al*., 2012). Low plasma levels of antioxidant vitamins A, C and E, and folic acid, and minerals such as zinc and chromium predispose to an increase in oxidative stress in poultry subjected to unfavourable environmental conditions (Sahin *et al.*, 2004). Environmental stress has been shown to reduce plasma protein concentration and the activity of antioxidant enzymes, such as paraoxonase, catalase and superoxide dismutase (Sahin *et al.*, 2004; Dai *et al*., 2012). It increases markedly blood glucose and cholesterol concentrations (Donkoh, 1989).

Transportation is a critical phase in animal production and often considered as one of the main causes of stress, raising considerable interest both in economic and animal welfare terms (Minka and Ayo, 2008a; Fisher *et al*., 2009; Hao *et al*., 2010). During road transportation, animals are exposed to a variety of potential stressors such as rough handling, loading, transportation and unloading, noise, introduction to a new environment, vibration and postural instability during the journey, long journey hours,

deprivation of feed and water, fear, vehicle motion and extreme climatic conditions, especially during the hot-dry season (Minka and Ayo, 2008b; Nazifi *et al*., 2009; Jones *et al.,* 2010). It has been established that transportation of animals during the hot-dry season imposes on the animals heat and transportation stresses, resulting in increased generation of free radicals in the body (Minka and Ayo, 2007; 2008b; Minka and Ayo, 2010a). Heat and transportation stress factors act concurrently on transported animals to induce oxidative stress; which impairs the normal body function, leading to increased morbidity, mortality, poor meat quality and decreased productivity (Kadim *et al.,* 2006; Minka and Ayo, 2007; Minka *et al*., 2009).

When broilers are exposed to stressors for extended durations, the birds have to adjust to the stressors, rather than attempt to avoid them (Virden *et al*., 2007). One of the main disadvantages of adjustment to high ambient temperature (AT) is the significant reduction in body weight, which results from the increased energy demand by thermoregulation and the decline in feed intake (Yahav and McMurtry, 2001; Dai *et al*., 2011). This is accomplished by the activation of the hypothalamic-pituitary-adrenal (HPA) system (Olanrewaju *et al*., 2007; Haldar *et al*., 2010). Stress responses are integrally associated with changes in acid-base balance (Altan *et al*., 2000b; 2003; Yalcin *et al*., 2004), performance (Puvadolpirod and Thaxton, 2000; Veldkamp *et al*., 2000; Post *et al*., 2003), immunocompetence (Boa-Amponsem *et al*., 2000; Korosi *et al*., 2012), body temperature (Ayo and Sinkalu, 2007), behaviour (Gerritzen *et al*., 2004; Ayo *et al*., 2006; Klaassens *et al*., 2012) and haematological parameters (Veldkamp *et al*., 2000; Olanrewaju *et al*., 2007; Minka and Ayo, 2008b).

To alleviate the detrimental effects of high environmental temperature on oxidative stress and performance of poultry, dietary manipulations are preferable to other methods because of their practicability and lower cost (Sahin *et al.*, 2004; Sinkalu *et al*., 2009; Singh *et al*., 2012). Previous studies have shown that diets enriched with antioxidant substances, such as vitamins C, E and A, zinc and chromium, may be used to attenuate the negative effects of environmental stress. This fact suggests that detrimental effects of environmental stress are largely due to induction of oxidative stress (Lin *et al.*, 2002; Sahin *et al.*, 2002; Singh *et al*., 2012).

Environmental variables influence pineal gland in many avian species (Sudhakumari and Haldar, 2001). Arendt (1988) suggested that the pineal gland acts as a photoneuroendocrine transducer that responds via melatonin release. Melatonin, or *N*-acetyl-5-methoxytryptamine, is the main neurohormone synthesized and released by pineal gland. It sets the internal biological clock that regulates different daily and seasonal cycles or rhythms in various physiological systems, including the cardiopulmonary, reproductive, excretory, thermoregulatory, behavioural, immune and neuroendocrine systems in birds (Brennan *et al*., 2002; Alarma-Estrany and Pintor, 2007; Melamud *et al*., 2012). It is released during the hours of darkness in response to the activity of serotonin-N-acetyltransferase, the enzyme that catalyzes the synthesis of melatonin in both the retina and pineal gland (Lewis *et al*., 2006; Gregory and Sadeh, 2012). During the day, the light-induced production of dopamine within the retina suppresses the production of serotonin in the photoreceptors and, as a consequence, inhibits the biosynthesis of melatonin (Morgan *et al*., 1995; Zawilska *et al*., 2007). Thus,

broiler chickens exposed to continuous lighting may be severely deficient in serum melatonin (Vanecek, 1998; Lewis *et al*., 2006).

Besides its primary function as synchronizer of the biological clock, melatonin exerts a powerful antioxidant activity (Maldonado *et al*., 2012). It easily crosses cell membranes and the blood-brain barrier due to its amphiphilicity (Hardeland, 2005; Hardeland and Pandi-Perumal, 2005). It is a direct scavenger of hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$) radicals, and nitric oxide (NO) (Poeggeler *et al*., 1994), and was reported to be more efficient than vitamin E in scavenging peroxyl radicals (Sahin *et al*., 2004). In addition to its ability to scavenge peroxyl radicals, hydroxyl radicals, and other reactive oxygen species, melatonin is also involved in many physiological functions such as immune response, energy metabolism, and temperature regulation (Sahin *et al.*, 2004). Furthermore, melatonin stimulates the activity of several enzymes related to the antioxidant defence system (Tan *et al.*, 2000a). The influence of dietary melatonin supplementation on the components of the antioxidant defence system of heat-stressed poultry may be evaluated by measuring biomarkers of oxidative stress, the serum levels of some antioxidant vitamins and minerals, and activities of antioxidant enzymes (Sahin *et al.*, 2004).

There is paucity of information in the available literature on diurnal variations of physiological parameters in broiler chickens and the impact of stresses imposed on the birds by heat, road transportation and continuous lighting. The effects of administration of melatonin to broilers, subjected to stress situations during the hot-dry season in the

Northern Guinea Savannah zone of Nigeria are currently lacking in the available literature.

1.2 JUSTIFICATION OF THE STUDY

In the Northern Guinea Savannah zone of Nigeria, the hot-dry season usually lasts from March to April (Igono *et al*., 1983) and may extend to the first week of June (Oladele *et al*., 2003). During this period which coincides with the Easter festive period, the effects of heat stress are pronounced with the attendant production losses and mortality in chickens (Ayo *et al*., 2011). This is an annual problem that occurs during the hot-dry season in the zone.

The use of evaporative cooling is an inefficient means of dissipating body heat since it is a mechanism that is capable of enhancing the generation of oxidants or free radicals in the body (Balnave, 1998). According to Sinkalu *et al.* (2008a), AT in poultry houses during the hot-dry season in the zone fluctuated markedly between 16 – 41 ^{o}C. Consequently, several physiological parameters of broilers raised during the season may vary markedly as the hour of the day increases. It is worth noting that whenever the homeostatic mechanisms of birds are activated, extra energy is expended in the process. The energy so expended is no longer available for production processes. Broilers typically react to heat stress conditions by eating less feed, thus naturally controlling the rise in deep body temperature caused by digestion. The resultant effect is impaired growth and productivity. Broiler chickens normally do not eat during darkness, as long as this period does not extend for more than about 12 h. Therefore, it is assumed that feed intake and growth are maximal for broilers that are reared in (nearly) continuous

illumination (Aperdoorn *et al*., 1999). Farmers who receive broiler chickens 5 – 6 weeks (about Late February – Early March) for rearing before Easter are compelled to increase lighting duration in the poultry house in order to increase feeding time. This practice depletes the reserves of the endogenous antioxidant, melatonin in broilers. This may result in oxidative stress and increased susceptibility of the birds to diseases. It has been shown that antioxidant supplementation provides beneficial effects against stress-induced tissue damages (Sen, 2001; Akinwande and Adebule, 2003; Tauler *et al*., 2003). Melatonin is a potent and non-toxic antioxidant (Vanecek, 1998; Korkmaz *et al*., 2008; Pandi-Perumal *et al*., 2008), which may be of value in the amelioration of stress in broiler chickens.

The establishment of poultry farms away from the target markets and the need to convince buyers about the health status of the broilers necessitate transportation of live broilers during the hot-dry season in the Northern Guinea Savannah zone of Nigeria. Transportation of live birds includes the processes of handling, loading and unloading. Unfortunately, handling and loading of animals are the most stressful procedures compared to the journey itself (Knowles *et al*., 1999; Minka and Ayo, 2008a). Quantitation of welfare assessment of farm animals (Bracke *et al*., 1999; Minka *et al*., 2009), especially during transportation is an important husbandry practice (Fazio and Ferlazzo, 2003). Physiological parameters used for the assessment include behavioural and haemato-biochemical parameters.

Of these parameters, the leucogram is of great importance in the evaluation of welfare in birds (Altan *et al*., 2000a). The leucogram is used in the routine evaluation of leucocytes

on the complete blood count (Gaunt, 2004a). The leucocyte count in the avian species is obtained using manual techniques because the presence of nucleated erythrocytes and thrombocytes interferes with the counting of leucocytes using electronic cell counters (Campbell, 1995). Evaluation of leucocytes involves interpretation of leucocyte parameters, including total and differential leucocyte counts, and leucocyte morphology (Barger, 2003). Up to date, avian Clinicians and Researchers in Nigeria do not routinely consider avian leucocyte value in diagnostic plans and experimental designs because avian blood is quite different from those of mammals, and so is the laboratory procedure required for its analysis. It is of utmost interest to obtain the blood picture of chickens and identify the associated variations, especially in birds subjected to stressful environmental conditions in Nigeria.

Economic losses world-over, and, especially in tropical zones of the world are incurred by the livestock industries because farm animals are raised in locations and seasons where effective temperature conditions venture outside their zone of thermal comfort. St-Pierre *et al.* (2003) reported that annual losses from heat stress averaged $897 million, $369 million, $299 million, and $128 million for dairy, beef, swine, and poultry industries, respectively in the US. It is conceivable that the results of this work may shed light to our current understanding of behavioural and physiological changes that occur in broiler chickens, subjected to heat stress and transported by road during the hot-dry season. Also, the supplementation of melatonin in broiler chickens transported by road during the season may improve their health and reduce high economic losses, incurred yearly in poultry production during the hot-dry season.

1.3 STATEMENTS OF RESEARCH HYPOTHESES

The following null hypotheses were drawn:

1. It is not possible to count the different types of leucocytes of avian blood in Nigeria.
2. There is no difference between the performance indices of broilers administered with melatonin and those of the controls during the hot-dry season.
3. The values of oxidative stress biomarkers (OSB) of broilers do not change as the hour of the day increases during the hot-dry season.
4. There is no difference between the behaviour of melatonin-treated broilers and those of the controls, transported by road during the hot-dry season.
5. There is no difference between the locomotor activity and tonic immobility of melatonin-treated broilers and those of the controls transported by road during the hot-dry season.
6. There is no difference between the values of OSB of melatonin-treated broilers and those of the controls transported by road during the hot-dry season.
7. Continuous lighting has no effect on the values of OSB of broilers subjected to heat and road transportation stresses

1.4 GENERAL AIM OF THE RESEARCH

The general aim of the study was to evaluate the impact of continuous lighting, meteorological and transportation stresses on the performance, behavioural and physiologic responses of broiler chickens administered with melatonin during the hot-dry season.

1.5 SPECIFIC OBJECTIVES OF THE RESEARCH

The study was conducted during the hot-dry season in the Northern Guinea Savannah zone of Nigeria to determine in broilers the:

1. Leucocyte count.
2. Diurnal fluctuations in OSB values.
3. Effect of melatonin supplementation on the fluctuations.
4. Role of melatonin supplementation on transportation stress.
5. Modulatory role of melatonin supplementation on behaviour.
6. Influence of melatonin supplementation on locomotor activity and tonic immobility.
7. Effect of continuous lighting on physiological responses

CHAPTER 2

LITERATURE REVIEW

2.1 SIGNIFICANCE OF PROPER LIGHTING SYSTEMS IN POULTRY HOUSES

Broiler production plays a major role in food security for the rapidly increasing human population (Al-Ghamdi, 2008). Broiler breeders are usually reared on 8-h day lengths followed by an initial increase of 3 – 4 h at 20 week and weekly increments of 30 min or 1 h, thereafter to a peak of 15 to 16 h between 24 and 27 week (Aviagen, 2001; Cobb-Vantress, 2003). The reports of broiler breeder responses to different size increments in photoperiod tend to have insufficient treatments to determine the optimal day length to use during the laying period (Robinson *et al.*, 1998; Ciacciariello and Gous, 2005; Lewis and Gous, 2006). The performance of broiler chickens is improved by intermittent lighting (IL) schedules compared with continuous lighting (CL) (Cave, 1981; Deaton *et al.*, 1981). Ohtani and Leeson (2000) conducted an experiment to compare the effects of IL schedule with repeated cycles of 1 h light and 2 h darkness with a CL schedule on the performance, metabolisable energy intake, and heat production of male broiler chickens. They found that body weight gain and feed intake were temporarily reduced after the changing from CL to IL; however, they were significantly higher in IL *vs* CL chickens during the subsequent period of 3 to 6 weeks of age.

For many years, broiler chickens have usually been reared under continuous or near continuous (23L:1D) photoperiods to maximize feed consumption and growth rate (Abbas *et al.*, 2008). However, several investigations showed that using CL programmes

induces sleep deprivation and causes severe physiological stress responses (Kliger *et al.*, 2000; Campo and Davila, 2002). Therefore, most of the recent researchers have focused on restricting light regimens to improve productivity of broiler chickens because the physical activity is very low during darkness and energy expenditure of activity is considerable (Rahimi *et al.*, 2005). Aperdoorn *et al.* (1999) reported that chickens under intermittent light (1L:3D) had lower total heat production during the second and third hour of the dark period than those under CL programme. Melatonin exerts a positive effect on broiler health and well-being (Gordon, 1994) as well as energy metabolism and immune function (Aperdoorn *et al*., 1999).

2.2 INFECTION, HEAT STRESS AND OXIDATIVE STRESS-INDUCED IMMUNOSUPPRESSION

The commercial broiler of today appears to have compromised humoral immunocompetence, which manifests in poorer antibody production, higher mortality and lower resistance to stressors (Cahaner *et al*., 1998; Beirao *et al*., 2012). Such correlated responses have been attributed to the intense selection for rapid growth to market weight (Boa-Amponsem *et al*., 2000). Crucial involvement of monocytes and macrophages in humoral and cell-mediated immunity makes them indispensable factors in host defence (Konjufca *et al*., 2004). Macrophages by way of phagocytosis eliminate necrotic cells, circulating immune complexes, tissue debris, and invading microorganisms (Qureshi, 2003; Levkut *et al*., 2011). Additional important functions of macrophages include antigen presentation and secretion of a number of cytokines, prostaglandins, and free radicals (Chuammitri *et al*., 2011). Increased presence of free radicals causes

oxidation of biomolecules, leading to cell injury and death involved in the pathogenesis of several disease states (Kehrer, 1993; Bhimaraj and Tang, 2012).

Stress in chickens suppresses phagocytic activity, cell-mediated immunity and antibody synthesis (Freeman, 1987; Siegel, 1995). Stressed birds show an increase in basophils and heterophils and a decrease in lymphocytes; and, consequently, an increase in the heterophil:lymphocyte (H:L) ratio (Chuammitri *et al.*, 2011). With time, the H:L ratio often returns to normal (Katanbaf *et al.*, 1988), but basophils remain elevated and, thus, the latter may be used to assess prolonged stress (Maxwell *et al.*, 1992). With thermal stress, thymus weight decreases and circulating T lymphocytes counts decline (Guo and Gu, 1988). Exposure to high AT modifies various components of immune function such as T cell counts, natural killer cell counts, cytolytic activity of the natural killer cells, cytokine secretion, lymphocyte proliferation and immunoglobulin concentrations (Shephard, 1998). Trout and Mashaly (1994) reported that helper T cells (CD4+) and cytotoxic T cells (CD8+) are decreased in the blood of heat-stressed birds. T lymphocytes are divided into two main populations: CD4+ T cells and CD8+ T cells. Briefly, the cells play an important role in both the humoral and cellular immunity. The main function of CD4+ is stimulating the proliferation of the antigen-specific B cells. CD8+ cells are important in killing infected cells (Hamal *et al.*, 2010).

The natural exposure of chickens to endemic pathogens in the production environment (due to intensive rearing) challenges the immune system and stimulates the production of cytokines by macrophages, which act on the central nervous system, causing anorexia, lethargy, and neuroendocrine alterations (Annamalai and Selvaraj, 2012). Phagocytes can

also liberate free radicals, with the objective of eliminating the invading infectious agent. However, the free radicals react aggressively in the living tissue of the animal itself, causing damages like lipid peroxidation and alteration in cell membrane permeability (Murakami *et al*., 2001). Cells generate small amounts of free radicals or reactive oxygen species (ROS) while performing their normal metabolic functions (Korkmaz *et al*., 2008; Kedzierska *et al*., 2012). External factors such as heat, trauma, ultrasound, infection, radiation, hyperoxia, toxins and exercise can lead to increased generation of free radicals and other ROS in the body (Elstner, 1991; Halliwell and Whiteman, 2004). Free radicals occur when oxygen molecules are transformed into a highly reactive unstable form of oxygen. One free radical can initiate many chain reactions which are damaging to the tissues, if the free radical 'quencher' is not available to stop them. In the living organisms, the ravaging effects of oxidative free radicals are 'quenched' by antioxidants (Akinwande and Adebule, 2003; Bhimaraj and Tang, 2012).

2.3 IMMUNNOSUPPRESSION IN MODERN POULTRY PRODUCTION

The domestic fowl is a homeotherm which can live comfortably only in a very relatively narrow zone of thermo-neutrality which ranges from 18 – 22 ^{o}C (Al-Ghamdi, 2008). Mahrous *et al.* (2008) reported that the bird immune system is a complex network of specialized organs, glands and cells, which when working properly protect the body from pathogens such as viruses, bacteria and fungi. This system is composed of three basic sub-systems, the humoural, cellular and phagocytic. These sub-systems have different methods of defending the body from diseases. Lymphoid organ weights are easily measured and reflect the body's ability to provide lymphoid cells during an immune response (Heckert *et al.*, 2002). The spleen and bursa of Fabricius are the important

lymphoid organs, involved in the development and differentiation of T or B lymphocytes (Cheema *et al.*, 2007). Immunocompetence and growth are influenced by genetic and non-genetic factors (Mahrous *et al.*, 2008).

The broiler industry is increasingly dependent on husbandry practices, including medicaments for controlling diseases to realise the objectives of selective breeding (Boa-Amponsem *et al.*, 2000). Birds are intensively raised and have been genetically selected to maximize output under environmental conditions, often less than optimal that lead to lowered immunity, thereby increasing the risk of disease (Abdukalykova *et al.*, 2008). Abdukalykova *et al.* (2008) reported that enhancing the immune response and resistance to pathogens of birds through nutrients are considered practical and efficient means of improving performance in modern poultry production.

2.4 ACUTE AND CHRONIC STRESS RESPONSES

In natural circumstances, the acute stress response comprises adaptive physiological and behavioural responses to a noxious stimulus, or stressor (Dickens *et al.*, 2010). Such responses to a single acute stressor, is generally beneficial to the animal (Sapolsky *et al.*, 2000). When an animal encounters a stressor, two arms of the acute stress response are activated: the fast fight-or-flight response and the slower glucocorticoid response. The immediately-initiated fight-or-flight response is mediated by the sympathetic nervous system and the release of catecholamines, adrenaline and nor-adrenaline (Axelrod and Reisine, 1984). The fight-or-flight response is then followed by the slower hormonal stress response, in which the adrenal gland secretes glucocorticoid hormones as an end result of a hormonal cascade along the hypothalamic-pituitary-adrenal (HPA) axis

(Sivonova *et al.,* 2004; Soleimani *et al*., 2012). The HPA axis begins with stimulation of the adrenocorticotropin hormone, which in turn stimulates the adrenal gland to release glucocorticoids (GCs) (Sapolsky *et al.*, 2000). The predominantly secreted GC is either cortisol or corticosterone, depending on the species. For most mammalian species, the predominant GC is cortisol, whereas in rodents, birds, amphibians and reptiles, the predominant GC is corticosterone. Negative feedback quickly suppresses GC release once the stressor diminishes or ceases (Sapolsky *et al.*, 2000; Dallman and Bhatnagar, 2001). The combined actions of the fight-or-flight response and the GC response that together make up the acute stress response mobilise the physiological and behavioural processes throughout the body to focus on immediate survival needs (Romero, 2004).

If a stressor persists or a series of acute stressors initiate multiple consecutive stress responses, an animal becomes chronically-stressed (Wingfield and Romero, 2001). In a chronically-stressed animal, the short-term physiological and/or behavioural changes crucial for alleviating the acute stressor no longer aid in survival and instead become detrimental, leading to pathological conditions (McEwen, 1998). The GC negative feedback signal is disrupted (Dallman *et al.*, 1992), meaning that individuals experience prolonged elevation of GC at stress-induced concentrations (Romero, 2004). Overall, chronic stress disrupts its own response system, which can lead to detrimental physiological and behavioural consequences (Dickens *et al.*, 2010) such as immunosuppression.

Capture and restraint, such as placing a bird in an opaque bag is used, on a wide range of species to elicit and study the maximal GC response to an acute stressor (Wingfield and

Romero, 2001) as well as the maximal fight-or-flight response (Cyr *et al.*, 2009). Capture myopathy, caused by over exertion and tearing of the muscles and exacerbated by the surge of adrenaline from the fight-or-flight response (Montane *et al.*, 2002), is a major source of mortality during capture of a wide range of species, including the avian species (Hanley *et al.*, 2005; Marco *et al.*, 2006). Even after release to the wild, a single capture and handling incident can have lasting effects, significantly decreasing the future capacity for an animal to mount an acute stress response (Lynn *et al.*, 2009). Capture myopathy can also have long-term effects and significantly affect overall body condition (Cattet *et al.*, 2008) or ability to escape predation following release (Montane *et al.*, 2002).

2.5 THE PARADOX OF STRESS

Due to the pioneering work of Selye (1956), the use of the word 'stress' has become popular all over the world (Korte *et al.*, 2005). Stress has mostly been associated with negative events and consequences, it can take its toll on physical and mental health. There is, however, no justification for the assumption that the expression of stress responses always compromise health and/or welfare. Indeed, the functional aspects of stress have been neglected too often. The paradox of stress lies in the simultaneity of its adaptive nature and its possible maladaptive consequences (Korte *et al.*, 2005). Recently, a new stress concept has been developed, called allostasis (McEwen, 2000; McEwen and Wingfield, 2003), which introduces new terminology that avoids the ambiguity of the word 'stress'. In this concept, allostasis is defined as the adaptive process for actively maintaining stability through change (Sterling and Eyer, 1988; Korte *et al.*, 2009). The brain plays a central role in allostasis. By controlling all the mechanisms simultaneously, the brain can enforce its command and incorporate influential factors such as experience,

memories, anticipation and re-evaluation of needs in anticipation of physiological requirements (Koob and LeMoal, 2001).

2.6 ALLOSTASIS AND ITS MEDIATORS

Darwin (1859) was the first to understand that animal populations consist of individuals that differ from one another in their adaptive qualities and limitations. This process is known as natural selection. It is important to realize that natural selection exerts benefits by maximizing reproductive success of the adapted organisms, even at the expense of individual happiness, health and longevity (Nesse, 2000, 2001). Korte *et al.* (2005) reported that stress research should consider a cost benefit analysis of the different behavioural and physiological stress responses. Such an analysis forms the basis of 'The Darwinian concept of stress', since the 'benefits of allostasis' and the costs of 'allostatic load' produce trade-offs in health and disease. Different personalities, each with associated differences in underlying physiology and behaviour, vary in their vulnerability to stress-related diseases. Further support for this dichotomy came from observations of the aggressive and non-aggressive mice in the forced swim test (Veenema *et al.*, 2003). The aggressive mice continued to display escape behaviour, more climbing and swimming, even when no escape was possible, whereas the non-aggressive mice showed relatively passive floating behaviour, which can be interpreted as very adaptive because it saves energy (Korte *et al.*, 1996, 2009).

The fight-flight response is characterized by high activation of the sympathetic adrenal-medullar system and hypothalamic-pituitary-gonadal (HPG) axis (Korte *et al.*, 1992a). On the other hand, the freeze-hide response is characterized by the activation of the

hypothalamic-pituitary-adrenal (HPA) axis (Korte *et al.*, 1995). More specifically, the hypothalamus produces a neuropeptide locally in the brain called corticotrophin-releasing factor (CRF) that, in turn, stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH) into the blood (Buwalda *et al.*, 1992). This stimulates the adrenal cortex to release corticosterone (in birds, rodents) or cortisol (in pigs, humans).

Interestingly, it has been suggested that increased levels of corticosterone operating via hippocampal mineralocorticoid receptors produces fear-induced freezing behaviour in rats (Korte *et al.*, 1995). There is mounting evidence that individuals that preferentially show the freeze-hide response are characterized by high parasympathetic reactivity (Buwalda *et al.*, 1992); this is particularly apparent at time of high attention during the orienting reflex that often precedes the freezing response (Graham and Clifton, 1966). Such individual differences in physiological traits have also been observed in chickens, (Van Hierden *et al.*, 2002). It is well known that all the hippocampal regions (CA1, CA2, CA3 and dentate gyrus) play important roles in the regulation of these neuroendocrine responses, but the hippocampus is also involved in behavioural adaptation during and after stressful conditions (Korte *et al.*, 2005). Hence, hippocampal functioning is highly dependent on the circulating levels of corticosteroids.

In neurones, corticosteroids may bind to two dramatically different intracellular receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul and de Kloet, 1985). Interestingly, both receptors are located in those brain structures (limbic system) that are involved in the regulation of allostasis, such as hippocampus, septum and amygdala (Fuxe *et al.*, 1985). In agreement, it has been

reported that different factors, such as foot shock, ether anaesthesia, cold exposure, heat and noise stress (Singh *et al.*, 1990) or systemic corticosterone administration enhance 5-HT turn-over in the hippocampus and medial amygdala in rats (Summers *et al.*, 2000). Corticosteroids, via GRs (Singh *et al.*, 1990), but not MRs (Singh *et al.*, 1994), in the dorsal raphe nucleus increase extracellular 5-HT levels by enhancing the catalytic efficiency of tryptophan hydroxylase (Azmitia *et al.*, 1993). In agreement, the corticosteroid synthesis blocker reduces 5-HT release in the dorsal hippocampus (Korte-Bouws *et al.*, 1996). Also, through another mechanism, corticosteroids acting via GRs in the dorasal raphe nucleus may restore 5-HT levels by functional desensitisation of 5-HT_{1A} autoreceptors, resulting in increased electrophysiological activity of 5-HT neurones (Laaris *et al.*, 1997). The activation of the HPA axis in diverse hostile natural environments plays a major role in the emergency life history stage via a rapid increase in corticosterone in order to maximize survival and reproductive success in the long-term (Korte *et al.*, 2005). Freezing behaviour itself may reduce the likelihood of detection and attack from an aggressive animal or a predator. If caught, freezing behaviour or a tonic immobility response may result in the predator or attacker losing interest and moving away (Jones, 1986). During freezing the animal remains very alert (Korte *et al.*, 2005).

Interestingly, besides glucocorticoids, catecholamines also play an important role in the formation of strong emotional memories. It has been reported that behavioural (muscle) activity is associated with higher noradrenaline levels, while emotional stimuli are more related to high adrenaline level (De Boer *et al.*, 1989; Korte *et al.*, 1992b). Glucocorticoids, so-named because of their ability to promote conversion of protein and lipids to usable carbohydrates, serve the body well in the short run by replenishing energy

reserves after a period of activity, like running away from a predator (Korte *et al.*, 2005). Higher HPA axis activity predisposes animals to easily store abdominal fat, resulting in obesity associated with altered or distorted metabolic profile (Barat *et al.*, 2007; Nieuwenhuizen and Rutters, 2008).

Increases in the levels of catecholamines result in elevated leucocyte numbers in the blood because it is known that catecholamines recruit the body's "soldiers" especially natural killer (NK) cells and granulocytes (Dhabhar *et al.*, 1995). HPA axis activation, on the other hand, results in the release of glucocorticoids that, in turn, induces a decrease in circulating leucocytes. This is not due to immunosuppression, but rather to redistribution of leucocytes; that is, leucocytes exit from the blood and take position at potential 'battle stations', such as skin and lymph nodes (Dhabhar and McEwen, 1996). It is well accepted that cytokines may produce 'sickness behaviour', which is not a maladaptive response, but rather an organized, evolved behavioural strategy to facilitate the role of fever in combating viral and bacterial infections (Hart, 1988).

Mediators of allostasis (such as adrenal hormones, neurotransmitters, and immuno-cytokines) act on receptors in various tissues and organs to produce effects that are adaptive in the short term, but can produce an allostatic state which may be damaging if the mediators are not shut off (Korte *et al.*, 2005). Allostatic state refers to a state of chronic deviation of the regulatory system from its normal mean operating level (Koob and LeMoal, 2001). Four types of allostatic states leading to allostatic load have been identified by McEwen and Wingfield (2003); (a) repeated challenges; (b) failure to habituate with repeated challenges, (c) failure to shut off the response after the challenge

is over; and (d) failure to mount an adequate response. It is worth noting that the body and the brain have a huge capacity for adaptive plasticity (Korte *et al.*, 2005). Glucocorticoids do not act alone, and their effects can be blocked by agents that interfere with excitatory amino acids, such as glutamate (Sousa *et al.*, 2000). Sodium channels and possibly also T-type calcium channels are activated during glutamate-induced excitation (Korte *et al.*, 2005).

2.7 ACTIVATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS BY STRESSORS

Studies on the effects of stress on poultry have established that corticosterone, produced during acute or chronic stress is one of the final hormones of the HPA axis cascade (Freeman, 1987; Carsia and Harvey, 2000; Haldar *et al.*, 2010), and plays a multi-functional role in the chicken's body through the alteration of neuro-endocrine and immune components (Siegel, 1995; Cheng and Muir, 2004). It is clear that the neuro-endocrine and immune systems interact to maintain homeostasis when an organism is under severe or chronic stress. These systems utilise neurotransmitters, hormones and cytokines for communication and regulation of biological systems (Shini *et al.*, 2009). Catecholamines, glucocorticoids and cytokines all respond at first to help the body to adapt when stressors activate the HPA axis. Normally, a feedback system protects the organism by down-regulating these mediators (Sapolski *et al.*, 2000), but continued elevation of stress mediators overwhelm the organism, resulting in biological consequences such as inhibition of immunity growth and reproduction (Ahmed *et al.*, 2007).

Brain catecholamines and acetylcholine have an important role in the regulation of several functions (Herlenius and Lagercrantz, 2001). It was reported that the central nervous system (CNS) serotonin (5-hydroxytryptamine, 5HT) and catecholamines play crucial roles in heat loss and heat production mechanisms in mammals (White *et al.*, 1985). On the other hand, cholinesterase inhibition elicits cholinergic stimulation in the CNS peripheral tissues and organs, which leads to marked homeostatic impairments, including thermoregulation (Gordon, 1996; Ahmed *et al.,* 2007). Che *et al*. (1995) and Fleckenstein (1997) found that drug-induced hyperthermia decreased the activity of tyrosine hydroxylase and tryptophan hydroxylase, the key enzymes responsible for catecholamines and serotonin formation. Short-term physical stress is a type of stressor that most organisms are often faced with. However, problems arise when a stress response is activated for too long or is too frequent, and this is referred to as a chronic stress response (Bulmer and Gil, 2008). In chronic stress, the hormonal output has been observed to decrease over time (Sapolsky, 1992).

In birds, several studies have noted that social conditions experienced by the mother influence egg corticosterone (CS) levels (Hayward and Wingfield, 2004; Rettenbacher *et al.*, 2004; Saino *et al.*, 2005). Handling methods, elevated temperatures and the presence of predators have been shown to increase CS levels in the eggs of laying hens (Saino *et al.*, 2005; Haldar *et al*., 2010).

Exposure to high levels of glucocorticoids inhibits food intake and evokes body weight loss, probably via increased hypothalamic corticotropin releasing factor levels which may act as catabolic signals (Kellendonk *et al.*, 2002). Abundant high calorie food together with inactivity and the lack of energy expenditure predispose the chronically elevated

glucocorticoids to inhibit the action of insulin in enhancing glucose uptake (Lieberman, 2003). One of the results of this interaction is that insulin levels increase, and, together, insulin and glucocorticoid elevations promote the deposition of body fat. Bray (1998) reported that high levels of glucocorticoids produce an allostatic load which may result in obesity that, if combined with inactivity, increases the risk of the metabolic syndrome or syndrome-X. Korte *et al.* (2005) described syndrome-X as a new term for the cluster of conditions, including insulin resistance, high blood sugar levels, high triglycerides, decreased HDL ('good') cholesterol, abdominal obesity and raised blood pressure.

2.8 THE CONCEPT OF OXIDATIVE STRESS AND ITS ASSOCIATED BIOMARKERS

Oxidative stress arises when the balance between pro-oxidatants and antioxidants is shifted towards the pro-oxidants (Halliwell and Gutteridge, 2007; Funchal *et al.*, 2010). The free fatty acids induce a state of oxidative stress, and also impair endogenous antioxidant defenses by decreasing intracellular glutathione (Bouwstra *et al.*, 2008; Sahoo *et al.*, 2009). Living organisms are able to adapt to oxidative stress by inducing the synthesis of antioxidant enzymes and damage removal or repair enzymes (Davies, 1995). The activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in lipid peroxidation prevention may sometimes decrease (Wohaieb and Godin, 1987; Ozkaya *et al.*, 2002) or increase (Huang *et al.*, 1999; Aliciguzel *et al.*, 2003). The increase in the activities of antioxidant enzymes such as SOD, CAT and reduced glutathione (GRd) is considered as a protective mechanism against heat-induced free-radical production and lipid peroxidation (Tatli Seven and Seven, 2008; Tatli Seven *et al.*, 2009). Thus, exposing birds to heat stress resulted in a significant increase in SOD

and CAT (Altan *et al.*, 2003). McArdle and Jackson (2000) have also demonstrated a significant increase in free-radical production together with an increase in the expression of antioxidant enzymes during a period of non-damaging exercise. The role of SOD is to accelerate the dismutation of the toxic superoxide radical, produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. The action of GSH-Px is to reduce H_2O_2, with coupled oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) (Tatli Seven *et al*., 2009).

Oxidative stress markers have been measured in studies designed to assess redox status in response to exercise (Nikolaidis *et al.*, 2007), nutrition (Boparai *et al.*, 2007), exposure to xenobiotics (Nandi *et al.*, 2006) and road transportation (Minka and Ayo, 2008b). Veskoukis *et al.* (2009) monitored and correlated several commonly used oxidative stress markers in blood and tissues in an attempt to provide guidelines in designing experiments in the free radical biology field. They concluded that several commonly used markers in blood, including xanthine oxidase, thiobarbituric acid-reactive substances (TBARS), protein carbonyls, GSH, oxidized glutathione, CAT and total antioxidant capacity are reliable indices of free-radical-mediated changes occurring in the same markers in skeletal muscle, heart and liver.

2.9 MALONDIALDEHYDE CONCENTRATION AS THE END-PRODUCT OF LIPID PEROXIDATION

Quantitative assessment of conjugated dienes, lipid hydroperoxides, alkanes, aldehydes and isoprostanes have been extensively studied, and the effects of free-radical damage have received increasing attention (Moore and Roberts, 1998; Freitas *et al*., 2012). Since

Yagi (1982) applied the thiobarbituric acid (TBA) assay to estimate lipoperoxide concentrations in human serum, the assay has been a popular method of TBARS detection. From the assay's initial low specificity as a measurement of malondialdehyde (MDA) levels by fluorometry, the introduction of high performance liquid chromatography (HPLC) after derivatisation has increased the specificity. As the measured levels of MDA/TBARS depend on a variety of factors, the correlations between studies have been difficult (Seljeskog *et al.*, 2006).

Malondialdehyde (MDA) is generated as a relatively stable end-product from the oxidative degradation of polyunsaturated fatty acids (PUFA) (Horton and Fairhurst, 1987; Vinagre *et al*., 2012). MDA as a part of TBARS is frequently used as an indicator of lipid peroxidation (Seljeskog *et al.*, 2006; Chou *et al*., 2012). This free radical-driven lipid peroxidation has been causatively implicated in heat stress (Altan *et al.,* 2003; Sinkalu *et al.*, 2009), in ageing process (Poon *et al.*, 2004; Schmitt-Schillig, 2005), atherosclerosis (Wiztum, 1994; Cavalca *et al.*, 2001), Alzheimer's disease (Markesbery and Lovell, 1998) and cancer (Niederhofer *et al.*, 2003). Plasma MDA has thus been used as a biomarker of lipid peroxidation and has served as an indicator of free-radical damage in health and disease (Veskoukis *et al.,* 2009; Vinagre *et al*., 2012).

Malondialdehyde (MDA) is a three-carbon dialdehyde that can exist in various forms in aqueous solutions, depending on the pH of the solution (Esterbauer *et al.*, 1991). At acidic pH (pH < 4.5), MDA is found mainly as β-hydroxyacrolein in equilibrium with a dicarbonyl form. In neutral and alkaline conditions, the predominant form of MDA is an enolate anion. Initial acid and basic hydrolysis of plasma samples contribute to the

measurement of free or total levels of TBARS, respectively (Seljeskog *et al.*, 2006). The measurement of plasma MDA has been carried out by the TBA test (Moore and Roberts, 1998). When heated under acidic conditions, TBA reacts with a number of chemical species (nucleic acids, amino acids, proteins, phospholipids and aldehydes (Nair *et al.,* 1986) to produce a pink chromophore that can be measured by UV or fluorescence detection. These substances are termed TBARS because MDA is the main aldehyde that reacts with TBA (Sinnhuber and Yu, 1958). Since TBARS measurements are usually correlated with standards made from pure MDA (acid hydrolysis of 1, 1, 3, 3-tetrahydroxypropane; TEP), researchers often use the term MDA when measuring TBARS (Seljeskog *et al.*, 2006; Chou *et al*., 2012; Freitas *et al*., 2012).

Measurements of TBARS using plasma obtained from ethylenediaminetetraacetic acid (EDTA)-treated blood seem to produce lower values than that obtained from plasma harvested from heparin- or citrate-treated plasma, and even serum samples (Nielsen *et al.*, 1997; Carbonneau *et al.*, 1991). This is probably due to the chelation of iron by EDTA that limits further *in vitro* peroxidation. Iron is necessary in the superoxide-driven Fenton reaction and in the iron-catalyzed Haber-Weiss reaction that produces hydroxyl radicals, leading to increased lipid peroxidation and increased levels of TBARS. Studies using EDTA plasma will, therefore, detect lower values of TBARS than those involving the use of heparin, citrate, or vials without anticoagulants when sampling blood. Knight *et al.* (1987) used blood with and without anticoagulants and found TBARS reference values for EDTA as 0.58 μmol/L, citrate - 0.88 μmol/L, heparin - 1.13 μmol/L and that of serum as 0.79 μmol/L.

In plasma, MDA may exist both in free and in hydrolysable bound forms. Pryor and Stanley (1975) proposed a possible mechanism for the formation of MDA from the precursors of PUFAS, arachidonic acid and docosahexaenoic acid break-down by thermal or acid-catalyzed reactions. For the acid hydrolysis, however, different acids have been used. Nielsen *et al.* (1997) used orthophosphoric acid, Sinnhuber and Yu (1958) used hydrochloric acid, whereas Carbonneau *et al.* (1991) used perchloric acid. Seljeskog *et al*. (2006) tested eight different acids at three different concentrations for the initial acidifying step of plasma. They found that the stronger the ability of an acid to oxidize, the more bound MDA will be liberated and more MDA may be produced by PUFA oxidation. Perchloric acid with the lowest pKa released the highest amount of MDA by hydrolysis. Hence, perchloric acid became the acid of choice for HPLC method. The eight different acids tested include CH_3COOH, HBr, PCA, H_3PO_4, H_3PO_2, HNO_3, H_2SO_4 and HCl. MDA is also formed by decomposition of lipid peroxides during the acid heating stage of the TBA assay. In studies with peroxidising fish oil as much as 98 % of the MDA that reacted in the TBA assay was formed during the acid heating (Sinnhuber and Yu, 1958). Deproteinisation and removal of lipids early in sample treatment may, thus, influence heavily on measurements.

2.10 COMPARATIVE EVALUATION OF PLASMA AND SERUM FOR BIOCHEMICAL ANALYSIS

Biochemical analyses may be conducted using either plasma or serum. Serum and plasma are similar; however, plasma contains clotting factors and an anticoagulant that are not present in serum. Values for most analytes are similar in mammalian serum and plasma when the blood is handled in a standardized manner and separated from the cells within 2

hours (Hrubec *et al.*, 2002). Unfortunately, analyte values in serum and plasma may not be the same in animals having nucleated red cells such as fish, reptiles and birds (Hrubec and Smith, 1999). Since the fluid component of blood is in contact with blood cells for a longer period of time for serum compared with plasma, the likelihood of artifactual changes in serum may be greater (Hrubec *et al.*, 2002).

Although it is commonly believed that prolonged contact of plasma with blood cells may change analyte values in avian species, there have been few studies to document the changes. Lumeij (1985) documented altered plasma potassium values that corresponded to the duration of time between blood collection and centrifugation. There was a 30 % decrease in potassium concentrations in pigeon's blood that remained at ambient temperature for 30 minutes prior to centrifugation and a 65 % decrease after 2 hours. In chickens, there was a 29 % decrease in plasma potassium concentrations when blood was centrifuged 2 hours after collection (Lumeij, 1985). Woerpel and Rosskopf (1984) reported that blood left in contact with erythrocytes overnight had decreased concentration of total protein and potassium, increased activities of amino transferase and lactate dehydrogenase, and no change in uric acid, creatinine, or thyroxine concentrations. Calcium values vary directly with the plasma protein concentrations because calcium is protein bound (Hrubec *et al*., 2002).

Nucleated erythrocytes have a higher metabolic rate than their non-nucleated mammalian counterparts, thus making metabolic changes more likely in samples collected from avian patients. Additionally, chicken erythrocytes use nucleosides and glutamine as energy sources instead of glucose (Mathew *et al.*, 1993). Therefore, artifacts in plasma or serum

that result from metabolic processes may occur at variable rates or may affect different analytes in the avian compared with mammalian blood. Artifactual changes appear to occur rapidly in avian serum, even when the blood is handled in a manner deemed appropriate. It is, therefore, recommended that blood chemistry values be determined from plasma (Hrubec *et al.*, 2002).

2.11 FACTORS AFFECTING THE ERYTHROCYTE OSMOTIC FRAGILITY

The findings on the effect of age on erythrocyte osmotic fragility (EOF) are inconsistent. It has been shown that EOF increases with age in humans (Meurs *et al.*, 2005), guinea fowls (*Numida meleagris*) (Oyewale, 1991) and layers or egg-typed chickens (Azeez *et al.*, 2009). However, in White England turkeys, Oyewale and Ajibade (1990a) reported that erythrocytes of the 6 – 8-week-old chickens were more fragile than those of the 20 – 22 weeks of age. Brzezinska-Slebodzinska (2001) also observed that erythrocytes in younger rabbits of 1 – 5 months' old are more susceptible to membrane peroxidation than those of 3.5 and 6 months' old rabbits.

In a comparative study, Olayemi and Oyewale (2002) found that the erythrocytes of the N'dama breeds of cattle were more fragile than those of the White Fulani cattle. Erythrocytes of ducks were observed to be more susceptible to osmotic haemolysis than those of the domestic fowl (Oyewale and Ajibade, 1990b). Deprivation of drinking water for 24 h increased EOF of ducks (Baloyi *et al.,* 2006), but no significant effect was observed in guinea fowl deprived of water for twice of that duration (48 h) (Mafuvadze *et al.,* 2008). In an experiment which examined the effect of anticoagulants and storage time

on EOF of adult ostriches, Mafuvadze and Erlwanger (2007) found that EDTA increased the EOF compared to heparin.

The EOF is of value in the prophylaxis, diagnosis, treatment and prognosis of poultry diseases, and in the adaptation to various environmental conditions, including seasons (Oladele *et al.,* 2003; Awodi *et al.,* 2005a). Awodi *et al.* (2005b) found that the administration of antioxidant vitamin C decreased haemolysis, induced by nitrite administration in Red Sokoto goats. Minka and Ayo (2010b) demonstrated that EOF may be used as a diagnostic tool in road transportation stress. Their results showed that handling, loading and transportation of goats induced significant increases in erythrocytes, haemoglobin and EOF, apparently due to an increase in ROS generation in the body. Ascorbic acid administration ameliorated all the changes.

2.12 CHANGES IN DIFFERENTIAL LEUCOCYTE COUNT OF HEAT-STRESSED BROILER CHICKENS

Peripheral blood leucocytes are comprised of cells with roles in innate/adaptive response and cell/humoral-mediated responses (Sandford *et al*., 2012). Lymphocytes are the predominant leucocytes in the peripheral blood of chickens and turkeys (Bounous *et al*., 2000; Schmidt *et al*., 2009). In chickens, heterophils represent key components of the innate cellular immunity and their biology resembles that of mammalian neutrophils (Harmon, 1998; Chuammitri *et al*., 2011). Heterophils are the major cell type involved in initial response to various pathogens and irritants (Swaggerty *et al*., 2005) via pathogen recognition and initiation of cytokine and chemokine production that relays information to other immune cell types (Swaggerty *et al*., 2004, 2006). High corticosterone levels in

chickens, resulting from heat stress increased H:L ratio (Shini *et al.,* 2008a; Rashidi *et al*., 2010; Sandhu *et al*., 2012), and changed heterophil morphology including size, shape, and granulation (Shini *et al.,* 2008b). It is noteworthy that during heat stress, the increase in the ratio is a physiological phenomenon and can be used as a stress indicator (Gross and Siegel, 1983; Mashaly *et al*., 2004). Shini *et al.* (2008b) demonstrated that corticosterone treatment induced release of immature heterophils from bone marrow into peripheral blood circulation. The fine structure of blood cells in broilers chickens before and after they had been subjected to heat stress was studied by Maxwell *et al.* (1992). They found out that the numbers of basophils from the birds significantly increased compared with those obtained from control birds, and there was a concomitant reduction in eosinophils. This latter response may be due to an increase in the levels of circulating adrenocorticotropic hormones. Monocytes also showed significant increases in cytoplasmic lipid droplets after heat stress compared with pre-heat treatment, suggesting the onset of possible early fatty degeneration. During stressful conditions, intestinal permeability increases, allowing bacteria to enter the bloodstream to cause infection (Soltan, 2009).

2.13 INDEX OF THYROID FUNCTION

Intact "haemoglobin free" membranes of erythrocytes bind both thyroxine (T_4) and triiodothyronine (T_3). The binding of thyroid hormones to red blood cells is used as a measure of thyroid function (Singh *et al.*, 1976). Thyroid hormones participate in numerous processes such as growth, maturation, development and metabolism of most tissues (Varas and John, 2005). Thyroid hormones affect sympathetic nervous system activity, basal metabolic rate, body fat mass, food intake and thermogenesis (Karakoc *et*

al., 2004). Hence, abnormal thyroid function gives rise to changes in body weight (Lin *et al.*, 2006a; Bunevicius *et al.*, 2008). Small fluctuations in thyroid hormone concentrations may be important for body weight (Bunevicius *et al.*, 2008). It has been demonstrated that thyroid autoimmunity is related to changes in body mass index (Smutck *et al.*, 2007), increased cardiovascular morbility in general (Asvold *et al.*, 2007) or to a specific cardiovascular dysfunction such as pulmonary arterial hypertension (Volzke *et al.*, 2007).

Thyroid hormones increase cardiac inotropy and chronotropy and also directly affect vascular smooth muscle cells, promoting relaxation of resistance arterioles and decreasing peripheral vascular resistance. Normal thyroid hormone concentrations are required for normal cardiovascular functioning, whereas an excess or deficiency of thyroid hormones disorganizes it (Klein and Ojamaa, 2001). The heart is the major target organ for thyroid hormones, and many changes occur in overt thyroid dysfunction (Kahaly and Dillmann, 2005). Thyroid hormones may cause haemodynamic changes, including increased myocardial contractility, a decrease in cardiovascular resistance, an elevation of cardiac output, and a widening of pulse pressure (Dorr *et al.*, 2005).

Most of the cardiovascular effects of thyroid hormones are measurable mainly in patients with overt hyperthyroidism. Cardiac output is higher in patients with hyperthyroidism than in normal subjects (Ozmen *et al.*, 2007). The increase is due to the combined effects of a decrease in systemic vascular resistance, an increase in resting heart rate, increases in left ventricular contractility and ejection fraction, and an increase in blood volume. The haemodynamic changes typical of hypothyroidism are opposite to those of

hyperthyroidism. Most common signs are bradycardia, mild hypertension, and narrowed pulse pressure (Klein and Ojamaa, 2001).

2.14 INFLUENCE OF OXIDATIVE STRESS ON THE PERFORMANCE OF BROILER CHICKENS

Since the free-radical theory of ageing was first proposed in 1956, an increasing body of evidence has accrued, implicating oxidative stress as an important factor in both age-associated diseases and declines in physiologic functions (Finkel and Holbrook, 2000; Radwan *et al*., 2008; Perez *et al.,* 2009; Ajuwon and Idowu, 2010). Li and Holbrook (2008) reported that at the cellular levels, oxidants elicit a wide spectrum of responses, ranging from proliferation to growth arrest, senescence and cell death. The particular outcome observed varies significantly with cell type and conditions, but largely reflects the strength of the stimulus and the balance between the activities of a variety of signaling pathways that are activated. While low concentrations of oxidants tend to promote proliferation, higher concentrations result in growth arrest and/or cell death. Among those pathways implicated in supporting proliferation and promoting survival in many cell types in response to oxidant stimulation is that leading to activation of the extracellular signal-regulated kinases (ERK), a subfamily of a large group of kinases known as the mitogen-activated protein kinases (MAPK) (Martindale and Holbrook, 2002). The ERK pathway constitutes a major pathway through which growth factor receptors transduce proliferative signals to the nucleus. The role of ERK in regulating cell growth has received much attention (Johnson and Lapadat, 2002; Stork and Schmitt, 2002). Schneider (2005) reported that fatty acid autoxidation is a process of radical reactions. Once an initial fatty acid radical is formed, it immediately reacts with oxygen;

the resulting peroxyl radical abstracts a hydrogen from another fatty acid giving the hydroperoxide product and a new fatty acid radical, resulting in propagation of the chain. The task and capability of the antioxidant are to efficiently interrupt this chain process (Veskoukis *et al.,* 2009).

The tropical zones of the world poses a great threat to the production performance of the poultry (Khan and Sardar, 2005). The hot seasons of the year impose severe stress on birds, which leads to poor performance and high mortality among chickens (Njoku, 1986). Heat stress occurs when birds have difficulty in balancing body heat production and body heat loss. At high environmental temperatures, birds rely on different mechanisms to regulate their body temperature within a zone of comfort, the thermoneutral zone (Simon, 2003). High ambient temperature is considered as a potent meteorological stressor, causing impaired antioxidant status in poultry (Donkoh, 1989). This status is characterized by an elevated lipid peroxidation or reactive oxygen species (ROS) in serum and tissues (Sahin *et al*., 2002; Maini *et al*., 2007).

2.15 FREE RADICALS AND THEIR PHYSIOLOGICAL ROLE

A free radical is any chemical species that contains one or more unpaired electrons. Thus, it is unstable and promotes electron transfers; that is, oxidations and reductions (Sorg, 2004; Pourova *et al*., 2010). Unpaired electrons act as electron acceptors from other molecules, leading to their oxidation. The most common cellular free radicals are hydroxyl radical, superoxide radical and nitric oxide (Migliore and Coppede, 2009). Free radicals and related molecules are classified as reactive oxygen species (ROS) due to their ability to induce oxidative changes within the cell (Lykkesfeldt and Svendsen,

2007). A wide variety of ROS, reactive nitrogen species (RNS) and reactive chlorine species (RCS) (Table 2.1) are produced in the course of the normal metabolism in biological systems. They have several important physiological functions, but their accumulation beyond the needs of the cell potentially damage lipids, proteins, and nucleic acids (Migliore and Coppede, 2009).

Sorg (2004) reported that the RNS, nitric oxide, besides being an intermediate in the formation of the strong oxidant peroxynitrite, has several physiological functions. In the nervous system, it acts as a neuromodulator and plays a role in synaptic plasticity and long-term memory; whereas in the vascular system, it controls blood pressure, inhibits platelet aggregation and kills certain pathogenic microorganisms. Activated neutrophils and macrophages produce several ROS as a defence mechanism against invading microorganisms. The cells possess an intricate network of defence mechanisms to neutralise excessive ROS accumulation, including antioxidant compounds (Elkheir *et al*., 2008; Migliore and Coppede, 2009) and antioxidant enzymes, including SOD, CAT, glutathione reductase and glutathione peroxidases (GPxs) (Krizanovic *et al*., 2008; Otitoju *et al*., 2008). Therefore, under physiological conditions, cells are able to cope with the flux of ROS (Ellis, 2007). In oxidative stress, cellular antioxidant defences are insufficient to keep the levels of ROS below a toxic threshold (Schulz *et al*., 2000).

2.16 OXIDATIVE STRESS AND THE KYNURENINE METABOLIC PATHWAY

Cytokines, including IFN-gamma and IFN-alpha, induce the enzyme indoleamine 2, 3-dioxygenase, which has various negative consequences in the brain (Wichers and Maes,

Table 2.1: Nomenclature of Reactive Chlorine, Nitrogen and Oxygen Species

Free radicals	Non-radicals
Reactive oxygen species (ROS)	
Superoxide, $O^{\bullet -}_2$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypobromous acid, HOBr
Hydroperoxyl, $HO^{\bullet}_2$	Hypochlorous acid, HOCl
	Ozone, O_3
Peroxyl, $RO^{\bullet}_2$	Singlet oxygen ($O^{\bullet}_2{}^1\Delta g$)
Alkoxyl, $RO^{\bullet}$	Organic peroxides, ROOH
Carbonate, $CO^{\bullet -}_3$	Peroxynitrite, $ONOO^-$
Carbon dioxide, $CO^{\bullet -}_2$	Peroxynitrous acid, ONOOH
Reactive chlorine species (RCS)	
Atomic chlorine, $Cl^{\bullet}$	Hypochlorous acid, HOCl
	Nitryl (nitronium) chloride, NO_2Cl
	Chloramines
	Chlorine gas (Cl_2)
Reactive nitrogen species (RNS)	
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO^{\bullet}_2$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, $NO_2{}^+$
	Alkyl peroxynitrites, ROONO
	Nitryl (nitronium) chloride, NO_2Cl

(Halliwell and Whiteman, 2004).

2004). First, this enzyme converts tryptophan along the kynurenine pathway into several neuroactive intermediates and then into kynurenine. This chain of events results in reduced levels of tryptophan, the precursor of 5-Hydroxytryptophan (5-HT), and, thus, to reduced central 5-HT synthesis. Second, kynurenine metabolites such as 3-hydroxy-kynurenine and quinolinic acid have toxic effects on brain function. The former metabolite can induce oxidative stress by increasing the production of ROS, while

quinolinic acid may produce over-stimulation of hippocampal N-methyl-D-aspartate (NMDA) receptors and thereby lead to apoptosis and hippocampal atrophy (Konsman *et al.*, 2002). Indeed, hippocampal atrophy can be caused by NMDA overstimulation and this has been associated with depression (Schwarez and Pelliciari, 2002).

2.17 INFLUENCE OF TRANSPORTATION STRESS ON BIRDS

Transport exposes animals to a wide range of stressors (Fazio and Ferlazzo, 2003; Minka and Ayo, 2010b). The act of transportation leads to increased glucocorticoids secretion across a range of species (Aoyama *et al.*, 2008; Nilsson *et al.*, 2008) and increased heart rate (Zapata *et al.*, 2004). The degrees of stress exposure and stimulation of the HPA axis may depend on the distance the animal is transported (Chacon *et al.*, 2005) or the time in transit. Long-distance transport appears to be a major, continuous stressor. Dickens *et al.* (2009) demonstrated a complete inability to mount a GC stress response immediately following 14-h transport in poultry. Such a lack of response may indicate "exhaustion" of the HPA (Selye, 1946), a condition potentially signifying that the animals had been activating the axis constantly throughout the journey such that the axis reached a point where it could no longer respond to stimulation.

2.18 IMPACT OF TRANSPORTATION STRESS ON THE PERFORMANCE OF BROILER CHICKENS

The demand for livestock to be slaughtered outside places where they are produced has necessitated animal transport all over the world. Transportation is a complex procedure and its various stages are associated with potent stressors that together give rise to the full deleterious effects of stress on domestic animals (Ayo and Oladele, 1996; Lay, 2004).

However, Fisher *et al*. (2009) argued that land transportation of livestock is neither inherently good nor inherently bad for their welfare. Rather, it is the way that the transportation is done, and the management of the risks involved that determine the level of welfare of the animals. Hence, animal welfare risks fall into three categories:

(1) Stress and fear due to handling, loading, and the conditions and novelty of transport;

(2) Hydration, energy and fatigue challenges that increase with transport duration; and

(3) Risk to the thermal comfort and physical integrity of the animals (Fisher *et al.,* 2009).

Transportation does not only affect animal welfare, but it also has negative influence on meat quality, and it may cause economic losses (Perez *et al*., 2002; Maria *et al*., 2006; Witte, 2009). Broiler chickens can travel for long periods from the farm to the processing plant for slaughter. In a survey of 9 million birds killed in four plants, the average time from the start of the journey to the end of the unloading was 2.7 h, but the maximum time recorded was over 12 h (Warriss *et al*., 1990). Cheng and Jefferson (2008) reported that chickens are transported at least 3 times during their life span.

Thermal loads in transit have been proposed as the major cause of mortality, injury and reduced welfare, although other aspects of the complex transport microenvironment may constitute important sources of physiological stress (Carlisle *et al*., 1998; Earley and Murray, 2010; Earley *et al.,* 2010). For example, vibration may induce fear, nausea, distress and fatigue because of the distribution of the oscillatory motions and forces

within the body (Randall *et al*., 1993; Nazifi *et al*., 2009). It is accepted that journey length is an aspect that may affect animal welfare during transport from the farm to the slaughter house (Gosalvez *et al*., 2006). In addition to transport time, other factors can influence animal welfare during road transport and subsequent meat quality, such as loading and uploading (Buil *et al*., 2004), stocking density (Warriss *et al*., 2005), weather conditions (temperature, air velocity and humidity) (Aksit *et al*., 2006), vehicle characteristics (Huff *et al*., 2006), novel environments (Vecerek *et al*., 2006; Voslarova *et al.,* 2007), food and water deprivation or mixing animals from different groups (Verga *et al*., 2009). These stressors may impair homeostasis, and, consequently, the well-being of birds.

From both animal welfare and economic points of view, it is necessary to control and minimize stress-inducing factors during transport and before slaughter (Van de Water *et al*., 2003). Handling during loading is a critical point that has not been adequately studied, especially for broilers (Minka and Ayo, 2008a; Mazzone *et al*., 2009). Vignola *et al*. (2008) found that stress parameters were more influenced by transport and handling than by specific conditions, related to different loading methods in rabbits transported to the abattoir. The complete set of transport events, in particular the loading phase, have been reported to determine stress stage and it affects meat quality in calves (Van de Water *et al*., 2003). The duration (Kannan *et al*., 1997) and method of crating (Duncan, 1989) are factors that most likely cause physical injuries in broilers and can also influence their stress response.

Transport has been observed to cause dehydration (Schaefer *et al*., 1997) as a result of factors such as time without water, increased respiration rates and urinary water loss. Dehydration is associated with both elevated packed cell volume (PCV) and plasma protein concentration (Schaefer *et al*., 1997; Broom, 2003). Lowe *et al*. (2002) found that the most rapid increase in plasma osmolarity occurred in animals that are both temporarily deprived of water and exposed to hot conditions. Thus, greater care needs to be taken in managing birds subjected to conconmitant water deprivation and transportation during hot weather. Acute leucocytosis sequel to transportation stress in domestic animals, including broiler chickens, is caused by the endogenous release of corticosteroids or adrenaline (Mazzone *et al*., 2009). Another useful measure of the sustained effect of stress in birds is the heterophil/lymphocyte (H:L) ratio, which increased due to road transport stress (Minka and Ayo, 2008b). Kegley *et al*. (1997) and Schaefer *et al*. (1997) also observed an increase in the neutrophil/lymphocyte (N:L) ratio with transportation stress in cattle. The H:L ratio has been used as a reliable index of stress in birds (Scope *et al*., 2002; Minka *et al*., 2004) and other livestock (Stull and Rodiek, 2000; Kannan *et al*., 2000).

According to Knowles *et al*. (1995), sheep transported for up to 24 hours tended to show an increase in plasma glucose during the journey, rather than a decrease that would indicate metabolic exhaustion. This plasma glucose increase was most probably due to the initial stress response inducing a mobilisation of body energy reserves. Kannan *et al*. (2000) also observed that as a response to stress, elevation of glucose concentration was preceded by an elevation of the cortisol concentration. Corticosterone is the major adrenal glucocorticoid, whose increase in all animals is attributed to the activation of

stress response (Szeto *et al*., 2004). Duncan (1989) found that broilers crated and transported in a vehicle for 40 minutes had higher plasma corticosterone concentrations than broilers that were crated and loaded onto the vehicle, but not transported. Cashman *et al*. (1989) reported that fear levels in broilers were mainly determined by transportation and not just by catching and loading. By contrast, in cattle, Warriss *et al*. (1995) and Pettiford *et al*. (2008) found that loading procedures and the initial stages of transport are the most stressful events of the pre-slaughter phase; after this period, animals adapt to the conditions. In general, loading has been shown to be more stressful than unloading, with more adverse effects on the animals' welfare (Maria *et al*., 2004; Minka and Ayo, 2008a).

Unloading does not appear to produce a significant stress response, with no increase in cortisol or body temperature reported in cattle (Warriss *et al*., 1995) or sheep (Broom *et al*., 1996). In cattle, a study by Pettiford *et al*. (2008) showed that plasma cortisol and body temperature peaked within the first 1 to 2 hours of a 6-hour journey conducted under good practice. However, it is important to recognise that a cortisol decline from an initial stress-induced peak may not mean that the animals have wholly adapted to the transport process. The initial response to handling and the novelty of the transport experience may have diminished, but other factors such as hydration and fatigue become more important as the journey continues. Furthermore, physiological feedback mechanisms can attenuate responses such as cortisol, despite animals remaining in conditions that are measurably and behaviourally stressful (Munksgard *et al*., 1999). Nnani Costa *et al*. (2002) showed that the loading methods had no significant effect on carcass traits of pigs transported to the abattoir. Transport duration is an important factor affecting animal welfare and meat quality in pigs (Lammens *et al*., 2007). The mortality

of pigs transported to slaughter rises as the transportation distance increases (Gosalvez *et al*., 2006).

Warriss *et al*. (1993) showed that transport of broilers for up to 6 h depleted liver and muscle glycogen, as did fasting for 10 h. Some of the glycogen depletion recorded during transport may, therefore, potentially contribute to fatigue (Warris *et al*., 1997). When broilers were transported for up to 6 h, a progressive reduction of liver glycogen occurred, which was measurable from 2 h (Warriss *et al*., 1993). After transportation for 1 h, 2 h or 4 h, respectively, blood plasma creatine kinase (CK), lactate dehydrogenase (LDH) increased and meat quality was lower after 2 h compared to1 h or 4 h of transport (Yu *et al*., 2009). A variety of enzymes associated with tissue damage during various forms of stress; for example, creatine kinase (CK; muscle), lactate dehydrogenase (heart muscle), aspartate aminotransferase (liver), alkaline phosphatase (turnover of bone tissue) have been applied to broilers (D'Eath *et al*., 2009). Raised CK was found in transported broilers suffering from heat stress (Mitchell *et al*., 1992). Earley and Murray (2010) demonstrated that in response to physical stress or exercise, the enzyme CK leaks from the sarcoplasm of muscle cells into the blood, due to increased permeability of the sarcolemma. Therefore, elevated plasma CK activity is a useful indicator of muscular activity or muscle damage. Plasma indicators of muscle exertion and damage, such as the enzyme CK, do not always increase with journey length, but tend to be more responsive to knocks and bruises. Zaidi *et al.* (2005) and Yousef *et al.* (2006) reported that the AST activity in blood significantly increased under stress. Similarly, in studies related to stress, oxidative damage increased the ALP activity (Kaur *et al.*, 2006; Manna *et al.*, 2006; Yousef *et al.,* 2006).

Animal genetics can also influence stress and fear responses to handling and transport. In cattle, for example, behavioural responses to handling can vary widely and have been shown to be heritable (Kadel *et al*., 2006). Research on transporting cattle of different temperaments has shown that more docile animals lose less weight as a result of transport and recover their weight more quickly than more flighty animals (Burrow *et al*., 1998; Colditz *et al*., 2006). Animals may lie down at the end of a journey, but this response may be variable. For example, in a New Zealand study, 6-month pregnant dairy cows transported for 9 hours did not spend any time lying in the 2 hours after arrival, but grazed instead (Fisher *et al*., 1999).This does not in itself prove that the animals were not tired, but suggests that eating was more of a priority for the animals at arrival than lying down and resting. The need for attenuating the adverse physiological and behavioural consequences associated with the inevitable transportation of broiler chickens is recognized within the poultry industry (Ghareeb *et al*., 2008).

2.19 HEAT STRESS ELICITS OXIDATIVE TISSUE DAMAGE IN POULTRY

Ambient temperature and relative humidity are important thermal environmental parameters which are crucial in poultry breeding and production (Tatli Seven *et al.*, 2009). Optimum temperature and humidity suitable for poultry range between 16 – 25 ^{o}C and 30 – 40 %, respectively (Cerci *et al.*, 2003). Values higher than these ranges result in heat stress. Heat stress results in the reduction of welfare and productivity of birds. It reduces feed intake, body weight gain and feed conversion (Tatli Seven, 2008). Heat stress enhances the formation of free radicals and ROS, resulting in oxidative stress in

cells. It increases lipid peroxidation as a consequence of increased ROS generation. The increased lipoperoxidation leads to increased MDA level in the blood and other tissues (Okutan *et al.*, 2005; Ates *et al.*, 2006). Plasma MDA concentration, is the end-product of lipid peroxidation which decreases antioxidants in tissues (Tatli Seven, 2008). Oxidative damage may be minimised by antioxidant defence mechanisms that protect the cell against cellular oxidants, and repair systems that prevent the accumulation of oxidatively damaged molecules. Antioxidant enzymes, CAT, SOD, glutathione peroxidase (GPx) protect the cells from damages due to harmful effects of ROS (Altan *et al.*, 2003).

2.20 EFFECT OF HEAT STRESS ON PERFORMANCE AND SOME BIOCHEMICAL PARAMETERS OF BROILERS

Howie *et al.* (2009) reported that intensively selected birds, including broilers, have altered food intake control mechanisms and may be constantly hungry, due to high demands of nutrients required to maintain fast growth rates. The standard harvest time for growing broilers is 35 – 42 d, which may be adversely affected by alarming stimuli (Al-Ghamdi, 2008; Ghareeb and Bohm, 2008). Zeitgebers such as light, temperature and feeding times have been shown to exert a modifying influence on performance of birds (Lewis *et al.*, 2001).

Genetics, antibiotics, probiotics, vitamin supplements, antibodies and pelleting of feed decrease the time an animal requires to reach market weight, reducing feed and overall cost (Cook, 2004). Genetic selection for growth and breast meat yield has resulted in the development of different commercial crosses, designed to address the various needs of the poultry market. Birds destined for fast food or roaster markets vary in growth rate,

feed conversion efficiency, meat yield and reproductive efficiency (Melnychuk *et al.*, 2004). Exposure of broiler chickens to chronic heat stress leads to the deterioration of most production parameters (Abbas *et al.*, 2007). In addition, exposure to high environmental temperatures compromises most immune functions in chickens and, thus, decreases total white blood cell (WBC) count in the circulation (Nathan *et al.*, 1976), CD4+ T and CD8+ T-cells in the circulation (Trout and Mashaly, 1994), antibody titres to sheep red blood cells (Bartlett and Smith, 2003), and T and B-cell mitogenic proliferation (Atta *et al.*, 1996). Furthermore, exposure to high environmental temperature activates the HPA-axis in chickens, culminating in increased plasma level of corticosterone (CS) (Nathan *et al.*, 1976; Soleimani *et al*., 2012), which suppresses many immune functions (McEwen *et al.*, 1997).

Heat stress is one of the most important factors adversely affecting overall poultry production in the tropics (Haldar *et al*., 2010). Aksit *et al.* (2006) reported that high ambient temperatures from 21 d to slaughter age resulted in a decrease in growth rate, meat yield and breast protein content in broiler chickens. High AT during rearing is also associated with an increase in the stress status of broilers, which is measured by H:L ratio and longer fearful behaviour, as indicated by the duration of tonic immobility (TI) (Yalcin *et al.,* 2003). Heat stress decreases lymphocyte percentages in birds (Borges *et al*., 2004), thus increasing H:L ratio. Heat stress affects the blood acid-base balance of poultry by decreasing the partial pressure of arterial blood carbon dioxide, which causes blood pH to become alkalotic (Koelkebeck and Odom, 1994). Studies in birds subjected to hyperthermic temperatures have also demonstrated that plasma levels of calcium,

sodium, inorganic phosphorus and magnesium are depressed (Koelkebeck and Odom, 1995).

After birds are exposed to a high ambient temperature, their body temperature rises above the normal value (Cable *et al.,* 2007). Cooper and Washburn (1998) reported that heat stress is induced in broilers, whenever the environmental temperature exceeds 32 ^{o}C. Corticosteroid concentrations (Aengwanich, 2008), the relationships between adrenocorticotropic hormone (ACTH) and leucocyte responses (Altan *et al.*, 2000a) and percentage of lymphocytes (Campo and Davila, 2002) have been used as measures of environmental heat stress in birds (Borges *et al.*, 2004).

When broilers are exposed to stressors for extended durations, they must adjust to the stressor, rather than attempting to combat or avoid it (Virden *et al*., 2007). One of the main disadvantages of adjustment to high AT is the significant reduction in body weight, which results from the increased energy demand by thermoregulation and the decline in feed intake (Yahav and McMurtry, 2001). This is accomplished by the activation of the HPA system (Siegel, 1980). When an animal experiences conditions that mediate the hypothalamic-hypophyseal-adrenal axis, it will exhibit a series of changes known collectively as physiological stress (Olanrewaju *et al*., 2007). It has long been recognised that stress responses are integrally involved in acid-base balance (Altan *et al*., 2000b; Yalcin *et al*., 2004), performance (Puvadolpirod and Thaxton, 2000; Veldkamp *et al*., 2000; Post *et al*., 2003), immunocompetence (Boa-Amponsen *et al*., 2000), body temperature (Ayo and Sinkalu, 2007), behaviour (Gerritzen *et al*., 2004; Minka and Ayo, 2010a) and haematological parameters (VeldKamp *et al*., 2000; Olanrewaju *et al*., 2007).

The acid-base balance is influenced by a range of internal and external factors, including the diet, environmental conditions and metabolism (Derjanti-Li *et al*., 2002). Collectively, these factors affect moment-to-moment regulation of pH in blood and tissues. Most final metabolites are acids that can be removed from the body by the kidneys and lungs (Olanrewaju *et al*., 2007). However, if these metabolites are left unregulated, they can accumulate in the body and alter the acid-base balance from its normal status (Ruiz-Lopez and Austic, 1993).

Integration of the HPA system stimulates the hypothalamus to produce corticotrophin-releasing factor, which causes the pituitary gland to release adrenocorticotropic hormone (ACTH) (Holmes and Phillips, 1976; Klemcke *et al*., 1989). The presence of ACTH in the circulation stimulates the adrenals to secrete CS (Holmes and Phillips, 1976). Chronically, elevated CS levels result in many deleterious effects on the performance of birds, with one of the most detrimental being the catabolism of structural protein through CS-induced gluconeogenesis (Baxter and Rousseau, 1979). Excessive concentrations of glucocorticoids result in catabolic effects in skeletal muscle (Tomas *et al*., 1979; Odedra *et al*., 1983). Elevated concentrations of glucocorticoids decrease the rate of synthesis (Hayashi *et al*., 1992) and increase the rate of breakdown of myofibrillar proteins, which leads to muscle wasting and growth retardation (Ohtsuka *et al*., 1992; Higuchi *et al*., 1996).

This corticosterone-induced growth inhibition is accompanied by increased lipid peroxidation, induced by free radicals. The oxidative stress accelerates muscle growth inhibition and atrophy (Taniguchi *et al*., 1999). This shift in metabolism is well

documented in birds treated with CS or ACTH, which typically demonstrate significant decreases in body weight (BW) (Siegel *et al*., 1989; Puvadolpirod and Thaxton, 2000; Post *et al*., 2003). Furthermore, a decrease in BW has been shown to be independent of increased feed intake (Siegel and van Kampen, 1984; Puvadolpirod and Thaxton, 2000). Studies involving physiological stress, induced through exogenous administration of stress hormones, demonstrate a general model for stress research. This is because the same hormonal cascade occurs during the stress response, regardless of the specific stressor responsible for the initiation of the sequence (Virden *et al*., 2007). However, the studies are, probably, more closely associated with environmental, non-temperature-related stressors because heat stress almost always results in a reduction in feed intake (Austic, 1985). High environmental temperatures decrease feed intake of broilers (Cooper and Washburn, 1998; Veldkamp *et al*., 2000). Moreover a reduced digestibility of some amino acids has also been recorded due to high environmental temperatures (Balnave and Oliva, 1991; Zuprizal *et al*., 1993). It has been suggested that high ambient temperature alters gut absorption of certain amino acids and induces an inbalance in plasma amino acids that may decrease broiler performance and thermal tolerance (Brake *et al*., 1994a).

2.21 THERMOREGULATION AND ITS AUTONOMIC AND BEHAVIOURAL MECHANISMS IN DOMESTIC ANIMALS

Thermal stress exerts profound effects and alters the physiological processes of absorption, distribution, metabolism and excretion (Leon, 2008). Physiological adjustments to heat exposure consist of a variety of autonomic and behavioural responses that are aimed at maintaining core body temperature at a non-lethal level. The primary autonomic responses enhancing heat dissipation include vasodilatation; that is, increased

blood flow from the core organs to the skin surface, sweating and increased ventilation (Leon, 2008). Panting is an effective method of evaporative heat dissipation in birds, dogs, sheep and rabbits. Although panting would appear to be a costly mechanism of cooling, in many species it is performed at a resonant ventilatory frequency that minimises energy expenditure (Calder and Schmidt-Nielsen, 1966). Behavioural homeostatic mechanisms of core body temperature vary slightly between species, but are used preferentially, when available and effective, over autonomic mechanisms as they require minimal energy expenditure to sustain. For example behavioural thermoregulatory mechanisms in humans include the seeking of shade or cool microclimates, the discontinuation of exercise or high metabolic activities, the consumption of cool fluids and removal of clothing (Leon, 2008).

2.22 ANIMAL WELFARE AND BEHAVIOUR

The production of broiler (meat) chickens in the UK by 2006 was around 800 million birds (Fazio and Ferlazzo, 2003). Over 2 million broilers are collected and transported daily in the UK for domestic consumption. On a typical transport vehicle carrying 6500 broilers, typically 100 kW of heat can be generated (Fazio and Ferlazzo, 2003). If this heat is not dispersed, then adverse conditions can develop quickly. The primary concern behind all handling and transport was always to improve the welfare of animals (Chacon *et al*., 2005; Minka and Ayo, 2008a).

Evaluation of an animal's mental state is a critical goal for welfare assessment, considerations of both positive feelings (what an animal 'likes') and resources that an animal is motivated to obtain (what an animal wants) appear to be important. However,

since animals may make choices that are not in their long-term interests, an assessment of positive feelings and resources should include an evaluation of any associated actual or potential harms, such as fear, distress, pain, injury and disease (Boissy *et al*., 2007). A review of current evidence suggests that positive welfare can be best assessed by resources (that is inputs) that are valued by an animal and by positive outcomes such as behavioural responses, influences on cognitive processes and physiological markers (Yeates and Main, 2008).

As Boissy *et al.* (2007) outlined, behaviour is of "crucial importance in gauging what animals want". A number of different approaches are available to infer whether an animal likes something, and they include physiological markers, behavioural markers and the more recently developed cognitive assessment (Yeates and Main, 2008). Behavioural studies appear practically useful. Positive welfare can improve the biological functioning of an animal and its health as well as its ability to cope with challenges and stress, thereby decreasing cortisol levels (Yeates and Main, 2008). Reduced activity is considered as a positive welfare indicator in broiler chickens (D'eath *et al*., 2009). Inactivity in overweight birds fed *ad libitum* may be due to lameness or an attempt to avoid generation of heat by locomotion (Nielsen *et al*., 2003), especially in aged individuals (Noble *et al*., 1996).

The objective of observing behaviour is to determine the influence of selection and the environment on ingestive (eating, drinking) and locomotor (walking *vs* standing or resting) behaviours (Fritz *et al*., 2012; Piccione *et al*., 2012). Noble *et al.* (1996) classified the activity of birds into the following five groups: eating, drinking, standing,

walking, or resting. A bird was considered to be eating when it was located at the feeder, manipulating feed with its beak. Likewise, a bird was considered to be drinking when it was at the waterer and dipping its beak in water. A bird was considered to be walking when moving from one place to another. Walking bouts were considered to have ended after a pause of more than 2s. A bird not in motion was classified as standing (upright) or resting (body on the surface) (Noble *et al*., 1996).

Noble *et al*. (1996) also showed that the rearing environment affected drinking, eating, and resting behaviour of turkeys. Meat-type and egg-type chickens differ in feeding behaviour: broilers spend half as much time eating as layers of the same age (Masic *et al.,* 1974), suggesting that layers spend more time in "non-productive" feeding behaviour (that is playing with feed) than do broilers. Altering the environment in which birds are placed may also alter behaviour. For example, tonic immobility response may be affected by cage *vs* floor rearing (Anderson and Adams, 1994), presence of perches during rearing (Brake *et al.,* 1994b), or the use of an intermittent lighting programme (Newberry and Blair, 1993). Intermittent lighting has been hypothesised to increase the behavioural activities of broilers (Simmons, 1982; Wilson *et al.,* 1984). Consistent behavioural changes have been associated with feed restriction: birds spend less time resting and more time foraging (scratching and pecking the litter), drinking and spot pecking than birds fed *ad libitum* (Hocking *et al.*, 2001, 2005).

Alternative measures of stress have been proposed, including the analysis of behavioural sequences (Rutherford *et al.*, 2003) and fluctuating asymmetry (FA) (Moller and Manning, 2003). Measures of developmental instability such as FA are important tools

for assessing the reaction of birds to their environment and, therefore, their welfare; they correlate well with stressful events and situations (Yngvesson and Keeling, 2001).

2.23 SOCIALITY OF CHICKENS

The chicken is a highly social bird that would normally live in small family groups or in larger mixed groups with a relatively stable social structure: indeed close attachments are often formed between brood mates or members of a group (Marin *et al*., 2001). The underlying levels of sociality (motivation to be with conspecifics) in birds can exert profound effects on all aspects of social interaction, including affiliation, aggression, dispersal, mating and social structure, as well as on their behavioural and physiological responses to social disruption (Marin *et al.,* 2001).

2.24 TEMPERAMENTS OF DOMESTIC CHICKENS

Broilers and layers have different temperaments; layers are regarded as flighty and broilers as docile. This difference in temperament is sometimes interpreted as a difference between the strains in underlying fearfulness (Keer-Keer *et al.,* 1996; Oden *et al*., 2005). The UK Farm Animal Welfare Council identified five freedoms as being important for safe-guarding animal welfare (Webster and Nicol, 1988). One of these is freedom from fear (Spinka, 2012). Fearfulness can be described as a propensity to be easily frightened by a variety of potentially alarming situations (Keer-Keer *et al*., 1996; Forkman *et al*., 2007). There has long been a consensus (Duncan, 1985; Jones, 1987a) that feeds, stocks and strains of birds show consistent differences in temperament (distinct nature and character). One of the most obvious traits is ‘flightiness’, which manifests as rapid movement or flight away from a recognised disturbing stimulus.

Stocks selected for meat production are regarded as less flighty than those selected for egg production (Siegel, 1984; Appleby *et al.,* 1992). Strain differences in flightiness have been interpreted in terms of differences in underlying fearfulness or emotionality (Appleby *et al*., 1992; Nordquist *et al*., 2011).

Jumping has been regarded as a flight reaction (Archer, 1973; Evans *et al*., 2012) or as an attempt to reinstate contact with companions (Gallup and Suarez, 1980; Faure *et al*., 1983). The more frequent jumping showed by birds could be interpreted in several ways. First, higher fear levels in birds may have elicited more escape behaviour. Secondly and conversely, high levels of fear-induced behavioural inhibition, apparently, reduced jumping in birds (Keer-Keer *et al*., 1996). Meat-type chickens have low locomotor activity (Gordon and Tucker, 1993) and they lie for 80 – 90 % of the time (Bizeray *et al*., 2002). Level of fearfulness is known to affect exploratory and locomotor activities in several behavour tests (Bizeray *et al*., 2002). For instance, duration of TI (Gallup *et al*., 1971) is correlated with locomotor activity and the number of jumps in meat-type chickens in a new, bare environment (open-field test) (Jones *et al*., 1982). Thus, the number of squares crossed in an open-field test has been widely used as an indicator of locomotor activity (Jones, 1987b; Moriarty, 1995; Al-badrany and Mohammed, 2007).

2.25 OPEN-FIELD ACTIVITY

Schrott *et al*. (1998) classified open-field activity as an unconditioned behaviour. Hall (1934) originally described the open-field test for the study of emotionality in rats. The open-field test has become so popular that its use has been extended to a great number of species (Prut and Belzung, 2003). The procedure consists of subjecting an animal to an

unknown environment from which escape is prevented by surrounding walls (Clarke and Jones, 2000). Increase of time spent in the central part and in the ratio of central/total locomotion or decrease of the latency to enter the central part are indications of anxiolysis (Prut and Belzung, 2003). Hence, it is a convenient procedure to measure anxiety-like behaviours, which are triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (as the arena is very large relative to the animal's breeding or natural environment or natural environment) (Prut and Belzung, 2003).

Some species seem inappropriate for the assessment of anxiolytic effects in the open field: for example, for chickens, most of the studies reported no effect or anxiogenic-like effects after treatment with anxiolytic compounds. Parenteral administration of full or partial agonists of 5-HT_{1A} receptors generally induces anxiolytic-like effects in animals, subjected to the open-field (Prut and Belzung, 2003). Prut and Belzung (2003) concluded that open-field may either be a model of the normal anxiety everyone is faced when confronted with a stressful or threatening situation, but not with the features of pathological anxiety.

The open-field test has been extensively used to assess emotionality in animals (Prut and Belzung, 2003; Al-badrany and Mohammed, 2007). In domestic fowls, open-field behaviour has been defined as an interaction or compromise between tendencies to reinstate contact with conspecifics and evade predation (Gallup and Suarez, 1980; Marin *et al.,* 1997b). It was observed that the time spent in isolation chamber was associated

with immobility; this motor inhibition of short duration might be a fear indicator (Marin *et al*., 1997b).

Several behavioural constructs, such as general activity, exploration, social motivation, and predator avoidance have been evoked to explain open-field behaviours (Jones and Faure, 1982; Faure *et al*., 1983). It was proposed that the response to open field primarily represents a compromise between opposing tendencies to reinstate contact with its social companions and to avoid detection by potential predators (Gallup and Suarez, 1980). Thus, the lower the ambulation latency, the higher the number of squares crossed, and the higher number of escape attempts can be regarded as socially motivated behavioural patterns that increase the likelihood of the isolated chick reinstating social contact in the open-field test (Suarez and Gallup, 1983).

2.26 TONIC IMMOBILITY

Heiblum *et al.* (1998) described tonic immobility (TI) as a catatonic-like, death-feigning, anti-predator response present in many species throughout the animal kingdom. The duration of the TI response to manual restraint is widely considered to be positively related to the antecedent fear state and to thereby represent a useful behavioural index of fear (Marin *et al*., 2001; Oden *et al*., 2005). TI is generally accepted as indicating the relative fearfulness of an individual, with more fearful individuals showing longer durations of TI (Gallup, 1979; Beuving *et al.,* 1989). In broiler chickens, selection for increased growth rate, body weight, feed intake, feed conversion efficiency (Siegel and Wisman, 1966) has affected many other traits (Siegel and Dunnington, 1987), including

reduced genetic diversity, compared with their red Jungle fowl ancestors (Siegel *et al.,* 1992) and altered physiology.

2.27 THE BEHAVIOUR OF BROILER CHICKENS DUE TO INTENSIVE GENETIC SELECTION

Broiler chickens are probably the most numerous farmed species: worldwide, some 40 billion are produced per annum (Weeks *et al.*, 2000). They have been subjected to increasingly intensive genetic selection for growth rate and food conversion efficiency during the last four decades. The result of this pressure has been a consistent decrease in the age at which slaughter weight (approximately 2 kg) is reached, which is at the rate of 1 day per year, and large relative increases in breast muscle size (Griffin and Goddard, 1994). In the short term, there was no sign of a decline in this rate, and it was anticipated that genetic changes resulting in enhanced performance will continue (Albers and Groot, 1998). Metabolic disorders are prevalent and they include heart failure (Scheele, 1997) and ascites (Mitchell, 1997; Mitchell and Kettlewell, 1998), major skeletal disorders (Thorp, 1994) and lameness (Kestin *et al.*, 1992).

The behaviour of broiler chickens is thus of interest from several perspectives. The consequences of attaining virtually adult body size whilst still very immature in many aspects of development may also be reflected in altered behaviour. Besides, the metabolic demands of extremely rapid growth may reduce the energy available for activity. Their behavioural repertoire and time budgets may change within a few generations or years to reflect the on-going, and comparatively rapid, changes in their genetic make-up and

physiology (Weeks *et al.,* 2000). Unlike layers and adult chickens, there is little evidence that group size alters the behaviour of broilers (Weeks *et al*., 2000).

A further benefit of quantifying behaviour is that activities may be ranked according to the amount of time spent on them. For broilers, lying (also described as sitting by some authors) predominates, with much of this time spent idling and sleeping (Buijs *et al*., 2010; Schwean-Lardner *et al*., 2012). In a study conducted by Weeks *et al.* (2000), 39 – 49-day-old broiler chickens spent 76 – 86 % of their time lying down. This proportion significantly increased as the birds grew heavier and older during the 10 days over which they were observed. Similar studies have found age-related increases in lying behaviour (Weeks *et al*., 1994). Apparently, the high levels of lying down are related to the body weight and fast growth rates of broiler chickens. Rest and sleep (defined as eyes closed and/or head tucked along back or under wing) are strongly associated with energy conservation, tissue restoration and growth (Blokhuis, 1984). It could be expected, therefore, that fast-growing broilers would rest and sleep more than other fowls. It has been found that broilers on some lighting programmes, especially those with a long photoperiod, have disturbed rest (Gordon, 1994). Selection for high breast meat yield has moved the centre of gravity forward for broilers, which may unbalance them, and predispose the birds to sitting behaviours (Weeks *et al*., 2000).

Although deemed an important activity for both layers and Jungle fowl, dust bathing occupied less than 0.5 % of time by broilers (Blokhuis, 1984). This agrees with low levels of dust bathing reported in previous studies on broilers (Weeks *et al.,* 1994). Unquestionably, the behaviour of poultry can be altered by genetic selection (Siegel,

1989). It seems highly likely that the drive to forage may have been considerably reduced in broilers that are more efficient and less wasteful feeders than layer strains (Masic *et al.,* 1974). Weeks *et al.* (2000) reported a reduction in the amount of walking with age in broilers. There was no effect on the total time spent drinking as this is an essential maintenance behaviour like feeding, which in total was also unaffected by age. Broilers are extremely inactive strain of chicken, quite distinct from their Jungle fowl ancestors. While some of the alterations in their time budgets may be directly linked with intensive genetic selection for faster growth rate and better food conversion efficiency, others are more likely to be a consequence of altered physiology (Week *et al*., 2000).

2.28 EFFECTS OF BEHAVIOURAL STRESS ON POULTRY

Hens on free-range system are able to roam around freely in open spaces (2.5 – 4 m^2 per hen) and exhibit a wide range of natural behaviours (Bulmer and Gil, 2008). However, those in this system are more likely to encounter cannibalistic events (Weeks and Nicol, 2006). Prolonged stress responses have been observed to alter immune functions and behaviour of animals (McEwen and Wingfield, 2003) and they are assumed to have negative effects on animal welfare (Broom and Johnson, 1993). Fear and anxiety are generally considered to be undesirable emotional states that may reduce welfare, growth and reproductive performance in farm animals (Salvatierra and Arce, 2001). In poultry the induction of a chronic state of anxiety or stress may cause damage to individual organisms and adversely affect egg production, growth rate, and food conversion efficiency (Mills and Faure, 1990; Jones, 1996). Thus, the reduction of fear in fowls is of ethical and economical significance.

Behavioural differences were observed not only among species, but also among individuals of the same species (Plomin, 1990). It has been reported that the persistence of individual differences in animal behaviour in reactions to various environmental challenges could reflect basic divergences in temperament, which might be used to predict details of adaptive response (Boissy, 1995). Salvatierra and Arce (2001) proposed that the inhibition to peck is induced by fear and neophobia and that the values of latency to peck correlate with the degree of fear and/or anxiety. Dunkley *et al.* (2008) quantified non-nutritive pecking, walking, drinking, feeder activity, preening, aggression and head movement as behavioural parameters for hens. Weeks *et al.* (2000) observed that, apparently, healthy or sound broilers spent 76 % of their time lying. Lying also increased with age. Although, the broilers spent only a minor part of the day on their feet, they spent significantly more time standing idle (7 %), standing preening (3.5 %) and standing eating (4.7 %) than lame birds. Walking declined with age, but occupied an average of 3.3 % of the time of a slaughter-weight broiler.

Prenatal stress in animals has been shown to impair the regulation of the HPA axis and predisposes to anxiogenic and depressive-like behaviour (Zagron and Weinstocks, 2006). Behavioural observations revealed that low-temperature exposure decreased distress, vocalisations and spontaneous activity. Low-temperature induced sleep-like behaviour in neonatal chicks. Active wakefulness was decreased, while standing or sitting motionless with eyes closed or open and sleeping posture rose significantly (Mujahid and Furuse, 2009). In poultry, a chronic state of anxiety or stress may cause damage to individual birds and adversely affects growth rate or disease resistance (Jones, 1989; Marin *et al*., 1997b).

2.29 BODY OXIDATIVE PROCESSES AND THE INTERVENTION OF ANTIOXIDANTS

The carotenoids, vitamins A, C, E, and, melatonin, certain enzymes and proteins are natural antioxidants (Sahin *et al*., 2004; Edem, 2009; Sinkalu *et al*., 2009; Singh *et al*., 2012), converting highly reactive and free fatty peroxyl radicals to less active species, thus protecting the body against unwanted oxidative events (Bhimaraj and Tang, 2012; Dai *et al*., 2012; Kedzierska *et al*., 2012). As the polyunsaturated lipids are oxidized, they form hydroperoxides, which are susceptible to further oxidation or decomposition to secondary reaction products such as short-chain aldehydes, ketones and other oxygenated compounds that may adversely affect the overall function of cells, tissues, organs and body systems (Radwan *et al.*, 2008). A major preventive measure of lipid oxidation is the use of natural and synthetic antioxidants that function either by scavenging chain-carrying peroxyl radicals or by diminishing the formation of the initiating lipid radicals (Yamamoto and Niki, 1990; Radwan *et al.*, 2008). The antioxidant system of the embryo and newly hatched chick is based on antioxidant enzymes such as SOD, GPx and CAT (Surai *et al.*, 2001, 2002; Radwan *et al.*, 2008). One of the most important enzymes that function as a cellular antioxidant is SOD, which rapidly converts superoxide radicals into hydrogen peroxide (H_2O_2) that is removed by CAT (Teixeira *et al.*, 1998). Oxidative stress causes down regulated synthesis or over-utilisation of the antioxidant enzymes (Irshad and Chaudhuri, 2002; Ajuwon and Idowu, 2010).

Depletion of glutathione from cellular pool (as a result of a stressor, a disease condition or an experimental administration of thiol inhibitors), has been shown to render cells and

living organisms more susceptible to the effects of oxidants (Rahman *et al.*, 1999; Ajuwon and Idowu, 2010). This shows that glutathione is important in conferring protection and preserving the integrity of living organisms (Korkmaz *et al.,* 2008). Lipid peroxidation is induced in cytomembranes by free radicals to form peroxyl radicals. Lipid peroxyl radicals damage cells by changing the fluidity and permeability of cell membrane or by directly attacking DNA and other intracellular molecules such as proteins (Mattie and Freedman, 2001).

Vitamin C, also known as ascorbic acid, is effective in scavenging free radicals, including hydroxyl radicals, aqueous peroxyl radicals and superoxide anions. It acts as a two-electron reducing agent and confers protection by contributing an electron to reduce free radicals. Thus, it neutralises these compounds in the extracellular aqueous environment prior to their reaction with biological molecules (Evans and Halliwell, 2001). Reduced glutathione (GSH) is an abundant non-protein thiol in living organisms and it plays a crucial role in intracellular protection against toxic compounds, such as ROS and other free radicals (Anderson and Luo, 1998; Ajuwon and Idowu, 2010). It can function as a nucleophile to form conjugates with many xenobiotics and/or their metabolism and can also serve as a reductant in the metabolism of hydrogen peroxides and other organic hydroperoxides (Korkmaz *et al.,* 2008). Vitamin C acts as an alternative sulphydryl nucleophile to GSH, thereby preventing its oxidation to oxidized glutathione (GSSG) in detoxification reaction against free radicals (Onunkwor *et al.*, 2004). It also regenerates other small molecule antioxidants such as glutathione, α-tocopherol and β-carotene (Evans and Halliwell, 2001).

2.30 MECHANISMS OF INTERVENTION OF ENDOGENOUS ANTIOXIDANT ENZYMES DURING OXIDATIVE STRESS

Reiter (2000) reported that classic antioxidative enzymes include the SODs, subspecies of which are located throughout the cell, and the GPx, GRd, and CAT. The GPx and CAT catalytically remove H_2O_2 and lipid hydroperoxides from the cell, thereby reducing the generation of the $^{\bullet}OH$. Thus, glutathione peroxidase converts reduced glutathione (GSH) to its oxidised product, reduced glutathione disulfide (oxidized glutathione) (GSSG). Since reduced glutathione reductase reduces GSSG to reduced GSH, it has an important role in the recycling of reduced GSH and thereby reducing free radical damage. Besides H_2O_2 removal from cells, glutathione peroxidase also reduces $ONOO^-$, thus having an additional catalytic function to lower oxidative stress (Korkmaz *et al.,* 2008).

2.31 PHYSIOLOGIC ANATOMY OF THE PINEAL GLAND

The pineal gland lies in the centre of the brain, behind the third ventricle (Brezinski, 1997), at the border between the mesencephalon and the diencephalon (Moller and Baeres, 2002). Neuroanatomically, the pineal gland is described as a part of the epithalamus and, therefore, as a component of the diencephalon (Moller and Baeres, 2002). The pineal gland mainly consists of two types of cells, the pinealocytes, which produce indolamines (mostly melatonin, N-acetyl-5-methoxytryptamine), and neuroglial cells (Vollrath, 1981). One of the most relevant aspects of melatonin biochemistry is its variable rate of synthesis during the day. Melatonin synthesis is elevated at night and reduced during the day (Tosini and Fukuhara, 2003). This circadian rhythm is generated by the suprachiasmatic nucleus (Lincoln *et al.*, 1985) and the superior cervical ganglion, the latter being responsible for adrenergic innervation of the gland (Arendt, 1998).

Melatonin synthesis is regulated by noradrenaline, which is released from nerves that innervate the gland exclusively at night (Schomerus and Korf, 2005). Noradrenaline acts through a dual α-β-drenergic cAMP/Ca^{2+} system to increase the activity of the arylalklamine-N-acetyl transferase (Klein *et al.*, 1997). Thus, the neural input to the gland is nor-adrenaline and the output is melatonin (Schomerus and Korf, 2005). Apart from the pineal gland and ocular tissues (Alarma-Estrany and Pintor, 2007), melatonin has been detected in the digestive tract of several species and it is, apparently, synthesized by enterochromaffin cells of the gut (Martin *et al.*, 1998).

2.32 MELATONIN: ITS HISTORY, BIOSYNTHESIS AND RELEVANCE TO RESEARCH

Melatonin is estimated to have evolved 2.5 – 3.0 billion years ago, coincident with the development of oxygen-based metabolism (Reiter, 2000). It was first isolated from the bovine pineals by Lerner *et al.* (1958). Underwood and Siopes (1985) highlighted three reasons why melatonin has been of interest to researchers: (1) melatonin was the first methoxyindole identified; (2) historically, melatonin was thought to be a unique pineal product since the enzyme responsible for the last step in its synthesis, hydroxyindole-O-methyltransferase (HIOMT) was thought to be confined to pineal tissue; and (3) melatonin has been shown to be involved in controlling circadian and photoperiodic systems. Melatonin is not stored within pineals, but is rapidly secreted into the circulation. Several sites other than the pineal have been shown to have melatonin-synthesizing capabilities, including the gut, the Harderian gland, and the eyes (Ralph, 1981; Hardeland, 1997). A prominent feature of melatonin synthesis in the pineal, the Harderian gland, and the eyes is the marked diurnal fluctuation observed in melatonin

synthesis. Higher melatonin levels occur at night than during the day, regardless of the habits (nocturnal or diurnal) of the animal (Underwood and Siopes, 1985). In rats (Lewy *et al.*, 1980) and baboon (Meyer *et al.*, 1981), pinealectomy abolished the blood melatonin rhythm; whereas in chicken (Reppert and Sagar, 1983) and trout (Gern *et al.*, 1978), blood levels are merely depressed after pinealectomy.

The chemical formula, molecular mass, bioavailability and half-life of melatonin are $C_{13}H_{16}N_2O_2$, 232.278 g/mol., 30 – 50% and 35 to 50 minutes, respectively. Several biologic functions and processes are affected by melatonin. In fact, this neurohormone is involved in cyclic mood disorders (Jan *et al.*, 1994), anti-ageing action (Oxenkrug *et al.*, 2001), and acts as an immunomodulator (Carrillo-Vico *et al.*, 2005). It participates also in sexual maturation and gonadal development (Kumanov *et al.*, 2005; Lewis *et al.*, 2006). Other actions mediated by melatonin include its role in reducing blood pressure, decreasing catecholamine levels (Sewerynek, 2002), protecting against oxidative damage (Reiter *et al.*, 2003) and reducing intraocular pressure (Pintor *et al.*, 2003).

Melatonin is the major neurohormone secreted during the dark hours at night by the vertebrate pineal gland. Tryptophan serves as the precursor for melatonin biosynthesis, and is taken up from the circulation and then converted into serotonin. Serotonin is then converted into N-acetyl-serotonin by the enzyme arylalkylamine-N-acetyl transferase while N-acetyl-serotonin is metabolized into melatonin by the enzyme hydroxyindole-O-methyltransferase (Axelrod and Wurtman, 1968). Once formed, melatonin is released into the capillaries and in higher concentrations into the cerebro-spinal fluid, and is then rapidly distributed to most body tissues (Cardinali and Pevet, 1998). Axelrod (1974)

demonstrated that the pinealocytes possess all the machinery necessary for the synthesis of melatonin. The biochemical cascade, described by Reiter (1991) and summarised by Macchi and Bruce (2004), is as follows (Fig. 2.1): Pinealocytes take up tryptophan from the blood and convert it to serotonin through hydroxylation and decarboxylation; serotonin is then converted to N-acetyl-serotonin by the rate-limiting enzyme N-acetyl transferase; N-acetyl-serotonin is methylated into melatonin by the enzyme hydroxyindole-O-methyl transferase (Macchi and Bruce, 2004).

2.33 MELATONIN IN THE GASTRO-INTESTINAL TRACT

Hardeland and Pandi-Perumal (2005) reported that the gastro-intestinal tract deserves particular attention; not only with regard to melatonin uptake, but even more, as an extrapineal site of melatonin biosynthesis. In the gut, this molecule is present in amounts exceeding those found in the pineal gland by several-hundred-fold, and there it is released into the circulation in a postprandial response, especially under the influence of high trytophan levels (Bubenik, 2002). Gastro-intestinal melatonin is also released to the lumen and participates in enterohepatic cycling (Bubenik *et al.*, 1999). Thus, nutrition is linked to melatonin by uptake and also by the influences of other food constituents and digestive factors on melatonin release. Tan *et al.* (2003) found that the removal of melatonin from feed caused a decrease in its plasma level and that melatonin may be regarded as a hormone, a tissue factor, and also as an antioxidant vitamin.

2.34 THE PHARMACOKINETICS OF MELATONIN

Macchi and Bruce (2004) described melatonin as a highly lipophilic molecule, as well as having hydrophilic properties; rather than being stored in the secretory organ upon

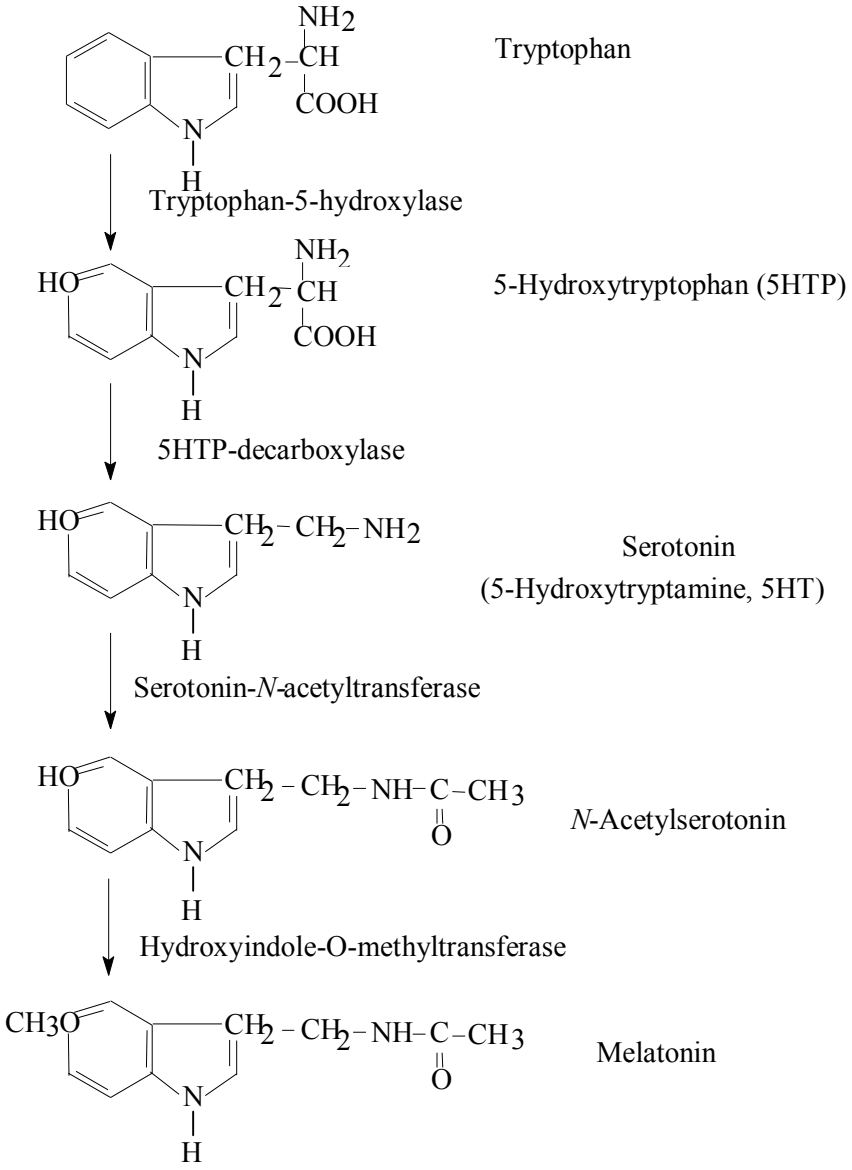


Figure 2.1: Chemical structure and synthesis of melatonin (Adapted from Macchi and Bruce, 2004)

biosynthesis, it is released into capillaries, where up to 70 % of it is bound to albumin. Its half-life is about 30 – 45 min. Melatonin is metabolized primarily in the liver, but also in the kidneys, undergoing hydroxylation and then conjugation into sulphate and glucuronide.

Intravenously administered melatonin exhibits a bi-exponential decay with a first distribution half-life of 2 minutes and a second metabolic half-life of 20 minutes (Claustrat *et al.*, 2005). Circulating melatonin is metabolized mainly in the liver, where it is first hydroxylated by cytochrome P_{450} monooxygenases and then conjugated with sulphate to form 6-sulphatoxymelatonin (Pandi-Perumal *et al.*, 2008). Melatonin is also metabolized by oxidative pyrrole-ring cleavage into kynuramine derivatives (Hirata *et al.*, 1974). The primary cleavage product is N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), which is deformylated, either by arylamine formamidase or haemoperoxidases to N^1-acetyl-5-methoxykynuramine (Hardeland *et al.*, 1993). Some evidence has suggested that pyrrole ring cleavage contributes to about one-third of the total melatonin catabolism, but the percentage may be even higher in certain tissues. It has been proposed that AFMK is the primitive and primary active metabolite of melatonin (Pandi-Perumal *et al.*, 2008).

2.35 EXPRESSION OF ENDOGENOUS ANTIOXIDANT ENZYMES UNDER GENOMIC REGULATION BY MELATONIN

Acuna-Castroviejo *et al.* (2001) reported that the expression of some enzymes, related mainly to the endogenous antioxidant system of the cell, such as GPx, GRd and SOD are under genomic regulation by melatonin. Two major findings suggest the involvement of melatonin in mitochondrial homeostasis. First, mitochondria produce high amounts of ROS and RNS. Secondly, mitochondria depend on the GSH uptake from the cytoplasm, although they have GPx and GRd to maintain GSH redox cycling. Thus, the antioxidant effect of melatonin and its ability to increase GSH levels may be of great importance for mitochondrial physiology (Acuna-Castroviejo *et al.*, 2001; Maldonado *et al.*, 2012).

2.36 ANTIOXIDANT VITAMIN-LIKE ACTIVITIES OF MELATONIN AND ITS SAFETY

The ingestion of high doses of antioxidant nutrients; for example, vitamins A, C and E, is considered a preventive measure against cell damage caused by immunological cells and free radicals (Murakami *et al*., 2001). Tan *et al.* (2003) found that the removal of melatonin from feed caused decreases in its plasma levels. They concluded that melatonin may not only be regarded as a hormone and a tissue factor, but also as an antioxidant vitamin. Melatonin acts as neurotransmitter, hormone, cytokine, metabolic modulator and antioxidant (Hardeland *et al*., 2011; Slominski *et al*., 2012). These vitamins, unlike antibiotics and other chemicals, are nutrients and are not known to cause any side-effects of welfare concern.

Melatonin is remarkably well tolerated (Korkmaz *et al*., 2011). Patients receiving daily very high doses of melatonin up to 60 mg per day, did not show any harmful side-effects (Jacob *et al.*, 2002; Hardeland and Pandi-Perumal, 2005). Apparently, melatonin could exert unfavourable effect by increasing the blood pressure, due to downregulation of NO synthase and NO scavenging by the indoleamine itself or by AMK. Melatonin was tested in clinical trials on hypertension and was reported to decrease blood pressure (Scheer *et al.*, 2004).

Antibiotic growth promoters for poultry diets have been banned for use in the European Union and pressure from consumer groups and major poultry buyers has threatened their removal from diets in the US (Soltan *et al.*, 2008). Therefore, the search for alternate products that can aid in growth promotion, improved feed utilisation and maintenance of

gut health continues (Hertrampt, 2001). Melatonin, a neurohormone with potent antioxidant property, is non-enzymatic in action and elicits no major side-effects when administered exogenously (Sharma *et al.*, 2005).

2.37 TIME OF ADMINISTRATION OF MELATONIN AND ITS DOSE

Morning administration of melatonin can delay the onset of evening sleepiness by delaying the phase of the circadian rhythms, while evening melatonin treatment can advance the circadian rhythms, including time of sleep onset (Rajaratnam, 2003). Melatonin has very low toxicity and no major abnormalities in behaviour or physiologic functions have been documented, even after ingestion of extremely high melatonin doses (Zhdanova, 2005). In humans, it has been reported that the physiologically and pharmacologically relevant daily doses of melatonin are 0.1 – 0.3 mg (0.001 – 0.004 mg/kg) and 2 – 10 mg (0.03 – 0.14 mg/kg), respectively (Zhdanova, 2005).

2.38 MECHANISMS UNDERLYING FREE RADICALS AND MELATONIN COMBAT

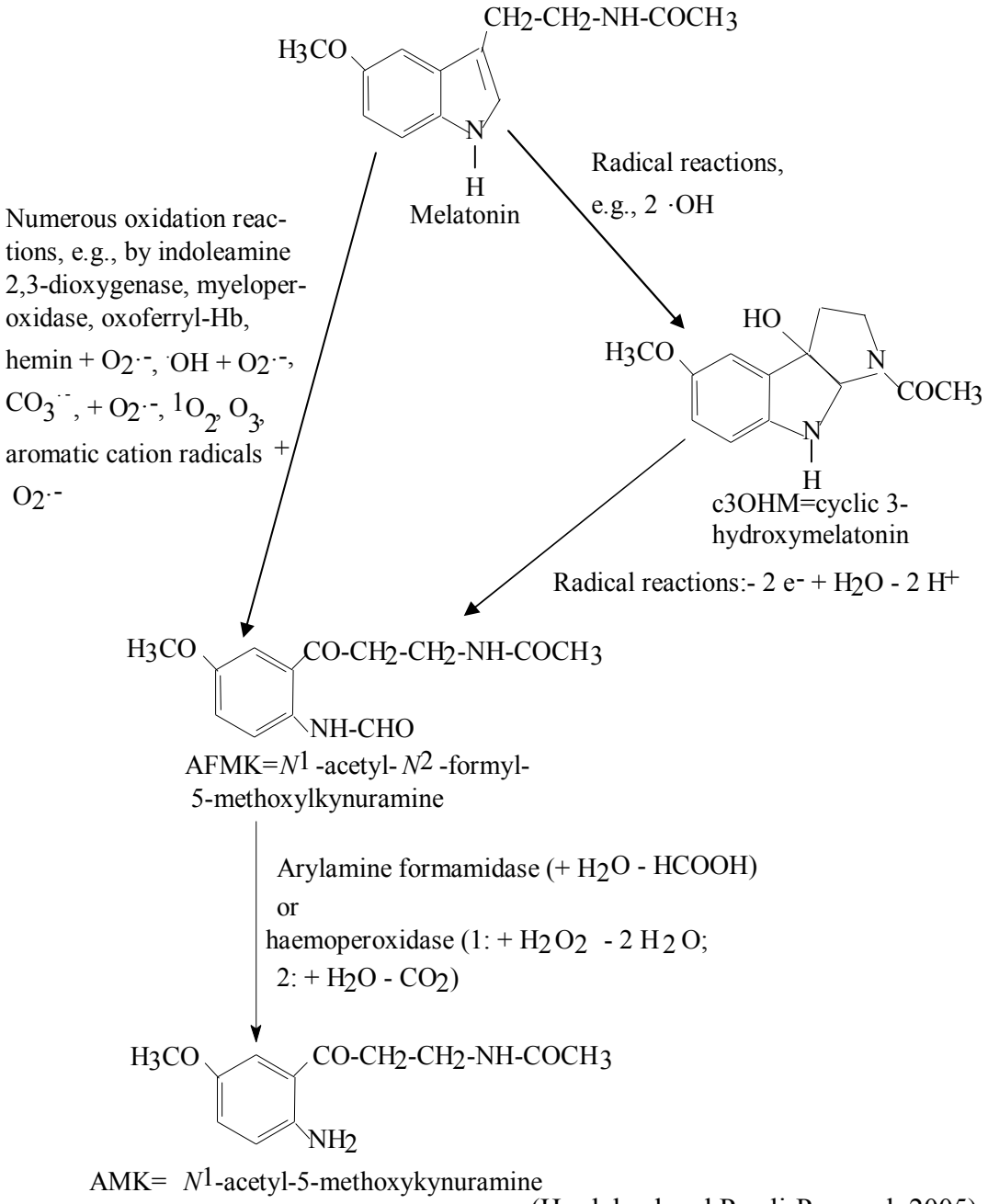
Free radicals can react with scavengers in different ways, either by abstraction of an electron, or a hydrogen atom, or by addition. In the case of melatonin, radical addition has been observed or predicted theoretically only for interactions with hydroxyl radicals (Tan *et al.*, 2002; Agozzino *et al.*, 2003) and nitric oxide (Turjanski *et al.*, 2001). Electron/hydrogen abstraction, however, is a common key step for interactions of melatonin with oxidising free radicals of both high and low reactivity and, therefore, reflects melatonin's property as a broad spectrum antioxidant. Electron abstraction was also concluded to be a primary step of melatonin oxidation in a pseudo-enzymatic

reaction, catalyzed by oxoferry (haemoglobin) (Tesoriere *et al.*, 2001). Hardeland and Pandi-Perumal (2005) reported that single-electron transfer reactions are involved in detoxification of resonance-stabilized free radicals, such as carbonate and aryl radicals, which are frequently underrated in their destructive potential. This is because they possess lower reactivity, compared to the hydroxyl radical. However due to their longer life-time, they can reach more distant sites than the extremely short-lived hydroxyl radical, which exists only for nanoseconds (Hardeland and Pandi-Perumal, 2005). The capability of melatonin of scavenging carbonate and certain aryl radicals may be of much higher significance and protective value. Newly discovered influences on sirtuins and downstream factors indicate that melatonin has a role in mitochondrial biogenesis (Hardeland *et al.*, 2011). Single-election exchange is thought to be the basis for interactions of melatonin with the mitochondrial respiratory chain (Hardeland *et al.*, 2003). The chain is assumed to require only very small, quasi-catalytic amounts of melatonin, which would convey antioxidative cell protection by radical avoidance rather than detoxification of radicals already formed (Hardeland *et al.*, 2003; Hardeland *et al.*, 2011).

Another highly interesting aspect of melatonin's antioxidant actions, which may be particularly important from the nutritional aspect, is its interactions with classic antioxidants. In both chemical and cell-free systems, melatonin has been repeatedly shown to potentiate the effects of ascorbate, Trolox (a tocopherol analogue), reduced GSH, or reduced nicotinamide adenine dinucleotide (NADH) (Gitto *et al.*, 2001; Tan *et al.*, 2003).

2.39 THE SCAVENGER CASCADE OF THE METABOLITES OF MELATONIN

Radicals derived from melatonin by interaction with a first, reaction-initiating radical, apparently, combine with superoxide anions so that the radical reaction chain is readily terminated (Hardeland and Fuhrberg, 1996). The product formed by oxidative pyrrole-ring cleavage is a substituted kynuramine, N-acetyl-N-formyl-5-methoxykynuramine (Fig. 2.2). These findings reflect the non-hepatic melatonin catabolism in vertebrates, but melatonin is also metabolized in the liver to 6-hydroxymelatonin followed by conjugation and excretion (Ferry *et al.*, 2005). The significance of pyrrole-ring cleavage in oxidative metabolism of tissue melatonin is particularly illustrated in the central nervous system, where a secondary product, N-acetyl-5-methoxykynuramine (AMK) derived from acetyl-formyl-5-methoxylkynuramine (AFMK) by deformylation, was identified as a main metabolite (Hirata *et al.*, 1974). AFMK and AMK were the only products formed from melatonin in the brain and no 6-hydroxymelatonin was detected. The two methoxylated kynuramines, AFMK and AMK, are of particular interest because of their own radical-scavenging and protective properties (Hardeland and Pandi-Perumal, 2005). Interestingly, AMK not only interacts with ROS, but also with RNS (Guenther *et al.*, 2005).



(Hardeland and Pandi-Perumal, 2005).

Figure 2.2: The Kynuric Pathway of Melatonin Metabolism.

2.40 MULTIPLE LEVELS OF ANTIOXIDATIVE PROTECTION BY MELATONIN

Antioxidative protection of melatonin is not only direct radical scavenging, but it up-regulates several antioxidant enzymes (Maldonado *et al*., 2012). Most frequently, this has been demonstrated for GPx (Reiter *et al.*, 2000; Rao and Chhunchha, 2010) and sometimes GRd (Rodriguez *et al.*, 2004), presumably indirectly via GSSG. In some tissues Cu, Zn- and/or Mn-SODs (Wang *et al.*, 2004) and, rarely, catalase (Tomas-Zapico *et al.*, 2002) are upregulated.

Melatonin also contributes to the avoidance of radical formation in several independent ways. It downregulates prooxidant enzymes, in particular 5- and 12-lipoxygenase (Reiter *et al.*, 2001) and NO synthases (Chang *et al.*, 2002; Rogerio *et al.*, 2002; Hardeland *et al.*, 2003). The widely observed attenuation of NO formation is particularly important in terms of limiting a rise in the strongly prooxidant metabolite peroxynitrite and of the free radicals, derived from this compound, namely $^{\cdot}NO_2$, carbonate ($CO_3^{\cdot-}$) and hydroxyl ($^{\cdot}HO$) radicals.

The effects of melatonin on the respiratory chain open new perspectives for diminishing radical formation, instead of seeking only antioxidant effects for the elimination of radicals already formed (Hardeland and Pandi-Perumal, 2005). Hardeland *et al.* (2003) proposed a model of radical avoidance in which electron leakage is reduced by single-electron exchange reactions between melatonin and components of the electron transport chain. In fact, mitochondrial H_2O_2 formation was found to be reduced by melatonin (Poeggeler, 2004). The basic idea of the model is that of a cycle of electron donation to

the respiratory chain, eventually to cytochrome C (Hardeland and Pandi-Perumal (2005), followed by reduction of the formed melatonyl cation radical by electron transfer from N_2 of complex I. The cation radical is assumed to act as alternate electron acceptor competing with molecular oxygen, thereby decreasing the rate of $O_2^{\cdot -}$ formation. In addition to the electrons being largely recycled, most of the melatonin is also recycled. Alternately, the melatonin metabolite AMK, which is also highly reactive and can undergo single-electron transfer reactions (Ressmeyer *et al.*, 2003), may act in the same way.

2.41 THE PECULIARITY AND SUPERIORITY OF MELATONIN TO OTHER ANTIOXIDANTS

Besides its primary function as synchronizer of the biological clock, melatonin also exerts a powerful antioxidant activity (Hardeland and Pandi-Perumal, 2005; Korkmaz *et al*., 2011). The discovery of melatonin as an antioxidant was made in 1993 (Tan *et al.*, 1993b). Melatonin is an antioxidant that can easily cross cell membranes and the blood-brain barrier (Hardeland, 2005). It is a direct scavenger of OH, O_2^-, and NO (Poeggeler *et al.*, 1994; Escames *et al*., 2012). Unlike other antioxidants, melatonin does not undergo redox cycling; that is, the ability of a molecule to undergo reduction and oxidation repeatedly. Redox cycling may allow other antioxidants (such as vitamin C) to regain their antioxidant properties. Melatonin, on the other hand, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals (Tan *et al.*, 2000b). Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan *et al.*, 2000a; Zisapel and Cardinali, 2008). The capacity to absorb free radicals extends at least to the quaternary metabolites of melatonin, a process

referred to as “the free-radical scavenging cascade”. This is not true of other conventional antioxidants (Tan *et al.*, 2007).

Experimental studies and clinical trials have shown that melatonin turned out to be more effective than classical antioxidants to reduce the oxidation of lipids, proteins and DNA (Rodriguez *et al*., 2007; Maldonado *et al.,* 2012). It also preserves the levels of glutathione in cells and mitochondria which helps to suppress oxidative damage at these sites (Acuna-Castroviejo *et al.,* 2011; Escames *et al*., 2012). Montilla-Lopez *et al*. (2002) showed that melatonin is considerably more efficient than vitamin C in reducing the extent of oxidative stress in Alzheimer’s disease, induced by okadaic acid in neuroblastoma cells. This phenomenon was demonstrated by the smaller dose of melatonin needed to obtain effects similar to those obtained with vitamin C on lipid peroxidation and by the protective effect of melatonin on antioxidant enzyme activity (Montilla-Lopez *et al*., 2002).

Melatonin has been demonstrated to prevent damage to DNA by some carcinogens, terminating the mechanism by which they cause cancer (Karbownik *et al.*, 2001). It has also been found to be effective in protecting against brain injury, caused by ROS released in experimental hypoxic brain damage in newborn rats (Tutunculer *et al.*, 2005). Melatonin has been shown to increase the average life span of mice by 20 % in some studies (Oaknin-Bendahan *et al.*, 1995; Anisimov *et al.*, 2003). It may also be useful in fighting infectious diseases (Maestroni, 2001), including viral, such as HIV, and bacterial infections, and potentially in the treatment of cancer (Maestroni, 1999). Jahnke *et al.* (1999) reported that melatonin has a very low toxicity in rats. They demonstrated that the

no-observable-adverse-effect level and lowest-observed-adverse-effect level were 100 and 200 mg/kg/day, respectively. Hence, large doses of melatonin can be counter productive (Lewy *et al.*, 2002). Melatonin is involved in the regulation of body weight, and may be of value in treating obesity, especially when combined with calcium (Barrenetxe *et al.*, 2004).

2.42 MELATONIN AND CIRCADIAN OR BIOLOGICAL RHYTHMICITY OF PHYSIOLOGICAL PARAMETERS

A circadian rhythm is a roughly-24-hour cycle in the biochemical, physiological or behavioural processes of living things, including plants, animals, fungi and cyanobacteria (Refinetti, 2006). The primary circadian "clock" in mammals is located in the SCN, a pair of distinct groups of cells located in the hypothalamus. The SCN receives the information on day length from the retina, interprets it, and passes it on to the pineal gland, a tiny structure shaped like a pine cone and located on the epithalamus. In response, the pineal secretes the hormone melatonin. Secretion of melatonin peaks at night and ebbs during the day. Melatonin is absent from the system or undetectably low during daytime. Its onset in dim light, called dim-light melatonin onset can be measured in the blood or the saliva and used as circadian markers. Melatonin metabolite, 6-sulphatoxymelatonin can be measured or detected in early morning urine samples (Laberge *et al.*, 1997).

Daily oscillation in the levels of physiological variables in animals has been described for a multitude of variables, including locomotor activity, body temperature, heart rate, blood pressure, hormonal secretion, haematological and haematochemcial parameters (Piccione

and Refinetti, 2003; Piccione *et al*., 2005). Circadian pacemaker located in the SCN of the hypothalamus generates daily rhythmicity, which is modulated by environmental cycles of light and darkness, food availability, ambient temperature and other factors (Helfrich-Forster, 2004, Giudice *et al*., 2009). Wright *et al*. (2006) reported that rhythms of the pineal hormone melatonin and body temperature are the most commonly used markers, representing the output of the circadian clock. Low melatonin and high body temperature levels represent the biological day, and high melatonin and low body temperature levels represent the biological night.

Among various avian species, it has been shown that the predominant circadian oscillator is located in the eye, pineal gland or SCN or multiple combination of these (Murakami *et al.*, 1994). Murakami *et al.* (2001) compared the effect of melatonin on circadian rhythm, body temperature, and locomotion in the intact house sparrow, Japanese quail and owl. They found that daily treatment with melatonin at a fixed time did not entrain the free-roaming rhythm of locomotor activity in the house sparrow and the disrupted rhythm in Japanese quail under constant dim light. However, melatonin clearly inhibited movement for several hours after treatment. The duration of resting after injection of melatonin was dose-dependent. Body temperature was significantly decreased after melatonin treatment, the effect being more potent during the active phase than in the resting phase.

2.43 AMPHIPATHICITY OR AMPHIPHILICITY OF MELATONIN

Melatonin is a very potent hydroxyl radical scavenger (Costa *et al.*, 1995). Its biological functions were shown to be dependent on protein plasma membrane receptors (Sudgen, 1992; Ebisawa *et al.*, 1994), though it has also been suggested that melatonin can cross

the cell membrane barrier and interact in the nucleus (Reiter *et al.*, 1993; Tan *et al.*, 1993a). Melatonin is highly soluble in aqueous media, being soluble and stable up to the concentration of 5 x 10^{-3} M (Shida *et al.*, 1994). Thus, after being secreted by the pineal gland, melatonin is transported by the blood stream to its target cells, without requiring protein transport. The aqueous solubility of melatonin explains its presence in the cytosol and, hence, its interaction with nuclear receptors. Costa *et al.* (1995) showed that melatonin has a relatively high lipid-partition coefficient and can easily cross lipid bilayers. Its ability to cross lipid membranes agrees with its reported effect at the cell nuclear level (Tan *et al.*, 1993a).

The multiple biological roles played by melatonin should be analyzed in the light of its ability of being almost everywhere in the cell, but preferentially in hydrophobic portion of the membrane. Its reported radical-scavenging action (Reiter *et al.*, 1993; Tan *et al.*, 1993b) should certainly be extended to the membrane lipid phase. Accordingly, it has been reported (Melchiorri *et al.*, 1995; Reiter *et al.*, 1995) that radicals formed by lipid peroxidation can be efficiently on-site destroyed by melatonin. Both human (Vijayalaxmi *et al*., 1995; 1996) and experimental animal studies (Vijayalaxmi *et al*., 1995; Take *et al*., 2009) have clearly shown that melatonin is a unique antioxidant and a DNA (Karbownik *et al*., 2000) and chromosome protector (Assayed, 2009) against a variety of harmful agents, including ionizing radiation (Vijayalaxmi *et al*., 2004). In the recent Japan's nuclear disaster, Korkmaz *et al*. (2011) reported that melatonin is the most feasible agent to reduce the risk of cancer and several other nuclear disaster- associated health problems, which will be observed in decades and even in subsequent generations.

Melatonin is an amphipathic molecule and can readily enter all cells (Korkmaz *et al*., 2011). There is no known biological barrier for melatonin, including the blood-brain barrier or cell and nuclear membranes. After oral administration, melatonin rapidly passes into blood stream, cerebrospinal fluid (Longatti *et al*., 2007), bile (Koppisetti *et al*., 2008), seminal, amniotic and ovarian follicular fluids (Tamura *et al*., 2009). Melatonin has been administered in both physiological and pharmacological amounts to humans and animals, and it is a non-toxic and non-teratogenic molecule (Seabra *et al*., 2000). It was also proven that melatonin is a more efficient protector than amifostine (Topkan, 2008) and another antioxidant, octreotide (Onal, 2011). Furthermore in the body, melatonin is not exclusively produced by pineal gland; every single cell with DNA may produce melatonin in small amounts, basically to protect themselves from the harmful effects of free radicals (Hardeland, 2011).

2.44 EFFECTS OF MELATONIN ON BODY FUNCTIONS

Being a highly lipophylic hormone, melatonin reaches every cell of the body. The wide distribution of melatonin receptors also suggests that melatonin affects various tissues and organs and, thus, is involved in multiple physiologic processes (Zhdanova, 2005). Thus, melatonin is known to modulate many body functions, including neuronal activity, sleep, body temperature, smooth muscle contractility and immune function (Macchi and Bruce, 2004). The secretion of melatonin is driven by the hypothalamic SCN, with circulating melatonin levels peaking during the night. With advanced ageing and certain age-related diseases, the endogenous secretion of melatonin drops markedly (Olcese *et al.*, 2009). Olcese *et al.* (2009) clearly showed that long-term melatonin administration protects the brain of Alzheimer's mice against cognitive impairment and oxidative stress.

Melatonin administration is followed by a decrease in core body temperature and an increase in surface body temperature, indicating increased heat loss (Macchi and Bruce, 2004).

2.45 FACTORS INFLUENCING POSSIBLE EFFECTS OF MELATONIN ADMINISTRATION

Zhdanova *et al.* (1998) reported that newborn babies do not secrete melatonin for approximately the first three months of life. Young children are reported to have the highest circulating melatonin levels, which start declining around the time of puberty. Mean circulating melatonin levels in aged individuals are significantly lower than in young healthy adults. Factors influencing possible effects following melatonin administration, highlighted by Zhdanova (2005) include different subject selection criteria, the melatonin doses and preparations used, time of treatment, circulating melatonin levels induced, co-medications, food intake around the treatment time and the environmental condition in a laboratory, home or institution.

2.46 DAILY RHYTHM OF MELATONIN SYNTHESIS AND ITS MODULATION BY PHOTOPERIODISM

In all species, the melatonin increase occurs during the night, regardless of whether they are day-active or night-active. This makes melatonin rhythm an endocrine marker of night (Vanecek, 1998). Melatonin is involved in synchronisation of circadian rhythms. The effect is most prominent in birds and reptiles where the pineal is an integral part of the circadian system (Vanecek, 1998).

Melatonin is immunomodulatory in both animals and in humans (Maestroni, 2001; Slominski *et al*., 2012). Inhibition of melatonin synthesis, for instance, suppresses both cellular and humoral responses in mice (Maestroni *et al.*, 1987; Pandi-Perumal *et al.*, 2008). Since pineal melatonin production occurs during the dark phase and is acutely suppressed by light, and, further, because melatonin is quickly cleared from the circulation following the cessation of its production, the time and duration of the melatonin peak reflect the environmental night period (Cardinali and Pevet, 1998). Plasma melatonin exhibits a circadian rhythm with high levels at night, and low levels during the day, attaining peak concentrations of plasma melatonin between 02:00 and 04:00 h. Longer nights are associated with a longer duration of melatonin secretion (Pandi-Perumal *et al.*, 2008).

2.47 MELATONIN, IMMUNE SYSTEM AND PHOTOPERIODISM

Modern broilers are intensively selected for growth rate (Rahimi *et al.*, 2005). Relatively little is known on the effects of the duration of light on stress status in birds (Campo and Davila, 2002). Much experimental evidence indicates that the HPA axis is activated in response to pathogens or environmental stressors (Kliger *et al.*, 2000). Chickens raised under intermittent light programmes are likely to have higher serum melatonin levels than those raised under constant light programmes. This is because melatonin is a hormone secreted in the dark from the pineal gland and sets the internal biological clock that governs different daily and seasonal cycles or rhythms in various physiological systems in birds (Pang *et al.*, 1996). Hamal *et al*. (2010) reported enhanced mitogenic activity for T and B lymphocytes as well as increased total WBC counts following the subcutaneous injection of melatonin in chickens. Adding melatonin to the drinking water improved

cellular and humoral immune responses of Japanese quails, raised under constant light conditions (Moore and Siopes, 2000). Furthermore, melatonin addition to the diet was reported to ameliorate the negative effects of heat stress on performance parameters in Japanese quail (Sahin *et al.*, 2003).

Melatonin stimulates lymphocytes proliferation (Hardeland, 1997; Kliger *et al.*, 2000), antibody production (Abbas *et al.*, 2007) and the interleukin 2 (IL-2) and interferon–• (1FN–•) (Garcia-Maurino *et al.*, 1997). Recent approaches in understanding the mechanism of pineal gland regulation of immune function have focused on either photoperiod or day length. Light-dark stimuli provide adequate environmental information necessary for physiological and behavioural adaptive changes (Abbas *et al.*, 2008). Moore and Siopes (2000) reported improved cellular and humoral immune responses for Japanese quail raised under continuous light condition.

Kliger *et al.* (2000) reported that increasing the dark period stimulated mitogen-induced splenocyte proliferation. This could be due to enhanced melatonin secretion during dark period (Abbas *et al.*, 2007). Kliger *et al.* (2000) showed that melatonin *in vitro* enhanced peripheral blood and splenic lymphocyte proliferation. Champney *et al.* (1997) suggested that melatonin disproportionately alters the number of blood splenic T- and B-lymphocytes, or modifies the intrinsic mitogen activity of each lymphocyte. Furthermore, melatonin may increase lymphocyte proliferation by enhancing synthesis and secretion of IL-2 and 1FN (Garcia-Maurino *et al.*, 1997) by activating T helper-1 cells (Maestroni, 1998; Lardone *et al.*, 2011).

Champney *et al.* (1998) obtained an increase in serum 1FN levels in Syrian hamsters after melatonin injection. Also, Maestroni *et al.* (1987) suggested that melatonin influences the immune response through opiatergic mechanisms. Another explanation was offered by Kuhlwein and Irwin (2001), who suggested that the increase in lymphocyte proliferation in response to the administration of physiological doses of melatonin may be due to decreases in the production of the inhibitory cytokine IL-10. Chickens exposed to a non-intermittent restricted light programme had significantly lower total WBC compared to intermittent light group or continuous lighting group (Abbas *et al.*, 2008). The effect of melatonin on immune function may be direct via melatonin receptors, located on immune tissues, including WBC (Calvo *et al.*, 1995), or may be indirect by acting through other endocrine hormones, most notably thyroid hormone (Poon *et al.*, 1994).

Raghavendra *et al.* (2001) found that chronic administration of melatonin activates T helper-2 response. T helper-2 cytokines enhance B cell activation and increase the production of antibody (Annamalai and Selvaraj, 2012). The negative effects of a non-intermittent restricted light regimen on the immune response could be due to corticosterone. Abbas *et al.* (2008) showed that a non-intermittent restricted light treatment induced the elevation of plasma corticosterone. The mechanisms through which corticosterone suppresses immune activities could be by initiating programmed lymphocyte death and inducing fragmentation of the cellular DNA, leading to cell death (Cohen and Duke, 1984). Trout *et al.* (1996) showed that both adrenocorticotropic hormone (ACTH) injection and heat stress caused significant decreases in the percentages of CD_3, CD_4 and CD_8 lymphocytes in the blood. Indeed, experiments with

mammals have indicated that both B and T lymphocytes are decreased in the circulation following glucocorticoid treatment (Fauci, 1975). Glucocorticoids have been reported to cause a redistribution of lymphocytes from the circulation to the secondary lymphoid tissues (Fauci, 1975).

Exposing broilers to 12-h darkness in a non-intermittent restricted light regimen seems to be a stressful programme, causing elevation of corticosterone (Abbas *et al.*, 2008). In general, HPA axis involves perception in the brain with the release of hypothalamic CRF and vasopressin, which stimulate the anterior pituitary to secrete ACTH. Circulating ACTH causes the adrenal cortex to produce glucocorticoids (Dohms and Metz, 1991). Abbas *et al.* (2008) found that both non-intermittent restricted and intermittent light programmes caused an increase in plasma triiodothyronine (T_3), compared to the continuous light group. This result could be due to both direct and indirect effects of melatonin on leptin hormone concentration. Abbas *et al.* (2007) showed that increasing dark period stimulates melatonin production. Furthermore, Mustonen *et al.* (2000) reported that melatonin increases the concentration of plasma leptin, involved in the regulation of energy balance, mainly by reducing food intake, body adiposity and increasing energy expenditure (Cerasale *et al*., 2011). In addition, Legradi *et al.* (1997) found that leptin raises the level of serum thyroxine (T_4) and prothyrotropin releasing hormone mRNA.

Long dark period in the non-intermittent restricted programme, 12 h, could be a stress and a main factor inducing elevation in corticosterone level. Corticosterone is a key player in increasing pro-inflammatory cytokines. Johnson (1997) reported that pro-

inflammatory cytokines, IL-1, IL-6 and TNF• inhibit growth by modulating the intermediary metabolism of carbohydrate, fat and protein substrates. Again, Abbas *et al.* (2008) demonstrated that body weight was significantly heavier by an average of 230 g/bird in the group that received the intermittent programme, compared to control. This result could be due to plasma triiodothyronine concentration, which was significantly higher in the intermittent light group compared to the continuous light group.

Although intermittent light programme (1L:3D) and non-intermittent restricted light programme (12L:12D) have the same period of darkness, the body weight of the chickens reared under intermittent light programme was heavier. In the intermittent light programme, the broilers eat to satiation during the light period and then do not expend much energy during dark period, causing greater body weight gain (Ingram and Hatten, 2000). Abbas *et al.* (2008) observed that the lowest value of feed intake was obtained significantly in the non-intermittent restricted light group, compared to the continuous or intermittent light groups. The reduction of activity during darkness may result in lower heat production and higher feed efficiency (Rahimi *et al.*, 2005).

2.48 MELATONIN AND ITS AMELIORATIVE EFFECTS ON BROILER CHICKENS SUBJECTED TO HEAT STRESS

It has been established that heat stress is evaluated by measuring the rectal temperature (RT), which is a true reflection of internal body temperature and a reliable index of thermal balance (Bianca, 1976; Ayo *et al*., 1998; Ozkan *et al*., 2003). Birds maintain a constant body temperature under normal conditions, but when the internal heat production and heat gain from the environment are greater than the rate of heat

dissipation from the birds, then the body temperature increases (Ozkan *et al*., 2003). Mineral excretion is increased under stressful conditions (Sahin *et al.*, 2004). El-Husseiny and Creger (1981) reported a low retention rate for Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn in birds subjected to a high ambient temperature.

Melatonin treatment significantly decreased body temperature in chickens exposed to chronic heat stress, compared to chicken exposed to chronic heat stress without melatonin administration (Rozenboim *et al.* 1998). Sejian and Srivastava (2010) reported that melatonin relieved thermal stress by modifying the adrenal cortex functions of Indian goats. Rozenboim *et al.* (1998) induced hypothermia in layers with a single melatonin injection in a dose-dependent manner. Injection of melatonin 30 minutes prior to heat exposure protected the layers against heat stress-induced hyperthermia, whereas melatonin injection 30 minutes post-heat exposure failed to induce such protection. Chronic melatonin treatment has been shown to attenuate adrenocortical secretory responses to acute and chronic stress (Konakchieva *et al.*, 1997). However, Abbas *et al.* (2007) showed that melatonin added to the diets of chickens subjected to chronic heat stress decreased their corticosterone levels. It has been suggested that immuno-suppression associated with the exposure of broiler chickens to chronic heat stress may be alleviated by applying an intermittent light programme or melatonin administration (Abbas *et al.*, 2007).

2.49 MELATONIN, SLEEP AND THE THERMOREGULATORY CENTRE

Both sleep and melatonin are normally associated with a decline in core body temperature and elevation of skin temperature due to a combination of increased heat loss

and reduced heat production (Hughes and Badia, 1997). This suggests that the effects of melatonin on cardiovascular system and/or thermoregulatory centres may underlie the effect of melatonin on sleep (Zhdanova, 2005). Physiologic (0.3 mg) and pharmacologic (3 mg) melatonin doses in humans show similar effects on sleep, but a significantly more prominent decline in core body temperature occurred after administration of the pharmacologic dose (Zhdanova *et al.*, 2001).

2.50 ANXIOLYTIC EFFECT OF MELATONIN ON CHICKEN BEHAVIOUR

Anxiolytic drugs such as melatonin might be expected to reduce fear associated with the new environment, and, hence, to induce suppressed behaviour (Marin *et al.,* 1997a). On the other hand, anxiogenic drugs enhance fearfulness in a variety of behavioural tasks (Stout and Weiss, 1994). Therefore, the effects of anxiogenic drugs should be the inhibition of reinstatement behaviour by increasing the fear response. Serotonin (melatonin precursor) has multiple functions in controlling the biological processes of an organism. In the CNS, 5-HT functions to inhibit aggression and modulates stress response, including social and environmental adaptability (Chen and Jefferson, 2008).

2.51 CONTROVERSIAL EFFECTS OF MELATONIN ON LOCOMOTOR ACTIVITY IN BIRDS

Zhdanova (2005) reported that melatonin is an exceptionally interesting hormone. Its evolutionary roots may be traced to unicellular organisms, invertebrates and lower vertebrates, which produce and use this small amine for time navigation. Every night, human and other mammals secrete melatonin and it conveys a message of darkness to every cell of the body. The responses to this message are, apparently, not the same in

different organisms. Night means activity for some and quietness for the others, foraging or building a shelter for nocturnal species and sleep for the diurnal ones (Zhdanova, 2005).

Important insights come from studies on the effects of melatonin in birds. Inhibition of locomotor activity following melatonin treatment was associated with a reduction in body temperature in diurnal birds, house sparrow and Japanese quails (Murakami *et al.*, 2001). In contrast, in the nocturnal owl, body temperature was reduced, but activity levels were preserved after melatonin treatment (Zhdanova, 2005). Melatonin administration reduced locomotor activity in the house sparrow and the Japanese quail (Murakami *et al.*, 2001). In pigeons, melatonin induced daytime sleep and increased EEG slow wave activity, without altering subsequent nighttime sleep (Mintz *et al.*, 1998). In contrast, melatonin does not change locomotor activity in the nocturnally active owl (Murakami *et al.*, 2001).

2.52 BEHAVIOURAL EFFECTS OF MELATONIN ADMINISTRATION

In contrast to common hypnotics, repeatedly underscored, melatonin induces a behavioural state that resembles quiet wakefulness, which normally predisposes to normal sleep initiation, rather than sleepiness or drowsiness (Zhdanova, 2005). Zhdanova (2005) reported that physiologic circulating melatonin levels (50 – 200 pg/ml) were achieved after ingestion of 0.1 – 0.3 mg preparations. Unlike humans, nocturnal animals sleep during the day, when their melatonin levels are low, and are at the peak of their activity at night, at the time of melatonin production by the pineal gland. Thus, melatonin is an unlikely candidate for being a sleep-promoting agent in nocturnally-active species (Zhdanova, 2005).

2.53 TONIC IMMOBILITY, PECKING AND CANNIBALISTIC BEHAVIOUR OF CHICKENS

Campo *et al*. (2008) categorized cannibalistic behaviour to include attacks to the back, wings, rump, tail, cloaca, legs and toes, while aggressive pecks involved head and neck attacks. There are a number of ways in which one bird can peck another. Vent pecking shares some of the features of feather pecking and could be considered as a precursor to feather pecking and cannibalism after the onset of lay (Potzsch *et al*. 2001). Feather pecking in laying hens and pullets is a behavioural disorder that is associated with reduced welfare in both actor and victim (Bestman *et al*., 2009; Van Zeeland *et al.,* 2009). Van Hierden *et al*. (2004) showed that feather pecking is triggered by low serotonin levels. Therefore, increasing serotonin level by increasing dietary tryptophan, which is a compound related to fear and stress from which serotonin and, subsequently, melatonin are synthesized decreases feather pecking. Campo *et al*. (2008) analysed the relationship between incidence of vent-pecking, fluctuating asymmetry, heterophil to lymphocyte ratio, and TI duration in chickens. They found that vent-pecked birds were more asymmetrical, stressed and fearful than non-vent-pecked birds, having increased relative fluctuating asymmetry, H:L ratio and TI duration.

2.54 EFFECTS OF MELATONIN ON THYROID FUNCTION

The relationship between the pineal and the thyroid gland has been shown in a number of studies (Wright *et al.*, 1996; Lewinski, 2002; Baltaci *et al.*, 2003). Preliminary studies on that topic reported that melatonin administration increased the stimulating effect of thyroid stimulating hormone (TSH) on thyroxine secretion (Csaba and Nagy, 1976). In

later studies general agreement developed that melatonin and the pineal gland had an inhibitory effect on thyroid hormones (Sewerynek *et al.*, 1999; Champney, 2001). However, it was claimed in some other studies that melatonin did not have an inhibitory effect on the thyroid gland (Ashley *et al.*, 1999). The effect of melatonin on thyroid function is affected by the hour of the day when the melatonin is administered. Both pinealectomy and exogenous melatonin application do not change thyroid function in the morning, while the inhibitor effect of the pineal gland on thyroid is observed at night (Kniazewski *et al.*, 1990a; Zwirska-Korczala *et al.*, 1991).

Melatonin application inhibits thyroid growth, but pinealectomy stimulates thyroid growth. Re-application of melatonin in pinealectomised rats reverses this effect (Wajs and Lewinski, 1992). Similar studies have shown that these findings are dose-dependent and that administration of high concentrations of melatonin inhibited the response of the thyroid gland to TSH, while lower doses of melatonin do not have such an effect (Wright *et al.*, 1997). Numerous studies have demonstrated that melatonin reduces the function and growth of thyroid gland (Wright *et al.*, 2000; Lewinski, 2002; Baltaci *et al.*, 2003). It was claimed that changes in the secretion of thyroid hormones resulted from actions that are stimulated by the pineal gland (Vaughan and Pruitt, 1985). It was reported that exogenous melatonin administration reduced thyroid hormones in rats with hyperthyroidism (Mogulkoc and Baltaci, 2002). However, it was shown in a study on dogs that oral melatonin application did not affect thyroxine levels (Ashley *et al.*, 1999).

2.55 RELATIONSHIP BETWEEN MELATONIN, ZINC AND THYROID FUNCTION

Several hormones and minerals affect zinc metabolism at physiological and biochemical levels (Prasad, 1985; Luo *et al*., 2011). The interaction is explained by several hormone receptors binding zinc (Valle and Falchuk, 1993; Voorhees *et al*., 2011). However, the hormonal control mechanism that plays a role in zinc homeostasis is still subject to research (Leblondel and Allain, 1989; Voorhees *et al*., 2011). One of the most important hormones, whose interaction with zinc has been investigated, is the thyroid hormone (Leblondel and Allain, 1989; Leblondel *et al.*, 1992; Rostami *et al*., 2011). The fact that zinc levels were reported to be low in hypothyroidism and high in hyperthyroidism (Leblondel *et al.*, 1992; Baltaci *et al.*, 1999) points to a direct relationship between zinc and thyroid hormones.

The presence of a number of elements is necessary for the normal function, synthesis and metabolism of the thyroid gland, and zinc has an important place among these elements (Arthur and Beckett, 1999; Hess, 2010). Zinc may play a role in thyroid hormone metabolism and in the conversion of T_4 to T_3 (Nishiyama *et al.*, 1994; Rostami *et al*., 2011). It was reported in a study conducted on rats that zinc chloride application lowered T_3 and the T_3/T_4 ratio and this inhibition was attributed to a decrease in the microsomal capacity to convert T_4 to T_3 (Moustafa, 2001). Similarly, it was claimed in an *in vitro* study that zinc inhibited the conversion of T_4 to T_3 in brown adipose tissue (Pavia *et al.*, 1999). Baltaci *et al.* (2003) found that plasma TSH levels of rats fed with zinc-deficient diet were lower than those in control group. This finding suggests that zinc deficiency suppresses TSH and leads to a reduction in thyroid hormones. It has been reported that

zinc administration increased thymic function, which in turn affected the hypophysis-thyroid axis and improved thyroid function (Napolitano *et al*., 1990; Kandhro *et al*., 2009).

Triiodothyronine requires zinc for its biological activity, and zinc deficiency affects the metabolic activity of this hormone in a negative way (Freake *et al.*, 2001; Rostami *et al*., 2011). It is also possible that changes in selenium, iodine and zinc in the thyroid gland affect thyroid function, depending on TSH; and, thus, lead to alterations in hormone levels (Bellisola *et al.*, 1998; Rostami *et al*., 2011). Morley *et al.* (1980) and Rostami *et al*. (2011) showed in a study that zinc deficiency caused a fall in T_3 and T_4 hormones as well as hypothalamic thyrotropin-releasing hormone content, while Gupta *et al.* (1997) demonstrated that zinc deficiency reduced thyroid hormones, depending on application duration.

Results of the studies on the relation between melatonin and thyroid gland showed that the findings were contradictory. For example, one study reported that thyroxine levels were unchanged shortly after melatonin administration (Csaba and Nagy, 1976). Parallel to this, it was claimed in a study on rabbits given melatonin for 3, 5 or 7 days that the treatment increased Type I monodeiodinase (MD) enzyme in the liver and Type II-5'-MD enzyme in the kidneys, thereby increasing serum T_4, T_3 and rT_3 levels (Brzezinska-Slebodzinska *et al.*, 1998; Skipor *et al*., 2010). Similar findings were reported in a study using minks (Mustonen *et al.*, 2000) as well as using an *in vitro* approach (Klocek-Gorka *et al*., 2010) that melatonin stimulated thyroid activity. The findings of Baltaci *et al.* (2003) contradicted those of the researchers, who claimed that melatonin administration

increased thyroid hormones. This is because of the questionable belief of Baltaci *et al.* (2003) that pinealectomy lowered melatonin levels, which led to significant increases in thyroid hormones. However, several researchers reported that melatonin administration inhibits thyroid hormones (Wajs and Lewinski, 1992; Sewerynek *et al.*, 1999; Champney, 2001).

It has been shown that exogenous melatonin application impaired the diurnal rhythm of T_3 and T_4 and that administration of melatonin, particularly at night, is more effective in obtaining this result (Zwirska-Korczala *et al.*, 1991; Kniazewski *et al.*, 1990b). It is claimed that melatonin administration, especially at later hours of the day, suppressed rates of mitotic activity and, thus, led to a strong inhibition on the thyroid gland (Wajs and Lewinski, 1992; Sewerynek *et al.*, 1999). Daily melatonin application to hamsters also resulted in a fall in thyroid hormones (Champney, 2001). In an *in vitro* study using frogs, Wright *et al.* (1997) found that administration of high concentrations of melatonin inhibited the thyroid's response to TSH. Studies investigating the relationship between melatonin and thyroid generally focus on the effects of melatonin administration on thyroid hormones. However, studies focusing on the relationship between pinealectomy and thyroid hormones, though are very few, are questionable, and they showed that pinealectomy increased thyroid hormones (Kniazewski *et al.*, 1990a and b).

It was demonstrated that melatonin administration increased the levels of zinc, while pinealectomy decreased them (Mocchegiani *et al.*, 1994; Brazao *et al*., 2011). Melatonin increases the absorption of zinc from the digestive system. The presence of specific binding sites for melatonin in the intestines seems to be a basic mechanism through

which this hormone increases the absorption of zinc in the digestive system (Mocchegiani *et al.*, 1994; Fabris *et al.*, 1997). The inhibition caused by pinealectomy on plasma zinc levels, in the work of Baltaci *et al.* (2003) is consistent with the findings of other researchers. The findings of Baltaci *et al.* (2003) showed that zinc deficiency decreases plasma melatonin levels, which is not entirely a surprising finding based on the role of zinc as a cofactor in the reaction, catalysed by l-amino acid decarboxylase that generates serotonin.

2.56 CONCLUDING REMARKS

The above comprehensive literature review revealed that the current approaches in the breeding programmes of chickens by exploitation of genetically diverse stocks for improving economic traits, especially body weights are associated with some unresolved problems, especially in the tropical zones of the world. Continuous lighting programmes, heat and road transportation stresses activate the HPA axis and inhibit melatonin synthesis, which, subsequently, decrease the overall performance of the broiler chickens. The literature clearly indicated that melatonin supplementation to broiler chickens reared in the tropical zones of the world, including Nigeria may improve their health and reduce high economic losses incurred yearly in modern poultry production.

CHAPTER 3

MATERIALS AND METHODS

3.1 EXPERIMENTAL SITES AND LOCATIONS

The experiment was performed in a poultry house, situated beside the Poultry Complex of Veterinary Teaching Hospital and belonging to the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria (11^{o} $10^{/}$ N, 07^{o} $38^{/}$ E), Kaduna State, located in the Northern Guinea Savannah zone of Nigeria. The premises of the poultry farms were utilised for experiments on tonic immobility and open-field. The birds were transported from the poultry house to Makarfi town (11^{o} $22^{/}$ N, 07^{o} $53^{/}$ E) located along Zaria-Kano road, a distance of about 50 km from the experimental site. They were immediately returned to the Faculty thereby covering a total distance of 100 km.

3.2 EXPERIMENTAL PERIOD AND FLOCK MANAGEMENT

The research was carried out between 2nd April, and 29th May, 2010 during the hot-dry season, prevailing in the Northern Guinea Savannah zone of Nigeria. Three hundred (300) broiler chicks belonging to the Marshall breed were obtained at a day-old from Fidan Farms, a branch of Fidan Investments Nigeria Limited, Ibadan, Oyo State, Nigeria on 2nd April, 2010. The Indian Poultry Equipment Manufacturer's Association (IPEMA, 2010) reported that the Marshall breed was developed in Nashik, India for high performance. The breed was launched in 2009 as a break-through in the Indian and, by extension, global broiler industry. Meticulous screening for feed conversion and meat yield along with severe selection pressures on growth rates have ensured that Marshall

breeds have an edge as broiler chickens. They are ultra-converters and have shown best performance efficiencies across multiple metrics, including feed conversion, liveability and growth rate. The breed is fast emerging as a leading broiler breed with sales in over 15 countries and a joint venture in Africa (IPEMA, 2010).

Complete routine vaccinations and appropriate medications were administered to the birds from day-old up to the end of the experiment. The broilers were raised on deep litter system and maintained on commercial feed obtained from Vital Feeds, Grand Cereals and Oil Mills Limited, Jos, Plateau State, Nigeria (Table 3.1). Starter diets were fed to the chicks from day-old to 21 d of life, after which they were placed on finisher diet up to the end of the experiment. They were given access to feeds and water *ad libitum*.

3.3 EXPERIMENTAL DESIGN

Simple random sampling was used to select and assign 300 broiler chicks into three groups, comprising hundred (100) chicks each. Group I was raised under natural photoperiod of about 12 h light and 12 h darkness cycle, without melatonin supplementation. Group II was kept under 24 h continuous lighting, without melatonin supplementation. Group III was raised under 24 h continuous lighting and administered daily with melatonin (ISI Brands, Inc. Utah, USA) at 0.5 mg/kg *per os* (Nisbet *et al*., 2008), via drinking water. Melatonin was administered once daily at 17:00 h (Deacon and Arendt, 1995). Heparinized blood samples collected at each hour of measurement were centrifuged at 400 *g* for 10 min and the separated plasma was frozen for further analysis. Most plasma constituents or parameters studied were determined with commercial kits

using appropriate microplate readers or the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany).

Table 3.1: Nutritive Contents of the Ration fed to Marshall Broiler Chickens during the Hot-dry Season

Nutrient Contents	Amount in % by Weight	
	Pelletised Starter Feed (1 – 21 d)	Pelletised Finisher Feed (22 – 56 d)
Calcium (%)	01.20	01.00
Available Phosphorous (%)	00.45	00.40
Metabolisable Energy (Kcal/kg)	2,800	2,900
*Proximate Analysis (%):		
a. Dry Matter	93.72	95.32
b. Crude Protein	22.00	18.56
c. Crude Fibre	03.94	04.27
d. Oil	05.09	04.23
e. Ash	10.47	07.91
f. Nitrogen Free Extract	58.50	65.03

*Analyzed in the biochemical laboratory, Department of Animal Science, Ahmadu Bello University, Zaria, Nigeria

3.4 METEOROLOGICAL DATA

The dry- and wet-bulb temperatures were taken concurrently with the hourly measurements for each treatment group at the experimental site and during the journey, using dry- and wet-bulb thermometer (Brannan, UK). From the data, RH and temperature-humidity index (THI) at each hour of measurement were calculated. The

THI was calculated using the following slightly modified equation of Avendano-Reyes *et al.* (2006):

$$THI = (0.41 \times AT) + RH\ (AT - 14.4) + 23.2$$

The data on meteorological parameters of maximum AT, minimum AT, wind direction, wind speed, rainfall and evaporation during the study period were collated from the Meteorological Unit of the Institute for Agricultural Research, Ahmadu Bello University, Samaru-Zaria, located at a distance of about 1 km from the experimental site.

3.5 EXPERIMENTAL PROCEDURES

3.5.1 Experiment I

3.5.1.1 Evaluation of Performance Indices

Live weight and feed consumption data for each of the groups were recorded at weekly intervals over a period of eight weeks. Live weight gain and feed conversion ratio during this period were also calculated as described by Avci *et al*. (2005). Water intake for each group was recorded daily, over a period of eight weeks as described by Hassan *et al.* (2009). Birds that died or culled were documented. Feed:gain ratio per group was corrected for body weight gain of the dead or culled birds. Dead birds were examined by post-mortem dissection on the day of death to determine cause of mortality. At 42 d of age, ten broilers per group that represented the average body weight were selected, slaughtered and processed as described by Veldkamp *et al.* (2000) to determine the yield of carcass parts. After dressing, carcasses were weighed and expressed as a percentage of live body weight and were cut into commercial-type of cut parts. Each part was weighed and expressed as a percentage of the carcass weight (Veldkamp *et al.,* 2000).

3.5.1.2 Broiler Meat Palatability Study

The consumer panel method of palatability measurements, described by Jackman *et al.* (2010) as the best approximation of public perception was used for this study. At 42 d of age, two birds from each of the groups were slaughtered and prepared for human consumption by an experienced and excellent cook, who was a final year Veterinary female student in the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The consumer panel consisted of ten members made up of staff and postgraduate students of the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. Sweetness, tenderness or softness, likeability or acceptability, satisfaction, flavour retention, structure or smoothness, bone edibility, bone marrow quantity, bone marrow delicacy and juiciness of the chicken cuts were assessed by the consumer panel. Three cuts, each from the groups were tasted by the same consumer who rinsed his or her mouth with clean water after eating each of the sample cuts. Consumer panel scored each of the cuts on a discrete scale ranging from 0 – 10 (0 was the lowest, while 10 was the highest scale).

3.5.2 Experiment II: Impact of Heat Stress on Broiler Chickens

A total of 300 broilers, consisting 100 birds per group was used for this research.

3.5.2.1 Base-line Haematological Analyses

At 28 d of age, 2 ml of blood in EDTA bottle was collected from a bird, each from the three groups and evaluated as described below. The blood was analysed using automated electronic cell counter (Abbott Haematological Analyser Cell-Dyn 1700, Illinois, USA) for haematocrit (Hct), haemoglobin (Hb), white blood cell count (WBC), erythrocyte count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH),

mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW_SD), thrombocytes or platelets (PLT), total and differential leucocyte counts in the haematology Department of Ahmadu Bello University Teaching Hospital, Shika-Zaria.

The leucocyte count in the avian species was also carried out according to the standard two-slide wedge technique used in mammalian haematology (Schalm *et al*., 1975; Coles, 1986). Leishman's staining technique was done as described by Cheesbrough (2000). Briefly, the slide was covered with the undiluted stain, and then diluted twice the volume with water and allowed to stain for 10 minutes. Giemsa's staining technique was carried out as described by Cheesbrough (2000). The blood films (methanol pre-fixed) were covered with the diluted stain (1 in 20) for 25 minutes. Wright's staining technique was carried out as reported by Altan *et al*. (2000a) with a slight modification. The slide was covered with the stain for 15 minutes. Thereafter for each technique, the slides were finally washed off the stain with tap water, air-dried and observed microscopically under oil immersion. 100 leucocytes, including heterophils, lymphocytes, monocytes, basophils and eosinophils were counted on each slide. Total leucocyte counts were calculated using the formula (Campbell, 1995):

$$\text{TWBC/mm}^3 = \frac{\text{Number of cells counted x 10 x 32 x 100}}{(\%\ \text{H} + \text{E}) \times 18}$$

Where TBWC = Total white blood cell count; H = Heterophils;
E = Eosinophils

Blood sample (2 ml) was collected from the wing vein of each bird into EDTA bottle and analysed immediately in the Clinical Laboratory of the Department of Veterinary

Pathology and Microbiology, Ahmadu Bello University, Zaria. Hct was determined using microhaematocrit method as described by Cheesbrough (2000). Briefly, heparinized capillary tubes were about three-quarter filled with blood sample by capillary action. The unfilled end was sealed using a pilot flame of a Bunsen burner. The tubes were slotted into the microhaematocrit rotor (Saitexiangyi, China) and centrifuged at 3, 000 x *g* for 10 minutes. Immediately after centrifuging, the Hct was read with the aid of the microhaematocrit reader (Hawksley, England). Hb values were obtained by dividing the corresponding Hct values obtained by 3 (Cheesbrough 2000; Abdelatif *et al*., 2009).

The erythrocyte osmotic fragility was determined according to the method described by Oyewale (1992). The procedure has been briefly described on page 102.

3.5.2.2 Determination of Parameters of Biorhythms

From 30-day old, diurnal fluctuations in the following oxidative stress biomarkers of broilers were determined at 6:00 h, 13:00 h and 19:00 h (GMT + 1) over a 24-h period for three days, two weeks apart. At each hour of measurement, fifteen (15) birds from each group were labeled and used throughout the study period, sparing the other subjects in the group for fear tests, behavioural and transportation studies:

Measurement of Rectal Temperature: Measurements of rectal temperature (RT) in broilers were taken according to routine method using standard digital clinical thermometers (The Hartman's Company PLC, UK) as described by the routine method of Sinkalu *et al*. (2009). The RT of 15 labeled broiler chickens from each group was measured at 06:00 h, 13:00 h and 19:00 h weekly, starting from the 2nd to 8th week of age.

Erythrocyte Osmotic Fragility Test: Erythrocyte osmotic fragility was determined according to the method described by Oyewale (1992). Briefly, 0.02 ml of blood collected from each bird from the groups was added to tubes, containing increasing concentrations (0, 0.1, 0.3, 0.5, 0.7 and 0.9 %) of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4. The tubes were gently mixed and incubated at room temperature (25 ^{o}C) for 30 minutes. The contents in each tube were then mixed and centrifuged at 400 *g* for 10 minutes and supernatant decanted. Optical density of the supernatant was determined spectrophotometrically at 540 nm using SM 22 PC Spectrophotometer (Surgienfield Instrument, England). Haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0 % NaCl) as 100 %. The percent haemolysis was calculated using the formula of Faulkner and King (1970):

$$\text{Percent (\%) haemolysis} = \frac{\text{Optical density of standard solution}}{\text{Optical density of test solution}} \times 100$$

The results were analysed and plotted in order to obtain osmotic fragility curves (fragiligrammes) as described by Oyewale (1992).

Haematological Studies: Blood sample (2 ml) in ethylenediaminetetraacetic acid (EDTA) bottle was collected at 6:00 h, 13:00 h and 19:00 h from each of the broiler chickens in each of the three groups and analysed as described earlier in section *3.5.2.1*.

Behavioural Studies: The behavioural events of the broilers, comprising standing idling, feeding, drinking and lying (resting/sleeping) were observed in each group for 10 min and recorded by an observer, invisible to the birds, as described by Gerritzen *et al*. (2004), Dunkley *et al*. (2008) and Minka and Ayo (2008b).

Fear Tests: (i) Tonic Immobility

Tonic immobility (TI) was induced starting from week 4 up to week 8 in 15 birds from each group at 06:00 h, 13:00 h and 18:00 h. The TI reaction was carried out on each bird as described by Ghareeb *et al*. (2008) in a separate room having the same conditions as the pen, where the birds were raised. Each bird, prevented from having auditory and visual contact with other birds, was laid on its back in a U-shaped cradle covered with cloth. The bird was then restrained with one hand resting on the sternum and fingers capped lightly over its beak for 25 s, while holding the head and neck by the other hand. Towards the end of the 25-s period, hand pressure was gradually lifted so that if the chick still moved, another induction period of 15 s was started, until movement ceased. When the bird lay still with one or both legs extended (although sometimes still peeping), it was then released and observed from a position about a metre away out of its sight (Heiblum *et al*., 1998). After removal of the hands, a stop-watch was used to record the latency and the duration of TI. The behaviour of each of the birds until self righting was also described and graded as follows: 1, if the process of self-righting was quick and spontaneous; 2, if the process was slow and without vigilance (peeping and vocalisation); and 3, if the process was very slow and with a prolonged period of vigilance. When duration was less than 3 s, additional induction trials of 25 s each were made, up to a maximum of four trials.

Fear Tests: (ii) Open-field Test

The open-field test was performed starting from week 4 up to week 8 in 15 birds from each group at 06:00 h, 13:00 h and 18:00 h. Open-field test involved placing each broiler alone in the centre of the arena of an open-field box (250 cm x 250 cm x 60 cm). The

arena was divided into 32 equal squares as described by Al-Badrany and Mohammad (2007). In the open-field test, the following behavioural patterns were measured within 5 min (Mohammad and Faris, 2006):

1. Latency to move from the centre square.
2. Number of lines crossed by both feet (ambulation).
3. Number of escape jumps.
4. Scoring of distress calls (vocalisation):

0 – No Calls

1 – 1 - 2 Calls

2 – 3 - 4 Calls

3 – 5 - Calls or >.

3.5.3 Experiment III: Biochemical Quantification of Transportation Stress

At 42 d of age, 60 broilers comprising 20 birds per group were used for this study. They were subjected to catching, handling, loading onto the floor of a commercial vehicle (Mitsubishi Bus L300, Japan), covered with wood shavings, and transported by road for about 2 h at a speed of 50 km/h. The covered distance was 100 km on a tarred road. The birds were transported from the poultry house to Makarfi town (11^{o} $22^{/}$ N, 07^{o} $53^{/}$ E) along Zaria-Kano road located at a distance of about 50 km from the experimental site. The birds were immediately returned to the Faculty from Makarfi, covering a total of 100 km at an average speed of 50 km/h. Blood samples from the birds were collected and analysed for the following biochemical parameters before and immediately after the journey.

3.5.3.1 Evaluation of Protein

The evaluation of TP was based on the biuret method, where peptide linkages reacted with cupric ions in an alkaline solution to form a violet-coloured compound. The samples were assayed using Bayer Diagnostics Total Protein Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 9 µL of the sample was added to 275 µL of the reagent and incubated for 4 min at 37 ^{o}C. The TP values in g/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 540 nm (Revision D of the Express Plus Applications Guide, 2000).

The concentration of TP was determined using microhaematocrit method as described by Cheesbrough (2000). Immediately after centrifuging, the capillary tube was neatly cut or broken at the level of plasma layer. The plasma was transferred directly to "glassy" surface of the hand refractometer (American Optical Inc., Keene, USA) from where TP concentrations were obtained.

Albumin bound to the anionic bromcresol green (BCG) dye to produce an increase in blue-green colour, which was measured at 600 nm. The samples were assayed using Bayer Diagnostics Albumin Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 3 µL of the sample was added to 300 µL of the reagent and incubated for 1 min at 37 ^{o}C. The albumin values in g/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 600 nm (Revision D of the Express Plus Applications Guide, 2000).

3.5.3.2 Evaluation of Blood Electrolytes

Determination of serum sodium (Na^{+}) and potassium (K^{+}) was carried out using the flame

photometry (Corning 410 Sherwood, UK) according to AOAC method (Jain, 1986; AOAC, 2000). The principle of the reaction is as follows: K^+ or Na^+ solutions under carefully controlled condition when finely sprayed (aspirated) into a burner, the flame dissolves the solution leaving solids (salts), which dissociate to give neutral ground state atoms. Some of these atoms are excited in the flame, thus moving into a higher energy state; when these excited atoms fall back to the ground state, light of characteristic wavelength is emitted (590 nm for Na^+ and 770 nm for K^+). The light then passes through a suitable filter onto a photosensitive element and the amount of current thus produced is measured – this is proportional to the amount of K^+ or Na^+ present in the original sample.

For Na^+, the Na^+ light filter was inserted and the galvanometer switched on. The gas supply was fully turned on, the flame ignited and the air supply was turned on. The air pressure was regulated to 517.15 millimeters of mercury. The gas was adjusted smoothly to obtain discrete cones of flame. The galvanometer scale with water was set at zero; then the galvanometer reading was set to 80 – using the working standard; and was reset to zero. The test was read, checking the standard after 2 – 3 test readings. The values of Na^+ were obtained by multiplying galvanometer reading by 2 in mmol/L.

For K^+, the K^+ light filter was inserted and the galvanometer with K^+ working standard was set to 70, and the procedure continued as for Na^+. The K^+ values were obtained by multiplying galvanometer reading by 0.1 in mmol/L.

The chloride method was based on the displacement of thiocyanate by chloride ions and the subsequent reaction of the liberated thiocyanate with ferric ions to form the colour complex. The samples were assayed using Bayer Diagnostics Chloride Reagent on the

Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 5 µL of the sample was added to 395 µL of the reagent and incubated for 3 min at 37 ^{o}C. The chloride values in mmol/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 510 nm.

3.5.3.3 Determination of Blood Urea Nitrogen (BUN)

This application was based on the following equations:

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{-Ketoglutarate} + \text{NADH} + H^+ \xrightarrow{\text{GLDH}} \text{L-Glutamate} + NAD^+ + H_2O.$$

Where NADH = Reduced nicotinamide adenine dinucleotide.

NAD^+ = Oxidized form of NADH.

GLDH = Glutamate dehydrogenase.

The samples were assayed using Bayer Diagnostics BUN Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 3 µL of the sample was added to 375 µL of the reagent and incubated for 1 min at 37 ^{o}C. The BUN values in mmol/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 340 nm (Revision D of the Express Plus Applications Guide, 2000).

3.5.3.4 Evaluation of Activities of Plasma Enzymes

The determination of alkaline phosphatase (ALP) was based on a kinetic procedure illustrated by the following equation:

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{p-nitrophenol} + H_3PO_4.$$

The samples were assayed using Bayer Diagnostics Alkaline Phosphatase Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 6 µL of the sample was added to 300 µL of the reagent and incubated for 1 min at 37 ^{o}C. The ALP values in IU/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 405 nm (Revision D of the Express Plus Applications Guide, 2000).

The determination of aspartate aminotransferase (AST) was based on a procedure illustrated by the following equations:

$$\text{L-aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AST}} \text{oxalacetate} + \text{L-glutamate}$$

$$\text{Oxalacetate} + \text{NADH} + H^{+} \xrightarrow{\text{MDH}} \text{L-malate} + NAD^{+} + H_2O$$

Where MDH = Malate dehydrogenase.

The samples were assayed using Bayer Diagnostics AST Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 25 µL of the sample was added to 250 µL of the reagent and incubated for 1 min 40 s at 37 ^{o}C. The AST values in IU/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 340 nm (Revision D of the Express Plus Applications Guide, 2000).

The determination of alanine aminotransferase (ALT) was based on a procedure illustrated by the following equations:

$$\text{L-alanine} + \text{2-oxoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{Lactate} + NAD^{+}$$

Where, LDH = Lactate dehydrogenase.

The samples were assayed using Bayer Diagnostics ALT Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 25 µL of the sample was added to 250 µL of the reagent and incubated for 1 min 40 s at 37 ^{o}C. The ALT values in IU/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 340 nm.

The determination of creatine kinase (CK) was based on a procedure illustrated by the following equations:

$$\text{ADP + creatine phosphate} \xrightarrow{\text{CK}} \text{creatine + ATP}$$

$$\text{ATP + glucose} \xrightarrow{\text{HK}} \text{ADP + glucose-6-phosphate}$$

$$\text{G-6-P + NADP}^{+} \xrightarrow{\text{G-6-PD}} \text{6-phosphogluconate + NADPH + H}^{+}$$

Where, ADP = Adenosine diphosphate.

G-6-P = Glucose 6-phosphate.

G-6-PD = Glucose 6-phosphate dehydrogenase.

ATP = Adenosine triphosphate.

HK = Hexokinase.

NADPH = Reduced nicotinamide adenine dinucleotide phosphate.

$NADP^{+}$ = Oxidized form of NADPH.

The samples were assayed using Bayer Diagnostics CK Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 13 µL of the sample was added to 260 µL of the reagent and incubated for 2 min at 37 ^{o}C. The CK values in IU/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at

340 nm (Revision D of the Express Plus Applications Guide, 2000).

Glutathione peroxidase (GPx; NWK-GPX01) was evaluated before and immediately after transportation using the Northwest Life Science Specialties (NWLSS), Vancouver assay. The NWLSS^{TM} glutathione peroxidase assay is an adaptation of the method of Paglia and Valentine (1967). Briefly, the assay was designed for use in 96-well microplate format. Samples were appropriately diluted with assay buffer and placed on ice. NADPH reagent was reconstituted with NADPH diluent as specified. 50 µL of diluted samples were added to appropriate wells of microplate. 50 µL of working NADPH and 50 µL of working H_2O_2 were then added to each of the wells. It was equilibrated for 1 min. The microplate was placed in the microplate reader (Biorad, Vienna, Austria) and measurements were taken at 340 nm every 30 seconds for 5 minutes. The GPx activity in the sample was calculated from the net rate.

Quantitative measurement of SOD activity (NWK-SOD02) was performed before and immediately after transportation using the NWLSS^{TM} assay (Northwest Life Science Specialties, Vancouver). The NWLSS^{TM} method used was based on monitoring the autoxidation rate of haematoxylin as originally described by Martin Jr. *et al*. (1987), with modifications to increase robustness and reliability. Briefly, 230 µL of assay buffer was added to wells of the microplate. Thereafter, 10 µL of assay buffer (for blank) and 10 µL of sample were added. The wells were properly shaken, mixed and incubated for two minutes. A multi-channel pipette was used to add 10 µL of haematoxylin reagent to begin the reaction. The content of each well was quickly mixed using the instrument's shaker

function, and immediately the absorbance at 560 nm was recorded every 10 seconds for 5 minutes. Finally, the SOD activity in the sample was calculated from the net rate.

3.5.3.5 Malondialdehyde Assay (NWK-MDA01) as an Index of Lipid Peroxidation

The NWLSS^{TM} (Northwest Life Science Specialties, Vancouver) kit used was based on the reaction of MDA with thiobarbituric acid (TBA) forming an MDA-TBA_2 adduct that was absorbed strongly at 532 nm.

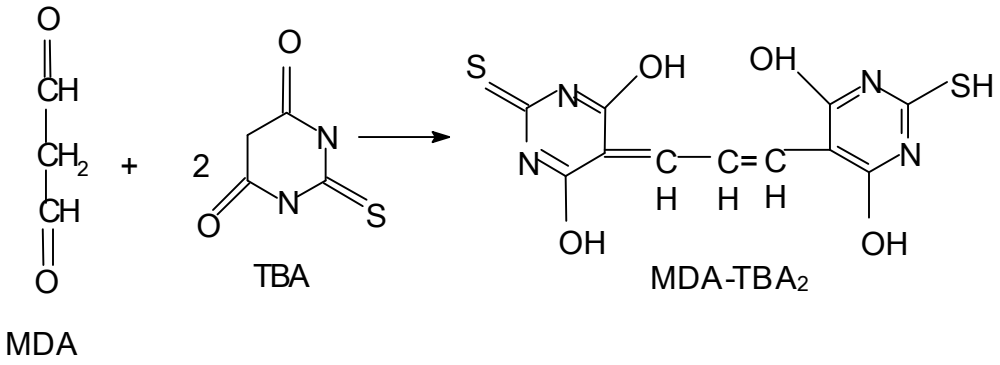


Figure 3.1: Reaction between Malondialdehyde (MDA) and Thiobarbituric acid (TBA)

The assay protocol, which was undertaken before and immediately after transportation, involved addition of 10 µL butylated hydroxytoluene (BHT) reagent to microcentrifuge vial. 250 µL of calibrator or sample, 250 µL of acid reagent and 250 µL of TBA reagent were added to the vial. The vial was vortexed vigorously (5-count) and incubated for 1 h at 60 ^{o}C. It was then centrifuged at 10,000 x *g* for 2 – 3 minutes. The reaction mixture was transferred to cuvette and placed in a spectrophotometer (Ultrospec 450, LKB Biochrom Ltd., Cambridge, UK). Spectra were recorded at 532 nm and 3rd derivative analysis was utilised to obtain the MDA concentration in the sample.

The plasma MDA level was analysed using the method of Draper and Hadley (1990), modified by Yavuz *et al*. (2004). Briefly, 2.5 ml of 100 g/L trichloroacetic acid solution (TCA) was added to 0.5 ml of plasma in the centrifuge tube and placed in boiling water bath for 15 minutes. Thereafter, it was cooled in running tap water for 5 minutes. The mixture was centrifuged at 1000 x *g* for 10 minutes, and 2 ml of the supernatant was added to 1 ml of 6.7 g/L TBA solution in a test tube, and placed in boiling water bath for 15 minutes. The solution was again cooled in running tap water for 5 minutes and its absorbance was measured using a UV spectrophotometer (Schimadzu, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 10^5/cm, and expressed in µmol/ml.

3.5.3.6 Determination of Total Cholesterol in Plasma

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4H_2O$$

The concentration of cholesterol in the sample was assayed before and immediately after transportation using Bayer Diagnostics Cholesterol Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 6 µL of the sample was added to 300 µL of the reagent, mixed and incubated for 6 min at 37 ^oC. The total cholesterol values in

mmol/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 510 nm.

3.5.3.7 Evaluation of Triglycerides in Plasma

The triglycerides were determined after enzymatic hydrolysis with lipases. The indicator was a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipases}} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone} + \text{Phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-Aminophenazone} + \text{Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + \text{HCl} + 4H_2O$$

Where GK = Glycerol kinase

GPO = Glycerol phosphate oxidase

POD = Phosphate oxidase-derived

The concentration of triglycerides in the sample was assayed before and immediately after transportation using Bayer Diagnostics Triglycerides Reagent on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 3 µL of the sample was added to 300 µL of the reagent, mixed and incubated for 8 min at 37 ^{o}C. The triglycerides values in mmol/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 540 nm.

3.5.3.8 Determination of High-Density Lipoprotein Cholesterol (CH 203) in Plasma

Low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and chylomicron fractions were precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the high density lipoprotein (HDL) fraction, which remained in the supernatant, was determined. The concentration of HDL cholesterol in the sample was assayed before and immediately after transportation using Bayer Diagnostics HDL cholesterol precipitant on the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany). 6 µL of the sample was added to 300 µL of the reagent, mixed and incubated for 6 min at 37 ^{o}C. The HDL concentration values in mmol/L were then obtained from the Bayer Express Plus Clinical Chemistry Analyser (Bayer, Germany) at 510 nm.

3.5.3.9 Evaluation of Triiodothyronine in Plasma

Triiodothyronine (T_3) ELISA kit (Cat. No. 3810-96; Clinotech, Canada) was used for the quantitative measurement of total T_3 in plasma before and immediately after transportation. The kit operated upon the competitive binding principle. The samples, assay buffer and T_3 enzyme conjugate were added to the wells coated with anti-T_3 monoclonal antibody. T_3 in the plasma competed with the enzyme conjugated T_3 for binding sites. Unbound T_3 and T_3 enzyme conjugate was washed off by washing buffer. Upon the addition of the substrate, the intensity of colour was inversely proportional to the concentration of T_3 in the samples. A standard curve was prepared relating colour intensity to the concentration of the T_3, from which the values of T_3 in the sample were extrapolated.

Precisely, 50 µL of T_3 standard, control and sample was pipetted into the coated strips in the holder. 100 µL of working T_3-enzyme conjugate was added to all wells and incubated for 1 h at room temperature (26 ^{o}C). Thereafter, liquid was removed from all wells and the wells were filled with working wash buffer. It was washed three times and blotted on absorbent paper towels. 100 µL of tetramethylbenzidine (TMB) substrate was added to all wells and incubated again for 15 minutes at room temperature. 50 µL of stop solution was added to all wells and the plate was gently shaken to mix the solution. Finally, absorbance was read on ELISA microplate reader (Biorad, Vienna-Austria) at 450 nm within 15 minutes of adding the stop solution. A calibration curve was plotted from which sample concentration was extrapolated.

3.5.3.10 Determination of Thyroxine in Plasma

The Microwell Thyroxine (T_4) EIA (Ref. No. 2210-96; Syntron, USA) used before and immediately after transportation was a solid-phase enzyme, utilising the competitive binding principle. The T_4 present in the sample competed with the enzyme-labeled T_4 conjugate, containing 8-anilino-1-naphtha-lenesulfonic for binding with anti-T_4 antibody, immobilized on the microwell surface. The amount of conjugate that bound to the microwell surface decreased in proportion to the concentration of T_4 in the sample.

The unbound sample and conjugate were then removed by washing and the colour development reagents (substrates) were added. Upon exposure to the enzyme, a colour change took place. The intensity of the colour reflected the amount of bound enzyme-T_4 conjugate and was inversely proportional to the concentration of T_4 in the sample within dynamic range of the assay. After stopping the reaction, the resulting colour was

measured at 450 nm using a spectrophotometer (Biorad, Vienna-Austria). The T_4 concentration in the sample was determined from the standard curve.

Briefly, 50 µL of standards, control and test sample was pipetted into the coated strips in the holder. 100 µL of enzyme conjugate was added to all wells and incubated for 1 h at 37 ^{o}C. Thereafter, all wells were decanted and washed five times with diluted washing buffer. The wells were dried by firmly tapping the plate on a clean paper towel to remove excess washing solution. 50 µL of substrate reagents A and 50 µL of substrate reagent B were dispensed into each well, mixed and incubated again at room temperature (28 ^{o}C) for 15 minutes. 50 µL of stopping solution was added to each well and the plate was gently rocked to mix the solution. Finally, the absorbance at 450 nm of each well was read within 30 minutes after stopping the reaction using a spectrophotometer (Biorad, Vienna-Austria). The T_4 concentration in the sample was determined from the standard curve.

3.5.3.11 Evaluation of Thyroid-Stimulating Hormone in Plasma

Thyroid stimulating hormone (TSH) enzyme-linked immunosorbent assay (ELISA) kit (Ref. No. 2211-96; Clinotech, Richmond, Canada) was used for the quantitative measurement of TSH in plasma before and immediately after transportation. The kit was a solid phase sandwich ELISA method. The samples and anti-TSH-HRP conjugate were added to the wells coated with Mab to TSH beta-subunit. TSH in the sample bound to anti-TSH MAb on the well and the anti-TSH second antibody then bound to TSH. Unbound protein and HRP conjugate were washed off by wash buffer. Upon the addition of the substrate, the intensity of colour was proportional to the concentration of TSH in

the samples. A standard curve was prepared relating colour intensity to the concentration of the TSH.

Precisely, 50 µL of TSH standards, control and sample was pipetted into the coated strips in the holder. 100 µL of ready-to-use enzyme conjugate was added to all wells and incubated for 1 h at room temperature (26 ^{o}C). Thereafter, liquid was removed from all wells and the wells were filled with working wash buffer. It was washed three times and blotted on absorbent paper towels. 100 µL of TMB substrate was added to all wells and incubated again for 15 minutes at room temperature. 50 µL of stopping solution was added to all wells and the plate was gently shaken to mix the solution. Finally, absorbance on ELISA microplate reader (Biorad, Vienna-Austria) at 450 nm was read within 20 minutes after adding the stop solution. A calibration curve was plotted from which TSH concentration was extrapolated.

3.5.3.12 Measurement of Corticosterone in Plasma

The kit (Cat. No. 900-097; Assay Designs Correlate-EIA^{TM}, USA) used before and immediately after transportation was a competitive immunoassay for the quantitative determination of corticosterone (Fig. 3.2) in plasma. The kit used a polyclonal antibody to corticosterone to bind, in a competitive manner, corticosterone in the standard or sample or an alkaline phosphatase molecule, which had corticosterone covalently attached to it. After a simultaneous incubation at room temperature (26 ^{o}C), the excess reagents were washed away and substrate was added. After a short incubation time of 10 min, the enzyme reaction was stopped and the yellow colour generated was read on a microplate reader (Biorad, Vienna-Austria) at 405 nm. The intensity of the bound yellow

colour was inversely proportional to the concentration of corticosterone in either standards or samples. The measured optical density was used to calculate the concentration of corticosterone.

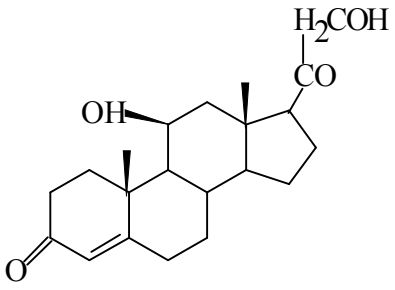


Figure 3.2: Structure of Corticosterone

The corticosterone assay procedure used followed the description of Chard (1990) and Tijssen (1985) on the principles and practice of immunoassays. Briefly, 100 µL of standard diluent (Assay Buffer 15) was pipetted into the non-specific binding (NSB) and the Bo (maximum binding) (0 ρg/mL standard) wells. 100 µL of standards No.1 through No.5 was pipetted into the appropriate wells. 100 µL of samples was pipetted into the appropriate wells. 50 µL of Assay Buffer 15 was pipetted into the NSB wells. 50 µL of blue conjugate was pipetted into each well, except the total activity (TA) and blank wells. 50 µL of yellow antibody was pipetted into each well, except the NSB, total activity (TA) and blank wells. Every well used turned green in colour, except the NSB wells which turned blue. The blank and TA wells were empty at this point and had no colour. The plate was incubated at room temperature (26 ^{o}C) on a plate shaker for 2 h at 2, 800 x *g*. The contents of the wells were emptied and washed thrice by adding 400 µL of wash solution to every well. After the final wash, the wells were emptied and the plate were firmly tapped on a lint free paper towel to remove any remaining wash buffer. 50 µL of

the blue conjugate was added to the TA wells. 200 µL of the p-nitrophenyl phosphate (pNpp) substrate solution was added to every well and incubated at room temperature for 1 hour without shaking. 50 µL of stop solution was added to every cell. This stopped the reaction, and the plate was read immediately on the microplate reader (Biorad, Vienna-Austria) at 405 nm. The measured optical density was used to calculate the concentration of corticosterone in the sample.

3.5.3.13 Brain Tissue Peroxidation Status

Brain sample before and immediately after transportation from each bird in all the groups were weighed, and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10 % homogenate, which was centrifuged at 6 000 x *g* for 10 minutes to obtain the supernatant. The supernatant was then used to assess the level of SOD, GPx, MDA and protein in the brain sample.

Quantitative measurement of SOD activity was performed using the NWLSS^{TM} assay kit [Northwest Life Science Specialties (NWLSS), Vancouver] on the supernatant. The NWLSS^{TM} NWK-SOD02 method used was based on monitoring the autoxidation rate of haematoxylin as originally described by Martin Jr. *et al*. (1987), with modifications to increase robustness and reliability as earlier described for plasma SOD in *3.5.3.4*.

Glutathione peroxidase (GPx) was evaluated using the NWLSS Vancouver assay on the supernatant. The NWLSS^{TM} glutathione peroxidase assay was an adaptation of the method of Paglia and Valentine (1967) as earlier described for plasma GPx in *3.5.3.4*.

Malondialdehyde was assayed using the NWLSS^{TM} NWK-MDA01 assay (Northwest Life Science Specialties, Vancouver) based on the reaction of MDA with TBA; forming an MDA-TBA_2 adduct was absorbed strongly at 532 nm as earlier described for plasma MDA in *3.5.3.5*.

The protein in the brain homogenate was evaluated using the method described by Lowry *et al*. (1951). The principle was based on the fact that the phenolic group of tyrosine and tryptophan residues (amino acid) in a protein produced a blue purple colour complex, with maximum absorption in the region of 660 nm wavelength, using Folin-Ciocalteau reagent, which consisted of sodium tungstate molybdate and phosphate. Thus, the intensity of colour depended on the amount of these aromatic amino acids present and, thus, varied for different proteins. Most proteins estimation techniques use bovine serum albumin (BSA) universally as a standard protein because of its low cost, high purity and ready availability. The method was sensitive down to about 10 µg/ml and was, probably, the most widely used protein assay, despite its being only a relative method, subject to interference from Tris buffer, EDTA, non-ionic and cationic detergents, carbohydrates, lipids and some salts. The incubation time was very critical for a reproducible assay. The reaction was also dependent on pH and a working range of pH 9 to 10.5 was essential.

Briefly, different dilutions of BSA solutions were prepared by mixing stock BSA solution (1 mg/ml) and water in the test tube. The final volume in each of the test tube was 5 ml. The BSA range was 0.05 to 1 mg/ml. From the different dilutions, 0.2 ml protein solution was added to different test tubes and 2 ml of alkaline copper sulphate reagent (analytical reagent) was also added. The solutions were thoroughly mixed thereafter, and incubated

at room temperature for 10 minutes. Then, 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 minutes. The colorimeter (Ultrospec 450, LKB Biochrom Ltd., Cambridge, UK) was zeroed with blank and optical density taken (absorbance measured) at 660 nm. The absorbance against protein concentration was plotted to obtain a standard calibration curve. The absorbance of the unknown sample was read and its concentration determined using the standard curve plotted.

3.6 Statistical Analyses

All data obtained, with the exception of fear tests and behavioural studies, were subjected to one-way analysis of variance (ANOVA) and Pearson's correlation analysis. Behavioural studies were analysed using Kruskal Wallis Test. Data were expressed as mean ± standard error of the mean (mean ± S.E.M.). Multiple means were compared by Duncan's (1955) multiple range test. Values of $P < 0.05$ were considered significant. The analyses were performed using a statistical package for social sciences software (Version 16.0; New York, USA).

CHAPTER 4

RESULTS

4.1 METEOROLOGICAL DATA INSIDE AND OUTSIDE THE POULTRY HOUSE DURING THE STUDY PERIOD

The results of the meteorological data during the study period are presented in Figures 4.1 – 4.2 and Tables 4.1 – 4.3. The mean ambient temperature (AT) ranged from the minimum value of 21.00 ± 0.69 ^{o}C at 1st week to the maximum mean value of 40.29 ± 0.36 ^{o}C, obtained at the 2nd week of the study (Table 4.1). The mean weekly meteorological parameters of relative humidity, maximum AT, minimum AT, sunshine duration, evaporation, wind speed and wind direction recorded outside the poultry house during the period of the study fluctuated significantly ($P < 0.05$). The overall average wind speed was 1.91 ± 0.12 m/s and the wind direction was predominantly south-west. The overall mean values of sunshine duration, rainfall and evaporation were 6.60 ± 0.44 h/day, 2.29 ± 0.85 mm and 9.79 ± 0.90 mm/day, respectively (Table 4.1).

Figure 4.1 shows that the weekly mean THI recorded inside the poultry house was lowest at 4th week of the study, with the value of 48.60 ± 0.08 ^{o}C, but highest at 1st week with the value of 53.48 ± 0.97 ^{o}C ($P < 0.0001$). Inside the poultry house where the birds were reared, the RH fluctuated significantly ($P < 0.001$) between 67.48 ± 1.33 % recorded at 19:00 h, and 72.38 ± 1.76 % obtained at 06:00 h, with an overall mean of 70.69 ± 1.61 % (Fig. 4.2). The mean AT values fluctuated significantly ($P < 0.0001$) as the hours of the day increased, with peak value of 38.61 ± 0.35 ^{o}C recorded at 13:00 h (Fig. 4.2).

The meteorological data during transportation are shown in Tables 4.2 and 4.3. The highest RH value of 87 % was recorded inside the poultry house 3 – 4 d after the journey, while the highest AT of 41 ^{o}C was obtained 1 h into the journey (Table 4.2). However, the highest THI value of 60.59 was recorded inside the vehicle immediately after the 2 h of transportation (Table 4.2). During the 4-day period of transportation study, the mean maximum AT, RH, sunshine duration, wind speed and evaporation values recorded outside the vehicle were 40.75 ± 0.48 ^{o}C, 67.75 ± 1.55 %, 8.3 ± 0.15 h/d, 1.50 ± 0.30 m/s and 11.80 ± 1.35 mm/d, respectively (Table 4.3).

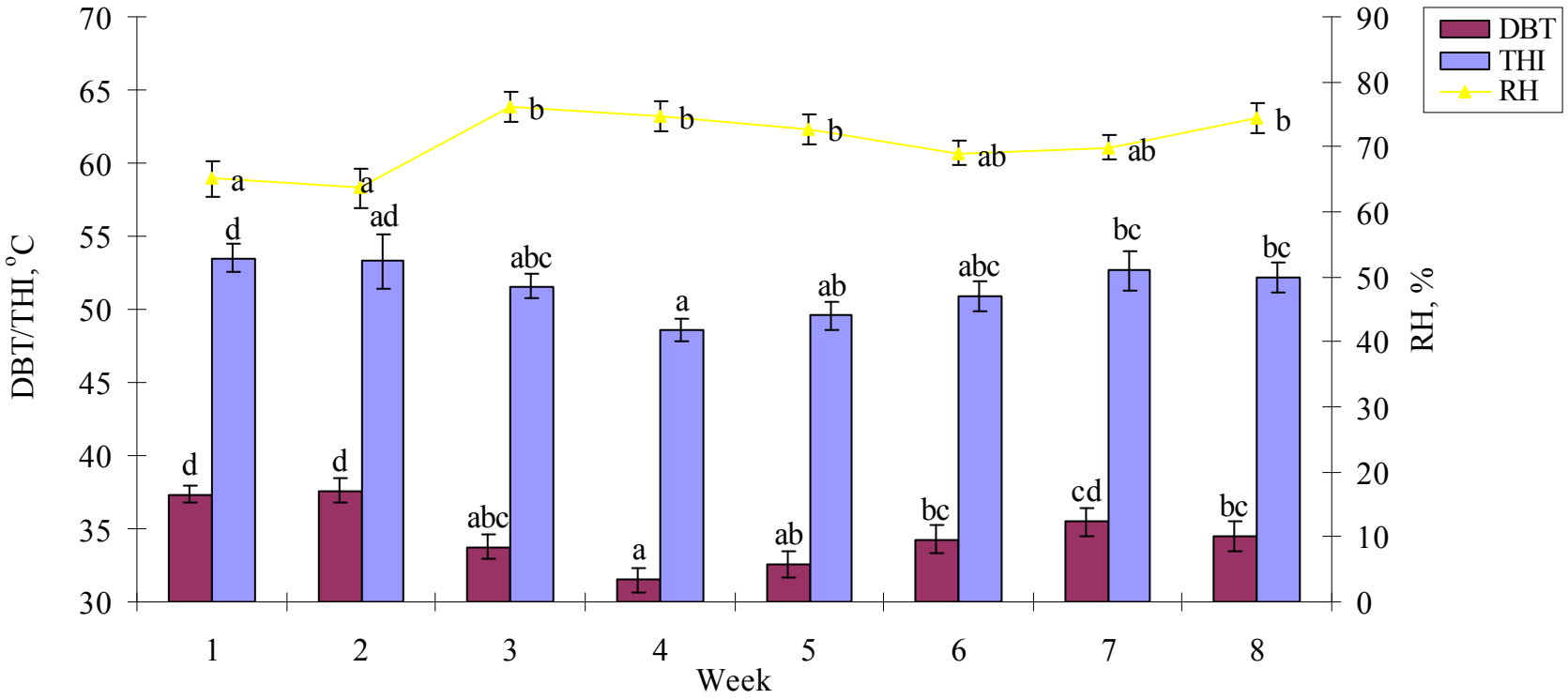


Figure 4.1: Weekly Thermal Environment Data Inside the Poultry House during the Study Period (Mean ± SEM); DBT = Dry-bulb temperature; THI = Temperature-humidity index; RH = Relative humidity, $^{a, b, c, d}$ = Means belonging to the same parameter and having different superscript letters are significantly ($P < 0.05$) different

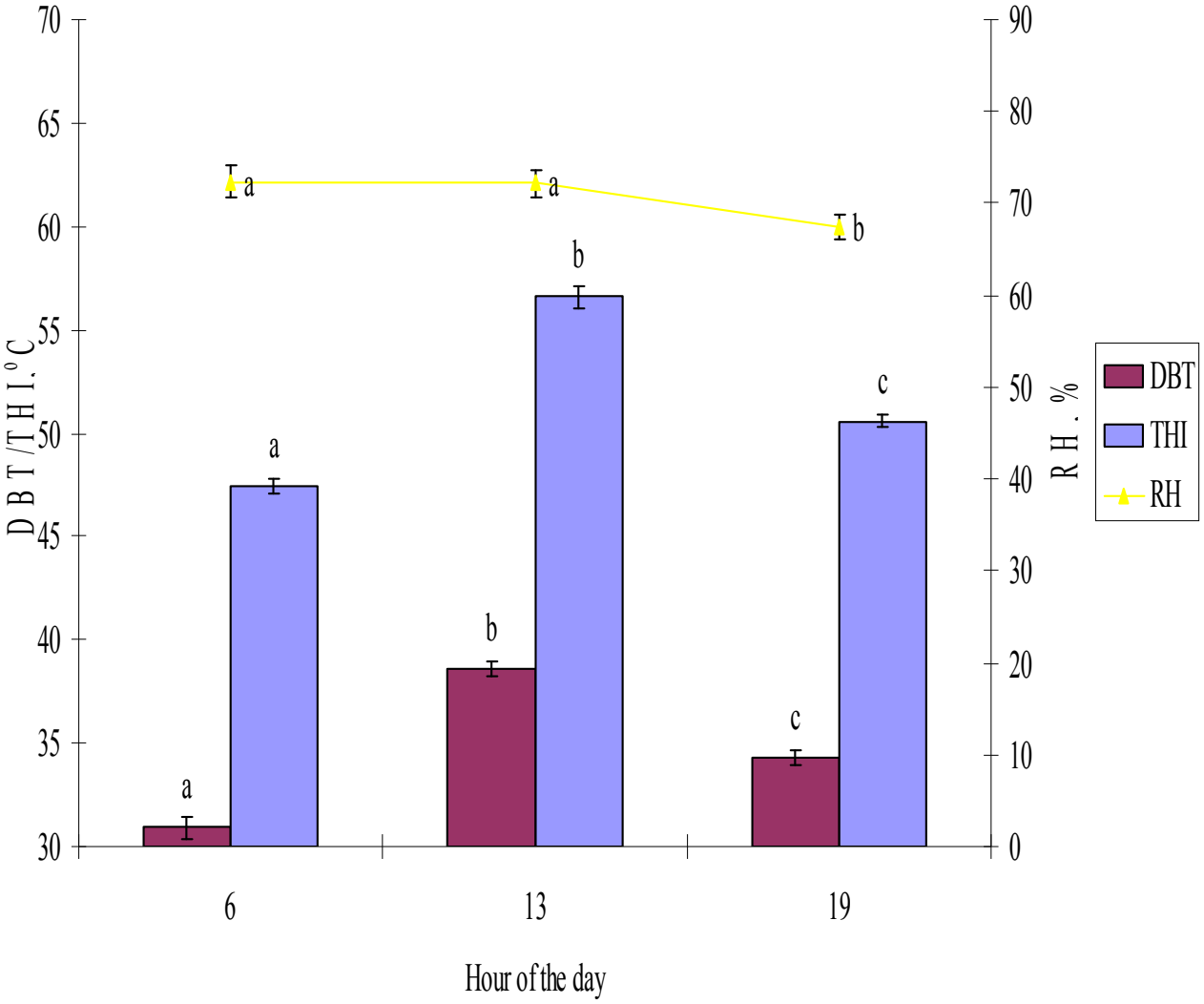


Figure 4.2: Diurnal Fluctuations in Dry-bulb Temperature (DBT), Temperature-humidity Index (THI) and Relative Humidity (RH) inside the Poultry House during the Study Period (Mean ± SEM); Note: $^{a, b, c, d}$ = Means belonging to the same parameter and having different superscript letters are significantly ($P < 0.05$) different

Table 4.1: Mean Weekly Meteorological Data outside the Poultry House during the Period of the Study (Mean ± SEM)

Week	RH, %	Ambient Temperature, °C		Sunshine Duration, h/d	Rainfall, mm	Wind Speed, m/s	Evaporation, mm/d	Wind Direction
		Maximum	Minimum					
1	07.29 ± 0.81[a] (5 – 11)	39.86 ± 0.34[c] (38 – 41)	21.00 ± 0.69[a] (19 – 24)	6.64 ± 0.76[ab] (0.9 – 8.6)	0.00 ± 0.00[a] (0.0 – 0.0)	1.40 ± 0.76[a] (0.9 – 1.8)	14.96 ± 0.52[b] (12.2 – 16.3)	SE
2	31.00 ± 7.25[b] (9 – 53)	40. 29 ± 0.36[c] (39 – 41)	23.43 ± 0.81[b] (21 – 26)	5.20 ± 1.20[a] (0.8 – 8.0)	0.00 ± 0.00[a] (0.0 – 0.0)	1.39 ± 0.13[a] (1.0 – 2.0)	11.31 ± 0.69[b] (9.1 – 13.2)	SE
3	53.57 ± 3.42[c] (41 – 67)	38.71 ± 1.34[bc] (32 – 43)	23.86 ± 0.46[b] (22 – 25)	7.69 ± 0.43[ab] (5.5 – 8.6)	0.47 ± 0.47[a] (0.0 – 3.3)	1.93 ± 0.12[b] (1.6 – 2.4)	10.19 ± 0.98[a] (5.6 – 13.6)	NW
4	61.71 ± 4.03[d] (50 – 80)	35.57 ± 1.0[ab] (31 – 38)	22.71 ± 0.78[ab] (20 – 25)	5.77 ± 1.14[ab] (0.0 – 8.2)	3.59 ± 1.85[a] (0.0 – 11.4)	2.28 ± 0.15[b] (1.9 – 3.11)	08.09 ± 1.19[a] (3.6 – 12.2)	NW/SW
5	67.57 ± 2.13[de] (58 – 74)	34.14 ± 1.24[a] (29 – 38)	22.86 ± 0.71[ab] (19 – 24)	5.74 ± 0.98[ab] (0.3 – 8.1)	4.89 ± 3.50[a] (0.0 – 24.0)	1.99 ± 0.22[b] (1.25 – 2.77)	09.74 ± 3.68[a] (4.6 – 31.6)	SW
6	69.29 ± 2.46[de] (60 – 77)	35.43 ± 0.37[ab] (34 – 36)	22.86 ± 0.51[ab] (20 – 24)	7.93 ± 0.61[ab] (5.1 – 9.3)	6.41 ± 6.41[a] (0.0 – 44.9)	2.02 ± 0.16[b] (1.5 – 2.5)	09.14 ± 1.31[a] (6.6 – 15.4)	SW
7	71.00 ± 2.80[de] (64 – 86)	35.86 ± 0.99[ab] (30 – 37)	22.86 ± 0.55[ab] (20 – 24)	8.33 ± 0.41[b] (7.2 – 10.1)	1.86 ± 1.46[a] (0.0 – 10.3)	2.15 ± 0.11[b] (1.8 – 2.7)	08.31 ± 0.46[a] (7.3 – 10.9)	NW/SW
8	73.14 ± 1.88[e] (64 – 80)	35.00 ± 0.69[ab] (33 – 38)	22.57 ± 0.57[ab] (20 – 24)	5.47 ± 1.05[ab] (0.1 – 8.7)	1.13 ± 0.73[a] (0.0 – 4.2)	2.11 ± 0.14[b] (1.5 – 2.8)	06.54 ± 0.83[a] (3.1 – 10.3)	SW
Mean ± SEM	54.32 ± 3.19	36.86 ± 0.84	22.77 ± 0.29	6.60 ± 0.44	2.29 ± 0.85	1.91 ± 0.12	09.79 ± 0.90	

[a, b, c, d, e] = Means in the same column and having different superscript letters are significantly ($P < 0.05$) different; RH = Relative humidity; SE = South-east; SW = South-west; NW = North-west; Data collated from the meteorological unit, Institute of Agricultural Research, Ahmadu Bello University, Zaria; Values in parentheses are Maximum – Minimum

Table 4.2: Meteorological Data within the Marshall Broiler Chickens' Microenvironment during the Period of Transportation (Mean ± SEM)

Day	Location	RH, %	THI, ^{o}C	DBT, ^{o}C
Day 1:	a) Inside the poultry house before loading	72	44.47	28
	b) Inside the vehicle before transport	73	46.89	30
	c) 1 h into the journey inside the vehicle	72	59.16	41
	d) Immediately after transport inside the poultry house	82	60.59	40
	e) 4 h post-transport inside the poultry house	59	50.70	36
Day 2:	Inside the poultry house after transport	62	54.44	39
Day 3:	Inside the poultry house after transport	87	59.31	38
Day 4:	Inside the poultry house after transport	87	59.31	38

RH = Relative humidity; THI = Temperature-humidity index; DBT = Dry-bulb temperature

Table 4.3: Meteorological Data Prevailing outside the Vehicle during the Period of Transportation (Mean ± SEM)

Meteorological Parameters	Day				Mean ± SEM
	1	2	3	4	
Ambient temperature, ^{o}C:					
maximum	42	41	40	40	40.75 ± 0.48
minimum	23	25	21	19	22.00 ± 1.29
range	19	16	20	21	19.00 ± 1.08
Relative humidity, %	66	72	68	65	67.75 ± 1.55
Sunshine duration, h/d	8.3	8.4	7.9	8.6	8.30 ± 0.15
Rainfall, mm	0	0	0	0	0.00 ± 0.00
Wind speed, m/s	1.55	2.31	1.02	1.1	1.50 ± 0.30
Evaporation, mm/d	8.6	11.7	11.7	15.2	11.80 ± 1.35
Wind direction	NW	NW	SE	SE	NA

SE = South-east; NW = North-west; NA = Not applicable. Data collated from the meteorological unit, Institute of Agricultural Research, Ahmadu Bello University, Zaria

4.2 EVALUATION OF PERFORMANCE INDICES

Body weight (BW), BW gain, feed consumption, water intake, feed conversion and mortality from day-old up to d 56 are shown in Tables 4.4 – 4.7. On d 42, BWs of broilers differed significantly ($P < 0.001$) between groups. The mean value of BW of 2420 ± 50 g was obtained in group III administered with melatonin, while in the other two groups the values were less than 2000 g (Table 4.4). Table 4.5 shows that the overall feed consumption was insignificantly different in all the groups. However, group II raised on CL without melatonin supplementation had the highest mean value of 117.63 ± 10.16 /bird. The highest overall amount of water intake was recorded in the melatonin-treated group (Table 4.5). The weekly water intake pattern between the groups showed clearly that all the groups administered with melatonin had the highest values starting from d 14 of age up to d 42. The rate and percentage mortality for all the broiler chicken groups are shown in Tables 4.6 – 4.7. Groups I, II and III had overall percentage mortality of 11, 14 and 3 %, respectively (Fig. 4.3).

Table 4.8 represents the gross margin analysis of the birds belonging to the various groups. A profit of ₦ 667:55 was made per bird on the melatonin-treated group as against ₦ 337:78 and ₦ 447:48 for birds on 12D:12L and 24CL treated groups, respectively. The results of the palatability study are shown in Tables 4.9 and 4.10. The ratings from the seven parameters studied across the groups on the meat (soft tissues) ranged from 5.80 ± 0.48 to 8.05 ± 0.37 (Table 4.9), while those on orthopaedics (bony or osteos tissues) were lower and varied from 5.25 ± 0.81 to 7.25 ± 0.48 across the groups (Table 4.10). The value of 6.90 ± 0.35 obtained under satisfaction in group III birds treated with melatonin, was not significantly ($P > 0.05$) higher than 6.50 ± 0.40, and it was not lower ($P > 0.05$) than 8.05 ± 0.37 obtained from birds in groups II and I, respectively. Bone strength as reflected by its edibility (inversely related)

was highest in group III administered with melatonin, with the value of 7.15 ± 0.42, and lowest in group II raised under CL, without melatonin administration (Table 4.10).

4.3 EFFECT OF METEOROLOGICAL FACTORS

The effect of meteorological factors on performance parameters of the broilers is shown in Tables 4.11 – 4.12. Maximum AT, DBT and evaporation rate were negatively, but significantly correlated with feed consumption and BW of birds in all the groups (Table 4.11 – 4.12). A similar effect was recorded for water intake and feed conversion ratio (FCR) across the groups. An exception was the relationship between evaporation rate and FCR obtained in group I, reared under 12D:12L, which was negative and insignificant ($P > 0.05$; Table 4.13). The relationships between the performance parameters were in general positively and strongly correlated (Table 4.14). Reverse effects were observed in the relationships between RH, DBT, THI and relative carcass weight, shown in Tables 4.17 and 4.18. Figures 4.4 – 4.10 indicate the Gompertz regression curves and associated equations for some of the significantly correlated parameters. The data on carcass characteristics presented in Table 4.15 show significant differences between the groups, especially in carcass, breast plate, thigh and liver weights ($P < 0.001$). The melatonin-treated group consistently ranked highest for each of the key parameters of broiler chicken production. Table 4.16 reveals that the liver weight values of 38.70 ± 2.14 g and 42.40 ± 1.63 g obtained in group III (CL + MEL) and group II (CL), respectively were significantly ($P < 0.05$) higher than 32.90 ± 1.42 g, recorded in group I subjected to 12D:12L cycle. Also, the highest ($P < 0.05$) dry feather weight of 37.10 ± 1.46 was recorded in group III reared on CL and administered with melatonin (Table 4.16).

Table 4.4: Mean Weekly Live Weights and Gain in Weights of Marshall Broiler Chickens administered with Melatonin (Mean ± SEM; n = 15)

Age in days	Live weights (g/bird)			Gain in weights (g/bird)		
	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
Day old	40.07 ± 0.36	40.13 ± 0.35	40.00 ± 0.38	NA	NA	NA
7	119.27 ± 0.42	119.33 ± 0.40	120.27 ± 0.37	79.20 ± 0.53	79.2 ± 0.45	80.27 ± 0.37
14	219.53 ± 0.38^{a}	319.60 ± 0.38^{b}	360.20 ± 0.38^{c}	100.27 ± 0.54^{a}	200.27 ± 0.48^{b}	239.93 ± 0.36^{c}
21	499.47 ± 0.62^{a}	600.13 ± 1.78^{b}	651.33 ± 1.56^{c}	279.93 ± 0.80^{a}	280.53 ± 1.79^{a}	291.13 ± 1.52^{b}
28	1150.00 ± 03.00^{a}	1200.00 ± 3.00^{b}	1300.00 ± 6.00^{c}	650.53 ± 3.47^{b}	599.87 ± 3.05^{a}	648.67 ± 5.79^{b}
35	1300.00 ± 40.00^{a}	1400.00 ± 20.00^{b}	1820.00 ± 30.00^{c}	150.00 ± 36.67^{a}	200.00 ± 18.36^{a}	520.00 ± 27.15^{b}
42	1470.00 ± 30.00^{a}	1907.00 ± 38.00^{b}	2420.00 ± 50.00^{c}	173.33 ± 40.79^{a}	506.67 ± 41.27^{b}	600.00 ± 49.45^{b}
49	1800.00 ± 20.00^{a}	2200.00 ± 30.00^{b}	2600.00 ± 30.00^{c}	326.67 ± 44.15	293.33 ± 60.53	180.00 ± 50.90
56	2000.00 ± 30.00^{a}	2500.00 ± 20.00^{b}	2800.00 ± 30.00^{c}	200.00 ± 42.54	300.00 ± 47.81	200.00 ± 36.52

$^{a, b, c}$ = Means in the same row having different superscript letters are significantly ($P < 0.05$) different;
12D:12L (12D:12L cycle) = Group I; CL (Continuous lighting) = Group II; and CL + MEL (CL + Melatonin administration) = Group III; NA = Not applicable

Table 4.5: Mean Weekly Feed Consumption and Water Intake of Marshall Broiler Chickens administered with Melatonin (Mean ± SEM; n = 15)

Age in weeks	Feed consumption (g/bird)			Water intake (g/bird)		
	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
I	12.86 ± 0.77[a]	15.29 ± 0.52[b]	14.14 ± 0.60[ab]	32.14 ± 4.66	35.00 ± 4.71	31.14 ± 4.69
II	22.43 ± 0.65[a]	25.14 ± 0.51[b]	24.86 ± 0.51[b]	84.14 ± 8.72	86.00 ± 8.78	93.00 ± 9.52
III	41.14 ± 0.46[a]	47.86 ± 0.83[c]	45.29 ± 0.36[b]	142.57 ± 10.99	154.00 ± 15.12	163.86 ± 12.99
IV	95.29 ± 05.61	104.00 ± 6.36	98.43 ± 5.55	239.00 ± 20.93	263.86 ± 24.02	272.14 ± 25.99
V	126.36 ± 1.59[a]	164.51 ± 5.75[c]	141.14 ± 1.39[b]	295.57 ± 8.83	302.14 ± 13.89	303.57 ± 10.32
VI	154.14 ± 2.76[a]	186.78 ± 1.57[c]	164.14 ± 1.52[b]	345.71 ± 5.19[a]	389.86 ± 6.35[b]	391.86 ± 1.90[b]
VII	146.17 ± 4.39[a]	190.71 ± 5.73[b]	163.15 ± 5.37[a]	442.43 ± 8.57	437.57 ± 7.69	439.71 ± 3.85
VIII	157.87 ± 1.45[a]	206.77 ± 7.82[b]	174.06 ± 6.93[a]	518.86 ± 9.65	532.86 ± 17.56	518.43 ± 14.61
Mean ± SEM	94.53 ± 7.74	117.63 ± 10.16	103.15 ± 8.51	262.55 ± 21.92	275.16 ± 22.56	276.71 ± 22.03

[a, b, c] = Means in the same row having different superscript letters are significantly ($P < 0.05$) different;
12D:12L (12D:12L cycle) = Group I; CL (Continuous lighting) = Group II; and CL + MEL (CL + Melatonin administration) = Group III

Table 4.6: Mean Weekly Feed Conversion Ratio and Mortality Rate of Marshall Broiler Chickens administered with Melatonin (Mean ± SEM; n = 15)

Age in weeks	Feed conversion ratio (g:g)			Mortality rate		
	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
I	1.14 ± 0.07[a]	1.35 ± 0.05[b]	1.23 ± 0.05[ab]	-	-	-
II	1.57 ± 0.05[c]	0.88 ± 0.02[b]	0.73 ± 0.12[a]	-		
III	1.03 ± 0.01[a]	1.19 ± 0.02[c]	1.09 ± 0.01[b]	-	-	-
IV	1.03 ± 0.06	1.21 ± 0.07	1.06 ± 0.06	0.14 ± 0.14	0.14 ± 0.14	-
V	5.91 ± 0.07[b]	5.75 ± 0.20[b]	1.90 ± 0.02[a]	0.29 ± 0.18	0.29 ± 0.18	-
VI	6.22 ± 0.11[c]	2.58 ± 0.02[b]	1.92 ± 0.02[a]	0.29 ± 0.18	0.43 ± 0.20	0.14 ± 0.14
VII	3.49 ± 0.11[a]	4.55 ± 0.14[b]	6.35 ± 0.21[c]	0.43 ± 0.20	0.57 ± 0.20	0.14 ± 0.14
VIII	5.52 ± 0.05[b]	4.82 ± 0.18[a]	6.09 ± 0.24[b]	0.43 ± 0.20	0.57 ± 0.20	0.14 ± 0.14
Mean ± SEM	3.24 ± 0.30	2.79 ± 0.25	2.55 ± 0.29	0.20 ± 0.05[b]	0.25 ± 0.06[b]	0.05 ± 0.03[a]

[a, b, c] = Means in the same row having different superscript letters are significantly ($P < 0.05$) different;
12D:12L (12D:12L cycle) = Group I; CL (Continuous lighting) = Group II; and CL + MEL (CL + Melatonin administration) = Group III

Table 4.7: Percentage Weekly Weight Gain and Mortality of Marshall Broiler Chickens administered with Melatonin (n = 100)

Age in weeks	Percentage weight gain (%)			Percentage mortality (%)		
	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
I	197.65	197.36	200.68	-	-	-
II	84.06	167.83	199.49	-	-	-
III	127.52	87.78	80.83	-	-	-
IV	130.24	99.96	99.59	1.00	1.00	-
V	13.08	36.21	32.97	2.02	2.02	-
VI	22.45	15.37	7.44	2.06	3.09	1
VII	11.11	13.64	7.69	3.16	4.26	1.01
VIII	4891.27	6129.75	6900	3.26	4.44	1.02
Mean ± SEM	684.67 ± 601.40	843.49 ± 755.56	941.09 ± 851.71	1.44 ± 0.49	1.85 ± 0.67	0.38 ± 0.18

12D:12L (12D:12L cycle) = Group I; CL (Continuous lighting) = Group II; and CL + MEL (CL + Melatonin administration) = Group III

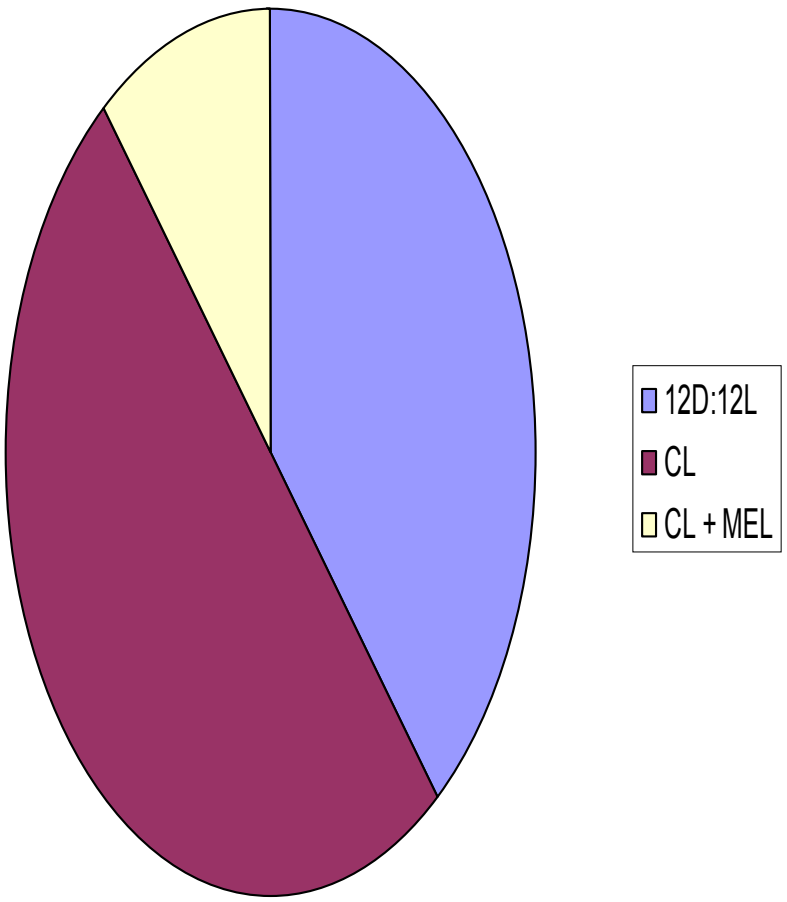


Figure 4.3: Percentage Overall Mortality of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season;
12D:12L (12D:12L cycle) = Group I (11 %); CL (Continuous lighting) = Group II (14 %); and CL + MEL (CL + Melatonin administration) = Group III (3 %)

Table 4.8: Gross Margin of Marshall Broiler Chickens administered with Melatonin from 0 – 42 d of Life

Item	Groups		
	I (12D:12L)	II (CL)	III (CL + MEL)
1. Cost/kg of Feed (Vital Feeds UAC Nig. Plc; ₦)	77.04	77.04	77.04
2. Feed intake/bird (kg)	3.17	3.81	3.42
3. Cost of feed consumed/bird (Product of items 1. and 2; ₦)	244.22	293.52	263.48
4. Cost/kg of dressed carcass (Mr. Biggs UAC Nig. Plc; ₦)	600	600	600
5. Dressed carcass/bird (kg)	0.97	1.41	1.92
6. Selling price/bird (Product of items 4. and 5; ₦)	582	846	1152
7. Cost of light supply during night time (₦)	0	105	105
8. Cost of melatonin administered/bird (₦)	0	0	115.97
9. Profit (Items 6. minus items 3, 7. and 8; ₦)	337:78	447:48	667:55

Note: All other costs were assumed constant; 12D:12L (12D:12L cycle) = Group I; CL (Continuous lighting) = Group II; and CL + MEL (CL + Melatonin administration) = Group III

Table 4.9: Palatability Attributes or Ratings of the Soft Tissues of Marshall Broiler Chickens (n = 10)

Groups	Sweetness	Softness	Acceptability	Satisfaction	Flavour retention	Juiciness	Structure/Smoothness (Amount of perceived connective tissue)
I (12D:12L)	7.15 ± 0.33	7.40 ± 0.34	7.10 ± 0.32	8.05 ± 0.37^{b}	7.10 ± 0.35^{b}	6.05 ± 0.33	6.50 ± 0.31
II (CL)	6.15 ± 0.37	6.75 ± 0.48	6.30 ± 0.34	6.50 ± 0.40^{a}	5.80 ± 0.48^{a}	5.95 ± 0.40	6.60 ± 0.34
III (CL + MEL)	6.95 ± 0.40	7.30 ± 0.45	6.50 ± 0.40	6.90 ± 0.35^{ab}	6.78 ± 0.49^{a}	5.90 ± 0.85	6.60 ± 0.54

$^{a, b, c}$ = Means for the same column having different superscript letters are significantly ($P < 0.05$) different;
12D:12L (12D:12L cycle) = Group I; CL (Continuous lighting) = Group II; and CL + MEL (CL + Melatonin administration) = Group III

Table 4.10: Palatability Attributes or Ratings of the Orthopaedic Tissues (Bone) of Marshall Broiler Chickens (n =10)

Groups	Bone marrow quantity	Bone marrow delicacy	Bone edibility
I (12D:12L)	5.25 ± 0.81	6.80 ± 0.57	7.20 ± 0.36
II (CL)	6.50 ± 0.64	6.20 ± 0.47	7.25 ± 0.43
III (CL + MEL)	6.25 ± 0.65	6.75 ± 0.47	7.15 ± 0.42

12D:12L (12D:12L cycle) = Group I; CL (Continuous lighting) = Group II; and CL + MEL (CL + Melatonin administration) = Group III

Table 4.11: The Relationships between Meteorological Paramaters, Body Weight and Live Weight Gain in Marshall Broiler Chickens (n = 15)

Meteorological parameters	Correlation with body weight			Correlation with live weight gain		
	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
Weeks	0.977**	0.990**	0.984**	0.155^{NS}	0.316**	0.159^{NS}
Relative humidity	0.221*	0.200*	0.179^{NS}	0.204*	0.094^{NS}	0.072^{NS}
Dry-bulb temperature	-0.235**	-0.196*	-0.188*	-0.268**	-0.273**	-0.348**
Temperature-humidity index	-0.117^{NS}	-0.086^{NS}	-0.087^{NS}	-0.186*	-0.239**	-0.335**
Maximum temperature	-0.656**	-0.606**	-0.612**	-0.257^{NS}	-0.353**	-0.334*
Minimum temperature	0.064^{NS}	0.045^{NS}	0.063^{NS}	0.117^{NS}	0.068^{NS}	0.175^{NS}
Sunshine duration	0.070^{NS}	0.087^{NS}	0.095^{NS}	-0.025^{NS}	-0.008^{NS}	-0.072^{NS}
Wind speed	0.531**	0.495**	0.462**	0.425**	0.352**	0.193^{NS}
Rainfall	0.133^{NS}	0.143^{NS}	0.137^{NS}	-0.078^{NS}	0.073^{NS}	-0.089^{NS}
Evaporation	-0.473**	-0.447**	-0.455**	-0.287*	-0.296*	-0.308*

^{NS} = Non-significant correlation (P > 0.05), * = P < 0.05, ** = P < 0.01, *** = P < 0.001;
12D:12L = 12 h darkness and 12 h light cycle; CL = Continuous lighting; and CL + MEL = CL + Melatonin

Table 4.12: The Relationships between Meteorological, Feed Consumption and Water Intake Parameters of Marshall Broiler Chickens (n = 15)

Meteorological parameters	Correlation with feed consumption			Correlation with water intake		
	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
Weeks	0.952**	0.958**	0.955**	0.984**	0.976**	0.978**
Relative humidity	0.333*	0.300*	0.315*	0.325*	0.344**	0.356**
Dry-bulb temperature	-0.633**	-0.597**	-0.615**	-0.569**	-0.581**	-0.598**
Temperature-humidity index	-0.613**	-0.581**	-0.597**	-0.527**	-0.528**	-0.545**
Maximum temperature	-0.672**	-0.655**	-0.653**	-0.556**	-0.587**	-0.575**
Minimum temperature	-0.026^{NS}	0.035^{NS}	0.048^{NS}	0.072^{NS}	0.073^{NS}	0.086^{NS}
Sunshine duration	-0.058^{NS}	0.065^{NS}	0.080^{NS}	0.068^{NS}	0.087^{NS}	0.078^{NS}
Wind speed	0.476**	0.457**	0.477**	0.468**	0.474**	0.486**
Rainfall	0.219^{NS}	0.190^{NS}	0.192^{NS}	0.157^{NS}	0.169^{NS}	0.168^{NS}
Evaporation	-0.432**	-0.437**	-0.438**	-0.439**	-0.455**	-0.468**

^{NS} = Non-significant correlation (P > 0.05), * = P < 0.05, ** = P < 0.01, *** = P < 0.001;
12D:12L = 12 h darkness and 12 h light cycle; CL = Continuous lighting; and CL + MEL = CL + Melatonin

Table 4.13: The Relationships between Meteorological Parameters, Feed Conversion Ratio and Mortality Rate of Marshall Broiler Chickens (n =15)

Meteorological parameters	Correlation with feed conversion ratio			Correlation with mortality rate		
	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
Weeks	0.755**	0.757**	0.821**	0.422**	0.522**	0.232^{NS}
Relative humidity	0.164^{NS}	0.202^{NS}	0.049^{NS}	0.127^{NS}	0.056^{NS}	0.078^{NS}
Dry-bulb temperature	-0.390**	-0.414**	-0.233^{NS}	-0.251^{NS}	-0.249^{NS}	-0.152^{NS}
Temperature-humidity index	-0.397**	-0.403**	-0.239^{NS}	-0.249^{NS}	-0.283*	-0.155^{NS}
Maximum temperature	-0.548**	-0.568**	-0.355**	-0.326*	-0.320*	-0.309*
Minimum temperature	-0.003^{NS}	-0.017^{NS}	0.029^{NS}	-0.088^{NS}	0.123^{NS}	-0.172^{NS}
Sunshine duration	-0.002^{NS}	-0.006^{NS}	0.118^{NS}	-0.121^{NS}	0.119^{NS}	-0.178^{NS}
Wind speed	0.237^{NS}	0.282*	0.294*	-0.153^{NS}	0.119^{NS}	0.172^{NS}
Rainfall	0.236^{NS}	0.121^{NS}	-0.016^{NS}	-0.092^{NS}	0.165^{NS}	0.118^{NS}
Evaporation	-0.258^{NS}	-0.289*	-0.312*	-0.064^{NS}	-0.303*	0.114^{NS}

^{NS} = Non-significant correlation (P > 0.05), * = P < 0.05, ** = P < 0.01, *** = P < 0.001;
12D:12L = 12 h darkness and 12 h light cycle; CL = Continuous lighting; and CL + MEL = CL + Melatonin

Table 4.14: The Relationships between Performance Parameters of Marshall Broiler Chickens (n = 15)

Correlated parameters	12D:12L cycle	CL	CL + MEL
Body weight and live weight gain	0.288**	0.389**	0.243**
Body weight and feed intake	0.971**	0.960**	0.973**
Body weight and water intake	0.960**	0.974**	0.968**
Body weight and feed conversion ratio	0.751**	0.739**	0.785**
Body weight and mortality	0.443**	0.492**	0.261^{NS}
Feed consumption and water intake	0.936**	0.953**	0.956**
Mortality and water intake	0.453**	0.508**	0.222^{NS}
Feed consumption and live weight gain	0.127^{NS}	0.290*	0.309*
Water intake and live weight gain	0.093^{NS}	0.309*	0.234^{NS}
Feed consumption and feed conversion ratio	0.831**	0.820**	0.716**
Water intake and feed conversion ratio	0.725**	0.730**	0.788**
Feed consumption and mortality	0.441**	0.533**	0.249^{NS}
Feed conversion ratio and live weight gain	-0.251^{NS}	-0.109^{NS}	-0.277*
Feed conversion ratio and mortality	0.365**	0.441**	0.143^{NS}

^{NS} = Non-significant correlation ($P > 0.05$), * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; and CL + MEL = CL + Melatonin

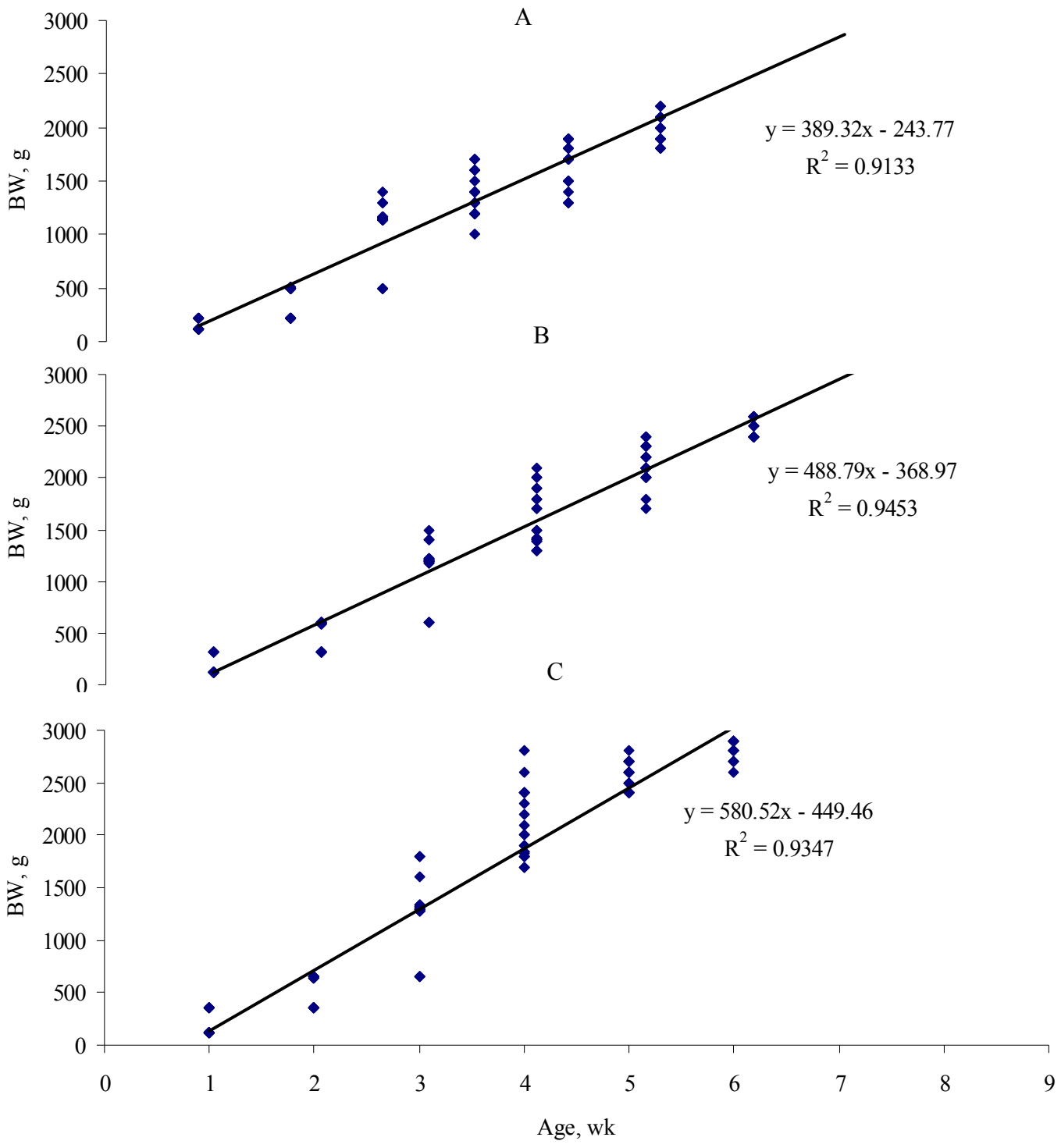


Figure 4.4: Relationships between Body Weight and Age of Marshall Broiler Chickens;
A = 12 h darkness and 12 h light cycle group; B = Continuous light (CL) group; and C = CL + Melatonin group; BW = Body weight (n = 15)

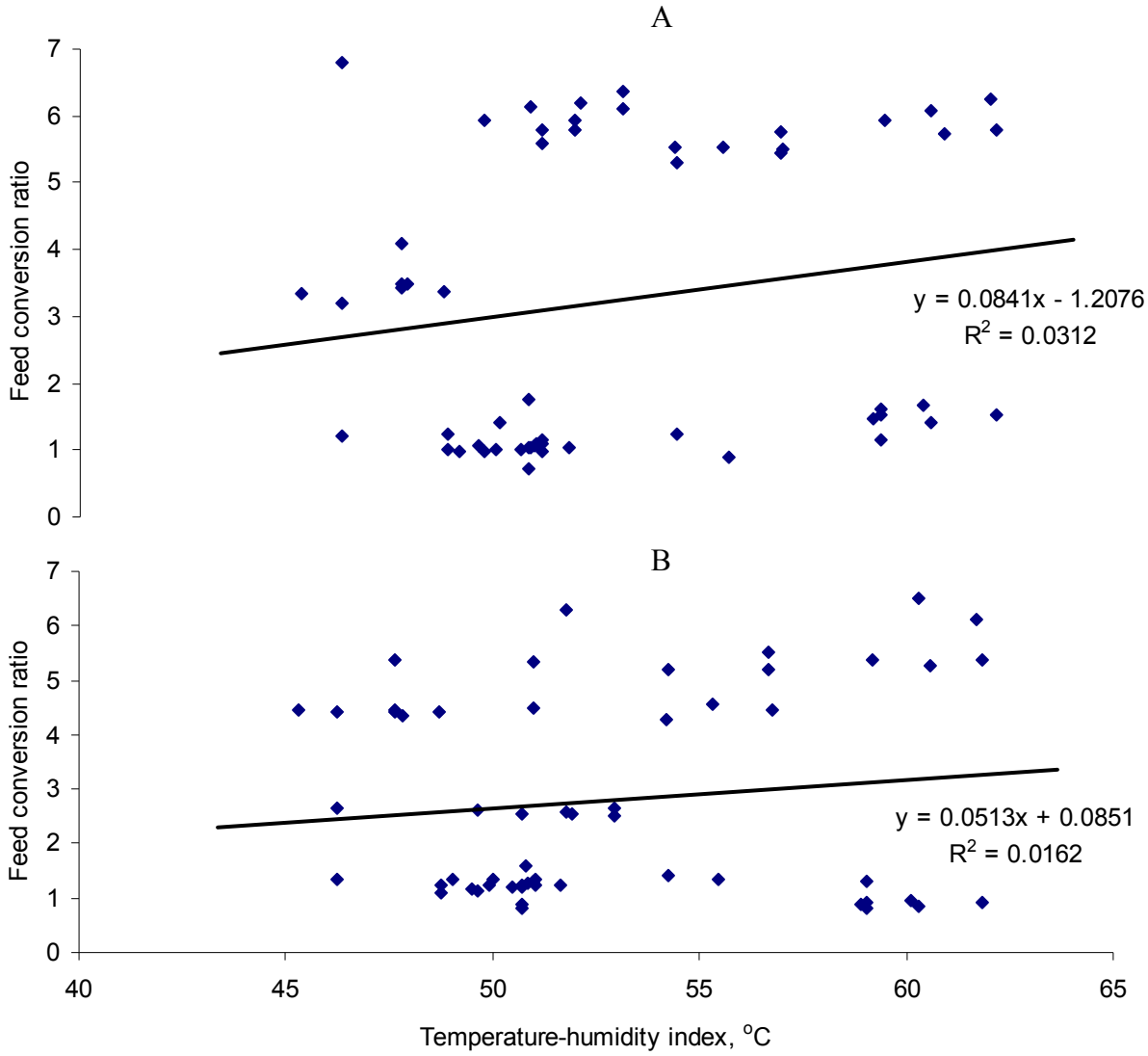


Figure 4.5: Relationships between Feed Conversion Ratio and Temperature-humidity Index of Marshall Broiler Chickens reared on 12 h darkness and 12 h light cycle (A) and Continuous lighting (B) (n = 15)

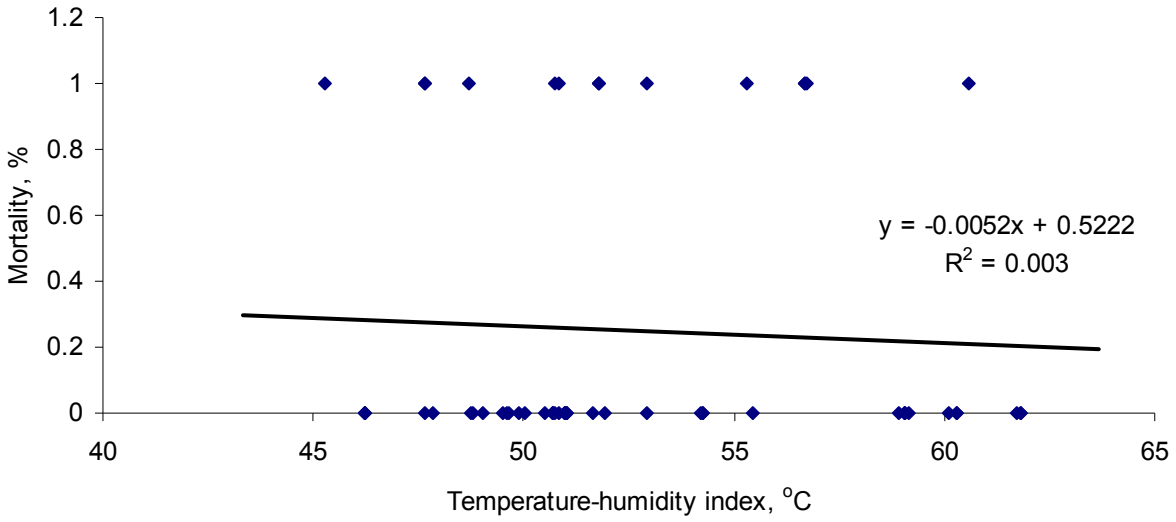


Figure 4.6: Relationships between Percentage Mortality and Temperature-humidity Index of Marshall Broiler Chickens reared on Continuous Lighting (n = 100)

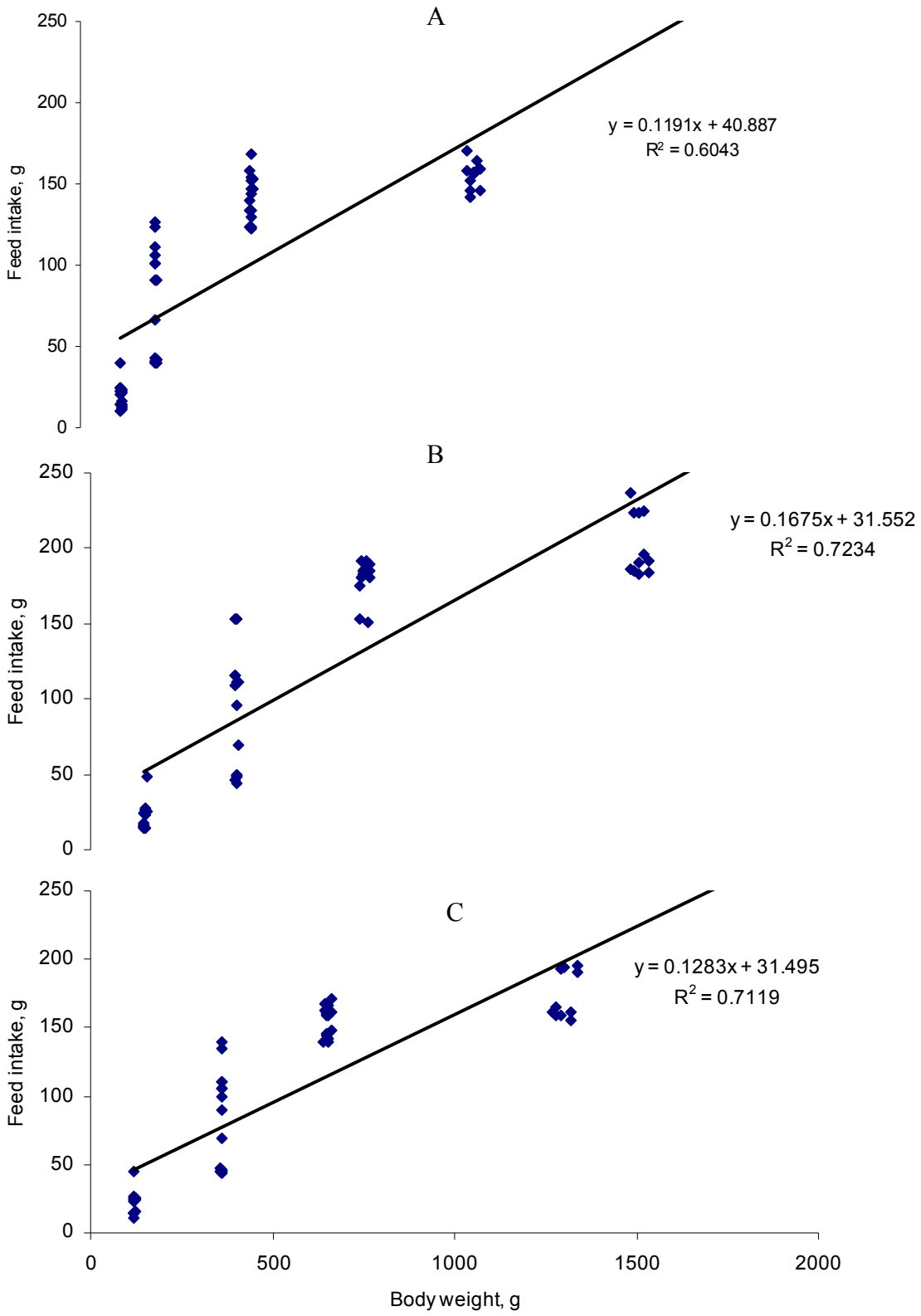


Figure 4.7: Relationships between Feed Intake and Body Weight of Marshall Broiler Chickens administered with Melatonin; A = 12 h darkness and 12 h light cycle group; B = Continuous lighting (CL) group; and C = CL + Melatonin group (n = 15)

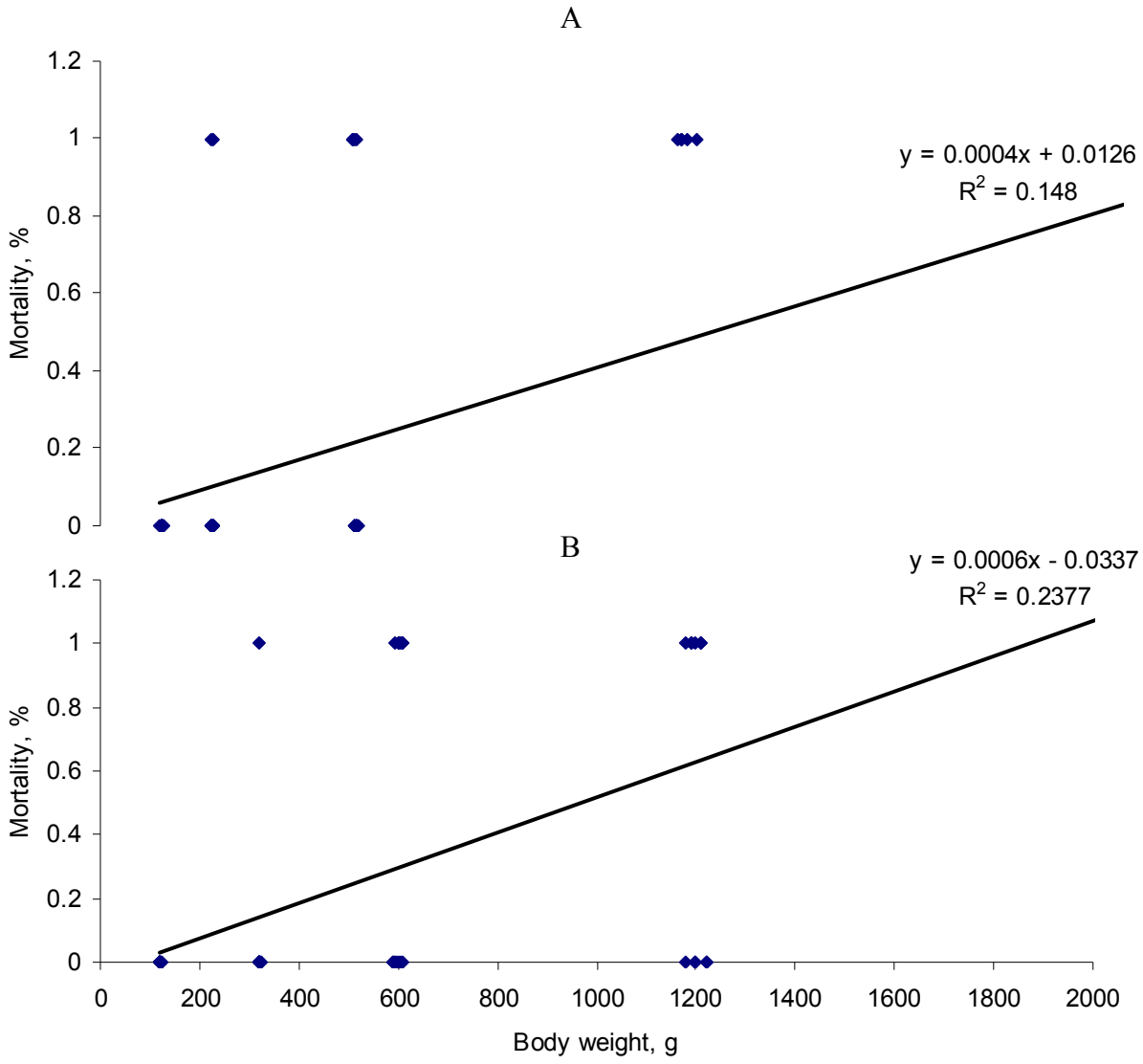


Figure 4.8: Relationships between Percentage Mortality and Body Weight of Marshall Broiler Chickens reared on 12 h darkness and 12 h light cycle (A), and Continuous lighting (B) (n = 100)

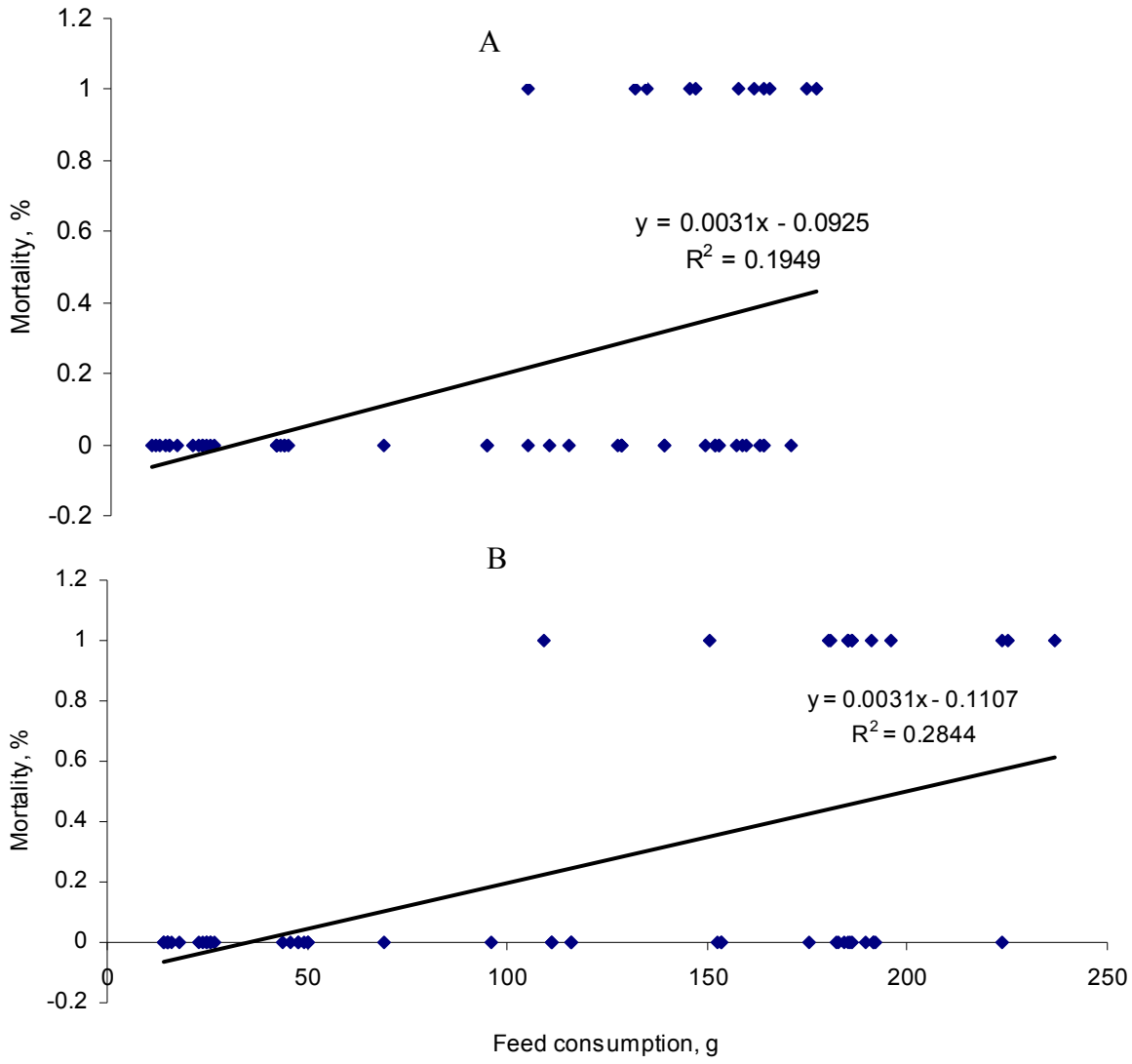


Figure 4.9: Relationships between Percentage Mortality and Feed Consumption of Marshall Broiler Chickens reared on 12 h darkness and 12 h light cycle (A), and Continuous lighting (B) (n = 100)

Table 4.15: Meat and Carcass Quality Parameters or Processing Yields (g) of Marshall Broiler Chickens administered with Melatonin at 42 d of Age

Parameter	Group		
	12D:12L	CL	CL+MEL
Live	1740.00 ± 48.00[a]	1970.00 ± 33.50[b]	2430.00 ± 68.40[c]
Carcass	1390.00 ± 18.00[a]	1740.00 ± 34.00[b]	1860.00 ± 61.80[b]
Breast	259.00 ± 10.53[a]	339.70 ± 16.43[b]	364.30 ± 13.46[b]
Thighs	306.50 ± 13.35[a]	350.30 ± 10.06[b]	368.10 ± 6.35[b]
Wings	126.30 ± 3.75[a]	138.80 ± 5.92[ab]	141.80 ± 5.11[b]
Neck	66.50 ± 3.52[a]	73.40 ± 2.98[a]	71.20 ± 4.40[a]
Legs	67.60 ± 3.41[a]	72.90 ± 2.82[a]	70.70 ± 2.68[a]
Head	34.30 ± 1.71[a]	37.40 ± 1.75[a]	38.80 ± 1.51[a]

[a, b, c] = Means in the same row and having different superscript letters are significantly ($P < 0.05$) different;
12D:12L = 12 h darkness and 12 h light cycle (Group I); CL = Continuous lighting (Group II); and CL + MEL = CL + Melatonin (Group III) (n = 15)

Table 4.16: Organ Processing Yield or Weight (g) of Marshall Broiler Chickens administered with Melatonin at 42 d of age.

	Organ weight					Feather	
Group	Heart	Liver	Gizzard	Brain	Intestine	Wet	Dry
I	17.10 ± 0.71[a]	32.90 ± 1.42[a]	66.60 ± 1.21[a]	2.54 ± 0.12[a]	152.90 ± 14.81[a]	95.60 ± 5.74[a]	31.20 ± 1.53[a]
II	19.10 ± 0.71[a]	42.40 ± 1.63[b]	72.30 ± 4.02[a]	2.52 ± 0.11[a]	146.30 ± 16.11[a]	125.50 ± 7.20[b]	36.40 ± 1.22[b]
III	17.40 ± 1.41[a]	38.70 ± 2.14[b]	74.00 ± 9.73[a]	2.37 ± 0.10[a]	121.40 ± 11.05[a]	121.10 ± 5.24[b]	37.10 ± 1.46[b]

[a, b] = Means in the same column having different superscripts are significantly different ($P < 0.05$). Group I = 12 h darkness and 12 h light cycle; Group II = Continuous lighting; and Group III = CL + Melatonin (n = 15)

Table 4.17: Relationships between Dry-bulb Temperature, Relative Humidity and Relative Carcass Weight of Marshall Broiler Chickens administered with Melatonin

	Dry-bulb temperature, ^{o}C			Relative humidity, %		
Relative carcass parameters, g	12D:12L	CL	CL + MEL	12D:12L	CL	CL + MEL
Live body weight	0.466^{NS}	0.634*	0.283^{NS}	0.180^{NS}	-0.311^{NS}	-0.240^{NS}
Carcass weight	0.539^{NS}	-0.101^{NS}	0.475^{NS}	0.180^{NS}	0.238^{NS}	0.236^{NS}
Breast plate	0.285^{NS}	0.328^{NS}	0.361^{NS}	0.126^{NS}	-0.040^{NS}	0.215^{NS}
Tighs	0.389^{NS}	0.209^{NS}	0.248^{NS}	0.261^{NS}	0.378^{NS}	0.277^{NS}
Wings	0.397^{NS}	0.009^{NS}	-0.253^{NS}	0.584^{NS}	-0.111^{NS}	0.205^{NS}
Head	0.824**	-0.123^{NS}	0.056^{NS}	0.377^{NS}	0.213^{NS}	-0.182^{NS}
Neck	0.186^{NS}	-0.340^{NS}	0.208^{NS}	0.377^{NS}	0.358^{NS}	0.513^{NS}
Intestine	0.795**	-0.280^{NS}	-0.250^{NS}	0.321^{NS}	-0.189^{NS}	-0.322^{NS}
Wet feather	0.774**	0.414^{NS}	-0.506^{NS}	-0.053^{NS}	0.179^{NS}	0.361^{NS}
Dry feather	0.403^{NS}	0.411^{NS}	-0.577^{NS}	-0.284^{NS}	0.121^{NS}	0.351^{NS}
Legs	0.594^{NS}	-0.320^{NS}	-0.049^{NS}	0.440^{NS}	0.071^{NS}	0.444^{NS}
Heart	0.636*	-0.535^{NS}	0.346^{NS}	0.079^{NS}	0.046^{NS}	0.427^{NS}
Liver	0.509^{NS}	0.270^{NS}	-0.098^{NS}	0.397^{NS}	-0.432^{NS}	-0.351^{NS}
Gizzard	0.178^{NS}	-0.321^{NS}	0.608^{NS}	0.055^{NS}	0.224^{NS}	0.424^{NS}
Brain	-0.278^{NS}	0.343^{NS}	0.095^{NS}	-0.043^{NS}	-0.169^{NS}	0.133^{NS}

^{NS} = Non-significant correlation (P>0.05), * = P<0.05, ** = P<0.01, *** = P<0.001. 12D:12L = 12 h darkness and 12 h light cycle (Group I); CL = Continuous lighting (Group II) and CL + MEL = CL + Melatonin (Group III) (n = 15)

Table 4.18: Relationships between Temperature-humidity Index and Relative Carcass Weight of Marshall Broiler Chickens administered with Melatonin (n = 15)

Relative carcass parameters, g	Temperature-humidity index, ^{o}C		
	12D:12L	CL	CL + MEL
Live body weight	0.424^{NS}	0.113^{NS}	-0.033^{NS}
Carcass weight	0.471^{NS}	0.129^{NS}	0.428^{NS}
Breast plate	0.281^{NS}	0.155^{NS}	0.371^{NS}
Tighs	0.447^{NS}	0.407^{NS}	0.388^{NS}
Wings	0.695**	-0.080^{NS}	0.027^{NS}
Head	0.783**	0.245^{NS}	0.416^{NS}
Neck	0.415^{NS}	-0.006^{NS}	-0.041^{NS}
Intestine	0.708**	-0.316^{NS}	-0.399^{NS}
Wet feather	0.413^{NS}	0.384^{NS}	0.594^{*}
Dry feather	0.024^{NS}	0.346^{NS}	0.600^{**}
Legs	0.688**	-0.110^{NS}	0.305^{NS}
Heart	0.443^{NS}	-0.245^{NS}	0.540^{*}
Liver	0.615^{**}	-0.173^{NS}	-0.338^{NS}
Gizzard	0.129^{NS}	0.026^{NS}	0.705**
Brain	-0.187^{NS}	0.054^{NS}	0.186^{NS}

^{NS} = Non-significant correlation (P > 0.05), * = P < 0.05, ** = P < 0.01, *** = P < 0.001;
12D:12L = 12 h darkness and 12 h light cycle (Group I); CL = Continuous lighting (Group II) and CL + MEL = CL + Melatonin (Group III)

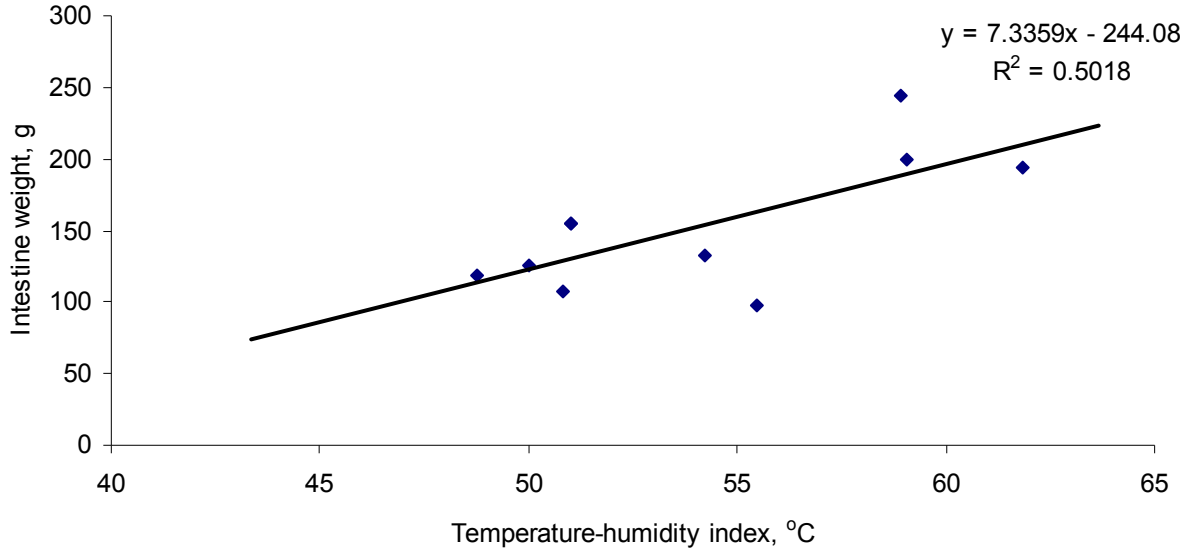


Figure 4.10: Relationships between Intestine Weight and Temperature-humidity Index of Marshall Broiler Chickens reared on 12 h Darkness and 12 h Light Cycle

4.4 BASE-LINE STUDIES ON THE AVIAN BLOOD

The results on haematology obtained using the electronic cell counters are shown in Table 4.19. The total RBC counts of 2.43 and 2.42 x 10^6/µL recorded from groups II and III, respectively were higher than the values of 2.29 x 10^6/µL, obtained in group I. The PCV, Hb, thrombocyte and total WBC values followed the pattern of the RBC count observed in all the groups. On the contrary, the MCV values of 135.5 fl obtained in group III birds was lower than that of groups I and II, with the values of 138.9 fl and 140.3 fl, respectively. Also, in a similar pattern, the MCH and MCHC values of 43.4 pg and 32 g/dl, respectively, recorded in group III broiler chickens were lowest, compared to the values obtained in birds from groups I and II (Table 4.19).

The leucocyte count in the avian species was obtained using manual techniques because the presence of nucleated erythrocytes and thrombocytes interfered with the counting of leucocytes using electronic cell counters (Table 4.19). The results of the various leucocyte components found in the present study are shown in Plates I – V. The following results were obtained after the counting of 100 leucocytes on a Leishman-stained slide:

Lymphocytes = 74; Heterophils = 18; Eosinophils = 4; Basophils = 2 and Monocytes = 2.

$$\text{Hence, TWBC/mm}^3 = \frac{\text{number of cells counted x 10 x 32 x 100}}{(\%\ \text{H} + \text{E}) \times 18}$$

$$= \frac{\text{number of cells counted x 1777.8}}{(\%\ \text{H} + \text{E})}$$

Therefore, TWBC = 8.1 x 10^3 /mm^3 or 8.1 x 10^9 /L.

The preliminary results obtained in the laboratory using the conventional methods to stain blood samples collected from groups II (CL; Control) and III (CL + Melatonin) are presented in Table 4.20. The PCV value of 39.00 ± 1.14 % obtained in the control group was higher ($P < 0.05$) than that of 36.79 ± 0.64 %, recorded in the melatonin-treated group. The range value of the monocyte recorded in the control group doubled that of the experimental group (Table 4.20). The eosinophil values of 8.79 ± 0.1 and 8.14 ± 1.2 $x10^3/\mu L$ obtained from the control and experimental group, respectively were not significantly ($P > 0.05$) different. The TWBC count of 6.36 ± 0.96 x 10^9 /L obtained in the experimental group was greater ($P < 0.05$) than the value of 5.407 ± 1.01 x 10^9 /L recorded from the control group. The fragiligrammes showed that maximum haemolysis for birds in groups I and II that were not administered with melatonin occurred at 0.1 % NaCl, but for melatonin–treated broiler chickens, it occurred at 0.3 % NaCl (Fig. 4.11).

Table 4.19: Haematological Values from the Base-line Studies on Marshall Broiler Chickens administered with Melatonin using Electronic Cell Counter

Parameters	Groups		
	I (12D:12L cycle)	II (CL)	III (CL + MEL)
RBC x $10^6/\mu L$	2.29	2.43	2.42
PCV, %	31.8	34.1	32.8
Hb, g/dl	10.3	11.1	10.5
MCV, fl	138.9	140.3	135.5
MCH, pg	45	45.7	43.4
MCHC, g/dl	32	32.6	32
Thrombocytes, x $10^3/\mu L$	3	10	9
WBC, x $10^3/\mu L$	21.9	22.9	22.6
Lymphocytes, x $10^3/\mu L$	95.9	-	-
Heterophils, x $10^3/\mu L$	-	-	-
Monocytes, x $10^3/\mu L$	-	-	-
Eosinophils, x $10^3/\mu L$	-	-	-
Basophils, x $10^3/\mu L$	-	-	-

RBC = Red blood cell count; PCV = Pack cell volume; Hb = Haemoglobin concentration; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; WBC = White blood cell count; 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + MEL = CL + Melatonin

Table 4.20: Haematological Values from the Base-line Studies on Marshall Broiler Chickens administered with Melatonin using the Conventional Staining Methods (n = 14)

Parameter	Group	Mean ± SEM	Maximum	Minimum	Range
Packed Cell Volume	Melatonin	36.79 ± 0.64[a]	40	33	07
	Control	39.00 ± 1.14[b]	46	33	13
Heterophil	Melatonin	11.79 ± 1.01[a]	19	5	14
	Control	09.57 ± 0.66[a]	14	5	9
Lymphocyte	Melatonin	79.86 ± 1.20[a]	85	71	14
	Control	81.36 ± 1.32[a]	90	71	19
Monocyte	Melatonin	00.14 ± 0.10[a]	1	0	1
	Control	00.36 ± 0.20[a]	2	0	2
Basophil	Melatonin	00.07 ± 0.07[a]	1	0	1
	Control	00.00 ± 0.00[a]	0	0	0
Eosinophil	Melatonin	08.14 ± 1.20[a]	18	2	16
	Control	08.79 ± 0.10[a]	19	4	15
Heterophil:Lymphocyte	Melatonin	0.15 ± 0.01[a]	0.25	0.06	0.19
	Control	0.12 ± 0.01[a]	0.18	0.06	0.12
Total leucocyte Count	Melatonin	6.36 ± 1.00[a]	9.9	1.1	8.8
	Control	5.41 ± 1.01[b]	9.4	1.1	8.3

[a, b] = Values with different superscript letters are significantly ($P < 0.05$) different

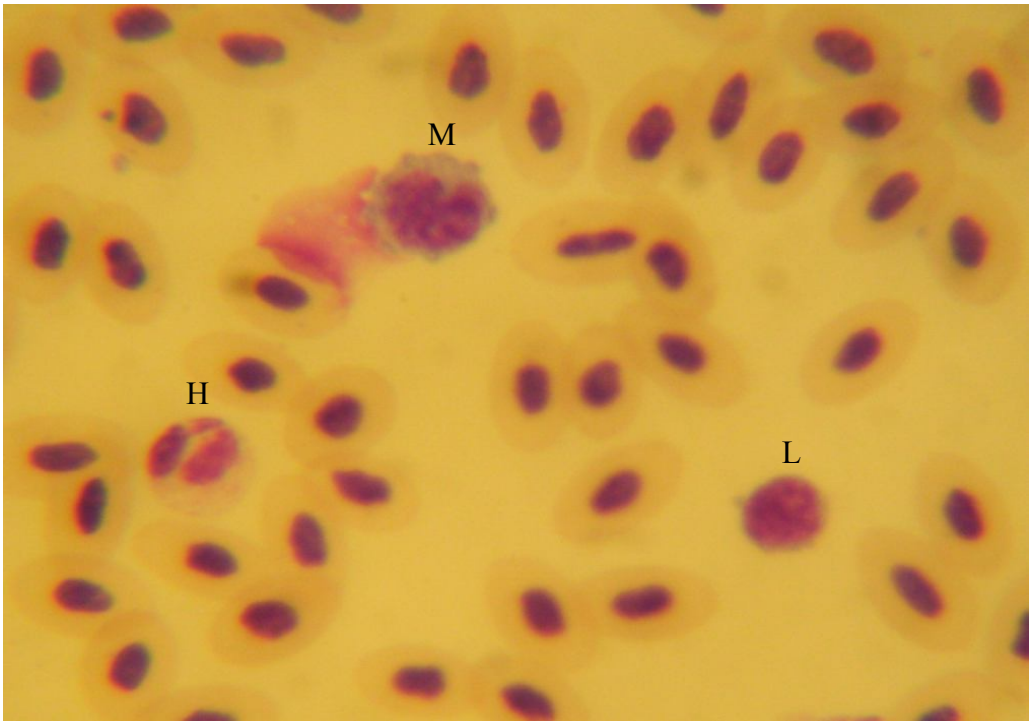


Plate I: Blood Smear from a Broiler Chicken on a Giemsa Stain (x 100); H = Heterophil; L = Lymphocyte; M = Monocyte

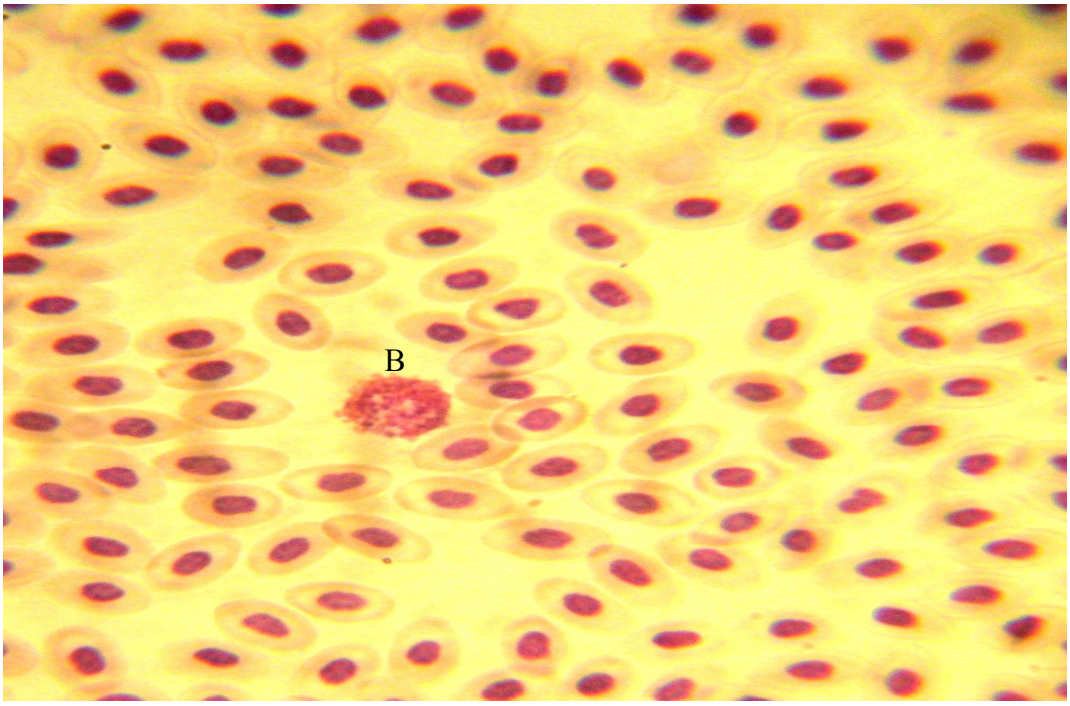


Plate II: Blood Smear from a Broiler Chicken on a Leishman-Wright's Stain (x 100); B = Basophil

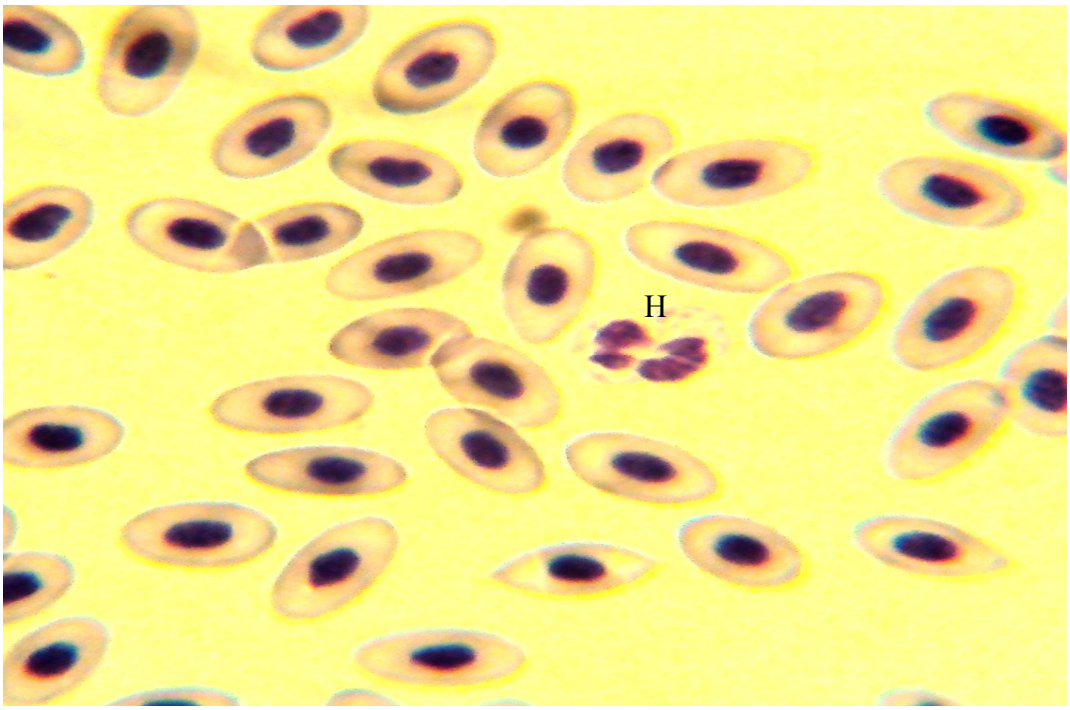


Plate III: Blood Smear from a Broiler Chicken on a Leishman's Stain (x 100); H = Heterophil

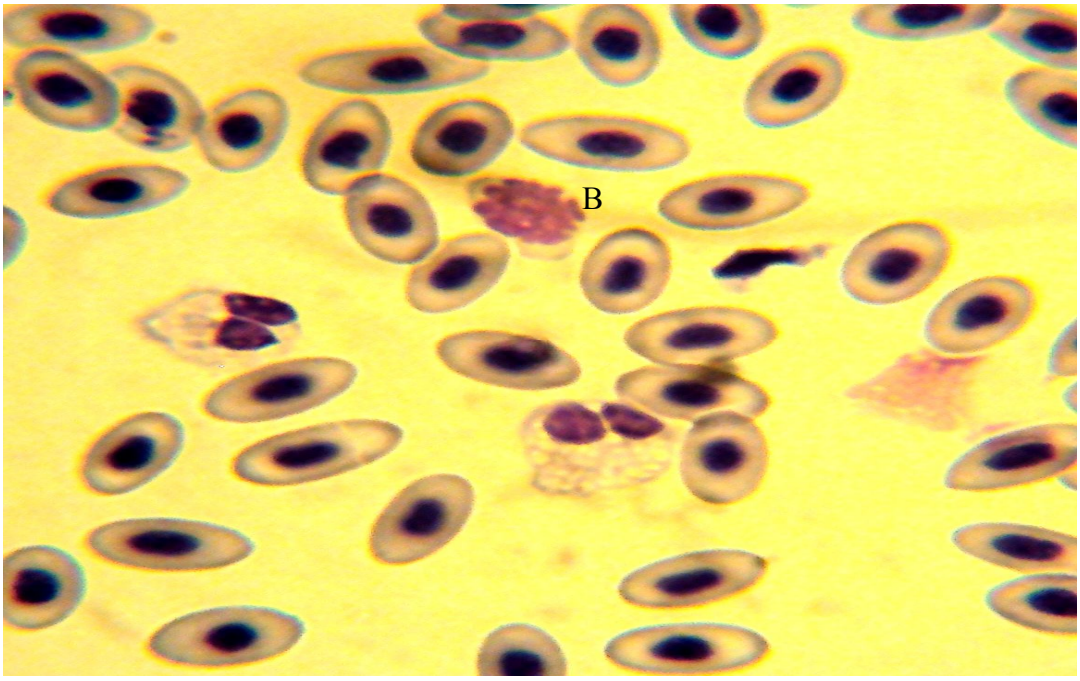


Plate IV: Blood Smear from a Broiler Chicken on a Leishman's Stain (x 100); B = Basophil

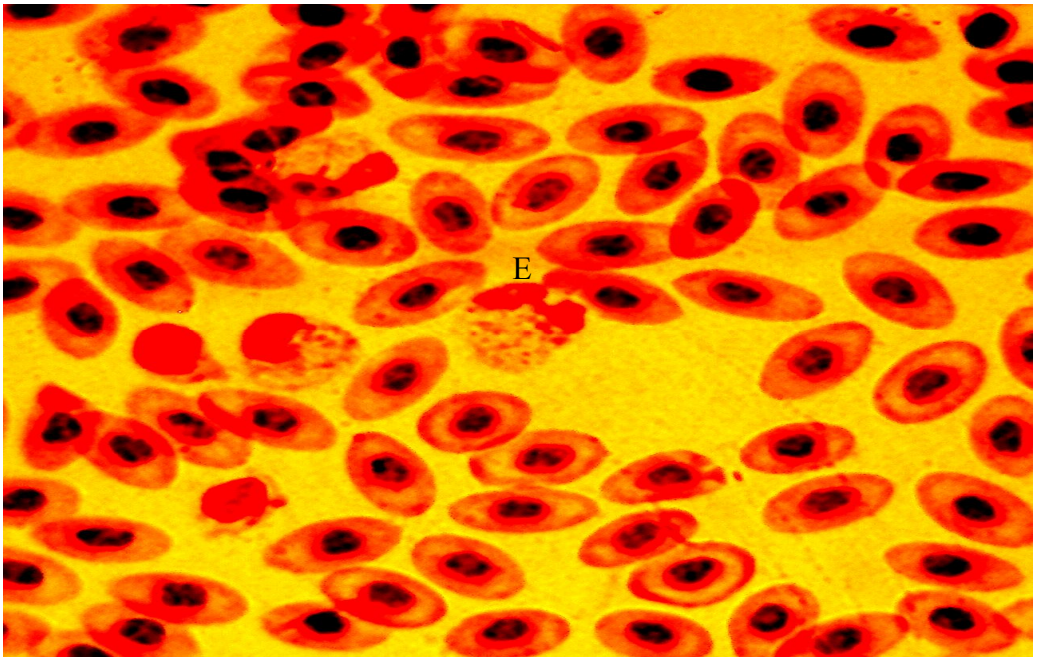


Plate V: Blood Smear from a Broiler Chicken on a Wright's Stain (x 100); E = Eosinophil

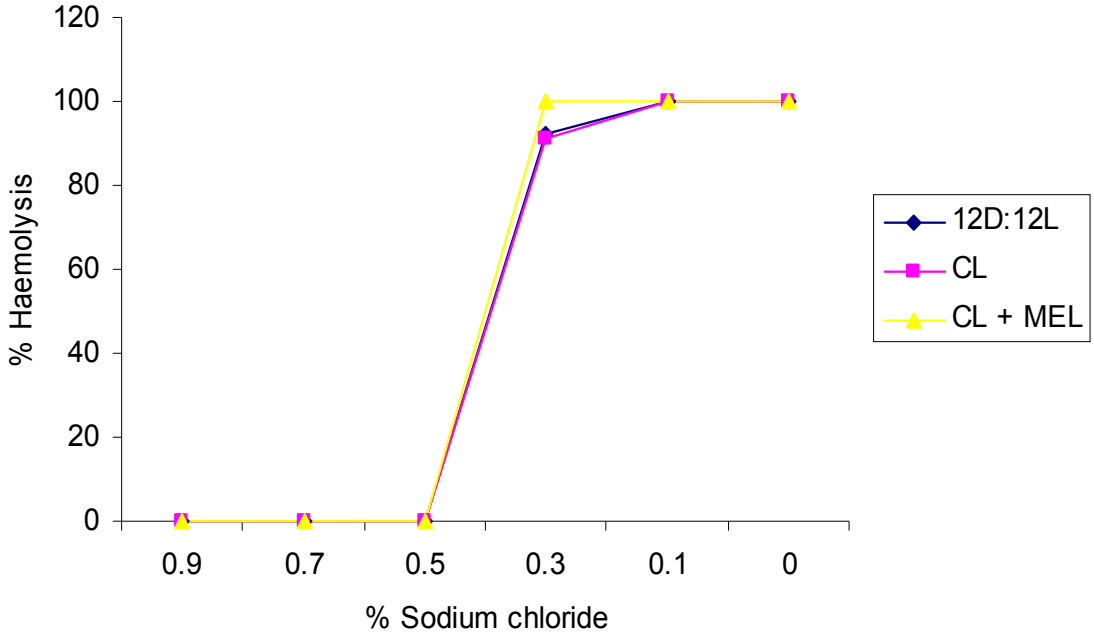


Figure 4.11: Effects of Melatonin administration and Lighting Regimens on Osmotic Fragility of Erythrocytes of Marshall Broiler Chickens during the hot-dry season;
12D:12L = 12 h darkness and 12 h light cycle (Group I); CL = Continuous lighting (Group II); and CL + MEL = CL + Melatonin (Group III)

4.5 RESULTS OF STUDIES ON BIOLOGICAL RHYTHMS

4.5.1 Rectal Temperature Fluctuations

The rectal temperature (RT) values fluctuated significantly in all the groups as the hour of the day increased (Tables 4.21 – 4.22; Fig. 4.12). The recorded mean hourly RT in 12D:12L cycle broilers (Table 4.21) was lowest at 06:00 h, with the value of 40.93 ± 0.05 ^{o}C when the birds were 28 days old, but the highest RT value of 42.15 ± 0.17 ^{o}C was obtained at 19:00 h ($P < 0.001$), when the broilers were 35 days old. Similarly, the mean RT value obtained in the CL broilers (Table 4.22) was lowest at 06:00 h on day 14 and highest at 13:00 h when the birds were 35 days' old, with the values of 41.16 ± 0.04 ^{o}C and 42.15 ± 0.07 ^{o}C, respectively. As shown in Table 4.21, the lowest RT value of 40.53 ± 0.66 ^{o}C was recorded in the melatonin-treated group at 19:00 h and at 49-days' old, but the highest value of 41.63 ± 0.06 ^{o}C was obtained, when the birds were 21 days' old at 13:00 h. The overall mean hourly RT value of 41.50 ± 0.02 ^{o}C, obtained in the 12D:12L cycle birds was significantly higher ($P < 0.001$) than the value of 41.14 ± 0.04 ^{o}C recorded in the melatonin-treated group, but lower than that of 41.65 ± 0.02 ^{o}C obtained in the CL birds (Fig. 4.13). The RT values, with the exception of those recorded in broiler chickens administered with melatonin, fluctuated significantly with age (Fig. 4.14).

4.5.2 Erythrocyte Osmotic Fragility Test

Diurnal variations in osmotic fragility of erythrocytes from broilers at different ages are shown in Fig. 4.15 – 4.20. The highest and significant ($P < 0.05$) percentage haemolysis with marked diurnal fluctuations was obtained in melatonin-treated broilers at day 28 (Fig. 4.15). In this group, the percentage haemolysis increased

Table 4.21: Diurnal Variations in Rectal Temperature of Marshall Broiler Chickens raised on 12D:12L cycle during the Hot-dry Season (Mean ± SEM, n = 15)

Hour	Age (weeks)							
	II	III	IV	V	VI	VII	VIII	Overall Mean ± SEM
06:00 h	41.25 ± 0.04[a]	41.29 ± 0.04[b]	40.93 ± 0.05[a]	41.34 ± 0.11[a]	41.21 ± 0.07[a]	41.16 ± 0.08[a]	41.21 ± 0.05[a]	41.20 ± 0.03[a]
13:00 h	41.77 ± 0.06[c]	41.74 ± 0.03[c]	41.67 ± 0.08[b]	42.01 ± 0.07[b]	41.84 ± 0.06[b]	41.78 ± 0.05[c]	41.70 ± 0.08[b]	41.79 ± 0.03[c]
19:00 h	41.33 ± 0.12[b]	41.16 ± 0.05[a]	41.58 ± 0.05[b]	42.15 ± 0.17[b]	41.36 ± 0.06[a]	41.54 ± 0.06[b]	41.37 ± 0.05[a]	41.50 ± 0.04[b]
Overall Mean ± SEM	41.45 ± 0.06[1]	41.40 ± 0.05[1]	41.40 ± 0.06[1]	41.83 ± 0.09[2]	41.47 ± 0.05[1]	41.49 ± 0.05[1]	41.43 ± 0.05[1]	41.50 ± 0.02

[a, b, c] = Means in the same column having different superscript letters are significantly ($P < 0.05$) different

[1,2] = Means in the same row having different superscript numbers are significantly ($P < 0.05$) different

Table 4.22: Diurnal Variations in Rectal Temperature of Marshall Broiler Chickens raised on Continuous Lighting during the Hot-dry Season (Mean ± SEM, n = 15)

Hour	Age (weeks)							
	II	III	IV	V	VI	VII	VIII	Overall Mean ± SEM
06:00 h	41.16 ± 0.04^{a}	41.23 ± 0.06^{a}	41.63 ± 0.06^{a}	41.33 ± 0.12^{a}	41.55 ± 0.07^{a}	41.34 ± 0.07^{a}	41.43 ± 0.09^{a}	41.38 ± 0.03^{a}
13:00 h	41.77 ± 0.09^{b}	41.72 ± 0.06^{b}	41.66 ± 0.07^{a}	42.15 ± 0.07^{b}	41.98 ± 0.07^{b}	41.94 ± 0.05^{c}	41.95 ± 0.08^{b}	41.88 ± 0.03^{c}
19:00 h	41.81 ± 0.05^{b}	41.57 ± 0.07^{b}	41.54 ± 0.05^{a}	42.00 ± 0.13^{b}	41.58 ± 0.04^{a}	41.61 ± 0.11^{b}	41.74 ± 0.11^{b}	41.69 ± 0.04^{b}
Overall Mean ± SEM	$41.58 \pm 0.06^{1,2}$	41.51 ± 0.05^{1}	$41.61 \pm 0.03^{1,2}$	41.83 ± 0.08^{3}	$41.70 \pm 0.05^{2,3}$	$41.63 \pm 0.06^{1,2}$	$41.71 \pm 0.06^{2,3}$	41.65 ± 0.02

$^{a, b, c}$ = Means in the same column having different superscript letters are significantly ($P < 0.05$) different

^{1,2,3} = Means in the same row having different superscript numbers are significantly ($P < 0.05$) different

Table 4.23: Diurnal Variations in Rectal Temperature of Marshall Broiler Chickens raised on Continuous Lighting and administered with Melatonin during the Hot-dry Season (Mean ± SEM, n = 15)

Hour	Age (weeks)							
	II	III	IV	V	VI	VII	VIII	Overall Mean ± SEM
06:00 h	40.83 ± 0.07^{a}	40.76 ± 0.04^{a}	41.07 ± 0.06^{a}	40.89 ± 0.12^{a}	40.92 ± 0.04^{a}	41.08 ± 0.06^{a}	41.18 ± 0.05^{a}	40.96 ± 0.03^{a}
13:00 h	41.59 ± 0.05^{b}	41.63 ± 0.06^{b}	41.11 ± 0.14^{a}	41.07 ± 0.10^{ab}	41.27 ± 0.05^{b}	41.27 ± 0.11^{a}	41.30 ± 0.07^{a}	41.32 ± 0.04^{b}
19:00 h	41.44 ± 0.10^{b}	41.23 ± 0.04^{c}	41.31 ± 0.06^{a}	41.26 ± 0.07^{b}	40.97 ± 0.04^{a}	40.53 ± 0.66^{a}	41.20 ± 0.03^{a}	41.14 ± 0.10^{ab}
Overall Mean ± SEM	41.28 ± 0.06^{2}	41.21 ± 0.06^{2}	41.16 ± 0.06^{2}	$41.07 \pm 0.06^{1,2}$	41.05 ± 0.03^{1}	$40.96 \pm 0.22^{1,2}$	41.23 ± 0.03^{2}	41.14 ± 0.04

$^{a, b, c}$ = Means in the same column having different superscript letters are significantly ($P < 0.05$) different

^{1,2} = Means in the same row having different superscript numbers are significantly ($P < 0.05$) different

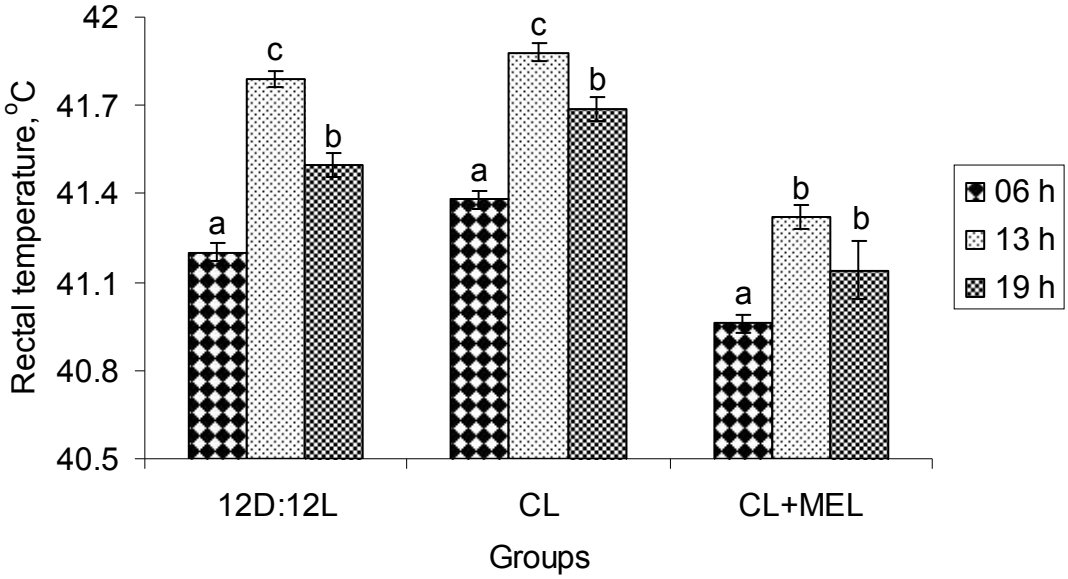


Figure 4.12: Diurnal Variations in Rectal Temperature of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season; [a, b, c] = Means for the same group having different superscript letters are significantly different ($P < 0.05$). 12D:12L = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + MEL = CL + Melatonin

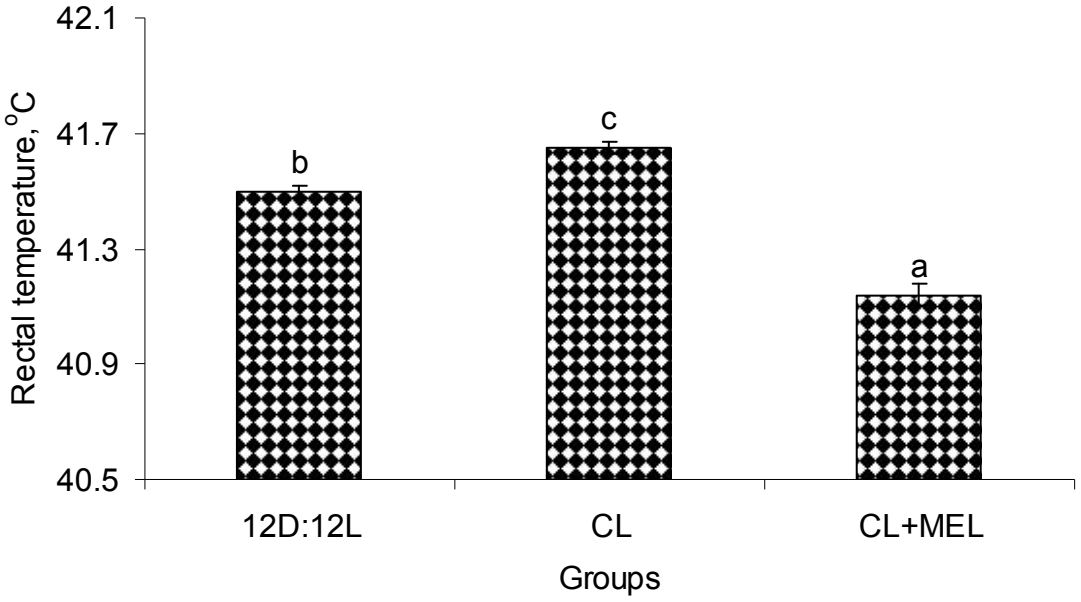


Figure 4.13: Overall Mean Rectal Temperature of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season;
[a, b, c] = Means having different superscript letters are significantly ($P < 0.05$) different

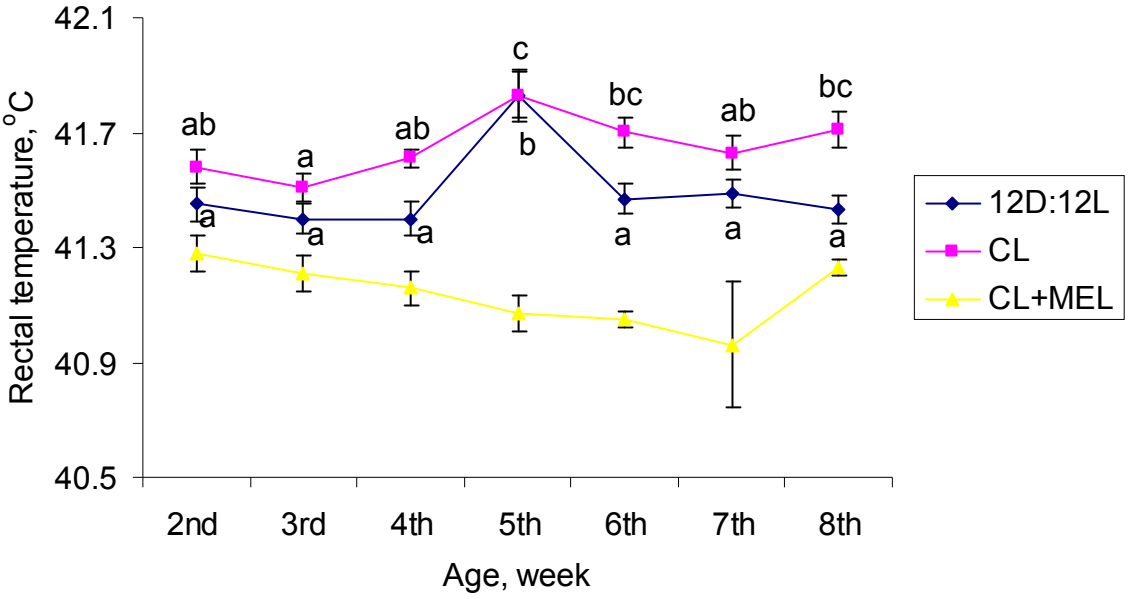


Figure 4.14: Age-related Changes in Mean Rectal Temperature of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season;
[a, b, c] = Means for the same parameters having different superscript letters are significantly different ($P < 0.05$)

consistently ($P < 0.05$) as the hour of the day increased, from 0.9 % up to 0.3 % NaCl concentration. On days 42 and 56 of the study, the percentage haemolysis recorded from the erythrocytes of birds in groups I and II were very similar and significantly higher than those obtained in broilers administered with melatonin (Fig. 4:16 and 4.17). At both ages, percentage haemolysis fluctuated significantly in groups I and II that were not administered with melatonin from 0.9 % to 0.3 % NaCl concentration, contrary to the results obtained in birds treated with melatonin in group III (Fig. 4:16 and 4.17). Fig. 4.18 shows that the diurnal variation in osmotic fragility of RBC from the birds was highest in those raised on CL, followed by those reared on 12D:12L cycle and lowest in the melatonin-treated broiler chickens. Between-group overall diurnal variation in haemolysis in the broilers showed that the CL birds had the highest ($P < 0.001$) percentage haemolysis at 06:00 h and 19:00 h, both at 0.5 % NaCl concentration (Fig. 4:19). Also, the highest percentage haemolysis at 13:00 h, was recorded in the 12D:12L group at 0.1 % NaCl concentration (Fig. 4:19). Fig. 4: 20 shows the overall percentage haemolysis between the three groups. The erythrocytes of the birds raised on CL was more osmotically fragile at 0.5 % NaCl concentration compared to the other two groups.

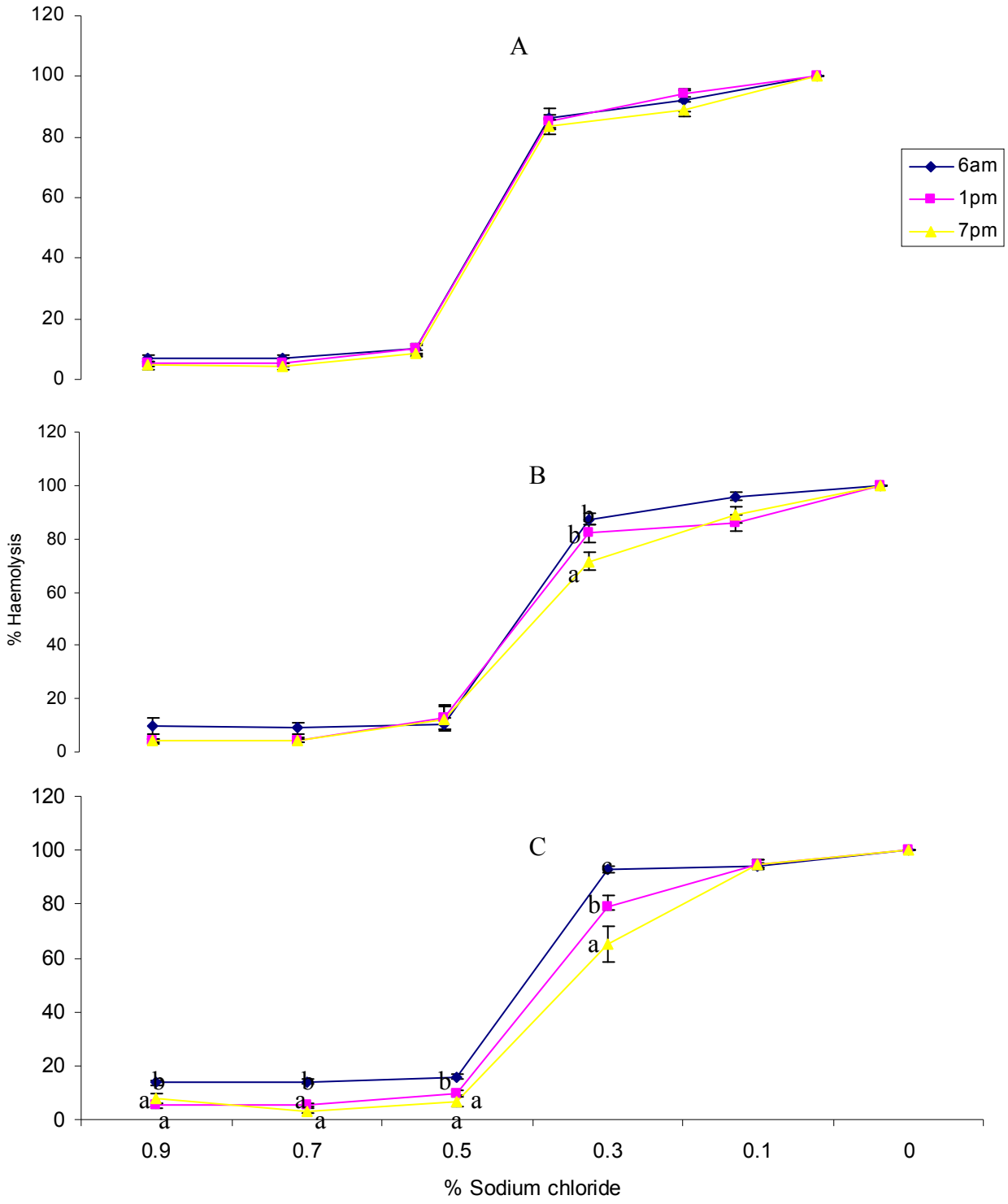


Figure 4.15: Diurnal Variations in Osmotic Fragility of Erythrocytes in Marshall Broiler Chickens administered with Melatonin at Day 28; A = Group I (12 h darkness and 12 h light cycle), B = Group II (Continuous lighting; CL), and C = Group III (CL + Melatonin); $^{a, b, c}$ = Means at the same NaCl concentration having different superscript letters are significantly ($P < 0.05$) different

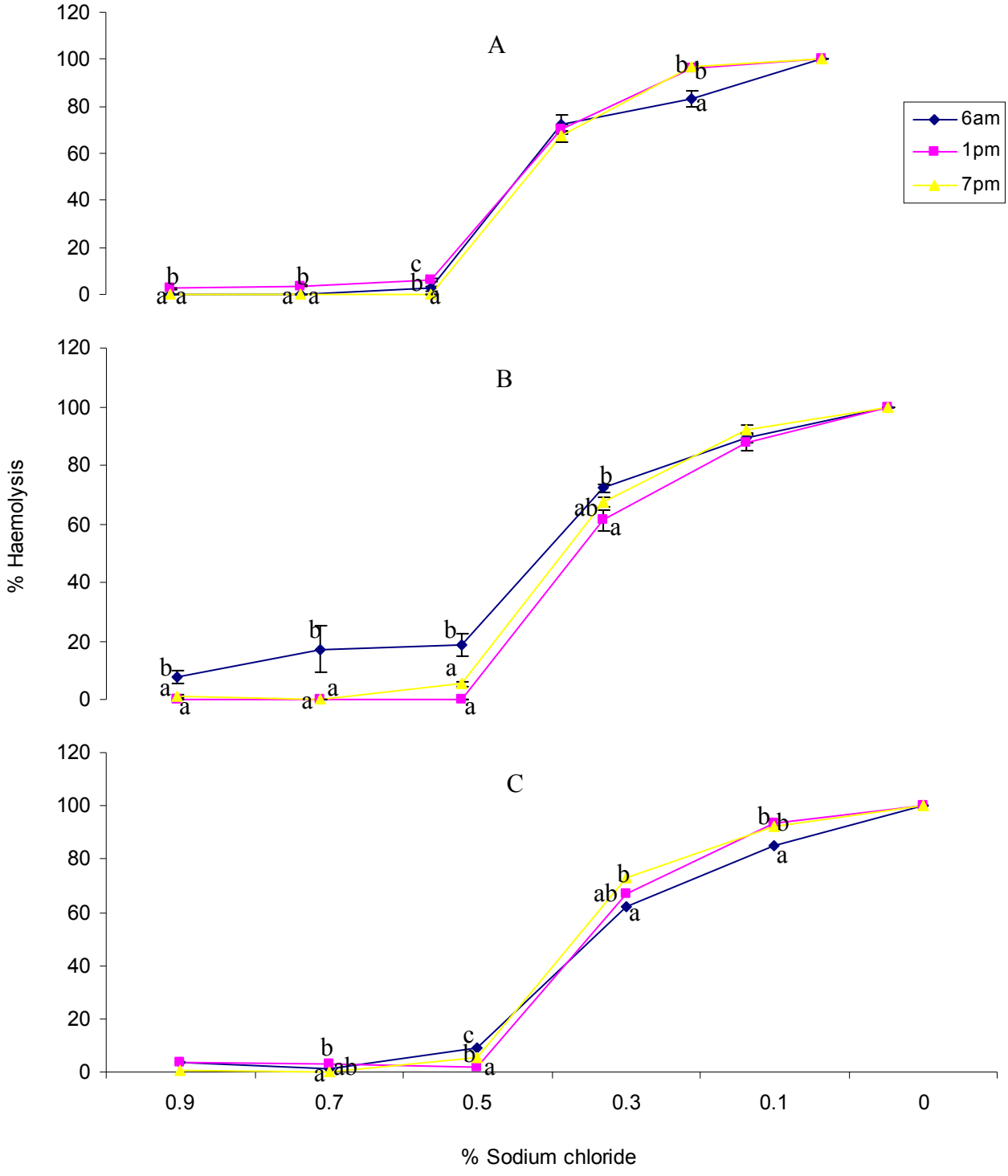


Figure 4.16: Diurnal Variations in Osmotic Fragility of Erythrocytes in Marshall Broiler Chickens administered with Melatonin at Day 42; A = Group I (12 h darkness and 12 h light cycle), B = Group II (Continuous lighting; CL), and C = Group III (CL + Melatonin); $^{a, b, c}$ = Means at the same NaCl concentration having different superscript letters are significantly ($P < 0.05$) different

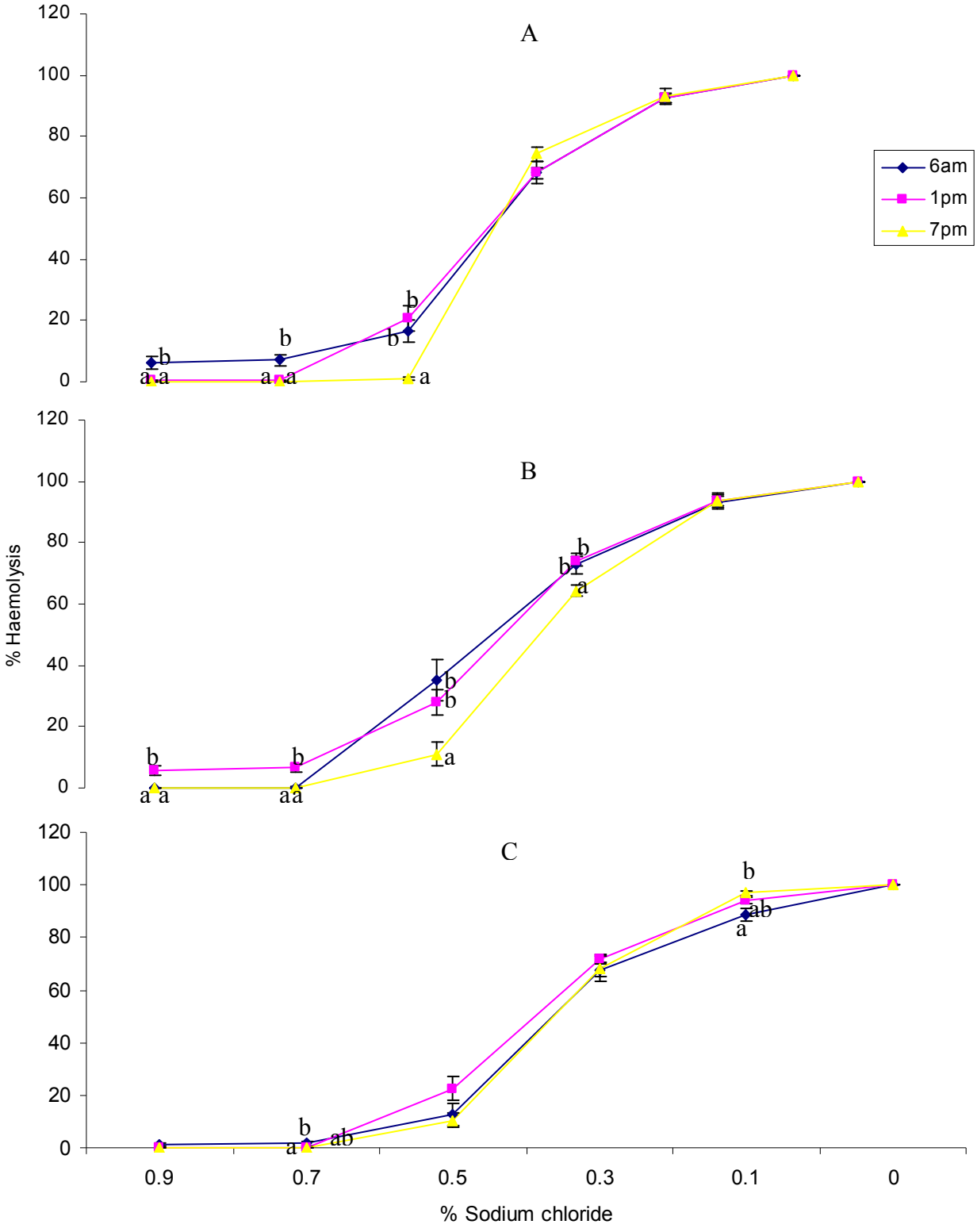


Figure 4.17: Diurnal Variations in Osmotic Fragility of Erythrocytes from Marshall Broiler Chickens administered with Melatonin at Day 56; A = Group I (12 h darkness and 12 h light cycle), B = Group II (CL), and C = Group III (CL + Melatonin); $^{a, b, c}$ = Means at the same NaCl concentration having different superscript letters are significantly ($P < 0.05$) different

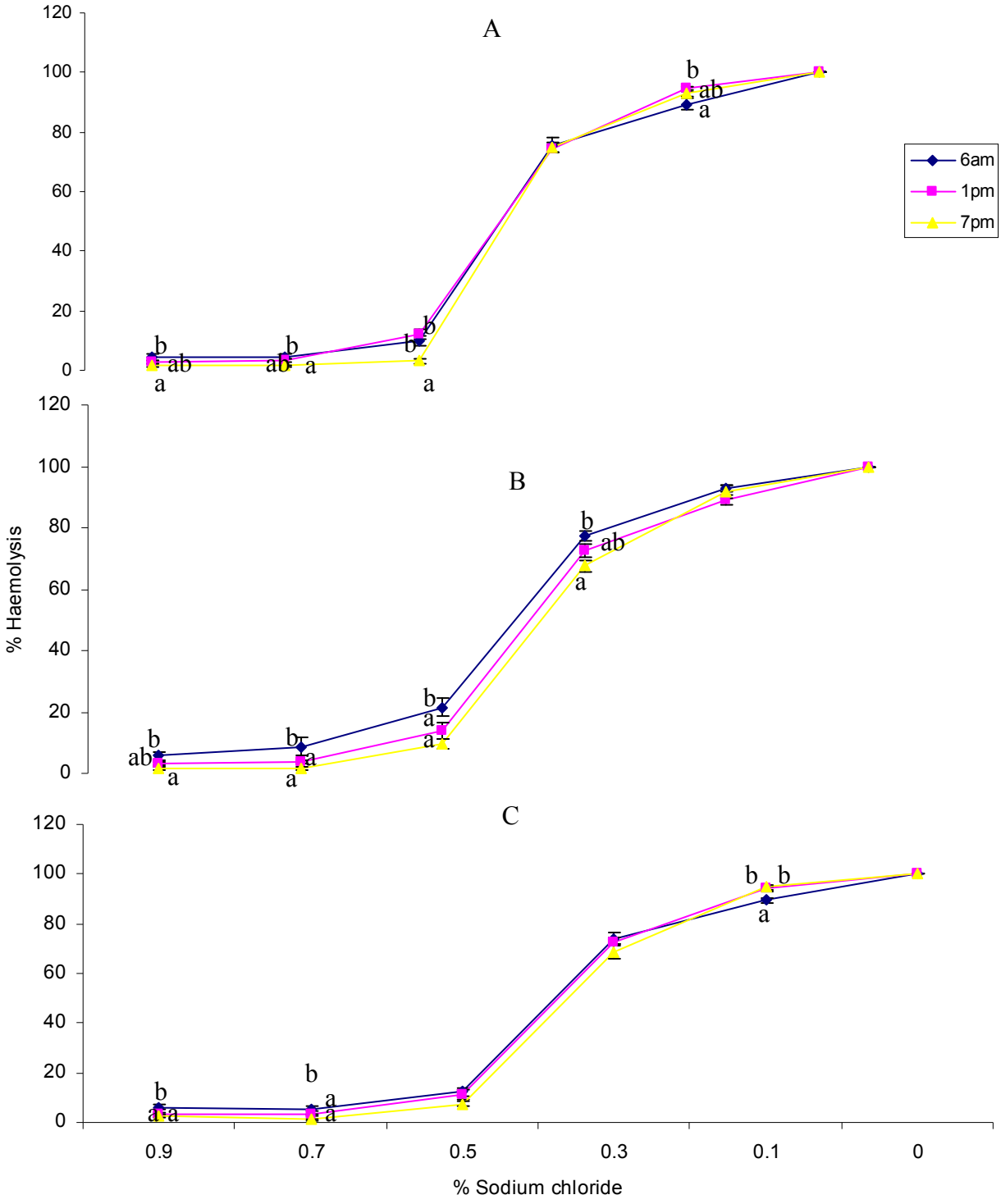


Figure 4.18: Overall Diurnal Variations in Osmotic Fragility of Erythrocytes from Marshall Broiler Chickens administered with Melatonin during the Study Period; A = Group I (12 h darkness and 12 h light cycle), B = Group II (Continuous lighting; CL), and C = Group III (CL + Melatonin); $^{a, b, c}$ = Means at the same NaCl concentration having different superscript letters are significantly ($P < 0.05$) different

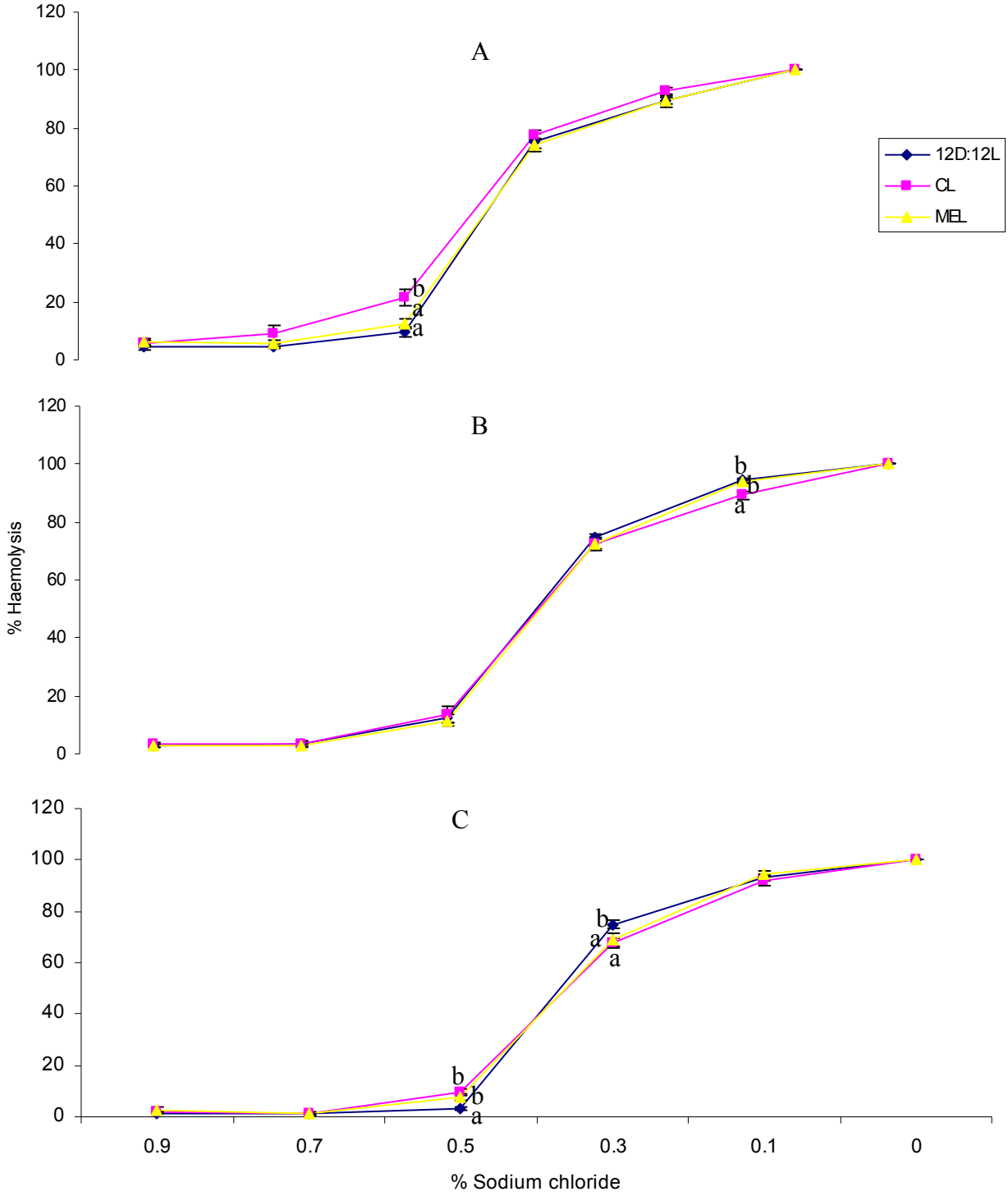


Figure 4.19: Between-group Overall Diurnal Variations in Osmotic Fragility of Erythrocytes from Marshall Broiler Chickens administered with Melatonin during the Study Period; A = 06:00 h; B = 13:00 h; C = 19:00 h; 12D:12L = Group I (12 h darkness and 12 h light cycle); CL = Group II (Continuous lighting; CL) and MEL (CL + Melatonin) = Group III; $^{a, b, c}$ = Means at the same NaCl concentration having different superscript letters are significantly ($P < 0.05$) different

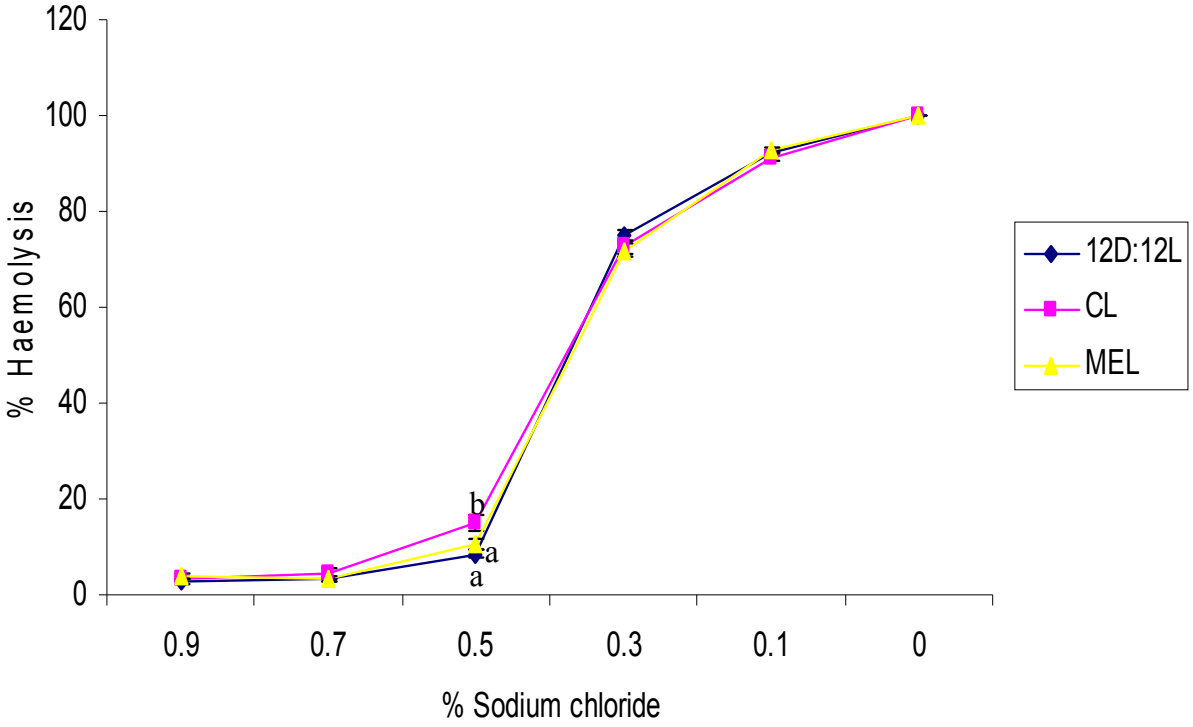


Figure 4.20: Between-group Overall Sodium Chloride Concentration Variations in Osmotic Fragility of Erythrocytes from Marshall Broiler Chickens administered with Melatonin during the Study Period;
12D:12L (12 h darkness and 12 h light cycle) = Group I; CL (Continuous lighting) = Group II and MEL (CL + Melatonin) = Group III;
[a, b, c] = Means at the same NaCl concentration having different superscripts are significantly ($P < 0.05$) different

4.5.3 Haematological Results from Studies on Biological Rhythms

The haematological parameters of Marshall broiler chickens obtained at different hours of the day are shown in Tables 4.24 – 4.28. The parameters, with the exception of thrombocytes and heterophil/lymphocyte ratio, fluctuated significantly ($P < 0.05$) as the hours of the day increased in all the groups, especially in group I raised under 12D:12L cycle (Tables 4.24 – 4.26). The highest and lowest mean values of erythrocyte count, Hb and PCV in all the groups were obtained at 06:00 h and 13:00 h, respectively. The values decreased significantly ($P < 0.05$) from these hours only in groups I and II, but insignificantly ($P > 0.05$) in group III birds administered with melatonin. The RDW_SD, MCV and MCH values recorded at 13:00 h were lower compared to those, recorded at either 06:00 h or 17:00 h in all the groups. On the contrary the RDW_CV and MCHC values obtained in all the groups were highest at 13:00 h. The data depicted in Tables 4.24 – 4.26 also indicate that total leucocyte and absolute lymphocyte counts recorded at 17:00 h were highest ($P < 0.05$), compared to the values obtained at the other hours of the day in all the groups.

The erythrocytic indices and leucocytic series of the Marshall broiler chickens recorded in the different groups are shown in Tables 4.27 – 4.28, respectively. The highest RBC, Hct, Hb and MCV values were obtained in CL group, followed by CL + Melatonin and the least values were obtained in birds raised on 12D:12L cycle. The melatonin-treated group had the highest MCH and MCHC values, followed by birds in the CL group, while 12D:12L group had the least values. The highest RDW_SD and RDW_CV values were recorded in the 12D:12L cycle group followed by CL group, and the lowest values were recorded in group III birds administered with melatonin. Finally, the absolute heterophil values and H:L ratio

obtained in the CL group were significantly ($P < 0.05$) higher compared to the values recorded in the other groups.

Table 4.24: Diurnal Variations in Haematological Values of Marshall Broiler Chickens raised on 12 h Darkness and 12 h Light Cycle during the Hot-dry Season (Mean ± SEM; n = 15)

Parameter	Hour of the day, h		
	06:00 h	13:00 h	19:00 h
Total leucocyte count ($x10^3/\mu l$)	21.32 ± 0.14^b	19.80 ± 0.54^a	22.00 ± 0.17^b
Lymphocytes ($x10^3/\mu l$)	20.42 ± 0.14^b	18.83 ± 0.50^a	20.81 ± 0.11^b
Heterophils ($x10^3/\mu l$)	0.91 ± 0.05^a	0.97 ± 0.07^{ab}	1.19 ± 0.14^b
Lymphocytes (%)	95.60 ± 0.23^a	95.07 ± 0.28^a	95.65 ± 0.13^a
Heterophils (%)	4.40 ± 0.23^a	4.93 ± 0.28^a	4.35 ± 0.13^a
Heterophil/lymphocyte ratio	0.05 ± 0.003^a	0.05 ± 0.003^a	0.06 ± 0.007^a
Thrombocytes ($x10^3/\mu l$)	16.93 ± 1.30^a	13.87 ± 1.80^a	18.47 ± 2.75^a
Erythrocyte count ($x10^6/\mu l$)	2.20 ± 0.02^b	1.91 ± 0.10^a	2.17 ± 0.04^b
Haemoglobin concentration (g/dl)	9.59 ± 0.09^b	7.83 ± 0.41^a	9.25 ± 0.19^b
Haematocrit (%)	31.01 ± 0.27^b	24.88 ± 1.26^a	30.47 ± 0.55^b
Mean corpuscular volume (fl)	140.83 ± 1.09^b	130.26 ± 0.33^a	140.41 ± 0.85^b
Mean corpuscular haemoglobin (pg)	43.55 ± 0.38^b	40.93 ± 0.22^a	42.61 ± 0.43^b
Mean corpuscular haemoglobin concentration (g/dl)	30.91 ± 0.11^b	31.42 ± 0.16^c	30.33 ± 0.19^a
Red cell distribution width_SD (fl)	32.51 ± 0.71^b	27.63 ± 0.64^a	32.49 ± 0.69^b
Red cell distribution width_CV (%)	9.91 ± 0.11^a	12.31 ± 0.33^b	9.83 ± 0.17^a

$^{a, b, c}$ = Means in the same row with different superscript letters are significantly ($P < 0.05$) different;
SD = Standard deviation; CV = Coefficient of variance

Table 4.25: Diurnal Variations in Haematological Values of Marshall Broiler Chickens raised on Continuous Lighting during the Hot-dry Season (Mean ± SEM; n = 15)

Parameter	Hour of the day, h		
	06:00 h	13:00 h	19:00 h
Total leucocyte count ($x10^3/\mu l$)	21.74 ± 0.43^a	20.82 ± 0.26^a	21.85 ± 0.31^a
Lymphocytes ($x10^3/\mu l$)	20.09 ± 0.37^a	19.46 ± 0.32^a	20.13 ± 0.20^a
Heterophils ($x10^3/\mu l$)	1.64 ± 0.23^a	1.36 ± 0.17^a	1.72 ± 0.28^a
Lymphocytes (%)	96.34 ± 0.31^b	94.07 ± 0.83^a	96.01 ± 0.21^b
Heterophils (%)	3.66 ± 0.31^a	5.93 ± 0.83^b	3.99 ± 0.21^a
Heterophil/lymphocyte ratio	0.08 ± 0.01^a	0.07 ± 0.01^a	0.09 ± 0.01^a
Thrombocytes ($x10^3/\mu l$)	17.87 ± 2.34^a	13.93 ± 0.80^a	14.93 ± 1.32^a
Erythrocyte count ($x10^6/\mu l$)	2.25 ± 0.04^b	2.03 ± 0.04^a	2.17 ± 0.06^{ab}
Haemoglobin concentration (g/dl)	9.72 ± 1.18^b	8.75 ± 0.20^a	9.45 ± 0.29^{ab}
Haematocrit (%)	31.68 ± 0.62^b	28.04 ± 0.60^a	30.68 ± 0.91^b
Mean corpuscular volume (fl)	140.79 ± 0.99^a	138.07 ± 0.95^a	140.77 ± 1.10^a
Mean corpuscular haemoglobin (pg)	43.22 ± 0.28^a	43.09 ± 0.35^a	43.45 ± 0.25^a
Mean corpuscular haemoglobin concentration (g/dl)	30.71 ± 0.19^a	31.19 ± 0.15^a	30.82 ± 0.20^a
Red cell distribution width_SD (fl)	30.56 ± 1.03^{ab}	28.37 ± 0.82^a	31.99 ± 1.3^b
Red cell distribution width_CV (%)	9.74 ± 0.20^a	11.85 ± 0.54^b	10.36 ± 0.37^a

$^{a, b, c}$ = Means in the same row with different superscript letters are significantly ($P < 0.05$) different;
SD = Standard deviation; CV = Coefficient of variance

Table 4.26: Diurnal Variations in Haematological Values of Marshall Broiler Chickens raised on Continuous Lighting and administered with Melatonin during the Hot-dry Season (Mean ± SEM; n = 15)

Parameter	Hour of the day, h		
	06:00 h	13:00 h	19:00 h
Total leucocyte count ($x10^3/\mu l$)	21.13 ± 0.21^a	21.05 ± 0.32^a	21.82 ± 0.11^b
Lymphocytes ($x10^3/\mu l$)	20.07 ± 0.08^{ab}	19.81 ± 0.28^a	20.59 ± 0.07^b
Heterophils ($x10^3/\mu l$)	1.06 ± 0.19^a	1.24 ± 0.15^a	1.23 ± 0.12^a
Lymphocytes (%)	96.06 ± 0.34^b	94.91 ± 0.34^a	95.41 ± 0.34^{ab}
Heterophils (%)	3.94 ± 0.34^a	5.09 ± 0.34^b	4.59 ± 0.34^{ab}
Heterophil/lymphocyte ratio	0.05 ± 0.009^a	0.06 ± 0.008^a	0.06 ± 0.01^a
Thrombocytes ($x10^3/\mu l$)	16.07 ± 1.67^a	13.00 ± 0.93^a	15.80 ± 1.08^a
Erythrocyte count ($x10^6/\mu l$)	2.19 ± 0.06^a	2.11 ± 0.05^a	2.13 ± 0.03^a
Haemoglobin concentration (g/dl)	9.52 ± 0.23^a	9.05 ± 0.28^a	9.30 ± 0.13^a
Haematocrit (%)	30.59 ± 0.72^a	28.82 ± 0.85^a	30.34 ± 0.32^a
Mean corpuscular volume (fl)	139.69 ± 1.26^{ab}	136.63 ± 1.28^a	142.44 ± 1.71^b
Mean corpuscular haemoglobin (pg)	43.47 ± 0.40^a	42.90 ± 0.46^a	43.65 ± 0.57^a
Mean corpuscular haemoglobin concentration (g/dl)	31.10 ± 0.10^{ab}	31.41 ± 0.20^b	30.64 ± 0.12^a
Red cell distribution width_SD (fl)	31.11 ± 1.11^a	27.95 ± 0.83^a	30.50 ± 1.37^a
Red cell distribution width_CV (%)	9.99 ± 0.19^a	11.87 ± 0.34^b	9.53 ± 0.22^a

$^{a, b, c}$ = Means in the same row having different superscript letters are significantly different ($P < 0.05$);
SD = Standard deviation; CV = Coefficient of variance

Table 4.27: Erythrocytic Indices of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM; n = 15)

Parameters	Groups		
	12D:12L cycle	CL	CL + MEL
RBC ($x10^6/\mu L$)	2.09 ± 0.04^a	2.15 ± 0.03^a	2.14 ± 0.03^a
Hct (%)	28.79 ± 0.62^a	30.13 ± 0.47^a	29.92 ± 0.39^a
Hb (g/dl)	8.89 ± 0.19^a	9.31 ± 0.14^a	9.29 ± 0.13^a
MCV (fl)	137.17 ± 0.87^a	139.88 ± 0.60^b	139.59 ± 0.88^{ab}
MCH (pg)	42.36 ± 0.26^a	43.25 ± 0.17^b	43.34 ± 0.28^b
MCHC (%)	30.89 ± 0.11^a	30.91 ± 0.11^a	31.05 ± 0.10^b
RDW_SD (fl)	30.88 ± 0.52^a	30.31 ± 0.64^a	29.86 ± 0.67^a
RDW_CV (%)	10.68 ± 0.21^a	10.65 ± 0.26^a	10.46 ± 0.21^a

$^{a, b, c}$ = Means in the same row with different superscript letters are significantly different ($P < 0.05$) different; 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration; SD = Standard deviation; CV = Coefficient of variance; RBC = Erythrocyte; Hct = Haematocrit; Hb = Haemoglobin; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; RDW = Red cell distribution width

Table 4.28: Leucocytic Counts of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM; n = 15)

Parameters	Group		
	12D:12L cycle	CL	CL + MEL
WBC ($x10^3/\mu L$)	21.04 ± 0.24^a	21.47 ± 0.21^a	21.33 ± 0.14^a
Lymphocyte (L; %)	95.44 ± 0.13^a	95.48 ± 0.33^a	95.46 ± 0.20^a
Heterophil (H; %)	4.56 ± 0.13^a	4.52 ± 0.33^a	4.54 ± 0.20^a
Lymphocyte ($x10^3/\mu L$)	20.02 ± 0.22^a	19.90 ± 0.18^a	20.16 ± 0.11^a
Heterophil ($x10^3/\mu L$)	1.02 ± 0.06^a	1.57 ± 0.13^b	1.18 ± 0.09^a
H:L ratio	0.05 ± 0.003^a	0.08 ± 0.007^b	0.06 ± 0.004^a
Thrombocyte	16.42 ± 1.19^a	15.58 ± 0.94^a	14.96 ± 0.75^a

[a, b, c]: Means in the same row with different superscript letters are significantly different ($P < 0.05$); 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration; WBC = Leucocyte

4.5.4 Behavioural Responses

The diurnal variations in stressful and beneficial behavioural events, expressed in percentages are shown in Tables 4.29 and 4.30, respectively. The behavioural responses fluctuated ($P < 0.05$) as the hour of the day increased ($P < 0.05$). Exceptions to this finding are the behavioural events of lying and two-fighting, obtained from broilers in 12D:12L cycle and melatonin-treated groups, respectively that fluctuated insignificantly ($P > 0.05$). Table 4.31 shows the overall behavioural events recorded from the three groups. The overall value of 21.83 ± 1.01 % for panting, obtained in the melatonin-treated birds was significantly (Kruskal Wallis Test = 17.45; $P < 0.0001$) lower than 69.72 ± 6.77 % and 67.87 ± 7.19 %, recorded in birds from 12D:12L cycle and CL groups, respectively. The overall mean value of 63.61 ± 2.11 % recorded for lying (resting/sleeping) in the melatonin-treated group was significantly (Kruskal Wallis Test = 12.25; $P < 0.005$) higher than the values of 43.56 ± 1.03% and 54.28 ± 4.52 %, obtained in broiler chickens in 12D:12L cycle and CL groups, respectively. The behavioural events of standing idle (Kruskal Wallis Test = 1.66; $P > 0.05$) and eating (Kruskal Wallis Test = 2.73; $P > 0.05$) across the groups were not different ($P > 0.05$) (Table 4.31).

Table 4.29: Diurnal Variations in Stressful or Risky Behaviour of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM; n = 15)

Behaviour	Hour of the day, h	Percentage of broiler chickens, %		
		12D:12L	CL	CL + MEL
Panting	06:00	46.5 ± 0.76[a]	42.17 ± 1.01[a]	23.83 ± 0.73[b]
	13:00	93.17 ± 1.42[c]	69.83 ± 1.30[b]	23.5 ± 0.76[b]
	19:00	69.50 ± 1.61[b]	91.67 ± 1.64[c]	18.17 ± 1.01[a]
Two fighting	06:00	6.5 ± 1.04[a]	18.0 ± 1.16[b]	0.00 ± 0.00[a]
	13:00	14.67 ± 1.17[b]	10.83 ± 0.83[a]	0.00 + 0.00[a]
	19:00	8.0 ± 1.16[a]	24.83 ± 2.89[c]	0.00 ± 0.00[a]
Dust bathing	06:00	66. 0 ± 3.46[b]	5.67 ± 1.2[a]	0.00 ± 0.00a
	13:00	58.17 ± 1.01[a]	5.5 ± 0.5[a]	5.17 ± 0.73[b]
	19:00	56.67 ± 1.92[a]	18.5 ± 1.89[b]	0.00 ± 0.00[a]
Preening	06:00	36.33 ± 0.88[b]	16.67 ± 1.76[a]	22.07 ± 1.10[b]
	13:00	38.17 ± 1.01[b]	17.17 ± 1.09[a]	11.17 ± 0.60[a]
	19:00	32.00 ± 1.16[a]	47.17 ± 1.48[b]	24.83 ± 0.83[b]
Running	06:00	43.83 ± 1.69[b]	21.33 ± 1.76[b]	0.00 ± 0.00[a]
	13:00	36.5 ± 0.76[a]	12.5 ± 1.8[a]	10.33 ± 0.88[b]
	19:00	54.33 ± 2.33[c]	54.33 ± 2.33[c]	0.00 ± 0.00[a]
Pecking	06:00	32.17 ± 1.17[b]	10.17 ± 1.17[a]	12.5 ± 1.44[b]
	13:00	16.17 ± 1.17[a]	20.17 ± 1.01[b]	5.17 ± 0.73[a]
	19:00	33.83 ± 0.73[b]	33.83 ± 0.73[b]	5.17 ± 0.60[a]

[a, b, c] = Means for the same column having different superscript letters are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

Table 4.30: Diurnal Variations in Stressless or Beneficial Behaviours of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM; n = 15)

Behavioural acitivity	Hour of the day, h	Percentage of broiler chickens, %		
		12D:12L	CL	CL + MEL
Standing	06:00	4.5 ± 1.04^{a}	16.33 ± 0.88^{ab}	20.0 ± 0.00^{b}
	13:00	14.17 ± 1.01^{b}	18.00 ± 1.16^{b}	7.67 ± 0.5^{a}
	19:00	29.17 ± 1.42^{c}	12.33 ± 1.45^{a}	42.0 ± 1.16^{c}
Eating	06:00	23.17 ± 2.24^{ab}	37.83 ± 1.30^{c}	17.67 ± 1.45^{a}
	13:00	16.5 ± 2.84^{a}	16.5 ± 0.87^{a}	33.5 ± 0.87^{b}
	19:00	24.83 ± 0.73^{b}	32.67 ± 1.45^{b}	17.83 ± 0.17^{a}
Drinking	06:00	20.83 ± 0.6^{a}	34.17 ± 1.01^{a}	20.0 ± 1.16^{a}
	13:00	24.83 ± 0.6^{b}	40.17 ± 1.30^{b}	28.00 ± 1.16^{b}
	19:00	24.0 ± 1.16^{b}	41.00 ± 1.53^{b}	24.00 ± 1.16^{ab}
Lying (Resting/sleeping)	06:00	45.17 ± 2.89^{a}	55.17 ± 2.89^{b}	62.17 ± 2.32^{a}
	13:00	43.5 ± 0.76^{a}	39.0 ± 3.4^{a}	5.83 ± 1.3^{a}
	19:00	42.00 ± 1.16^{a}	68.67 ± 2.03^{c}	70.83 ± 1.64^{b}

[a, b, c]: Means for the same column having different superscript letters are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

Table 4.31: Effects of Lighting Regimens and Melatonin Administration on Overall Percentage Behavioural Responses of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM; n = 15)

Behavioural event	Percentage of broiler chickens, %		
	12D:12L	CL	CL + MEL
Panting	69.72 ± 6.77^{b}	67.87 ± 7.19^{b}	21.83 ± 1.01^{a}
Two fighting	9.72 ± 1.38^{b}	17.89 ± 2.23^{c}	0.00 ± 0.00^{a}
Dust bathing	60.28 ± 1.87^{c}	9.89 ± 2.25^{b}	1.72 ± 0.89^{a}
Preening	35.5 ± 1.05^{b}	27.0 ± 5.10^{ab}	19.36 ± 2.13^{a}
Running	44.89 ± 2.73^{c}	25.66 ± 4.63^{b}	3.44 ± 1.74^{a}
Pecking	27.39 ± 2.86^{c}	16.00 ± 1.62^{b}	7.61 ± 1.32^{a}
Standing	15.94 ± 3.64^{a}	15.56 ± 1.03^{a}	23.22 ± 5.05^{a}
Eating	21.5 ± 1.66^{a}	29.0 ± 3.27^{a}	23.0 ± 2.67^{a}
Drinking	23.22 ± 0.74^{a}	38.44 ± 1.26^{b}	24.00 ± 1.29^{a}
Lying (Resting/Sleeping)	43.56 ± 1.03^{a}	54.28 ± 4.52^{b}	63.61 ± 2.11^{c}

[a, b, c]: Means for the same row having different superscript letters are significantly different ($P < 0.05$). 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

4.5.5 Tonic Immobility Responses

The number of induction trial attempts fluctuated insignificantly ($P > 0.05$) as the hours of the day increased (Table 4.32). The highest numbers of induction trial attempts were 2.13 ± 0.34 and 2.15 ± 0.22 in 12D:12L cycle and CL groups, respectively, and they were recorded at 13:00 h, when the birds were 56 days' old. The overall mean values of induction trial attempts differed significantly ($P < 0.0001$) between the groups, with the lowest mean values of 1.22 ± 0.4 recorded in group III birds administered with melatonin (Table 4.35).

Table 4.33 shows that the TI duration fluctuated as the hours of the day increased during the study period. At d 42, the lowest mean TI duration of 101.87 ± 10.24 s obtained in the CL group was recorded at 06:00 h, and the value rose ($P < 0.001$) to 184.07 ± 23.69 s at 13:00 h. The overall mean values of TI duration differed significantly ($P < 0.0001$) between the groups, with the highest mean duration of 167.82 ± 8.35 s being recorded in group III broiler chickens administered with melatonin (Table 4.35).

The results of the vigilance behavioural rankings of the broilers are shown in Table 4.34. Unlike the results obtained on TI duration, similar to that recorded in the number of induction attempts, no significant difference was found in the values of mean vigilance behaviour rankings at the different hours of the day. The overall mean vigilance behaviour ranking values of 1.85 $\pm$ 0.07 and 1.70 $\pm$ 0.08, obtained in 12D:12L cycle and CL broiler chickens, respectively were higher ($P < 0.05$) than the value of 1.44 $\pm$ 0.05 recorded in melatonin-treated birds (Table 4.35).

Table 4.32: Effects of Lighting Regimens and Melatonin Administration on Diurnal Variations in the Number of Induction Attempts of Marshall Broiler Chickens during the Hot-dry Season (Mean ± SEM; n = 15)

Age, weeks	Hour of the day, h	Group		
		12D:12L	CL	CL+ MEL
IV	06:00	1.87 ± 0.19	1.27 ± 0.12	1.07 ± 0.07
	13: 00	1.93 ± 0.32	1.40 ± 0.16	1.23 ± 0.09
	19:00	1.67 ± 0.19	1.74 ± 0.25	1.13 ± 0.09
VI	06:00	1.73 ± 0.18	1.18 ± 0.09	1.20 ± 0.11
	13: 00	1.89 ± 0.24	1.40 ± 0.16	1.49 ± 0.19
	19:00	1.67 ± 0.19	1.47 ± 0.17	1.33 ± 0.16
VIII	06:00	2.07 ± 0.3	1.67 ± 0.19	1.24 ± 0.11
	13: 00	2.13 ± 0.34	2.15 ± 0.22	1. 27 ± 0.12
	19:00	1.75 ± 0.21	2.00 ± 0.22	1.21 ± 0.22

12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

Table 4.33: Effects of Light Regimens and Melatonin Administration on Diurnal Variations in Tonic Immobility Duration in Marshall Broiler Chickens during the Hot-dry Season (Mean ± SEM; n = 15)

Age, weeks	Hour of the day, h	Group		
		12D:12L	CL	CL + MEL
IV	06:00	43.33 ± 9.5	83.73 ± 8.25	117.40 ± 19.93
	13: 00	73.60 ± 15. 99	88.27 ± 9.08	162.93 ± 26.79
	19:00	53.27 ± 11.03	95.20 ± 9.53	112.07 ± 14.80
VI	06:00	76.27 ± 11.37	101.87 ± 10.24[a]	120.27 ± 10.40
	13: 00	98.2 ± 13.21	184.07 ± 23.69[b]	135.47 ± 21.22
	19:00	92.6 ± 14.78	118.07 ± 14.21[a]	115.73 ± 27.06
VIII	06:00	54.33 ± 8.42	192.80 ± 29.34	226.0 ± 20.63
	13: 00	68.53 ± 10.87	154. 67 ± 27.55	254.27 ± 20.07
	19:00	61.53 ± 9.4	151.53 ± 30.09	266.27 ± 15.61

[a, b] = Means in the same column having different superscript letters are significantly ($P < 0.05$) different. 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

Table 4.34: Effects of Age, Light Regimens and Melatonin Administration on Diurnal Fluctuations in Vigilant Behaviour of Marshall Broiler Chickens during the Hot-dry Season (Mean ± SEM; n = 15)

Age, weeks	Hour of the day, h	Group		
		12D:12L	CL	CL + MEL
IV	06:00	1.73 ± 0.21	1.60 ± 0.19	1.53 ± 0.19
	13:00	1.83 ± 0.24	1.67 ± 0.18	1.35 ± 0.13
	19:00	1.72 ± 0.18	1.66 ± 0.16	1.47 ± 0.13
VI	06:00	2.07 ± 0.18	1.40 ± 1.13	1.48 ± 1.2
	13:00	1.93 ± 0.23	1.47 ± 0.17	1.20 ± 0.15
	19:00	2.21 ± 0.23	1.93 ± 0.23	1.33 ± 0.13
VIII	06:00	1.65 ± 0.24	1.86 ± 0.19	1.55 ± 0.19
	13:00	1.67 ± 0.21	2.27 ± 0.52	1.60 ± 0.21
	19:00	1.78 ± 0.18	1.49 ± 0.13	1.53 ± 0.19

12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

Table 4.35: Effects of Age, Light Regimens and Melatonin Administration on Overall Mean Tonic Immobility Responses in Marshall Broiler Chickens during the Hot-dry Season (Mean ± SEM; n = 15)

Parameter	Age, weeks	Group		
		12D:12L	CL	CL + MEL
TI induction attempts	IV	1.82 ± 0.14	1.47 ± 0.11	1.11 ± 0.05
	VI	1.78 ± 0.12	1.33 ± 0.08	1.33 ± 0.09
	VIII	1.98 ± 0.16	1.93 ± 0.12	1.22 ± 0.06
	Overall mean	1.85 ± 0.80^{c}	1.58 ± 0.06^{b}	1.22 ± 0.4^{a}
TI duration, s	IV	56.73 ± 7.29	89.07 ± 5.11	130.8 ± 12.38
	VI	89.02 ± 7.57	134.67 ± 10.99	123.82 ± 11.77
	VIII	61.47 ± 5.50	166.33 ± 16.61	248.84 ± 10.96
	Overall mean	69.07 ± 4.11^{a}	130.02 ± 7.33^{b}	167.82 ± 8.35^{c}
Vigilance behaviour	IV	1.76 ± 0.11	162.0 ± 0.10	1.44 ± 0.09
	VI	2.09 ± 0.12	1.60 ± 0.11	1.31 ± 0.08
	VIII	1.71 ± 0.11	1.87 ± 0.19	1.56 ± 0.11
	Overall mean	1.83 ± 0.07^{b}	1.70 ± 0.08^{b}	1.44 ± 0.05^{a}

$^{a, b, c}$ = Means in the same row having different superscript letters are significantly ($P < 0.05$) different; TI = Tonic Immobility; 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

4.5.6 Open-field Activity

The hourly fluctuations in the open-field activities of Marshall broiler chickens, obtained at different ages are shown in Tables 4.36 – 4.38). Escape jumps, first calls, number of steps and especially latency to move fluctuated significantly ($P < 0.05$) as the hours of the day increased in birds raised on 12D:12L cycle and those on CL with melatonin administration (Tables 4.36 and 4.38). However, no significant hourly fluctuation was recorded when the birds in both groups were 6 weeks' old. The value of the hourly fluctuation recorded in the groups raised on CL was 200 % higher than that obtained from either the group raised on 12D:12L cycle or CL with melatonin administration (Table 4.39). Unlike the results obtained from the 12D:12L and CL+MEL groups, the values recorded in the CL group showed significant ($P < 0.05$) hourly fluctuations in all the ages.

The agoraphobia of the broiler chickens differed significantly ($P < 0.001$) between the different groups in the present study (Table 4.39). The duration of time before first call (vocalisation) and first step taken (latency to move) was significantly ($P < 0.0001$) higher in broiler chickens administered with melatonin than that obtained in either 12D:12L or CL group, not administered with melatonin. Conversely, the numbers of lines crossed, steps, escape jumps, distress calls and faecal boluses were significantly ($P < 0.001$) higher in birds belonging to the two groups that were not administered with melatonin, compared to the melatonin-treated group (Table 4.39). Broiler chickens belonging to the CL groups were more active compared to those on 12D:12L cycle (Table 4: 39). 12D:12L cycle birds significantly ($P < 0.001$) crossed more lines, made more steps and escape attempts than those in any other group. However, the highest distress call with a score of 2.62 ± 0.07 was obtained in broiler chickens belonging to the 12D:12L cycle. Table 4.39 shows that the

numbers of faecal boluses of 1.00 ± 0.08 and 1.23 ± 0.10 obtained in 12D:12L cycle and CL groups, respectively were higher ($P < 0.05$) than 0.67 ± 0.07, recorded in broiler chickens treated with melatonin.

Table 4.36: Diurnal Variations in Open-field Activity of Marshall Broiler Chickens Raised on 12 h Darkness and 12 h Light Cycle at 4, 6 and 8 Week of Age during the Hot-dry Season (Mean ± SEM; n = 15)

Age, weeks	Hour of the day, h	LM, s	LC	NS	EJ	FC, s	DC	FB
IV	06:00	21.40 ± 3.79	4.60 ± 0.58	9.93 ± 0.95	0.40 ± 0.13[a]	6.87 ± 0.68	2.80 ± 0.11	1.27 ± 0.18
	13:00	28.47 ± 8.21	9.07 ± 2.84	10.40 ± 0.58	0.80 ± 0.20[ab]	7.00 ± 0.66	2.80 ± 0.11	1.27 ± 0.21
	19:00	26.53 ± 4.81	6.80 ± 1.17	12.07 ± 1.63	1.13 ± 0.31[b]	7.00 ± 0.58	2.87 ± 0.09	1.73 ± 0.28
	Overall Mean ± SEM	25.47 ± 3.37	6.82 ± 1.06	10.80 ± 0.66	0.78 ± 0.13	6.96 ± 0.36	2.82 ± 0.06	1.42 ± 0.13
VI	06:00	34.27 ± 5.78	5.27 ± 0.54	8.40 ± 0.65	0.60 ± 0.13	9.53 ± 1.15	2.93 ± 0.07	0.80 ± 0.22
	13:00	45.80 ± 9.14	10.00 ± 2.47	9.67 ± 0.94	0.60 ± 0.21	12.33 ± 1.27	2.53 ± 0.26	1.00 ± 0.20
	19:00	29.20 ± 5.35	8.13 ± 1.10	7.60 ± 0.61	1.00 ± 0.24	11.60 ± 1.15	2.73 ± 0.15	0.93 ± 0.25
	Overall Mean ± SEM	36.42 ± 4.07	7.80 ± 0.94	8.56 ± 0.44	0.73 ± 0.12	11.16 ± 0.70	2.73 ± 0.10	0.91 ± 0.13
VIII	06:00	36.80 ± 5.55[ab]	6.33 ± 0.53	7.47 ± 0.68	0.73 ± 0.23	11.07 ± 1.08[a]	2.33 ± 0.25	0.53 ± 0.19
	13:00	42.53 ± 5.67[b]	9.13 ± 1.67	10.67 ± 1.61	0.60 ± 0.21	15.93 ± 2.29[ab]	2.40 ± 0.27	0.87 ± 0.24
	19:00	24.20 ± 5.05[a]	6.93 ± 0.49	8.67 ± 1.12	0.73 ± 0.23	17.27 ± 2.24[b]	2.20 ± 0.28	0.60 ± 0.21
	Overall Mean ± SEM	34.51 ± 3.27	7.47 ± 0.62	8.93 ± 0.71	0.69 ± 0.13	14.76 ± 1.17	2.31 ± 0.15	0.67 ± 0.12

LM: Latency to move; LC: Lines crossed; NS: Number of steps; EJ: Escape jumps; FC: First call; DC: Distress calls (scores); FB: Faecal boluses; [a, b, c] = Means in the same column having different superscript letters are significantly ($P < 0.05$) different

Table 4.37: Diurnal Variations in Open-field Activity of Marshall Broiler Chickens Raised on Continuous Lighting at 4, 6 and 8 Week of Age during the Hot-dry Season (Mean ± SEM; n = 15)

Age, weeks	Hour of the day, h	LM, s	LC	NS	EJ	FC, s	DC	FB
IV	06:00	28.33 ± 7.70	6.60 ± 1.30^{a}	10.60 ± 1.25	1.07 ± 0.21	8.93 ± 0.71^{b}	2.60 ± 0.24	1.47 ± 0.32
	13:00	48.67 ± 8.90	10.80 ± 1.31^{ab}	7.73 ± 0.88	1.00 ± 0.26	6.13 ± 0.69^{a}	1.93 ± 0.30	1.73 ± 0.27
	19:00	39.07 ± 9.02	11.33 ± 1.93^{b}	9.80 ± 1.29	0.73 ± 0.18	7.73 ± 0.86^{ab}	2.07 ± 0.30	1.53 ± 0.35
	Overall Mean ± SEM	38.69 ± 4.99	9.58 ± 0.93	9.38 ± 0.68	0.93 ± 0.13	7.60 ± 0.46	2.20 ± 1.16	1.58 ± 1.18
VI	06:00	42.40 ± 7.62^{b}	9.00 ± 1.32^{a}	12.53 ± 1.31	0.87 ± 0.22^{a}	8.73 ± 0.61^{a}	1.87 ± 0.32	1.20 ± 0.34
	13:00	15.53 ± 2.04^{a}	12.60 ± 1.43^{ab}	9.73 ± 0.83	1.07 ± 0.21^{ab}	12.93 ± 1.25^{b}	2.33 ± 0.21	0.87 ± 0.22
	19:00	27.87 ± 5.20^{ab}	14.47 ± 1.35^{b}	10.80 ± 0.95	1.67 ± 0.30^{b}	10.53 ± 0.77^{ab}	2.33 ± 0.25	1.33 ± 0.27
	Overall Mean ± SEM	28.60 ± 3.49	12.02 ± 0.85	11.02 ± 0.62	1.20 ± 0.15	10.73 ± 0.58	2.18 ± 0.15	1.13 ± 0.16
VIII	06:00	48.40 ± 6.99^{b}	8.07 ± 0.93	12.53 ± 0.80^{a}	1.13 ± 0.22	8.53 ± 0.95^{a}	2.40 ± 0.25	1.33 ± 0.42
	13:00	30.40 ± 6.99^{ab}	11.07 ± 1.50	15.53 ± 1.06^{b}	0.87 ± 0.22	11.33 ± 1.31^{ab}	2.73 ± 0.15	1.00 ± 0.24
	19:00	26.67 ± 4.09^{a}	11.27 ± 1.54	10.53 ± 0.68^{a}	1.07 ± 0.27	14.93 ± 1.79^{b}	2.53 ± 0.24	0.60 ± 0.21
	Overall Mean ± SEM	35.33 ± 3.76	10.13 ± 0.79	12.87 ± 0.58	1.02 ± 0.13	11.60 ± 0.88	2.56 ± 0.13	0.98 ± 0.18

LM: Latency to move; LC: Lines crossed; NS: Number of steps; EJ: Escape jumps; FC: First call; DC: Distress calls (scores); FB: Faecal boluses; $^{a, b, c}$ = Means in the same column having different superscript letters are significantly ($P < 0.05$) different

Table 4.38: Diurnal Variations in Open-field Activity of Marshall Broiler Chickens Raised on Continuous Lighting and administered with Melatonin at 4, 6 and 8 Week of Age during the Hot-dry Season (Mean ± SEM; n = 15)

Age, weeks	Hour of the day, h	LM, s	LC	NS	EJ	FC, s	DC	FB
IV	06:00	87.07 ± 5.77[b]	7.40 ± 1.15	8.13 ± 1.09	0.33 ± 0.13	26.07 ± 4.5	1.20 ± 0.30	1.00 ± 0.28
	13:00	64.40 ± 7.16[a]	8.07 ± 0.76	9.80 ± 0.75	0.27 ± 0.12	21.93 ± 4.07	1.47 ± 0.34	0.53 ± 0.17
	19:00	82.47 ± 6.05[ab]	8.60 ± 0.94	9.13 ± 0.89	0.33 ± 0.13	28.13 ± 4.88	1.47 ± 0.31	0.60 ± 0.19
	Overall Mean ± SEM	77.98 ± 3.87	8.02 ± 0.55	9.02 ± 0.53	0.31 ± 0.07	25.38 ± 2.57	1.38 ± 0.18	0.71 ± 0.13
VI	06:00	92.93 ± 6.05	6.67 ± 0.86	6.07 ± 1.03	0.53 ± 0.13	35.47 ± 5.91	0.87 ± 0.26	0.60 ± 0.19
	13:00	93.87 ± 6.99	7.00 ± 1.10	7.93 ± 1.23	0.40 ± 0.13	25.73 ± 4.84	0.93 ± 0.30	0.53 ± 0.17
	19:00	85.80 ± 6.85	8.00 ± 1.06	8.00 ± 1.00	0.47 ± 0.13	31.13 ± 4.89	1.00 ± 0.29	0.80 ± 0.24
	Overall Mean ± SEM	90.87 ± 3.79	7.22 ± 0.58	7.33 ± 0.63	0.47 ± 0.08	30.78 ± 3.01	0.93 ± 0.16	0.64 ± 0.12
VIII	06:00	107.13 ± 4.96[b]	4.53 ± 0.58	6.00 ± 0.59[a]	1.13 ± 0.22	38.33 ± 6.12	1.20 ± 0.33	0.67 ± 0.23
	13:00	89.40 ± 5.81[a]	6.07 ± 0.67	8.73 ± 0.75[b]	0.87 ± 0.22	22.87 ± 4.78	0.93 ± 0.32	0.67 ± 0.19
	19:00	98.07 ± 6.20[ab]	6.27 ± 0.69	9.00 ± 0.76[b]	1.07 ± 0.27	25.00 ± 4.86	1.00 ± 0.29	0.60 ± 0.24
	Overall Mean ± SEM	98.20 ± 3.38	5.62 ± 0.38	7.91 ± 0.45	1.02 ± 0.13	28.73 ± 3.16	1.04 ± 0.18	0.64 ± 0.12

[a, b, c] = Means in the same column having different superscript letters are significantly ($P < 0.05$) different; LM: Latency to move.
LC: Lines crossed; NS: Number of steps; EJ: Escape jumps; FC: First call; DC: Distress calls (scores); FB: Faecal boluses

Table 4.39: Overall Open-field Activity of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM; n = 15)

Parameter	Group		
	12D:12L cycle	CL	CL + MEL
Latency to move, s	32.13 ± 2.10^{a}	34.21 ± 2.40^{a}	89.01 ± 2.23^{b}
Lines crossed	7.36 ± 0.51^{a}	10.58 ± 0.50^{b}	6.96 ± 0.31^{a}
Number of steps	9.43 ± 0.36^{b}	11.09 ± 0.38^{c}	8.09 ± 0.32^{a}
Escape jumps	0.73 ± 0.07^{a}	1.05 ± 0.08^{b}	0.60 ± 0.06^{a}
First call, s	10.96 ± 0.54^{a}	9.98 ± 0.41^{a}	28.30 ± 1.69^{b}
Distress calls (Scores)	2.62 ± 0.07^{c}	2.31 ± 0.09^{b}	1.12 ± 0.10^{a}
Faecal boluses	1.00 ± 0.08^{b}	1.23 ± 0.10^{b}	0.67 ± 0.07^{a}

$^{a, b, c}$ = Means in the same row having different superscript letters are significantly ($P < 0.05$) different; 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration

4.6 RESULTS OF STUDIES ON TRANSPORTATION STRESS

4.6.1 Effects of Transportation by Road on Plasma Biochemical Parameters of Marshall Broiler Chickens

The results of plasma hormone profiles in Marshall broiler chickens are presented in Table 4.40. The mean values of T_3, T_4, T_3:T_4 ratio and TSH recorded before and after transportation were consistently higher in group III broilers, administered with melatonin than the corresponding values recorded in groups I and II that were not administered with melatonin. The mean corticosterone levels, unlike the results of thyroid hormones were lower in the broilers treated with melatonin in group III, compared to their counterparts in groups I and II that were not administered with melatonin. In all the groups, no significant ($P > 0.05$) difference was found between pre- and post-transportation corticosterone values. In group I broilers, post-transportation values of T_3:T_4 ratio and TSH were significantly ($P < 0.05$) higher than pre-transportation values. The post-transportation T_4 value of 72.13 ± 1.76 ng/ml recorded in group II birds, raised on continuous lighting without melatonin administration, was significantly higher than that of 61.93 ± 3.71 ng/ml, obtained pre-transportation. Post-transportation values of 1.50 ± 0.13 ng/ml, 0.02 ± 0.001 and 3.34 ± 0.09 miμ/ml recorded for T_3, T_3:T_4 and TSH, respectively in group III birds, treated with melatonin, were significantly higher than their corresponding pre-transportation values (Table 4.40).

Table 4.41 shows that the pre- and post-transportation values recorded for total protein, albumin, urea, HDL and total cholesterol did not differ significantly ($P > 0.05$) in all the groups. The post-transportation MDA values of 2.99 ± 0.20 μmol/ml and 2.57 ± 0.21 μmol/ml recorded in CL and CL + melatonin groups, respectively were significantly ($P <$

0.05) higher than the respective pre-transportation values. A significant ($P < 0.05$) decrease in triglyceride values was recorded after transportation in groups I and III (Table 4.41). When the results were compared between the groups, the least significant mean values of MDA, triglyceride, total cholesterol, albumin and total protein were obtained in group III broiler chickens, raised on CL and administered with melatonin. Similarly, the highest significant ($P < 0.05$) values of urea and HDL of 4.66 ± 0.20 mmol/l and 1.28 ± 0.06 mmol/l, respectively were recorded in broilers reared on CL and administered with melatonin.

Table 4.42 shows that pre-transportation Na^+ concentration of 137.73 ± 0.04 mmol/l decreased significantly ($P < 0.05$) to 136.87 ± 0.92 mmol/l following transportation in group II birds that were raised on CL without melatonin administration. Conversely, K^+ concentration increased from 3.81 ± 0.15 mmol/l to 4.07 ± 0.10 mmol/l post-transportation in group I broiler chickens, raised on 12D:12L cycle without melatonin administration (Table 4.42). Unlike the results obtained from birds in groups I and II, road transportation had no significant effect on all the plasma electrolytes in group III birds, raised on CL and administered with melatonin.

Acitivities of plasma enzymes, including AST, ALP, ALT, CK, SOD and GPx in Marshall broiler chickens, administered with melatonin are presented in Table 4.43. Generally, AST, ALT, CK and ALP activities rose significantly ($P < 0.05$) after transportation in all the groups. However, the activities of the endogenous enzymes, with the exception of SOD in group III birds treated with melatonin, were not significantly ($P > 0.05$) altered by road transportation (Table 4.43). Group III birds raised on CL and administered with melatonin consistently had lower ($P < 0.05$) AST, ALP, ALT and CK values, but consistently higher (P

< 0.05) GPx and SOD values, compared to the corresponding activities recorded in groups I and II, not administered with melatonin.

4.6.2 Effects of Transportation by Road on the Brain Superoxide Dismutase Activities and Malondialdehyde Concentration of Marshall Broiler Chickens

The brain SOD and GPx activities did not differ significantly ($P > 0.05$) before and after road transportation in all the three groups (Table 4.44). Tissue protein increased significantly ($P < 0.05$) after transportation from 5.68 ± 0.51 g/dl, 7.40 ± 0.22 g/dl and 7.10 ± 0.14 g/dl to 8.18 ± 0.21 g/dl, 7.58 ± 0.10 g/dl and 7.36 ± 0.20 g/dl in 12D:12L, CL, and CL + melatonin groups, respectively. The MDA concentration of 3.53 ± 0.11 μmol/ml obtained after transportation in group III broiler chickens administered with melatonin was not significantly ($P > 0.05$) different from the pre-transportation concentration of 2.69 ± 0.11 μmol/ml. Brain MDA post-transportation concentration of 4.07 ± 0.12 μmol/ml, recorded in 12D:12L group was significantly higher ($P < 0.05$), but the post-transportation concentration of 3.83 ± 0.09 μmol/ml, recorded in the CL group was significantly ($P < 0.05$) lower than the corresponding concentrations obtained pre-transportation (Table 4.44). The brain of broilers in group III administered with melatonin had higher ($P < 0.05$) SOD and GPx activities, but significantly ($P < 0.05$) lower MDA values, when compared to the corresponding values obtained in the other groups not administered with melatonin.

4.6.3 Comparison between Brain and Blood Oxidative Stress Biomakers in Marshall Broiler Chickens.

Table 4.45 compares blood plasma and brain activities of SOD, GPx, and MDA and protein concentration in broiler chickens before transportation. The results obtained in all the groups showed that SOD activity was consistently higher ($P < 0.05$) in the plasma than in the brain. However, brain GPx activities were significantly ($P < 0.05$) higher in all the groups, when compared to the corresponding activities of the enzyme obtained in the plasma of the same birds, either pre- or post-transportation. Higher MDA concentrations were obtained from the brain samples, especially in group I broilers raised on 12D:12L cycle, and the brain activity of 2.49 ± 0.06 IU/ml, recorded was significantly ($P < 0.05$) higher than that of 2.41 ± 0.18 IU/ml obtained in the plasma. No significant difference was recorded between the plasma and brain protein concentrations, recorded in melatonin-treated birds. In group II birds raised on CL, brain protein concentration of 7.40 ± 0.22 g/dl was significantly ($P < 0.05$) higher than that of 6.97 ± 0.11 g/dl, obtained in the plasma. Conversely, plasma protein concentration of 7.36 ± 0.13 g/dl was higher ($P < 0.05$) than the concentration of 5.68 ± 0.51 g/dl recorded in the brain of group I birds, raised on 12D:12L cycle (Table 4.45). Table 4.46 shows that after the transportation, the SOD activities of 2.63 ± 0.12 IU/ml and 2.05 ± 0.07 IU/ml obtained in the blood and brain, respectively in group II broilers were not different ($P > 0.05$).

The GPx activities recorded after transportation, with higher concentration in brain tissues, follows a similar pre-transportation pattern as shown in Table 4.45. The MDA concentrations were higher in brain tissues than plasma, especially in groups II and III broiler chickens, where the difference in the values was highly significant ($P < 0.001$) (Table 4.46). When

compared to the results obtained for GPx and MDA in the present study, the brain protein values were higher than the corresponding values recorded in all the groups, especially in group III that were raised on CL and administered with melatonin, where a significant ($P < 0.05$) difference was recorded (Table 4.46). The relationships between plasma and brain for each biomarker are as shown in Table 4.47. Significant correlation was found only between plasma and brain SOD activities after transportation in group III Marshall broiler chickens, administered with melatonin (Fig. 4.18).

Table 4.40: Concentrations of Plasma Hormone Profiles in Marshall Broiler Chickens transported by Road and administered with Melatonin during the Hot-dry Season (n = 15)

Parameter	Period	Group		
		12D:12L cycle	CL	CL + Melatonin
Triiodothyronine, (T_3), ng/ml:				
	Pre-transportation	$0.76 \pm 0.07^{a(1)}$	$0.79 \pm 0.06^{a(1)}$	$0.91 \pm 0.05^{a(1)}$
	Post-transportation	$1.13 \pm 0.09^{a(1)}$	$0.87 \pm 0.07^{a(1)}$	$1.50 \pm 0.13^{b(2)}$
Thyroxine,(T_4), ng/ml:				
	Pre-transportation	$58.73 \pm 3.67^{a(1)}$	$61.93 \pm 3.7^{a(1)}$	$73.2 \pm 2.94^{b(1)}$
	Post-transportation	$67.27 \pm 3.40^{a(1)}$	$72.13 \pm 1.76^{a(2)}$	$84.33 + 3.20^{b(1)}$
T_3:T_4, ng/ml:				
	Pre-transportation	$0.10 \pm 0.001^{a(1)}$	$0.01 \pm 0.001^{a(1)}$	$0.01 \pm 0.001^{a(1)}$
	Post-transportation	$0.02 \pm 0.001^{b(2)}$	$0.01 \pm 0.001^{a(1)}$	$0.02 \pm 0.001^{b(2)}$
Thyroid-stimulating hormone, miu/ml:				
	Pre-transportation	$2.23 \pm 0.25^{a(1)}$	$2.37 \pm 0.17^{a(1)}$	$2.87 \pm 0.30^{a(1)}$
	Post-transportation	$2.32 \pm 0.10^{a(2)}$	$2.46 \pm 0.17^{a(1)}$	$3.34 \pm 0.09^{b(2)}$
Corticosterone, ρg/ml:				
	Pre-transportation	$683.80 \pm 30.29^{b(1)}$	$634.47 \pm 20.13^{b(1)}$	$538.60 \pm 14.02^{a(1)}$
	Post-transportation	$745.73 \pm 22.86^{b(1)}$	$772.73 \pm 17.74^{b(1)}$	$680.73 \pm 22.35^{a(1)}$

^{a,b,c} = Means in the same row having different superscript letters are significantly ($P < 0.05$) different; ^{1,2} = Means within the same column having different superscript numbers are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + Melatonin = Continuous lighting with melatonin administration

Table 4.41: Concentrations of Plasma Protein and Lipid Profiles in Marshall Broiler Chickens transported by Road and administered with Melatonin during the Hot-dry Season (n = 15)

Parameter	Period	Group		
		12D:12L cycle	CL	CL + Melatonin
Total protein, g/dl:				
	Pre-transportation	$7.36 \pm 0.13^{b(1)}$	$6.97 \pm 0.11^{a(1)}$	$6.85 \pm 0.10^{a(1)}$
	Post-transportation	$7.20 \pm 0.17^{b(1)}$	$6.73 \pm 0.12^{a(1)}$	$6.82 \pm 0.10^{a(1)}$
Albumin, g/dl:				
	Pre-transportation	$4.05 \pm 0.10^{b(1)}$	$3.93 \pm 0.10^{ab(1)}$	$3.71 \pm 0.13^{a(1)}$
	Post-transportation	$3.81 + 0.11^{a(1)}$	$3.77 \pm 0.10^{a(1)}$	$3.52 \pm 0.14^{a(1)}$
Urea, mmol/l:				
	Pre-transportation	$3.34 \pm 0.22^{a(1)}$	$3.48 \pm 0.24^{a(1)}$	$4.66 \pm 0.20^{b(1)}$
	Post-transportation	$3.33 \pm 0.16^{a(1)}$	$3.21 \pm 0.19^{a(1)}$	$3.93 \pm 0.10^{b(1)}$
High-density lipoprotein, mmol/l:				
	Pre-transportation	$0.90 \pm 0.08^{a(1)}$	$1.13 \pm 0.06^{ab(1)}$	$1.28 \pm 0.06^{b(1)}$
	Post-transportation	$1.03 \pm 0.06^{a(1)}$	$1.08 \pm 0.05^{a(1)}$	$1.18 \pm 0.08^{a(1)}$
Total cholesterol, mmol/l:				
	Pre-transportation	$3.95 \pm 0.13^{b(1)}$	$3.79 \pm 0.12^{b(1)}$	$3.35 \pm 0.15^{a(1)}$
	Post-transportation	$4.20 \pm 0.12^{b(1)}$	$4.01 \pm 0.13^{b(1)}$	$3.50 \pm 0.14^{a(1)}$
Triglycerides, mmol/l:				
	Pre-transportation	$1.28 \pm 0.08^{b(2)}$	$1.10 \pm 0.07^{ab(1)}$	$1.00 \pm 0.08^{a(2)}$
	Post-transportation	$1.15 \pm 0.05^{b(1)}$	$1.04 \pm 0.06^{ab(1)}$	$0.90 \pm 0.06^{a(1)}$
Malondialdehyde, µmol/ml:				
	Pre-transportation	$2.41 \pm 0.18^{b(1)}$	$2.08 \pm 0.12^{b(1)}$	$1.51 \pm 0.11^{a(1)}$
	Post-transportation	$3.66 \pm 0.18^{b(1)}$	$2.99 \pm 0.20^{a(2)}$	$2.57 \pm 0.21^{a(2)}$

^{a,b,c} = Means in the same row having different superscript letters are significantly ($P < 0.05$) different; ^{1,2} = Means within the same column having different superscript numbers are significantly ($P < 0.05$) different;

12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + Melatonin = Continuous lighting with melatonin administration

Table 4.42: Concentrations of Plasma Electrolytes in Marshall Broiler Chickens transported by Road and administered with Melatonin during the Hot-dry Season (n = 15)

Parameter	Period	Group		
		12D:12L cycle	CL	CL + Melatonin
Na^+, mmol/l:				
	Pre-transportation	$136.33 \pm 0.83^{a(1)}$	$137.73 \pm 0.48^{ab(2)}$	$139.47 \pm 0.76^{b(1)}$
	Post-transportation	$138.73 \pm 0.77^{a(1)}$	$136.87 \pm 0.92^{a(1)}$	$138.73 \pm 0.55^{a(1)}$
K^+, mmol/l:				
	Pre-transportation	$3.81 \pm 0.15^{a(1)}$	$3.81 \pm 0.14^{a(1)}$	$3.80 \pm 0.11^{a(1)}$
	Post-transportation	$4.07 \pm 0.10^{a(2)}$	$3.95 \pm 0.14^{a(1)}$	$3.53 \pm 0.13^{a(1)}$
Cl^-, mmol/l:				
	Pre-transportation	$95.73 \pm 0.99^{a(1)}$	$96.40 \pm 0.68^{a(1)}$	$98.67 \pm 0.50^{b(1)}$
	Post-transportation	$98.40 \pm 0.76^{b(1)}$	$96.00 \pm 0.98^{a(1)}$	$99.47 \pm 0.66^{b(1)}$

^{a,b,c} = Means in the same row having different superscript letters are significantly ($P < 0.05$) different; ^{1,2} = Means within the same column having different superscript numbers are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + Melatonin = Continuous lighting with melatonin administration

Table 4.43: Activities of Plasma Enzymes in Marshall Broiler Chickens transported by Road and administered with Melatonin during the Hot-dry Season (n = 15)

Parameter	Period	Group		
		12D:12L cycle	CL	CL + Melatonin
AST, iu/l:				
	Pre-transportation	19.13 ± 1.33$^{a(1)}$	16.73 ± 0.57$^{a(1)}$	16.33 ± 1.26$^{a(1)}$
	Post-transportation	24.47 ± 2.22$^{b(2)}$	28.60 ± 1.72$^{b(2)}$	17.87 ± 1.84$^{a(1)}$
ALT, iu/l:				
	Pre-transportation	38.80 ± 3.04$^{a(2)}$	36.47 ± 1.58$^{a(1)}$	32.27 ± 1.69$^{a(1)}$
	Post-transportation	38.00 ± 1.98$^{a(1)}$	55.87 ± 3.35$^{b(2)}$	36.13 ± 1.98$^{a(1)}$
ALP, iu/l:				
	Pre-transportation	61.47 ± 3.01$^{ab(1)}$	64.27 ± 2.98$^{b(1)}$	54.27 ± 2.06$^{a(1)}$
	Post-transportation	62.80 ± 2.81$^{a(2)}$	71.87 ± 1.93$^{b(1)}$	63.40 ± 1.45$^{a(2)}$
CK, iu/l:				
	Pre-transportation	132.73 ± 2.63$^{b(1)}$	141.00 ± 5.97$^{b(2)}$	119.53 ± 2.60$^{a(1)}$
	Post-transportation	157.07 ± 6.22$^{b(2)}$	125.53 ± 1.73$^{a(1)}$	124.73 ± 4.73$^{a(2)}$
GPx, iu/ml:				
	Pre-transportation	32.33 ± 1.71$^{a(1)}$	34.60 ± 1.73$^{ab(1)}$	38.13 ± 1.41$^{b(1)}$
	Post-transportation	36.40 ± 2.17$^{a(1)}$	39.87 ± 1.92$^{ab(1)}$	43.67 ± 1.88$^{b(1)}$
SOD, iu/ml:				
	Pre-transportation	1.56 ± 0.18$^{a(1)}$	1.77 ± 0.14$^{ab(1)}$	2.18 ± 0.14$^{b(1)}$
	Post-transportation	2.36 ± 0.14$^{a(1)}$	2.63 ± 0.12$^{a(1)}$	3.42 ± 0.38$^{b(2)}$

^{a,b,c} = Means in the same row having different superscript letters are significantly ($P < 0.05$) different; ^{1,2} = Means within the same column having different superscript numbers are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + Melatonin = Continuous lighting with melatonin administration

Table 4.44: Effects of Road Transportation on Oxidative Stress Biomarkers in Brain Homogenates of Marshall Broiler Chickens administered with Melatonin under different lighting regimens

Parameter	Period	Group		
		12D:12L cycle	CL	CL + Melatonin
SOD, IU/ml:				
	Pre-transportation	$1.42 \pm 0.06^{(1)a}$	$1.40 \pm 0.05^{(1)a}$	$2.07 \pm 0.07^{(1)b}$
	Post-transportation	$1.27 \pm 0.08^{(1)a}$	$2.05 \pm 0.07^{(1)b}$	$2.28 \pm 0.06^{(1)c}$
GPx, IU/ml:				
	Pre-transportation	$33.80 \pm 0.94^{(1)a}$	$34.87 \pm 1.13^{(1)a}$	$48.13 \pm 1.31^{(1)b}$
	Post-transportation	$39.53 \pm 1.01^{(1)a}$	$41.13 \pm 1.05^{(1)a}$	$51.53 \pm 0.60^{(1)b}$
MDA, µmol/ml:				
	Pre-transportation	$2.49 \pm 0.06^{(1)a}$	$4.17 \pm 0.20^{(2)b}$	$2.69 \pm 0.11^{(1)a}$
	Post-transportation	$4.07 \pm 0.12^{(2)b}$	$3.83 \pm 0.09^{(1)ab}$	$3.53 \pm 0.11^{(1)a}$
Tissue Protein, g/dl:				
	Pre-transportation	$5.68 \pm 0.51^{(1)a}$	$7.40 \pm 0.22^{(1)b}$	$7.10 \pm 0.14^{(1)b}$
	Post-transportation	$8.18 \pm 0.21^{(2)b}$	$7.58 \pm 0.10^{(2)a}$	$7.36 \pm 0.20^{(2)a}$

^{a,b,c} = Means in the same row having different superscript letters are significantly ($P < 0.05$) different; ^{1,2} = Means within the same column having different superscript numbers are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + Melatonin = Continuous lighting with melatonin administration. SOD = Superoxide dismutase; GPx = Glutathione peroxidase; MDA = Malondialdehyde

Table 4.45: Comparison between some Brain and Plasma Oxidative Stress Biomarkers in Marshall Broiler Chickens administered with Melatonin before Road Transportation

Parameter		Group		
		12D:12L cycle	CL	CL + Melatonin
SOD, IU/ml:	*Plasma*	1.56 ± 0.18^{b}	1.77 ± 0.14^{b}	2.18 ± 0.13^{b}
	Brain	1.42 ± 0.06^{a}	1.40 ± 0.05^{a}	2.07 ± 0.07^{a}
GPx, IU/ml:	*Plasma*	32.33 ± 1.71^{a}	34.60 ± 1.73^{a}	38.13 ± 1.40^{a}
	Brain	33.80 ± 0.94^{b}	34.87 ± 1.13^{b}	48.13 ± 1.31^{a}
MDA, µmol/ml:	*Plasma*	2.41 ± 0.18^{a}	2.08 ± 0.12^{a}	1.51 ± 0.11^{a}
	Brain	2.49 ± 0.06^{b}	4.17 ± 0.20^{a}	2.69 ± 0.11^{a}
Protein, g/dl:	*Plasma*	7.36 ± 0.13^{b}	6.97 ± 0.11^{a}	6.85 ± 0.10^{a}
	Brain	5.68 ± 0.51^{a}	7.40 ± 0.22^{b}	7.10 ± 0.14^{a}

^{a,b} = Means in the same column having different superscript letters are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + Melatonin = Continuous lighting with melatonin administration. SOD = Superoxide dismutase; GPx = Glutathione peroxidase; MDA = Malondialdehyde

Table 4.46: Comparison between some Brain and Plasma Oxidative Stress Biomarkers in Marshall Broilers administered with Melatonin after Road Transportation

Parameter		Group		
		12D:12L cycle	CL	CL + Melatonin
SOD, IU/ml:				
	Plasma	2.36 ± 0.14^{b}	2.63 ± 0.12^{a}	3.42 ± 0.38^{b}
	Brain	1.27 ± 0.08^{a}	2.05 ± 0.07^{a}	2.28 ± 0.06^{a}
GPx, IU/ml:				
	Plasma	36.40 ± 2.17^{a}	39.87 ± 1.91^{a}	43.67 ± 1.88^{a}
	Brain	39.53 ± 1.01^{b}	41.13 ± 1.05^{b}	51.53 ± 1.60^{a}
MDA, µmol/ml:				
	Plasma	3.66 ± 0.18^{a}	2.99 ± 0.20^{a}	2.57 ± 0.21^{a}
	Brain	4.07 ± 0.12^{a}	3.83 ± 0.09^{b}	3.53 ± 0.11^{b}
Protein, g/dl:				
	Plasma	7.20 ± 0.17^{a}	6.73 ± 0.12^{a}	6.82 ± 0.09^{a}
	Brain	8.18 ± 0.21^{a}	7.58 ± 0.10^{a}	7.36 ± 0.20^{b}

^{a,b} = Means in the same column having different superscript letters are significantly ($P < 0.05$) different;
12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL + Melatonin = Continuous lighting with melatonin administration. SOD = Superoxide dismutase; GPx = Glutathione peroxidase; MDA = Malondialdehyde

Table 4.47: Relationships between Plasma and Brain Biomarkers of Oxidative Stress in Marshall Broiler Chickens before and after Road Transportation

Group		SOD	GPX	MDA	Protein
12D:12L cycle:	*Plasma vs brain pre-transportation*	0.425^{NS}	0.121^{NS}	0.216^{NS}	0.305^{NS}
	Plasma vs brain post-transportation	0.329^{NS}	-0.042^{NS}	0.484^{NS}	-0.285^{NS}
CL:	*Plasma vs brain pre-transportation*	0.222^{NS}	0.130^{NS}	0.088^{NS}	0.038^{NS}
	Plasma vs brain post-transportation	0.314^{NS}	-0.063^{NS}	-0.113^{NS}	-0.043^{NS}
CL+MEL:	*Plasma vs brain pre-transportation*	-0.031^{NS}	0.364^{NS}	0.128^{NS}	-0.059^{NS}
	Plasma vs brain post-transportation	0.515*	0.101^{NS}	0.089^{NS}	-0.242^{NS}

^{NS} = Non-significant correlation ($P < 0.05$); * = $P < 0.05$; 12D:12L cycle = 12 h darkness and 12 h light cycle; CL = Continuous lighting; CL+MEL = Continuous lighting with melatonin administration; SOD = Superoxide dismutase; GPx = Glutathione peroxidase; MDA = Malondialdehyde

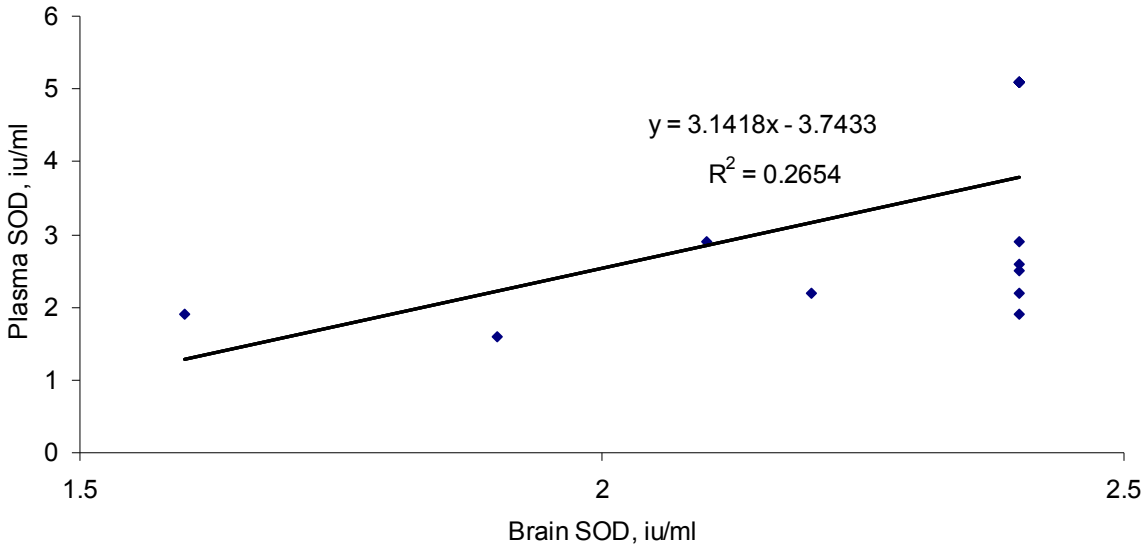


Figure 4.21: The Relationship between Plasma and Brain Superoxide Dismutases (SODs) of Marshall Broiler Chickens reared on 12 h Darkness and 12 h Light Cycle without Melatonin Administration during the Hot-dry Season

CHAPTER 5

DISCUSSION

5.1 METEOROLOGICAL PARAMETERS DURING THE STUDY PERIOD

The meteorological data obtained inside the poultry house and those from the transportation study were similar, but they were characterized by high THI, AT and RH, established for the hot-dry season in the Northern Guinea Savannah zone of Nigeria (Igono *et al*., 1983; Ayo *et al*., 1998). The AT recorded during the study period were outside the established thermoneutral zones of 12 – 24 ^{o}C (Selyansky, 1975; Plyaschenko and Sidorov, 1987) and 20.9 – 28.5 ^{o}C (Donkoh, 1989; Prinzinger *et al*., 1991) in poultry species reared in the temperate and tropical regions of the world, respectively. The finding agreed with the results obtained by Ryder *et al*. (2004) and Nardone *et al*. (2010) that mortality and poor performance of broiler chickens adversely affect the economy of broiler producers in regions in which ambient temperatures approach or exceed 40 ^{o}C. Similarly, the extremely high RH recorded in this study was about two times higher than the values of 30 to 40 %, established for chickens raised in the tropics (Oluyemi and Roberts, 2000). This factor may be predominantly responsible for the undesired heat stress the birds were subjected to, as demonstrated by the high THI obtained in the study. The high THI showed that the thermal environmental conditions, prevailing during the study period were uncomfortable for the raising of broiler chickens in the zone. This finding agrees with the report of Marai *et al.* (2002) that animals raised under conditions with THI higher than 29 °C suffer heat stress. Therefore, measures aimed at alleviating the high THI recorded in the present study are of paramount importance in order to reduce economic losses due to the adverse effects of heat

stress on broilers. This is because during high environmental temperatures and high relative humidity, heat dissipation by evaporative cooling is impeded (Yahav *et al*., 1995; Keim *et al.,* 2002). Brown-Brandl *et al.* (1997) demonstrated in a study on physiological responses of male (tom) turkeys to temperature and humidity change with age that RH had a stronger effect than temperature. The harsh weather conditions of high AT reported in this work triggered panting, but elevated RH made evaporative cooling practically impossible (Keim *et al*., 2002). The meteorological conditions prevailing during the hot-dry season were unfavourable for the rearing of the broilers in the Northern Guinea Savannah zone of Nigeria. Hence, measures aimed at decreasing the adverse effects of the conditions on birds may enhance their performance and productivity in the zone.

The increased meteorological factors of sunshine duration and evaporation, but decreased rainfall and wind speed recorded outside the poultry house and outside the vehicle during the transportation study, during the present study show that heat was, apparently, transferred from the environment to the body of the Marshall broiler chickens via conduction, convection, radiation and evaporation. Increased sunshine duration, decreased rainfall and wind speed increased the heat load in the poultry house, thereby increasing the AT during the day. Increased evaporation of water from the drinkers, body and faecal droppings of the broilers may be responsible for the extremely high RH recorded inside the poultry house during the present study, which prevented effective evaporative cooling. The finding agrees with the report of Keim *et al.* (2002) that meteorological parameters affecting heat transfer are a combination of air temperature, wind speed, relative humidity and radiation. The deleterious effects of these meteorological parameters and heat transfer from the environment to the body of the animal, has been reported to increase body temperature and respiratory rate

in broilers (Kassim and Norziha, 1995; Nardone *et al.,* 2010). In addition, the meterological effects include alteration in physiological characteristics of various circadian rhythms (Cable *et al*., 2007), impairment in performance, metabolic and health status, and immune response (Ayo *et al.,* 1996; Nardone *et al.,* 2010; Ondruska *et al*., 2011).

5.2 EVALUATION OF PERFORMANCE INDICES

The results of the present study demonstrated that groups II and III broiler chickens that were raised on continuous lighting photoperiods consumed more feed and grew faster than their group I broiler counterparts that were reared on 12D:12L cycle. The lower feed consumption and faster growth rate recorded in group III broilers, administered with melatonin compared to group II birds that were not given melatonin showed the beneficial effects of the antioxidant, melatonin on broiler chickens subjected to management or production stress during the hot-dry season. The result demonstrated that the concomitant effects of the high AT and high RH, classical of the hot-dry season and inducing heat stress (Ayo *et al.,* 2011), and the continuous lighting regimens in the poultry house were alleviated by melatonin administration. The finding is in agreement with the result obtained by Sinkalu *et al.* (2009) and Minka and Ayo (2012) that antioxidant vitamins alleviate stress in birds. The finding of the present study has, thus, demonstrated that the increases in internal heat energy generation from feed metabolism, associated with rearing broilers under continuous photoperiods (Abbas *et al.,* 2008) may be ameliorated by melatonin.

The results obtained in this research suggest that melatonin administration to broiler chickens enhanced their growth and productivity. The marked improvement in the overall growth, carcass characteristics and performance observed in group III may be attributed to the ability

of melatonin to effectively counter the adverse effect of oxidative stress, induced by heat stress, continuous lighting (CL) and feed metabolism. This is because CL programme induces sleep deprivation and causes severe physiological stress responses (Campo and Davila, 2002). Interestingly, the group reared under CL programme had consistently higher BW compared to those on 12L:12D. This finding contradicts the result of Rahimi *et al.* (2005), who obtained no significant difference in body weights at 42 d between lighting schedule treatment groups. The idea is that restricting light regimens improves productivity of broiler chickens because physical activity is very low during darkness and energy expenditure of activity is considerable.

The values of weekly feed consumption were insignificant between the groups. In spite of the significantly higher BW of broilers in melatonin-treated groups at different stages of growth, the difference in feed consumption of birds in various groups was insignificant. However, the lowest feed consumption was recorded in broiler chickens, treated with melatonin, indicating melatonin induced higher efficient utilization of nutrients than in those in other groups, resulting into higher live weight in melatonin-treated group. The lowest overall feed consumption obtained in the melatonin-treated group, compared to 12L:12D and 24 CL groups suggested that melatonin reduces feed consumption in broiler chickens. This finding agreed with the results of Apeldoorn *et al*. (1999), who subjected broiler chickens to varied lighting schedule of continuous 23L:1D and intermittent 1L:3D photoperiods and repeatedly found that the amount of feed consumed by melatonin-treated groups was consistently the lowest. Bermudez *et al.* (1983) found that following melatonin injection, feed intake in domestic fowl was reduced.

In studying the effect of melatonin on energy metabolism, Apeldoorn *et al*. (1999) did not record any significant interactions between lighting schedule and melatonin on energy metabolism in chickens. However, they found that heat production related to activity was reduced when melatonin was supplemented to the diet. Melatonin, apparently, reduced feed intake in order to minimise internal body heat generation. Besides its calming effects, melatonin depresses appetite in heat-stressed broiler chickens (Apeldoorn *et al*., 1999), probably by increasing the quantity of water intake as demonstrated in the present study. The amount of water consumed per bird at 6 weeks of age in the present study was 36.11 % greater than that recorded by Hassan *et al.* (2009), who reared broilers under an artificial AT of 33 ± 2 ^{o}C. The higher volume of water consumed in the present study was attributed partly to higher AT and RH values, but more importantly to the marked diurnal fluctuations of the thermal environmental parameters as the hour of the day increased. Overall the amount of water consumed by broiler chickens depends on the prevailing weather conditions at that particular period of time. Hence, greater quantity of water will be required by broiler chickens for maximum performance in hot-dry season than any other seasons of the year.

In a study by Onyimonyi *et al.* (2009), the corrected average profit of broiler chickens fed varying dietary levels of Neem leaf meal, containing antioxidants, was ₦528:85 for five treatment groups. The monetary value was higher than those obtained in 12D:12L cycle (₦337:78) and 24 CL (₦447:48), but lower than ₦667:55 recorded in melatonin-treated group in the present study. The higher profit obtained in the melatonin-treated group further agrees with the observation that broiler chickens administered with melatonin had significantly better performance than those from either of the two groups that were not treated with melatonin.

The satisfaction of the panel utilised in any palatability study is representative of consumer acceptance (Jeremiah, 1996; Jackman *et al*., 2010). It is interesting to note that the overall palatability rankings of soft tissues in the present study were consistently higher than those reported for beef in previous studies (Jeremiah and Gibson, 2003; Jeremiah *et al*., 2003). This finding suggests that chicken meat may be more delicious and may be preferred by most people. However, the difference between the result of the present study and that reported for beef may be due to the fact that different cooking methods were used, and the species evaluated were different. In addition, Jeremiah *et al.* (2003) admitted that the physical and chemical composition of animals have changed considerably over the past five to six decades. The results of the present study showed that rapid growth was compromised for palatability in the 12D:12L cycle group of broiler chickens. This may be because virtually all the values of palatability parameters studied were higher in the 12D:12L cycle groups, characterized by lower rate of growth. The results of the palatability study showed that melatonin administration harmonized the two traits of rapid growth and enhanced palatability. The hardest bone was observed in the melatonin-treated groups, suggesting that the antioxidant hormone, melatonin may be involved in vitamin D, phosphorous and calcium metabolism. Thus, melatonin may be of value in clinical conditions and diseases or disorders involving osteoporosis. This finding showed that melatonin administration may ameliorate adverse effects of heat stress on birds, including increased mineral excretion (Sahin *et al.,* 2004) and low-retention rate for calcium and phosphorous (El-Husseiny and Creger, 1981). The result of the present study suggests that melatonin may be of value in retaining minerals, especially calcium and phosphorous in broiler chickens subjected to heat stress.

The considerable effects of the meteorological factors on broiler performance obtained in the present study, as evidenced by strong and significant relationships between temperature-humidity index and live weight gain, suggest that the meteorological conditions prevailing during the hot-dry season imposed a threat to the homeostatic mechanisms of the birds and, consequently, their survival and productivity. This finding agrees with those of Sinkalu *et al.* (2009) and Minka and Ayo (2012) who showed that the meteorological conditions of the hot-dry season in the zone exerted significant thermal load on birds. The massive white feathering observed in the melatonin-treated broiler chickens was in addition a compensatory and adaptation mechanism, which enhanced heat reflection and, thus, reduced thermal load on the birds. The extremely low mortality rate recorded in the melatonin-treated group showed for the first time that melatonin may be capable of preventing death due to heat stress or stroke, and may be rightly called or named "the vitamin of life".

5.3 BASE-LINE STUDIES ON THE AVIAN BLOOD

The higher values of erythrocytes, PCV, Hb, thrombocytes and total WBC obtained in groups II and III using the electronic cell counter may be attributed to increased feed consumption. This is because birds in the groups were raised on CL programmes, unlike those in group I that were restricted to 12D:12L cycle. From the present study, the MCV, MCH and MCHC values were higher in groups I and II that were not administered with melatonin. This finding suggests that the birds in these groups had more immature erythrocytes in systemic circulation, which may be a response to an intravascular haemolysis or a pending blood crisis. The haemolytic crisis probably resulted from a free-radical-induced oxidative damage to the erythrocytes induced by heat stress. The results obtained on erythrocyte osmotic fragility supports this finding because, unlike the melatonin-treated birds, the erythrocytes of

broiler chickens in groups I and II were more osmotically resistant. The finding of the present study agrees with those of Hanzawa and Watanabe (2000), Minka and Ayo (2008b, 2010b, 2012) and Asala *et al*. (2011) that changes in the osmotic fragility of the erythrocytes during stressful conditions are important indicators of intravascular haemolysis. The presence of greater number of reticulocytes associated with higher MCV values in systemic circulation has been reported to be responsible for erythrocyte resistance to osmotic fragility test in birds (Durotoye and Oyewale, 1988) and other species of domestic animals (Olusanya and Adepoju, 1979). Durotoye and Oyewale (1988) concluded that the erythrocytes of the guinea fowl were more osmotically fragile than those of the Nigerian domestic fowl because the latter had higher MCV values. Therefore, the results of the present study demonstrated that melatonin maintained the vigor of the cellular constituents of blood by preventing rapid ageing and free-radical-induced oxidative damage to the cells as a result of heat stress. This finding is consistent with that of Tesoriere *et al*. (1999), who reported that melatonin inhibited the formation of free radicals within the human erythrocyte membranes *in vitro*.

The traditional or conventional methods, successfully used in the laboratory for the first time in the present study, are of practical value in the differential counting of leucocytes and have increased the arsenal of diagnostic, therapeutic and prophylactic tools, currently used in disease control in avian species in Nigeria. The leucocytes are part of the body's defence system. Five types of leucocytes were found in the birds: heterophils, lymphocytes, eosiniphils, monocytes and basophils. The appearance of the cells was consistent with their morphological descriptions by Gaunt (2004a) and Latimer and Bienzle (2010). The method of determining the differential leucocyte count, developed in the present study is an important and powerful diagnostic tool. This is because the differential leucocyte count is

used as a marker of stress (especially the H to L ratio), to monitor the response to therapy, determine the severity of a disease or as a starting point for formulating a list of differential diagnoses (Barger, 2003). The leucocyte count in the present study was obtained using manual techniques because the presence of nucleated erythrocytes and thrombocytes in the avian species interfered with the counting of leucocytes using electronic cell counters (Campbell, 1995). Based on the developed method of determination of differential leucocyte count, the evaluation of leucocytes involved the interpretation of leucocyte parameters, including total and differential leucocyte counts, and leucocyte morphology. Such evaluation may enhance the efficiency of diagnosis and management of diseases and stressful conditions in avian species in Nigeria. The results of the present study demonstrated, for the first time in our local laboratory, Giemsa, Leishman's and Wright's staining techniques for counting avian leucocytes. This is of great value as it will henceforth enhance the efficiency of Veterinarians in the diagnosis, therapy, prognosis and prophylaxis of avian diseases in Nigeria. Veterinarians may perform differential leucocyte counting techniques in birds in the local laboratories by following any of the three procedures demonstrated in this study. It is recommended that Clinicians and Researchers in Nigeria use the techniques adopted in the present study and consider avian leucocyte values in their diagnostic plans and research.

The result of the present study showed that broiler chickens in all the groups were lymphocytic. The finding was consistent with the results of Bounous *et al*. (2000) and Schmidt *et al*. (2009), who independently reported that lymphocyte was the predominant leucocyte in the peripheral blood of chickens and turkeys. Monocytes of broiler chickens have been reported to show significant increases in cytoplasmic lipid droplets after heat stress compared with pre-heat treatment (Maxwell *et al.,* 1992). The lower monocyte values

obtained in the melatonin-treated birds in the present study demonstrated that melatonin administration mitigated the adverse effects of heat stress on the broiler chickens during the hot-dry season. The insignificant eosinophil values recorded in this work agrees with the finding of Altan *et al*. (2000a) that exposure of broilers to acute heat stress decreases monocyte and lymphocyte proportions, whereas eosinophil and haematocrit values were not affected. The results of the present study demonstrated, for the first time, the ability of melatonin, by increasing total leucocyte count to enhance the immunity of broiler chickens and reduce their susceptibility to diseases during the hot-dry season.

Overall, melatonin administration alleviated the deleterious effects of heat stress on broilers. Its oral administration may enhance the health and productivity of broiler chickens in the tropical zones of the world.

5.4 STUDIES ON BIOLOGICAL RHYTHMS

5.4.1 Diurnal Fluctuations in Rectal Temperature

The RT values in all the groups showed distinct diurnal fluctuations with peak values at 13:00 h. This finding agrees with the report of Ayo and Sinkalu (2007), who recorded the RT values of pullets from 06:00 h to 19:00 h and found that the highest values occurred between 13:00 h and 15:00 h. Core body temperature is used routinely by veterinary doctors for the assessment of health status (Moller, 2010). It is often the marker rhythm of choice in chronobiology due to ease of measurement, particularly in field conditions (Cable *et al*., 2007). The increase in body temperature is caused by an imbalance between the heat loss by evaporative and sensible means, and heat caused by digestion, production, maintenance, activity and panting (Brown-Brandl *et al.,* 1997). This mechanism probably explains why the

RT values obtained in the CL broilers were significantly ($P < 0.001$) higher than those raised on 12D:12L cycle. An increase in body temperature also causes an increase in heat production from the van't Hoff effect, which, as described by Brody (1945), states that the rate of reactions in the body increases logarithmically with body temperature. Again, Brown-Brandl *et al.* (1997) reported that the air temperature at which the imbalance occurs between heat production and heat loss is not absolute, but changes with many factors, such as species, age and prior thermal exposure. In the present study, the marked decrease in the RT value in melatonin-treated broilers, compared to that obtained in either of the two groups not administered with melatonin demonstrated the hypothermic effect of melatonin. This effect became obvious at week 2 after its administration to the birds from day old. Thus, the RT values in melatonin-treated birds were maintained at a relatively constant value, fluctuating insignificantly only within a narrow range up to the 8^{th} week of age. This observation suggests that melatonin is a potent antioxidant (Slominski *et al*., 2012), effective in ameliorating the deleterious effects of oxidative stress in broilers, induced by heat stress.

5.4.2 Diurnal Fluctuations in Erythrocyte Osmotic Fragility

From the fragiligrammes obtained in the present study, both age and melatonin administration altered significantly the EOF in broiler chickens. In all the groups and at different ages, an inverse relationship existed consistently between the percentage haemolysis in the broiler chickens and percentage concentration of NaCl. This finding agrees with the results obtained by Oladele *et al.* (2003) and Awodi *et al.* (2005a), who showed that haemolysis in birds rose with a decrease in the percentage concentration of NaCl. The erythrocytes obtained from birds raised on CL and 12D:12L cycle were osmotically more fragile with age; and this finding agrees with the result that the EOF increases with age in

humans (Meur *et al*., 2005), guinea-fowl (*Numida maleagris*) (Oyewale, 1991) and layers or egg-typed chickens (Azeez *et al*., 2009). However, the erythrocytes of birds in melatonin-treated group were more osmotically resistant with increase in the age of the broiler chickens. This finding is consistent with that of Oyewale and Ajibade (1990a), who showed that the erythrocytes of 6 – 8-week-old White England turkeys were more fragile than those of 20 – 22 weeks of age. Brzezinska-Slebodzinska (2001) also observed that the erythrocytes in younger rabbits of 1 – 5 months' old are more susceptible to membrane peroxidation than those of 3.5- and 6-months-old rabbits. Awodi *et al.* (2005b) demonstrated that the antioxidant vitamin C reduced nitrite-induced haemolysis in Red Sokoto goats.

The results of the present study showed for the first time that melatonin exerts a strong protective effect on the integrity of the erythrocyte membranes of broiler chickens as they grow, apparently by preventing oxidative damage to lipids and proteins in the erythrocyte membrane. Concurrent higher EOF with growth has been shown to be associated with oxidative damage, resulting from ageing and dehydration emanating from heat stress (Baloyi *et al*., 2006). The proximate mechanism involved in melatonin-induced haemolysis was not investigated in the present study. However, it has been shown that melatonin increased erythrocyte osmotic resistance, partly by preventing lipoperoxidation of the cell membranes caused by the pecking action of free radicals, and partly by preventing massive fluid and electrolyte losses from animals subjected to heat stress (Rozenboim *et al*., 1998; Slominski *et al*., 2012).

5.4.3 Diurnal Fluctuations in Haematological Parameters

The marked hourly fluctuations in haematological parameters of broiler chickens observed during the present study showed that the birds were raised under stressful conditions. The diurnal variations recorded, which were within the normal physiological ranges, demonstrated that the birds maintained homeostasis, inspite of the unfavourable meteorological factors acting on them during hot-dry season. This finding agreed with that of Lin *et al.* (2006a), who showed that homeostasis is constantly challenged by internal and external stressors. The resultant effect is that energy meant for production purposes; that is feed conversion and weight gain, is diverted to maintain homeostasis. The results clearly showed that melatonin administration minimized hourly fluctuations in haematological parameters of broiler chickens.

The least values of erythrocytes, Hct, Hb and MCV values obtained in the group subjected to 12D:12L cycle were attributed to reduced dietary intake. The finding agrees with that of Abdelatif *et al.* (2009), who associated low values of erythrocyte count, PCV and Hb concentration in goats with placement on low level of nutrition. The insignificantly higher level of these values in broiler chickens raised on CL may be attributed to greater impact of dehydration on the birds, which occurs when animals are exposed to heat stress. Dehydration is associated with elevated PCV (Schaefer *et al.,* 1997; Broom, 2003). Lowe *et al.* (2002) found that the most rapid increase in PCV occurred in animals that were both temporarily deprived of water and exposed to hot conditions. This fact again demonstrates that melatonin administration alleviated the adverse effects of heat stress on the broiler chickens. The fact that the highest ($P < 0.05$) value of MCH was obtained in the melatonin-treated group showed that melatonin administration enhanced oxygen-carrying capacity of the erythrocytes

in broilers during the hot-dry season. The highest RDW value obtained in broilers raised under 12D:12L cycle was an evidence of marked anisocytosis, related to iron-deficiency anaemia. This finding suggests that melatonin improved avian blood via iron and/or mineral metabolism, and it is consistent with that of Ersahin *et al.* (2009), who reported that melatonin improves cardiovascular function.

The highest H:L obtained in birds in the CL groups showed that the group was most adversely affected during the study period. This finding agrees with the results of Shini *et al*. (2009), who found the highest H:L in hens administered exogenously with the hormone of stress, corticosterone. The results of the present study demonstrated that oral melatonin administration to broiler chickens resulted in a decrease in the H:L ratio. This finding is consistent with that of Moore and Siopes (2000), who showed that melatonin increased the counts of total white blood cells (WBC) and lymphocytes, but decreased heterophil count and H/L ratio.

5.4.4 Diurnal Fluctuations in Behavioural Events

The marked fluctuations in the behavioural events observed in the present study demonstrated that the hot-dry season prevailing in the Northern Guinea Savannah zone of Nigeria is stressful to broiler chickens. The results disagree with the findings of Hollosy *et al.* (2004), who recorded only slight fluctuations from morning to afternoon in the general behaviour of domestic chickens in England. The difference in the results is an evidence that the meteorological conditions in the zone are more severe and unfavourable to poultry production in the zone, compared to those of England during summer.

The significantly decreased level of panting observed in group III demonstrated that melatonin administration alleviated the adverse effects of heat stress on broiler chickens by reducing heat generation. Panting is considered beneficial on a short term, but may induce respiratory alkalosis if prolonged (Marder and Arad, 1989; and Yahav *et al*., 1995). Although it is an effective method of evaporative heat dissipation in animals, it is a very costly mechanism of cooling. This is because it is performed at a resonant ventilatory frequency (Calder and Schmidt-Nielsen, 1966); and if prolonged, may increase energy expenditure and enhance oxidative stress mechanisms (Spaich *et al*., 1968; Yahav *et al*., 2011). The results of the present study suggest that melatonin prevented respiratory alkalosis in broiler chickens by decreasing panting behaviours. This finding agrees with the reports of Marder and Arad (1989) and Yahav *et al*. (1995), who demonstrated that panting induced respiratory alkalosis, resulting in inhibition of growth in heat-stressed chickens. The present study, for the first time, showed that melatonin promoted growth and reduced mortality in heat-stressed broilers, apparently, by preventing respiratory alkalosis, resulting from hyperventilation due to prolonged panting.

The findings on lying behaviour ranked the group III broilers treated with melatonin first, followed by those on CL, and 12D:12L cycle group was the least. Weeks *et al.* (2000) reported that selection for high breast meat yield has moved broilers' centre of gravity forward, which unbalances and predisposes them to sitting behaviours. It is likely that sternal recumbency is the most comfortable, and least physically-demanding posture for broiler chickens. Therefore, reduced activity is considered a positive welfare indicator (D'eath *et al*., 2009), and it suggests the avoidance of heat generation in broiler chickens (Nielsen *et al.,* 2003). Therefore, the increased lying behaviour recorded in the melatonin-treated birds was

attributed to higher body weight gain, resulting from adequate rest and sleep, despite the unfavourable conditions of production, which the broiler chickens were subjected to during the hot-dry season. The results of the present study agree with the finding of Blokhuis (1984), who demonstrated that rest and sleep are strongly associated with energy conservation, tissue restoration and growth. It was expected, therefore, that fast-growing broilers rest and sleep more than other fowls. Hence, disturbed rest (Gordon, 1994) found in broilers on CL was mitigated by melatonin administration in the present study.

The lower number of TI induction attempts and longer TI duration recorded in the melatonin-treated group demonstrated that melatonin induced hypoactivity in the treated broiler chickens. This finding agrees with the result of Zhdanova (2005) that melatonin promotes sleep in healthy humans and other diurnal animals, if administered during habitual hours of wakefulness. Sleep is influenced by both ambient and body temperatures (Kumar *et al.,* 2009). Based on behavioural observations, Mujahid and Furuse (2009) and Moller (2010) reported that low-temperature exposure decreased distress, vocalisations and spontaneous activity, and induced sleep-like behaviour in neonatal chicks. In the present study, active wakefulness decreased, while standing or sitting motionless with eyes closed or open and sleeping posture rose significantly. It has been shown that behavioural effects of melatonin differ from those of common hypnotics. Zhdanova (2005) reported that in contrast to common hypnotics, which have been repeatedly underscored, melatonin induces a behavioural state that resembles quiet wakefulness, which normally predisposes to normal sleep initiation, rather than sleepiness or drowsiness. Thus, melatonin-induced calming effect, hypo- and inactivity, demonstrated to enhance performance and productivity in broiler chickens (Marin *et al*., 1997a; Murakami *et al*., 2001; Zhdanova, 2005). Indeed, it has been

established that inadequate rest is stressful to birds and drastically decreases feed intake, feed conversion ratio and, consequently, the overall weight gain of the birds (Blokhuis, 1984; Gordon, 1994; Melnychuk *et al*., 2004). The findings of the present study are consistent with the result of Campo and Davila (2002) that CL programmes inhibit sleep, which may increase physiological stress and, consequently, reduce performance.

The results of the present study showed that the melatonin-treated broilers exhibited less vigilance behaviour, compared to those raised on CL and 12D:12L cycle that were not administered with melatonin. This finding showed for the first time that melatonin markedly reduced fear in the broiler chickens. Furthermore, the results of the present study agree with the report of Brooks *et al*. (2011) that TI is an inherently stressful experience, which magnifies the degree of perturbation. The finding also agrees with the result obtained by Oden *et al.* (2005) that female chickens in groups mixed with males were fearless because they had less vigilance behaviour, compared to their counterpart females in all-female groups. The findings of the present study show that the administration of melatonin in broiler chickens elicited boldness and confidence via freezing behaviour suppression. Overall, melatonin administration, by alleviating behavioural stress responses, may reduce the production losses incurred during the inevitable stressful management practices, involving rigorous feeding, continuous lighting and subjecting the broiler chickens to thermally stressful meteorological conditions of the hot-dry season in the hot-humid regions of the world.

The significant hourly fluctuations in the open-field activities in all the groups, especially the CL groups, showed that the thermal micro-environment conditions prevailing during the

study period in the hot-dry season were thermally stressful to the broiler chickens, and that they exert significant behavioural effects, especially, on their locomotor activities. The hot-dry season of the Northern Guinea Savannah zone of Nigeria is characterized by severe fluctuations in high AT and RH which have been reported to adversely affect the health and productivity of livestock (Igono *et al.,* 1983; Sinkalu *et al.,* 2008b). The energy utilised in the open-field activities decreased the amount of energy available for production purposes. The result of the present study showed that melatonin effectively reduced the magnitude of the hourly fluctuations, and its administration may enhance the performance of broiler chickens during the hot-dry season.

General behavioural activities of the chickens demonstrated that those administered with melatonin were less active in a novel environment, compared to their counterparts in any of the other two groups. Birds in the melatonin-treated group exhibited less anxiety, as evidenced by the decrease in the number of lines crossed, number of steps taken, escape jumps, distress calls and the number of defaecation boluses recorded in the present study. Thus, the finding demonstrated that melatonin inhibited anxiety in Marshall broiler chickens, subjected to stress due to heat and continuous lighting during the hot-dry season. The findings agree with the report of Chen and Jefferson (2008) that in the central nervous system, 5-hydroxytryptophan (5-HT) functions to inhibit aggression and modulates stress response, including social and environmental adaptability. Anxiety or emotional reactivity is a damaging stressor (Ferre *et al*., 1995). Higher anxiety or emotionality predisposes animals to increased stressor-induced corticosterone and heart attacks, resulting from either tachycardia or bradycardia (Castanon and Mormede, 1994; Gentsch *et al.,* 1995). Heart attack due to anxiousness may be partly responsible for the high percentage mortality of

broiler chickens, recorded annually by poultry farmers during the hot-dry season of the Northern Guinea Savannah zone of Nigeria. The results of the present study agree with the findings of Prut and Belzung (2003) that parenteral administration of full or partial agonists of 5-HT_{1A} receptors generally induces anxiolytic-like effects in animals subjected to open-field activities. Locomotor inhibition or hypo-activity associated with increased time of first call and latency to move in the melatonin-treated group may be beneficial to the broiler chickens. This is because such a response may enhance feed utilisation and prevent energy wastage through excessive motor activity in stressed broiler chickens.

In the present study, melatonin significantly inhibited stress-induced abnormal defaecation. The finding, for the first time, suggests that a negative feedback mechanism exists between melatonin and its precursor serotonin in intestinal epithelial cells of animals subjected to stressors. Hirata *et al.* (2008) reported that central corticotrophin-releasing factor, administered exogenously or secreted in response to stress, enhances the release of endogenous 5-HT, which, in turn, acts on the 5-HT_3 receptor. This results in abnormal colonic water transport in addition to intestinal motility and defaecation.

5.5 STUDIES ON TRANSPORTATION STRESS

Post-transportation T_3, T_4 and TSH values recorded in the present study were higher than the respective pre-transportation values in all the groups. The results demonstrated that road transportation of broiler chickens during the hot-dry season was stressful and placed a major metabolic demand on the birds. Thyroid hormone stimulates numerous metabolic processes, including food intake, energy expenditure and the metabolism of lipids, carbohydrates, proteins and minerals (Varas and John, 2005; Ishii *et al*., 2008). It has also been reported to

stimulate sympathetic nervous system activity (Karakoc *et al*., 2004). Increased energy expenditure and mineral excretion may be the possible cause of high mortality of broiler chickens subjected to road transportation during the hot-dry season.

The significantly increased plasma concentrations of T_3, T_4, T_3:T_4 ratio and TSH in broiler chickens administered with melatonin demonstrated the ability of the birds to sustain metabolic processes, while mitigating the adverse effects of heat and transportation stresses during the hot-dry season. Heat and transportation stresses decreased the plasma concentrations of thyroid hormones in broilers not administered with melatonin in the present study. The finding agrees with that of Lin *et al.* (2006a), who demonstrated that acute heat stress decreased plasma concentration of T_3 and T_3:T_4 ratio in broiler chickens; but disagreed with that of Gupta and Premabati (2002), who reported that T_3:T_4 ratio decreased following melatonin treatment, irrespective of time of administration and seasons. The decrease in plasma T_3 level observed in the groups not administered with melatonin in the present study may be related to the effect of inefficient nutrient utilisation (Buyse *et al*., 2002). Thyroid hormones accelerate the basal metabolic rate by increasing the mitochondria mass and mitochondria cytochrome content (Varas and John, 2005; Ishii *et al*., 2008). The findings of the present study demonstrated that melatonin enhanced metabolic processes and growth in Marshall broiler chickens by increasing the levels of circulating thyroid hormones. The findings are consistent with the results obtained by Gupta and Premabati (2002), who reported that the plasma concentrations of total T_4 increased following evening injections of higher doses of melatonin in the air-breathing fish, *Clarias gariepinus,* during both breeding and quiescent phases. Furthermore, Brzezinska-Slebodzinska *et al.* (1998) reported that melatonin administration to rabbits for up to 7 days increased Type I monodeiodinase

enzyme in the liver and Type II – 5' monodeiodinase enzyme in the kidneys, thereby increasing serum T_4 and T_3 levels.

Concentrations of corticosterone increased insignificantly in all the broiler chickens transported by road for 2 h. Due to a depletion effect, the increment failed to attain significant levels because the birds were raised under heat stress for 42 days prior to transportation. The severity of the heat stress experienced may elicit a negative feedback inhibition of corticosterone release. Normally, a feedback system protects the birds by down-regulating corticosterone (Sapolski *et al*., 2000), but continued elevation of the stress hormone, corticosterone overwhelmed the broiler chickens, resulting in biological consequences such as inhibition of immunity, growth and reproduction. The findings agreed with the results obtained by Wang *et al*. (2012), who worked on predator stress-induced emotional arousal in rats. They found that plasma corticosterone increased dramatically 1 h after the stress and gradually decreased 1 day later, and then returned to the basal level at week 1. Corticosterone promotes the conversion of proteins and lipids to usable carbohydrates, which serve the body well in the short run by replenishing energy reserves after a period of activity; like running away from a predator (Korte *et al*., 2005). The significantly decreased corticosterone levels recorded in broiler chickens administered with melatonin demonstrated that the neuro-hormone prevented oxidative stress in the birds. Oxidative stress (Lin *et al.,* 2006b), total protein (Aoyama *et al.,* 2008; Nilsson *et al.,* 2008), handling methods, elevated temperatures (Saino *et al.,* 2005) and the presence of predators (Cockrem, 2007) have been shown to increase corticosterone secretion in birds. Duncan (1989) found that broilers crated and transported in a vehicle for 40 minutes had higher plasma corticosterone concentrations than those crated and loaded onto the vehicle, but not

transported. The ability of melatonin to lower corticosterone levels in the present study shows, for the first time, that its administration to broiler chickens shortly before transportation may be of value in preventing production losses associated with transportation stress, especially during the hot-dry season.

The insignificant ($P > 0.05$) values of total protein, albumin and urea obtained from all the groups before and after road transportation showed that the birds successfully prevented changes in their blood osmolarity. This is because total protein, albumin and urea are key elements in the regulation of blood osmotic pressure and fluid distribution between different compartments. The decreased plasma total protein, albumin and urea in all the groups after transportation, compared to the pre-transportation values (depletion effect) demonstrated the involvement (consumption or utilization) of total protein, albumin and urea as antioxidants in alleviating the road transportation-induced oxidative stress in broiler chickens. Depletion of antioxidants from cellular pool (as a result of a stressor or a disease condition), has been shown to render cells and living organisms more susceptible to the effects of oxidants (Rahman *et al.*, 1999; Ajuwon and Idowu, 2010). The results of the present study agreed with those of Roche *et al*. (2008), Simunovic *et al*. (2009) and Conde *et al*. (2011), who reported that total protein, albumin and urea, respectively possess antioxidant properties. The findings indicated that farmers in tropical zones of the world may find Marshall broiler chickens suitable and more profitable. This is because the innate ability of Marshall broiler chickens to prevent marked fluctuations in their blood osmolarity, as obtained in the present study, may decrease their susceptibility to diseases, enhance their health, adaptation, survival and productivity. However, the increased total protein and albumin values obtained in groups I and II that were not administered with melatonin showed greater dehydration and higher

corticosterone levels compared to group III broiler chickens administered with melatonin. Conversely, the depleted urea values in groups I and II, reflected greater impact of oxidative stress on the broiler chickens, compared to the melatonin-treated birds in group III. The results suggested that urea in broiler chickens may be involved in antioxidant defence mechanisms. The findings agreed with the results of Simunovic *et al*. (2009), who concluded that urea possesses antioxidant activities because it inhibited lipid peroxidation *in vitro*.

In all the groups, road transportation stress has no significant effect on high-density lipoprotein and total cholesterol levels. However, triglyceride values, unlike the results obtained in group II birds that were not administered with melatonin, decreased significantly post-transportation in the melatonin-treated broiler chickens. The finding demonstrated that pre-transportation administration of melatonin to the broilers chickens mitigated the deleterious effects of road transportation stress during the hot-dry season. The results of the present study agreed with that of Boullart *et al.* (2011) who reported in a review that environmental factors can induce severe hypertriglyceridaemia with the associated risk of cardiovascular diseases.

The significantly high post-transportation MDA concentration observed in this study, including broilers in group III administered with melatonin, showed that oxidative stress occurred in all the broilers following road transportation stress. Oxidative degradation of polyunsaturated fatty acids produces MDA (Horton and Fairhurst, 1987), which according to Seljeskog *et al.* (2006) is frequently used as an indicator of lipid peroxidation. However, the results of the present study showed that melatonin significantly reduced the amount of MDA, but increased the level of antioxidant urea produced in group III broiler chickens, compared

to the results of the other two groups. The findings agree with the reports of Hardeland and Pandi-Perumal (2005), Korkmaz *et al.* (2011) and Maldonado *et al.* (2012) that, the antioxidative protection of melatonin is not only by direct radical scavenging, but it also up-regulates several antioxidants including urea.

The present study revealed that supplementation with melatonin increased high-density lipoprotein (HDL), but decreased triglycerides and total cholesterol compared to the other two groups. The finding is similar to the results of Mondal *et al.* (2007), who reported that supplemental Cu decreased plasma LDL-cholesterol, triglyceride, total lipid and phospholipids, but increased HDL-cholesterol in broiler chickens. It was found that in the other two groups, not administered with melatonin, triglycerides and total cholesterol levels were elevated and were associated with low concentration of HDL. Lefevre *et al.* (1986) suggested that a primary biochemical lesion underlying the hypercholesterolaemia is a reduction in hepatic HDL binding site, which may result in a slower turn-over of HDL and fat accumulation. Transportation has been observed to cause dehydration (Schaefer *et al.,* 1997) as a result of factors such as water deprivation, increased respiration rates and urinary water loss. The lower concentrations of Na^{+} and Cl^{-} observed in groups I and II broilers suggest increased mineral excretion, compared to the melatonin-treated birds. The results agree with the finding of Sahin *et al.* (2004), who reported that mineral excretion increases under stressful conditions. El-Husseiny and Creger (1981) reported a low retention rate for Ca^{2+}, Cu^{+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, P^{3+} and Zn^{2+} in birds subjected to a high ambient temperature. The insignificantly ($P > 0.05$) higher K^{+} concentrations obtained in the two groups not administered with melatonin were probably due to attempts to maintain electrical gradient in the blood of the affected broiler chickens.

The elevated plasma AST, ALT, ALP and CK activities in all the groups after transportation demonstrated that the journey was very stressful to the broiler chickens. The result is consistent with the finding that the AST (Yousef *et al.*, 2006), ALP (Kaur *et al*., 2006), CK (Earley and Murray, 2010) and ALT (D'Eath *et al.,* 2009) activities in blood significantly increased under stressful conditions, including road transportation (Yu *et al.,* 2009). However, the plasma concentrations of these muscle and bone enzymes were consistently and significantly ($P < 0.05$) lower in the melatonin-treated birds, compared to their counterparts that were not administered with melatonin. This finding showed that melatonin administration alleviated significantly the adverse effects of road transportation stress on the broiler chickens during the hot-dry season. Its administration, therefore, may improve the integrity and quality of muscle, liver and bones of broilers subjected to the adverse effects of road transportation stress.

The results of the present study showed that transportation stress elicited an insignificant ($P > 0.05$) increase in the activities of GPx in all the three groups studied. The increment in SOD activity was very significant ($P < 0.05$) only in broilers treated with melatonin. This is probably due to the physiological response of the broilers to scavenge the offensive free radicals, generated and propagated as a result of oxidative stress induced by road transportation and heat stress. The findings of this study demonstrated that birds administered with melatonin and subjected to 24-h lighting regimens had higher levels of the endogenous antioxidant enzymes, compared to their counterparts that were not administered with melatonin. The results agree with the findings of Ozen *et al*. (2009) that melatonin co-administration increased tissue GSH levels of chicks compared to the aflatoxin-treated chicks

without melatonin administration. In a comparative study, Montilla-Lopez *et al*. (2002) found that melatonin has a greater protective effect than vitamin C on antioxidant enzyme activity. The lower activities of antioxidant enzymes recorded in the present study show that the impact of transportation stress was greater in groups I and II birds that were not administered with melatonin during the hot-dry season. Oxidative stress causes down-regulated synthesis or over-utilisation of the antioxidant enzymes (Irshad and Chaudhuri, 2002; Ajuwon and Idowu, 2010). More importantly, Acuna-Castroviejo *et al.* (2001) reported that the expression of some enzymes, related mainly to the endogenous antioxidant system of the cell, such as GPx and SOD are under genomic regulation by melatonin.

The results show for the first time that road transportation of broiler chickens up to 2 hours may not affect the endogenous antioxidant enzymes status or activities of the brain. The significantly ($P < 0.05$) higher level of the enzymes, including SOD and GPx, in melatonin-treated broilers shows that melatonin has the potential to prevent oxidative damage to the brain. The finding is consistent with the report of Hardeland (2005) that melatonin easily crosses cell membranes and the blood-brain barrier to exert its powerful antioxidant activity. Therefore, melatonin administration to broiler chickens during the hot-dry season may greatly improve their behavioural and physiological activities.

The protective effects of melatonin on the brain of Marshall broiler chickens subjected to road transportation stress during the hot-dry season was demonstrated by the insignificantly ($P > 0.05$) stable MDA recorded from pre- and post-transportation values. The MDA concentrations obtained in the two groups not administered with melatonin increased significantly ($P < 0.05$), when the pre- and post-transportation concentrations were compared

with those recorded in melatonin-treated broiler chickens. The decrease in MDA concentration obtained in CL + melatonin birds showed that minimal oxidative processes or damage occurred in the brain of broilers administered with melatonin and transported by road during the hot-dry season. The finding agrees with the results of Ozen *et al.* (2009), who reported reduced kidney and liver MDA levels in chicks suffering from aflatoxicosis and treated with melatonin, compared to those without melatonin treatment. The finding of the present study agrees with that of Ozen *et al*. (2009) that melatonin effectively reduced lipid peroxidation and that it has a protective effect against tissue damage in birds. Furthermore, Bayir *et al*. (2011) showed that the treatment of acute organophosphate poisoning in rabbits with melatonin reduced considerably both erythrocyte and brain tissue MDA levels.

The significantly ($P < 0.05$) increased level of protein in the brain obtained in all the groups was associated with dehydration due to the adverse effects of the transportation stress on the broiler chickens. The highest increment of 44 % recorded in birds, raised on 12D:12L cycle without melatonin administration showed that the broilers were more adversely affected than birds in any other group. Also, the significantly ($P < 0.05$) decreased protein content of the brain tissues in 12D:12L cycle birds, compared to the other groups before transportation may be due to nutritional or dietary effects. Overall, the present study demonstrated that melatonin administration to broilers exposed to transportation stress enhanced the integrity of brain tissues, and prevented excessive fluid loss from the body, including the brain due to dehydration.

The findings of the present study demonstrated for the first time that SOD and GPx possess reverse activities comparing their antioxidant roles in the blood and the brain. The results

showed that the activity of SOD was predominant in the brain, while that of GPx was in the blood. The consistently higher brain MDA concentrations obtained in the present study demonstrated clearly that brain tissues are more susceptible to oxidative damage, resulting from free-radical attacks than the blood. The results on tissue proteins also suggest that the protein content of the brain in broiler chickens reared on continuous lighting regimens and subjected to 2-h of road transportation stress during the hot-dry season was significantly higher than the values obtained in the blood. The lack of significant correlation between blood and brain tissues in each biomarker of oxidative stress indicated that the blood does not reflect directly the brain tissue oxidative stress in broiler chickens. Hence, biomarkers measured in the blood failed to provide a reliable indication about the redox status in the brain tissues. This finding partially contradicted the results of Veskoukis *et al.* (2009), who reported that blood of rats reflects tissue oxidative stress depending on the kind of biomarker and tissue studied.

CHAPTER 6

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

The AT ranged from the minimum value of 21.00 ± 0.69 ^{o}C at 1st week to the maximum mean value of 40.29 ± 0.36 ^{o}C, obtained at the 2nd week of the study. The mean weekly meteorological parameters of relative humidity, maximum AT, minimum AT, sunshine duration, evaporation, wind speed and wind direction recorded outside the poultry house during the period of the study fluctuated significantly ($P < 0.05$). The overall average wind speed was 1.91 ± 0.12 m/s and the wind direction was predominantly south-west. The overall mean values of sunshine duration, rainfall and evaporation were 6.60 ± 0.44 h/day, 2.29 ± 0.85 mm and 9.79 ± 0.90 mm/day, respectively. The weekly mean temperature-humidity index (THI) recorded inside the poultry house was lowest at 4th week of the study, with the value of 48.60 ± 0.08 ^{o}C, but highest at 1st week with the value of 53.48 ± 0.97 ^{o}C ($P < 0.0001$). Inside the poultry house where the birds were reared, the RH fluctuated significantly ($P < 0.001$) between 67.48 ± 1.33 % recorded at 19:00 h and 72.38 ± 1.76 % obtained at 06:00 h, with an overall mean of 70.69 ± 1.61 %. The mean AT values fluctuated significantly ($P < 0.0001$) as the hours of the day increased, with peak values of 38.61 ± 0.35 % recorded at 13:00 h.

On day 42 of age, body weights (BWs) of broilers differed significantly ($P < 0.001$) between groups. The mean value of BW of 2420 ± 50 g/bird was obtained in group III administered with melatonin, while BW values recorded in the other two groups were less than 2000 g. The overall feed consumption were insignificantly different in all the groups. However,

group II, raised on continuous lighting without melatonin supplementation, had the highest mean values of 117.63 ± 10.16 g/bird. The highest overall amount of water intake was recorded in the melatonin-treated group. The weekly water intake pattern between the groups showed that the group administered with melatonin had the highest values from the 2nd week of age up to d 42, when the birds were 6 weeks old. Groups I, II and III had overall mortality percent of 11, 14 and 3 %, respectively. Maximum AT, dry-bulb temperature and evaporation rate were negatively, but significantly correlated with feed consumption and BW of birds in all the groups. A similar effect was recorded for water intake and feed conversion ratio (FCR) across the groups. An exception was the relationship between evaporation rate and FCR obtained in group I, reared under 12D:12L, which was negative and insignificant ($P > 0.05$).

The results of the present study demonstrated, for the first time in the local laboratory, Giemsa, Leishman's and Wright's staining techniques for identification of broilers' leucocytes, including heterophils, lymphocytes, monocytes, basophils and eosinophils.

In the present study, the rectal temperature (RT) values fluctuated significantly in all the groups as the hour of the day increased. The overall mean hourly RT value of 41.50 ± 0.02 ^{o}C, obtained in the 12D:12L cycle birds was significantly higher ($P < 0.001$) than the value of 41.14 ± 0.04 ^{o}C recorded in the melatonin-treated group, but lower than that of 41.65 ± 0.02 ^{o}C obtained in birds belonging to the continuous lighting group. The RT values, with the exception of those recorded in broiler chickens administered with melatonin, fluctuated significantly with age.

On days 42 and 56 of the study, the percent haemolysis recorded from the erythrocytes of birds in groups I and II were very similar and significantly higher than those obtained in broilers administered with melatonin. At both ages percent haemolysis fluctuated significantly in groups I and II that were not administered with melatonin from 0.9 % to 0.3 % NaCl concentration, contrary to the results obtained from the erythrocytes of birds treated with melatonin in group III. The diurnal variation in osmotic fragility of erythrocytes from the birds was highest among those raised on continuous lighting, followed by those reared on 12D:12L cycle and lowest in the melatonin-treated broiler chickens. Between-group overall diurnal variation in osmotic fragility of erythrocytes from the broilers showed that the continuous lighting birds had the highest ($P < 0.001$) percent haemolysis at 06:00 h and 19:00 h both at 0.5 % NaCl concentration.

The haematological parameters, with the exception of thrombocytes and heterophil/lymphocyte ratio, fluctuated significantly ($P < 0.05$) as the hours of the day increased in all the groups, especially in group I raised under 12D:12L cycle. The highest and lowest mean values of erythrocyte count, Hb and PCV in all the groups were obtained at 06:00 h and 13:00 h, respectively. The values decreased significantly ($P < 0.05$) at these hours only in groups I and II, but insignificantly ($P > 0.05$) in group III birds administered with melatonin. The RDW_SD, MCV and MCH values recorded at 13:00 h were lower compared to those of other hours of the day in all the groups. On the contrary the RDW_CV and MCHC values obtained in all the groups were highest at 13:00 h. The highest RBC, Hct, Hb and MCV values were obtained in CL group followed by CL + MEL and the least values were obtained in birds raised on 12D:12L cycle. However, the melatonin–treated group had the highest MCH and MCHC values, followed by birds in the CL group, while 12D:12L

group had the least values. Highest RDW_SD and RDW_CV values were recorded in the 12D:12L cycle group followed by continuous lighting group, and the lowest values were recorded in group III birds administered with melatonin. Finally, the absolute heterophil values and H:L ratio obtained in the continuous lighting group were significantly ($P < 0.05$) higher compared to the values recorded in the other groups.

The behavioural responses fluctuated ($P < 0.05$) as the hour of the day increased ($P < 0.05$). Exceptions to this finding are the behavioural events of lying and two-fighting, obtained from broilers in 12D:12L cycle and melatonin-treated groups, respectively that fluctuated insignificantly ($P > 0.05$). The overall value of 21.83 ± 1.01 % for panting obtained in the melatonin-treated birds was significantly (Kruskal Wallis Test = 17.45; $P < 0.0001$) lower than 69.72 ± 6.77 % and 67.87 ± 7.19 %, recorded in birds from 12D:12L cycle and continuous lighting groups, respectively. The overall mean value of 63.61 ± 2.11 % recorded for lying (resting/sleeping) in the melatonin-treated group was significantly (Kruskal Wallis Test = 12.25; $P < 0.005$) higher than the values of 43.56 ± 1.03% and 54.28 ± 4.52 %, obtained in broiler chickens in 12D:12L cycle and continuous lighting groups, respectively. The overall mean values of induction trial attempts of tonic immobility differed significantly ($P < 0.0001$) between the groups, with the lowest mean values of 1.22 ± 0.4 recorded in Group III birds administered with melatonin.

The open-field activities of escape jumps, first calls, number of steps and especially latency to move in Marshall broiler chickens at different ages fluctuated significantly ($P < 0.05$) as the hours of the day increased in birds raised on 12D:12L cycle and those on continuous lighting with melatonin administration. However, no significant hourly fluctuation was

recorded when the birds in both groups were 6 weeks' old. The hourly fluctuation recorded in the groups raised on continuous lighting was 200 % higher than those obtained from either the group raised on 12D:12L cycle or CL with melatonin administration.

The mean values of triiodothyronine (T_3), thyroxine (T_4), T_3:T_4 ratio and thyroid stimulating hormone recorded before and after transportation were consistently higher in group III broilers administered with melatonin than the corresponding values recorded in groups I and II that were not administered with melatonin. The mean corticosterone levels unlike the results of thyroid hormones were lower in the broilers treated with melatonin in group III, compared to their counterparts in groups I and II that were not administered with melatonin. The pre- and post-transportation values recorded for total protein, albumin, urea, HDL-cholesterol and total cholesterol did not differ significantly ($P > 0.05$) in all the groups. The post-transportation MDA values of 2.99 ± 0.20 µmol/ml and 2.57 ± 0.21 µmol/ml recorded in continuous lighting without melatonin treatment and continuous lighting with melatonin administration groups, respectively were significantly ($P < 0.05$) higher than the corresponding pre-transportation values. Pre-transportation Na^+ concentration of 137.73 ± 0.04 mmol/l decreased significantly ($P < 0.05$) to 136.87 ± 0.92 mmol/l following transportation in group II birds that were raised on CL without melatonin administration. Conversely, K^+ concentration increased from 3.81 ± 0.15 mmol/l to 4.07 ± 0.10 mmol/l post-transportation in group I broiler chickens raised on 12D:12L cycle without melatonin administration. Aspartate aminotransferase, alanine aminotransferase, creatine kinase and alkaline phosphatase activities rose significantly ($P < 0.05$) after transportation in all the groups. However, the activities of the endogenous enzymes, with the exception of SOD in group III birds treated with melatonin were not significantly ($P > 0.05$) altered by road

transportation. The brain SOD and glutathione peroxidase (GPx) activities did not differ significantly ($P > 0.05$) before and after road transportation in all the three groups. The results obtained in all the groups showed that SOD activity was consistently higher ($P < 0.05$) in the plasma than the brain. However, brain GPx activities were significantly ($P < 0.05$) higher in all the groups, when compared to the corresponding activities obtained in the plasma of the same birds, either pre- or post-transportation. The MDA concentrations were higher in brain tissues than plasma, especially in groups II and III broiler chickens, where the values were highly significant ($P < 0.001$).

6.2 CONCLUSIONS

1. The harsh meteorological conditions, especially of high AT, RH and prolonged sunshine duration prevailing during the hot-dry season were unfavourable for the rearing and transporting of broiler chickens in Northern Nigeria.
2. The amount of water consumed by broiler chickens depended on the prevailing weather conditions.
3. Broiler chickens raised on continuous lighting and administered with or without melatonin gained more weight, performed better and yielded greater profit than those raised on 12D:12L cycle.
4. Broiler chickens raised on continuous lighting without melatonin administration recorded higher mortality resulting from heat stroke than those raised on continuous lighting but treated with melatonin.
5. Broiler chickens raised on 12D:12L cycle, were more delicious than those raised on continuous lighting and treated with or without melatonin.

6. Melatonin administration is of value in retaining minerals, especially calcium and phosphorous in broiler chickens subjected to heat stress.
7. The conventional methods, involving the Leishman's, Giemsa's and Wright's staining technique, was successfully demonstrated for the first time in our clinical pathology laboratory for the counting of broilers' heterophils, lymphocytes, eosinophils, monocytes and basophils.
8. Melatonin administration alleviated the deleterious effects of heat stress on broiler chickens by maintaining a relatively constant RT value, fluctuating insignificantly only within a narrow range up to the 8^{th} week of age during the hot-dry season.
9. Melatonin had a strong protective effect on the integrity of the erythrocyte membranes of broiler chickens as they grow, apparently by preventing oxidative damage to lipids and proteins in the erythrocyte membrane.
10. Melatonin administration mitigated the adverse effects of heat stress by increasing mean corpuscular haemoglobin in order to enhance the erythrocytes' oxygen-carrying capacity of broiler chickens during the hot-dry season.
11. Melatonin induced hypo-activity or locomotor inhibition observed during behavioural studies might be responsible for better nutrient utilisation and greater weight gain in the treated broiler chickens.
12. The significantly high post-transportation MDA concentration, aspartate aminotransferase, alanine aminotransferase, creatine kinase and alkaline phosphatase activities in all the groups, showed that oxidative stress occurred in all the broiler chickens following 2 h of road transportation.

13. The protective effects of melatonin on the brain of Marshall broiler chickens subjected to road transportation stress during the hot-dry season was demonstrated by the insignificantly ($P > 0.05$) stable MDA concentration recorded pre- and post-transportation.
14. The findings of the present study demonstrated for the first time that SOD and GPx possess reverse activities comparing their antioxidant roles in the blood and the brain. The results showed that the activity of SOD was predominant in the brain, while that of GPx was in the blood.
15. The consistently higher brain MDA concentrations obtained in the present study demonstrated clearly that brain tissues of broilers were more susceptible to oxidative damage, resulting from free-radical attacks than the blood.
16. The lack of significant correlation between blood and brain tissues in each biomarker of oxidative stress indicated that the blood does not reflect directly the brain tissue oxidative stress in broiler chickens. Hence, biomarkers measured in the blood failed to provide a reliable indication about the redox status in the brain tissues.

6.3 RECOMMENDATIONS

1. Farmers should not attempt raising broiler chickens during the hot-dry season without putting in place adequate measures aimed at decreasing the risk of adverse effects of heat stress on the birds in order to enhance their performance and productivity in the zone.

2. Broiler chickens' producers in developing nations of the world should improve on the lighting programmes on their farms beyond the natural photoperiods to 15 – 18 h daily.
3. Adequate amounts of drinking water should be provided for broiler chickens for maximum performance during the hot-dry season.
4. Oral administration of melatonin may enhance the adaptation, performance and productivity of broiler chickens in the tropical zones of the world.
5. Melatonin should be administered prior to road transportation in order to enhance the health status and survival of broiler chickens after the journey in the tropical zones of the world.
6. The Government of the Federal Republic of Nigeria should ensure that melatonin is readily available and highly subsidized to poultry farmers.

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APPENDICES

Appendix 4.1: Weekly Thermal Environment Data inside the Poultry House during the Study Period (Mean ± SEM)

Week	Relative humidity, %	Temperature-humidity index, ^{o}C	Dry-bulb temperature, ^{o}C
1	65.10 ± 2.81^{a}	53.48 ± 0.97^{d}	37.33 ± 0.57^{d}
2	63.67 ± 3.03^{a}	53.29 ± 1.9^{d}	37.62 ± 0.84^{d}
3	76.05 ± 2.28^{b}	51.58 ± 0.85^{abc}	33.76 ± 0.82^{abc}
4	74.62 ± 2.34^{b}	48.60 ± 0.80^{a}	31.48 ± 0.84^{a}
5	72.71 ± 2.27^{b}	49.57 ± 0.97^{ab}	32.57 ± 0.95^{ab}
6	69.05 ± 1.97^{ab}	50.92 ± 1.06^{abc}	34.29 ± 0.93^{bc}
7	69.91 ± 1.78^{ab}	52.65 ± 1.32^{bc}	35.48 ± 0.95^{cd}
8	74.43 ± 2.42^{b}	52.18 ± 0.97^{bc}	34.48 ± 1.06^{bc}
Mean ± SEM	70.69 ± 1.62	51.53 ± 0.62	34.63 ± 0.76

$^{a, b, c, d}$ = Means for the same column having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.2: Diurnal Fluctuations in Dry-bulb Temperature, Temperature-humidity Index and Relative Humidity inside the Poultry House during the Study Period (Mean ± SEM)

Hour of the day, h	Relative humidity, %	Temperature-humidity index, ^{o}C	Dry-bulb temperature, ^{o}C
06:00	72.38 ± 1.76^{a}	47.45 ± 0.40^{a}	30.95 ± 0.54^{a}
13:00	72.21 ± 1.46^{a}	56.58 ± 0.58^{b}	38.61 ± 0.35^{b}
19:00	67.48 ± 1.33^{b}	50.56 ± 0.31^{c}	34.32 ± 0.34^{c}
Mean ± SEM	70.69 ± 1.61	51.53 ± 2.68	34.63 ± 2.22

$^{a, b, c}$: Means for the same column having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.3: Percentage Overall Mortality of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season

Group	Percentage Overall Mortality, %
I	11
II	14
III	3

12D:12L = 12 h darkness and 12 h light cycle (Group I); CL = Continuous lighting (Group II) and CL + MEL = CL + Melatonin (Group III)

Appendix 4.4: Effects of Lighting Regimens and Heat Stress on Osmotic Fragility of Erythrocytes from Marshall Broiler Chickens administered with Melatonin

Percent NaCl Solution, %	Group Percent Haemolysis, %		
	I	II	III
0.9	0.00	0.00	0.00
0.7	0.00	0.00	0.00
0.5	0.00	0.00	0.00
0.3	92.31	91.30	100
0.1	100	100	100
0.0	100	100	100

12D:12L = 12 h darkness 12 h light cycle (Group I); CL = Continuous lighting (Group II) and CL + MEL = CL + Melatonin (Group III)

Appendix 4.5: Diurnal Variations in Rectal Temperature of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM)

Hour, h	Group		
	I	II	III
06:00 h	41.20 ± 0.03[a]	41.38 ± 0.03[a]	40.96 ± 0.03[a]
13:00 h	41.79 ± 0.03[c]	41.88 ± 0.03[c]	41.32 ± 0.04[b]
19:00 h	41.50 ± 0.04[b]	41.69 ± 0.04[b]	41.14 ± 0.10[ab]

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting and Group III = Continuous lighting + Melatonin;
[a, b, c] = Means for the same column having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.6: Overall Mean Rectal Temperature of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM)

Group	Overall Mean Rectal Temperature
12 h darkness 12 h light cycle	41.50 ± 0.02^{b}
Continuous lighting	41.65 ± 0.02^{c}
Continuous lighting and administered with melatonin	41.14 ± 0.04^{a}

[a, b, c] = Means for the same column having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.7: Age-related Changes in Mean Rectal Temperature of Marshall Broiler Chickens administered with Melatonin during the Hot-dry Season (Mean ± SEM)

Age, week	Group		
	I	II	III
2nd	41.45 ± 0.06[a]	41.58 ± 0.06[ab]	41.28 ± 0.06
3rd	41.40 ± 0.05[a]	41.51 ± 0.05[a]	41.21 ± 0.06
4th	41.40 ± 0.06[a]	41.61 ± 0.03[ab]	41.16 ± 0.06
5th	41.83 ± 0.09[b]	41.83 ± 0.08[c]	41.07 ± 0.06
6th	41.47 ± 0.05[a]	41.70 ± 0.05[bc]	41.05 ± 0.03
7th	41.49 ± 0.05[a]	41.63 ± 0.06[ab]	40.96 ± 0.22
8th	41.43 ± 0.05[a]	41.71 ± 0.06[bc]	41.23 ± 0.03

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting and Group III = Continuous lighting + Melatonin;
[a, b, c] = Means for the same column having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.8: Diurnal Variations in Osmotic Fragility of Erythrocytes in Marshall Broiler Chickens administered with Melatonin at Day 28 (Mean ± SEM)

NaCl Solution, %	Percent Haemolysis, %								
	Group I			Group II			Group III		
	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h
0.9	6.85 ± 1.27	5.12 ± 1.00	4.65 ± 1.32	9.65 ± 3.08	4.31 ± 0.86	4.10 ± 0.67	13.75 ± 0.95^{b}	5.37 ± 0.48^{a}	7.60 ± 1.84^{a}
0.7	6.85 ± 1.27	5.56 ± 1.14	4.20 ± 0.98	8.85 ± 2.35	4.56 ± 0.81	4.23 ± 1.10	14.10 ± 1.07^{b}	5.60 ± 0.47^{a}	3.09 ± 0.91^{a}
0.5	10.11 ± 1.36	10.02 ± 1.84	8.64 ± 1.10	10.63 ± 2.30	13.08 ± 4.62	12.38 ± 2.47	15.81 ± 0.96^{b}	9.76 ± 1.32^{a}	6.62 ± 2.01^{a}
0.3	86.10 ± 3.33	84.92 ± 2.24	83.33 ± 2.20	87.18 ± 2.30^{b}	82.13 ± 3.26^{b}	71.38 ± 4.86^{a}	92.65 ± 1.14^{c}	79.14 ± 3.83^{b}	65.11 ± 6.46^{a}
0.1	92.12 ± 3.75	94.42 ± 1.01	89.06 ± 2.30	95.70 ± 1.47	86.06 ± 3.03	89.14 ± 5.35	94.35 ± 1.20	94.89 ± 1.83	94.82 ± 1.42
0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting; and Group III = Continuous lighting + Melatonin;
$^{a, b, c}$ = Means for the same row of the same group having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.9: Diurnal Variations in Osmotic Fragility of Erythrocytes in Marshall Broiler Chickens administered with Melatonin at Day 42 (Mean ± SEM)

NaCl Solution, %	Percent Haemolysis, %								
	Group I			Group II			Group III		
	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h
0.9	0.00 ± 0.00^{a}	2.52 ± 0.35^{b}	0.00 ± 0.00^{a}	7.82 ± 2.14^{b}	0.00 ± 0.00^{a}	0.95 ± 0.51^{a}	3.54 ± 0.80	3.52 ± 1.49	0.77 ± 0.41
0.7	0.00 ± 0.00^{a}	3.71 ± 0.6^{b}	0.00 ± 0.00^{a}	17.25 ± 8.11^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.00 ± 0.52^{ab}	3.15 ± 1.48^{b}	0.00 ± 0.00^{a}
0.5	2.58 ± 0.9^{b}	6.12 ± 0.68^{c}	0.00 ± 0.00^{a}	18.75 ± 3.76^{b}	0.00 ± 0.00^{a}	5.21 ± 0.74^{a}	8.92 ± 2.59^{c}	1.88 ± 0.48^{a}	5.46 ± 1.26^{b}
0.3	72.39 ± 4.06	70.43 ± 1.61	67.21 ± 2.12	72.07 ± 1.21^{b}	61.12 ± 3.57^{a}	67.44 ± 1.64^{ab}	61.98 ± 2.27^{a}	66.94 ± 1.96^{ab}	73.19 ± 2.08^{b}
0.1	83.12 ± 3.48^{a}	96.47 ± 1.18^{b}	96.75 ± 1.11^{b}	89.11 ± 1.20	87.72 ± 2.87	92.32 ± 1.52	85.23 ± 2.1^{a}	93.20 ± 0.95^{b}	92.52 ± 1.46^{b}
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting and Group III = Continuous lighting + Melatonin;
$^{a, b, c}$ = Means for the same row of the same group having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.10: Diurnal Variations in Osmotic Fragility of Erythrocytes in Marshall Broiler Chickens administered with Melatonin at Day 56 (Mean ± SEM)

NaCl Solution, %	Percent Haemolysis, %								
	Group I			Group II			Group III		
	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h
0.9	6.27 ± 1.96^{b}	0.29 ± 0.29^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	5.63 ± 1.58^{b}	0.00 ± 0.00^{a}	1.11 ± 0.61	0.22 ± 0.22	0.00 ± 0.00
0.7	7.01 ± 1.86^{b}	0.29 ± 0.29^{a}	0.00 ± 0.00^{a}	0.22 ± 0.22^{a}	6.47 ± 1.28^{b}	0.00 ± 0.00^{a}	1.62 ± 0.74^{b}	0.22 ± 0.22^{ab}	0.00 ± 0.00^{a}
0.5	16.65 ± 3.74^{b}	20.63 ± 4.10^{b}	0.83 ± 0.57^{a}	35.18 ± 6.96^{b}	28.00 ± 4.15^{b}	11.06 ± 3.72^{a}	12.51 ± 4.26	22.59 ± 4.72	10.48 ± 2.67
0.3	68.18 ± 3.74	68.22 ± 1.76	74.35 ± 2.23	73.10 ± 3.36^{b}	73.10 ± 1.71^{b}	64.26 ± 1.92^{a}	67.81 ± 2.94	71.75 ± 1.97	68.24 ± 4.88
0.1	92.52 ± 1.77	92.40 ± 1.34	93.25 ± 2.33	93.29 ± 2.33	93.82 ± 2.31	93.75 ± 1.56	88.55 ± 2.26^{a}	93.91 ± 1.14^{ab}	96.80 ± 1.02^{b}
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting and Group III = Continuous lighting + Melatonin;
$^{a, b, c}$ = Means for the same row of the same group having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.11: Overall Diurnal Variations in Osmotic Fragility of Erythrocytes from Marshall Broiler Chickens administered with Melatonin during the Study Period (Mean ± SEM)

NaCl Solution, %	Percent Haemolysis, %								
	Group I			Group II			Group III		
	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h	06:00 h	13:00 h	19:00 h
0.9	4.37 ± 0.89^{b}	2.64 ± 0.47^{ab}	1.55 ± 0.54^{a}	5.83 ± 1.37^{b}	3.31 ± 0.69^{ab}	1.68 ± 0.38^{a}	6.13 ± 0.94^{b}	3.04 ± 0.61^{a}	2.80 ± 0.80^{a}
0.7	4.62 ± 0.88^{b}	3.19 ± 0.54^{ab}	1.40 ± 0.44^{a}	8.78 ± 2.94^{b}	3.67 ± 0.64^{a}	1.41 ± 0.47^{a}	5.57 ± 1.02^{b}	2.99 ± 0.61^{a}	1.03 ± 0.37^{a}
0.5	9.78 ± 1.59^{b}	12.25 ± 1.75^{b}	3.16 ± 0.71^{a}	21.52 ± 3.09^{b}	13.69 ± 2.66^{a}	9.55 ± 1.55^{a}	12.41 ± 1.71	11.41 ± 2.06	7.52 ± 1.21
0.3	75.56 ± 2.39	74.53 ± 1.54	74.97 ± 1.58	77.45 ± 1.71^{b}	72.42 ± 2.12^{ab}	67.69 ± 1.84^{a}	74.15 ± 2.37	72.61 ± 1.72	68.85 ± 2.76
0.1	89.25 ± 1.88^{a}	94.43 ± 0.71^{b}	93.02 ± 1.22^{ab}	92.70 ± 1.06	89.20 ± 1.63	91.74 ± 1.90	89.38 ± 1.22^{a}	94.00 ± 0.77^{b}	94.72 ± 0.79^{b}
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting and Group III = Continuous lighting + Melatonin;
$^{a, b, c}$ = Means for the same row of the same group having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.12: Between-group Overall Diurnal Variations in Osmotic Fragility of Erythrocytes from Marshall Broiler Chickens administered with Melatonin during the Study Period (Mean ± SEM)

NaCl Solution, %	Percent Haemolysis, %								
	06:00 h			13:00 h			19:00 h		
	Group I	Group II	Group III	Group I	Group II	Group III	Group I	Group II	Group III
0.9	4.37 ± 0.89	5.83 ± 1.37	6.13 ± 0.94	2.64 ± 0.47	3.31 ± 0.69	3.04 ± 0.61	1.55 ± 0.54	1.68 ± 0.38	2.80 ± 0.80
0.7	4.62 ± 0.88	8.78 ± 2.94	5.57 ± 1.02	3.19 ± 0.54	3.67 ± 0.64	2.99 ± 0.61	1.40 ± 0.44	1.41 ± 0.47	1.03 ± 0.37
0.5	9.78 ± 1.59[a]	21.52 ± 3.09[b]	12.41 ± 1.71[a]	12.25 ± 1.75	13.69 ± 2.66	11.41 ± 2.06	3.16 ± 0.71[a]	9.55 ± 1.55[b]	7.52 ± 1.21[b]
0.3	75.56 ± 2.39	77.45 ± 1.71	74.15 ± 2.37	74.53 ± 1.54	72.42 ± 2.12	72.61 ± 1.72	74.97 ± 1.58[b]	67.69 ± 1.84[a]	68.85 ± 2.76[a]
0.1	89.25 ± 1.88	92.70 ± 1.06	89.38 ± 1.22	94.43 ± 0.71[b]	89.20 ± 1.63[a]	94.00 ± 0.77[b]	93.02 ± 1.22	91.74 ± 1.90	94.72 ± 0.79
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting and Group III = Continuous lighting + Melatonin;
[a, b, c] = Means for the same row of the same hour having different superscript letters are significantly ($P < 0.05$) different

Appendix 4.13: Between-group Variations in Osmotic Fragility of Erythrocytes of Marshall Broiler Chickens administered with Melatonin during the Study Period (Mean ± SEM)

Percent NaCl Solution, %	Group Percent Haemolysis, %		
	I	II	III
0.9	2.86 ± 0.39	3.61 ± 0.54	3.99 ± 0.47
0.7	3.07 ± 0.39	4.62 ± 1.04	3.20 ± 0.44
0.5	8.40 ± 0.88[a]	14.92 ± 1.51[b]	10.45 ± 0.99[a]
0.3	75.02 ± 1.08	72.52 ± 1.14	71.87 ± 1.34
0.1	92.23 ± 0.80	91.21 ± 0.91	92.70 ± 0.58
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Group I = 12 h darkness 12 h light cycle; Group II = Continuous lighting; Group III = CL + Melatonin;
[a, b, c] = Means for the same row having different superscript letters are significantly ($P < 0.05$) different