EFFECT OF ESTRADIOL BENZOATE ON SERUM BIOCHEMISTRY, SPLEEN HISTOMORPHOMETRY AND SHELL GLAND HISTOLOGY IN LOHMANN BROWN HENS

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DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Abdussamad Muhammad Abdussamad and has not been presented anywhere for the award of a degree or certificate. All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this dissertation and the subsequent write-up (Kabiru Muhammad SPS/14/MAS/00019) were carried out under my supervision.

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APPROVAL

This dissertation has been examined and approved for the award of Masters in (Animal Science).

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This research work is dedicated to my family for their encouragement and support.

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ABSTRACT

The present study determined the effect of Estradiol Benzoate (EB) on serum chemistry, spleen histomorphometry and shell gland histology in eighteen Lohmann Brown hens. Completely randomized design was used with each treatment (0, 0.2, 0.4, 0.6, 0.8 and 1 mg per bird) administered intramuscularly via the breast muscle and replicated thrice for six weeks. Data were analyzed using GraphPad InStat® statistical package. Results revealed non-significant ($P>0.05$) effect of EB on median serum glucose, blood urea nitrogen, creatinine, cholesterol, total protein, albumin, globulin, calcium, phosphorus, aspartate and alanine aminotranferases, and alkaline phosphatase. Similarly, proportion of spleen vascular skeleton and B follicles were not significantly ($P>0.05$) affected. There was an increase in fat infiltration (1-3, 6-10 and >10 fat cell aggregates) of the shell gland across 0, 0.2 and 0.4 mg EB treatments, respectively. Beyond 0.4 mg, the same magnitude (6-10 fat cell aggregates) of shell gland fat infiltration was recorded. Mean proportion of splenic red pulp in control birds was 25% significantly ($P<0.05$) less than the corresponding proportion in birds administered 0.8 mg EB. However, mean proportion of ellipsoids and peri-arteriolar lymphatic sheath (PALS) in control birds was 18% significantly ($P<0.05$) greater than the corresponding value in the 0.8 mg EB-administered birds. In conclusion, EB has effect on shell gland fat infiltration and proportion of spleen red pulp and ellipsoids and PALS.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION

Poultry are group of birds generally reared for their flesh, eggs and leather (Oluyemi & Roberts, 2000). Based on the principles of avian classification and domestication, Oluyemi and Roberts (2000) classified Birds into: *Galliforms, Anseriforms* and *Columbiforms*. Domesticated birds of economic importance in the tropics include ducks, geese, quails, chickens, turkeys and guinea fowls (Singh, 1981).

Poultry refers to all domesticated birds that are reared for the production of meat and eggs for human consumption, as well as for economic benefits (Kumar, Saiful & Ashraful, 2013). Poultry are adapted to most environments that humans have exploited, especially the tropics (Mogesse, 2007). Moreover, poultry are able to adapt to most areas of the world and have short generation interval (Smith, 2001). They are resources that are available to even the poorest family (Barnejee, 1998). Poultry also help in contributing to household security throughout the world which leads to diversifying income and providing quality food and energy (Sonaiya & Swan, 2004).

Lohmann Brown is an egg-laying strain of chicken selectively bred from New Hampshire the Lohmann Brown hen starts laying at about 18 weeks of age, producing about 300 eggs/year these strains were kept for egg production and were also used to upgrade the indigenous chickens (Feltwell, 2011).

The spleen is the principal organ of systemic immunity and its importance in disease resistance is accentuated by the scarcity of avian lymph nodes (Moore, Mumaw & Schoenberg, 1964). The avian spleen functions as a major blood filtering organ and is the major source of

antibody production (Cross & Mercer, 1993). It does not function as a reservoir of blood as in mammals and its function is not oriented towards supply of oxygen (Jeurissen, 1991). The spleen also plays an important role in erythrocyte destruction, phagocytosis and antigen-antibody interactions (Burke & Simon, 1970).

Hormones are indispensable in carrying out reproductive function in farm animals (Sharp & Gow, 1983). Estradiol is the main reproductive hormone affecting growth, development, maturation and functioning of the reproductive tract as well as sexual differentiation and behavior (Balthazart *et al.,* 2009). Estrogens are synthesized and produced by gonads during avian embryonic development and control growth and differentiation of accessory sex structures (Johnson, 1990). This hormone is produced in the ovary by developing follicles and secreted by theca interna cells of Graaffian follicles under control of gonadotropins (Osinowo, 2016).

According to Elnagar and Abd-Elhady (2009), Estradiol Benzoate when administered to birds results in significantly higher body weight at sexual maturity and increase in number of egg, egg weight, shell gland size and serum calcium level. Estradiol plays important role in calcium metabolism especially shell formation (Dacke, 2000). Administration of exogenous estradiol increases plasma calcium levels in laying hens (Bar *et al.,* 1996). Calcium plays a vital role during reproduction by providing structural strength to the skeleton it also plays a role in biochemical reactions within the body via its concentration in the extracellular fluid (Dacke, 2000).

Aspartate (AST) and alanine aminotransferases (ALT) are a group of enzymes that catalyze the interconversion of amino acids by transfer of amino groups (Lumeij, 1987). Blood urea nitrogen is produced during protein catabolism and amino acids are converted to urea in the liver by the action of enzymes (Harr, 2006). Albumin is the most abundant protein found in

plasma and urine; its main function is the maintenance of colloid oncotic pressure in the intravascular and extravascular spaces (Stokol, Tarrant & Scarlett, 2001). Alkaline phosphatase has been identified in cell membranes in the liver, kidney, intestine and bone (osteoblasts) (Hochleithner *et al.,* 1994). High and low levels of alkaline phosphatase have been identified in chicks and also in the liver of pigeons (Lumeij & Remple, 1992). Transport of cholesterol in blood occurs via lipoproteins and cholesterol is synthesized and degraded in the liver (Harr, 2006).

1.2 PROBLEM STATEMENT

About 30% of laying hens experience fractures either during the production period or during the unloading of birds from cages at the end of egg production period, mostly due to low level of calcium which is used in the formation of egg shell (Gregory & Wilkins, 1989). At the end of the laying period, the hen produces larger eggs with reduced shell quality (Al-Batshan *et al.,* 1994).

Cracked shells, due to age-related reduction of shell quality, are a costly problem for the industry (Anna *et al.,* 2014). Parallel to reduced shell quality, the skeleton becomes brittle resulting in bone fractures calcium, a main prerequisite for both eggshell and bone, is regulated by estrogen in a complex manner (Anna *et al.*, 2014). Carbonic anhydrase is involved in providing major constituents for shell formation (Eastin & Spaziani, 1978). It is well known that estrogen is implicated in shell formation indirectly by acting on organs involved in calcium metabolism and an injection of estradiol increases the circulating levels of calcium in plasma (Bar *et al*., 1996).

In Nigeria, almost 3% or 3.8 million kilo of eggs is ruined yearly due to cracked eggshells leading to large economical losses (Mahmoud *et al.*, 1996). The eggshell is formed by

the shell gland which is an expanded pouch-like part of the oviduct, and during shell formation the blood flow to the shell gland increases 4-5 fold (Wolfenson, Frei & Berman, 1982). Eggshell consists mainly of calcium carbonate ($CaCO_3$) and the ionic precursors which are supplied by the blood through trans-epithelial transport. Blood flow through the shell gland increases dramatically during shell formation (Wolfenson *et al*., 1982).

1.3 JUSTIFICATION FOR THE STUDY

Estrogen concentration is very high before onset of the laying period which may be essential for synthesis of yolk protein and medullary portion of long bone and egg shell formation (Williams, 1994). Administration of an acute dose of exogenous estradiol has been shown to induce premature ovulation of a mature follicle at a specific time during the ovulatory cycle in normal laying chicken hens (Nakada, Koja & Tanaka, 1994).

Estradiol Benzoate initiates messenger ribonucleic acid (mRNA) expression and synthesis of albumen proteins from the oviduct (Stevens, 1991; Salomaa *et al.*, 1992). It has an indirect effect on liver insulin-like growth factor-1 (IGF-1) synthesis. IGF-1 has stimulatory effect on ovalbumin synthesis in the presence of estradiol (Pennequin, Robins & Schimke, 1978). Estradiol Benzoate affects liver function and stimulates the egg yolk precursors, vitellogenin (VTG) and very-low-density lipoprotein (VLDL), by increasing capacity for *de novo* fatty acid synthesis (Dashti *et al.*, 1983). Biochemical blood parameters, therefore, provide valuable information on avian health status and are often helpful in revealing health disorders (Harr, 2002).

The avian spleen has been frequently used to study immune status in birds (Smith & Hunt, 2004). Injection of estradiol in Japanese quail during the growth phase resulted in greater population of trabecular arteries and veins in the spleen (Sultana *et al.*, 2005). Exogenous

estradiol leads to maturation of spleen B-lymphocytes and production of immunoglobulins (Seko *et al.,* 2005).

1.4 OBJECTIVES

The specific objectives of the study were:

i. To determine changes in serum biochemistry as affected by administration of Estradiol Benzoate in Lohmann Brown layers.

ii. To evaluate spleen histomorphometry in Lohmann Brown layers following administration of Estradiol Benzoate.

iii. To assess histological changes in the shell gland of Lohmann Brown hens after administration of Estradiol Benzoate.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 LOHMANN BROWN

The Lohmann Brown is an egg-laying strain of chicken which is of hybrid origin and also due to advance in genetic selection make today's commercial layers quite different from those of years ago (Feltwell, 2011). These strains are selected from New Hampshire and other brown egg laying breeds and also, they start laying at about 18 weeks of age, laying about 1 egg per day and laying up to 300 brown eggs a year (Feltwell, 2011). Their eggs are laid daily, normally at dawn or dusk they are a very inquisitive breed of chicken most Lohmann browns have a caramel/brown shade of feathers, with white feathers in a pattern round their necks, and white feathers at the tips of their tail feathers (Feltwell, 2011). According to Vits, Weitzenburger, Hamann and Distl (2005), percent egg production, egg weight, shell thickness, and shell breaking strength of Lohmann Brown are better than that of Lohmann Selected Leghorn (LSL).

2.2 EXOGENOUS ESTROGENS

Estrogens (Estradiol-17ß) are synthesized and secreted by the gonads during development of the avian embryo, estradiol-17ß regulates growth and differentiation of the sex accessory structures (Johnson, 1990). This hormone stimulates the avian liver to produce the yolk precursors such as vitellogenin and very-low density lipoprotein (VLDL), which are the primary sources of yolk protein and lipid, respectively (Wallace, 1985). The production of these yolk precursors occurs not only in females forming eggs, but also in males and immature females treated with estradiol (Wiskocil *et al*., 1980). Estrogens stimulate feed intake and the deposition of calcium within the medullary portion of long bones (Bacon, Brown & Musser, 1980).

Estradiol Benzoate has effects on the growth of specific organs being responsible for the massive growth of the oviduct during sexual maturation (Scanes, Brant & Ensminger, 2004).

2.3 SERUM BIOCHEMISTRY

Serum is the component that is neither blood cell (serum does not contain white or red blood cells) nor a clotting factor and have blood plasma that have absence of fibrinogen (Liu *et al.,* 2010). Serum includes all proteins absent in blood clotting (coagulation) and electrolytes, antibodies, antigens, hormones and other exogenous substances (drugs and microorganisms) (Liu *et al*., 2010).

2.3.1 Serum Proteins, Calcium and Phosphorus

Serum albumin often refers to blood albumin (globular protein) found in vertebrate blood (Harr, 2006). According to Harper, Rodwell and Mayer (2012), serum albumin is essential for maintaining the oncotic pressure needed for proper distribution of body fluids between blood vessels and body tissues. Hypoalbuminemia will reduce the quantity of bound calcium and result in a decreased total calcium concentration without reducing biologically active calcium (ionized fraction) (Hochleithner, 1989; Lumeij, 1990; Hochleithner, 1991 as cited in Hochleithner, 1994).

Low protein levels may indicate malnutrition, malabsorption, chronic disease, renal disease, liver disease, or stress (Sakas, 2002). The hypoproteinemia that occurs with dehydration may result in an increased total calcium concentration (Hochleithner, 1994). Advancing age has been associated with increases in total protein in several bird species (Gylstorff & Grimm, 1987; Clubb *et al*., 1990, Clubb *et al*., 1991a and Clubb *et al*., 1991b as cited in Hochleithner, 1994). Hormones can have either an anabolic or catabolic effect on total protein (Hochleithner, 1994). In general, hormonal effects on total protein are minimal. However, testosterone, estrogen and growth hormone were found to increase total protein in chickens; thyroxine decreased

concentrations (Kaneko, 1989). Total calcium should always be interpreted along with albumin concentration (Hochleithner, 1994). Hypoalbuminemia will reduce the quantity of bound calcium and result in a decreased total calcium concentration without reducing biologically active calcium (ionized fraction) (Hochleithner, 1989; Lumeij, 1990; Hochleithner, 1991 as cited in Hochleithner, 1994).

Changes in inorganic phosphorus concentration can occur with several diseases, but not on a consistent basis (Hochleithner, 1994). No changes in inorganic phosphorus levels were noted in laying hens (Lewandowski, Campbell & Harrison, 1986).

2.3.2 Serum Glucose

Normal serum glucose for most birds ranges between 200 and 450 mg% (Sakas, 2002). Different effects of estrogen have been reported in relation to glucose (Nematbakhsh *et al*., 2009 as cited in Elkhier *et al.,* 2017). While Verma *et al.* (2005) showed a lowered glucose level in ovariectomized rats following treatment with estradiol-17β, Nagira *et al*. (2006) found an increase in glucose level. Reports by Barros *et al.* (2009) and Foryst-Ludwig and Kintscher (2010) suggest that estrogens had profound modulating effects on systemic glucose homeostasis. Plasma glucose levels are higher in juvenile than adult budgerigars (Hochleithner, 1989 as cited in Hochleithner, 1994). Variations also occur due to time of day and amount of environmental stress (Lewandowski *et al.*, 1986).

2.3.3 Serum Cholesterol

Elevated and decreased cholesterol concentrations may occur from a number of physiologic influences and different diseases; however, the diagnostic value of this test in birds appears to be poor (Hochleithner, 1994). Cholesterol concentrations will vary with a birds' diet.

Carnivorous birds have higher concentrations, whereas fruit or grain-eating birds have lower concentrations (Lewandowski *et al*., 1986).

2.3.4 Blood Urea Nitrogen

Urea is present in very small amounts in avian plasma, and determining urea level has generally been considered of little value (Hochleithner, 1994). However, investigations have shown good correlation between increased plasma concentrations and renal disease in pigeons (Lumeij, 1987 as cited in Hochleithner, 1994). In other avian species, urea may have little value in detecting renal disease but can be used as a sensitive indicator of dehydration.

2.3.5 Serum Enzymes

Alanine aminotransferase is an enzyme that is found mainly in the liver, but also found in red blood cells, heart cells, muscle tissue and other organs, such as the pancreas and kidneys (Huang *et al.,* 2006). Any abnormal increase in serum levels of alanine aminotransferase and alkaline phosphatase may signify liver damage (Yalcin *et al.,* 2010). When body tissues or an organ such as the liver or heart is diseased or damaged, additional aspartate aminotransferase are released into the blood stream causing level of the enzyme to rise in the blood stream (Thapa & Walia, 2007).

High levels will damage the liver cells and leak enzymes into the blood stream (Parmar *et al.,* 2012). Additional AST and ALT are released into the bloodstream, causing levels of these enzymes to rise (Huang *et al.,* 2006). Liver diseases caused by infection increases AST and other liver function enzymes as a result of challenges encountered by the liver to overcome toxins released by pathogens (Hashem & Mohamed, 2009; Huang *et al.,* 2006). Alkaline phosphatase is an enzyme responsible for dephosphorylation of a substrate which is produced in all types of tissues in the body, but gets activated in alkaline environment (Senanayake *et al.,* 2015).

Elevated levels of alkaline phosphatase can be mostly seen in liver damages (Mauro, Renze & Wouter, 2006).

Activities of serum transaminases were not affected by exogenous estradiol-17β injection in Japanese quails during different stages of production (El-Ghalid, 2009). Alanine Aminotransferase (ALT) is mainly found in the liver and kidney in pigeons (Lumeij, de Bruijne, Slob, Wolfswinkel & Rothuizen, 1988). It has been reported to exhibit low activity in plasma, high activity in erythrocytes and seasonal variation with limitation in terms of its use for diagnostic purposes (Lewandowski *et al.*, 1986; Lumeij & Westerhof, 1987). Aspartate Aminotransferase (AST) is widely distributed in avian tissues and its relative distribution varies from one genus to another (Lumeij & Westerhof, 1987). Alkaline Phosphatase (ALP) is a well established marker of bone diseases, but it is not specific for bone only (Pardhe *et al*., 2017). Bone-specific ALP measurement is, therefore, indicated in order to differentiate liver disease from osteoporotic activity (Bikle, 1997).

2.3.6 Serum Creatinine

There is a slim margin between the physiologic and pathologic levels of creatinine (Hochleithner, 1994). Excretion of creatinine is solely via the kidneys (Hochleithner, 1994). It is freely filtered and reabsorbed in the tubules (Gylstorff & Grimm (1987) as cited in Hochleithner, 1994). In birds, creatine is excreted in urine before it is conversion to creatinine (Bell & Freeman, 1971). The urinary excretion of creatine may be one of the reasons that creatinine level does not provide an accurate assessment of avian renal function.

2.4 SPLEEN

Payne (1971) described spleen in avian species as a round or oval structure lying dorsal to and on the left side of the proventriculus. Eerola, Veromaa and Toivanen (1987) highlighted

that, the splenic development occurs after hatching, upon encounter to antigens. Spleen is the most important organ of systemic immunity and disease resistance; it is pronounce by the limited number of avian lymph nodes (John, 1994).

The avian spleen serves as a filter of blood and provider of antibodies (Jeurissen, 1991). Burke and Simon (1970) reported the roles of spleen in erythrocyte destruction, phagocytosis and antigen-antibody interactions. The spleen also contains a sizable population of natural killer T (NKT) cells, which sense lipid antigens and are involved in a broad range of immune responses by secreting cytokines and inducing downstream activation of adaptive immune cell types (Bronte & Pittet, 2013). Olah and Glick (1982) indicated that in chicken, the periarterial lymphatic sheath consisted of densely packed mass of medium and large sized lymphocytes and macrophages. The splenic red pulp is composed of pulp cords consisted of erythrocytes, reticular cells and lymphocytes of various sizes, macrophages, granulocytes, plasma cells and mast cells (Kannan *et al.,* 2015). Olah and Glick (1982) studied the chicken spleen and found that the ellipsoid was made up predominantly of reticular cells. The reticular cells were extremely polymorphic. Reticular fibres were seen between the reticular cells. King and Mc Lelland (1981) as cited in Kannan *et al* (2015) described white pulp of spleen as Islands enclosed by red pulp and the distinction between the two pulps are not marked in chicken. White pulp in the chicken is divided into four elements, such as periarterial lymphatic sheath (PALS), perivenous lymphatic tissue, periellipsoidal lymphatic tissue and germinal centres. The first elements appeared two days after hatching. The second, third and fourth elements appeared on the sixth day, third week and fourth week, respectively (Ogata, Sukumura & Kudo, 1977).

2.5 SHELL GLAND

Rahman (2013) mentioned that the shell gland (also referred to as the uterus) of hens is about 7 cm long, reddish in colour and pouch-like in structure. The egg remains in the uterus for 19-20 hours of the laying cycle (Rahman, 2013). This phase is largely under the control of progesterone (Ottinger & Bakst 1995).

Mucosa in shell gland was reported to deliver calcium carbonate during egg shell formation (Eastin & Spaziani, 1978 as cited in Wistedt, Ridderstrale, Wall & Holm, 2014). Shell gland length increased significantly by 17% due to estradiol injection (Elnagar & Abd-Elhady, 2009). Bar *et al.* (1996), as cited in Wistedt *et al.* (2014), indicated that estrogen is implicated in shell formation indirectly by acting on organs involved in calcium metabolism and an injection of estradiol increases the circulating levels of calcium in the plasma. It has been established that estrogen has some regulatory influence on membrane-bound calcium activity in the shell gland, which is supported by the finding that estradiol up-regulates membrane-bound calcium in H9C2 cells (Liu *et al.*, 2007 as cited in Wistedt *et al.*, 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 EXPERIMENTAL SITE

The experiment was conducted at the Poultry unit, Teaching and Research Farm of the Department of Animal Science, Bayero University, Kano (GPS Coordinates: 11.97643^0N, 008.42995^0E, with an elevation of 469 m above sea level). The birds were managed intensively in a battery cage. The cage was housed in a building flanked by high walls by the western and eastern sides to prevent direct sun rays and rain splash. The cage comprised of 18 separate compartments coupled with aluminum SP drinkers and feeders of half-opened plastic pipes.

3.2 EXPERIMENTAL BIRDS AND THEIR MANAGEMENT

A total of 18 Lohmann Brown hens aged twenty four weeks were used for this study. They were purchased from SOVET International Farm, Kano at rate of ₦1200 each. The birds were allowed to stabilize for two weeks. During this period, they were fed *ad libitum* with layer mash (Sovet Super Layer®) containing 16.0 % crude protein, 3.5 % calcium, 5.0 % fibre, 0.4 % phosphorus, 5.0 % fat and 2600 kcal/kg energy and were provided with water throughout the experimental period. The experimental birds were given multivitamins for 1 day (Anupco®, Anglia Nutrition Products Company, UK) at 0.5 g/liter as anti-stress as well as the antibiotic for 3 days Oxytetracycline HCl (Oxywin®, Sellwell Pharmaceuticals Ltd, India) at 1 g/liter via drinking water against secondary bacterial infections.

3.3 EXPERIMENTAL DESIGN

The experiment was laid out in a Single Factor Completely Randomized Design. Estradiol Benzoate was administered at six dose levels of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/bird

which were designated as treatments A, B, C, D, E and F, respectively. Normal saline (1ml) were given to Treatment A which served as control birds. Each treatment was replicated three times.

3.4 EXPERIMENTAL PROCEDURE AND DATA COLLECTION

3.4.1 Estradiol Benzoate Administration

Estradiol Benzoate Injection (Super estradiol®, Hebei New Century Pharmaceutical Company Limited, China) was administered twice weekly via the breast muscle using a 2 ml hypodermic syringe in the morning between 10:00 and 11: 00 am throughout the six weeks of experimentation.

3.4.2 Blood Collection and Serum Extraction

On the last day of the experiment, blood was taken during slaughter. Blood was immediately transferred into labeled plain bottles and kept for two hours at room temperature to allow for clotting. A wooden stick was used to dislodge the blood clot and content was centrifuged at 4000 revolutions per minute (RPM) for 30 minutes (Centrifuge 800D®, Techmel & Techmel, USA). The recovered sera were transferred into clean labeled plain tubes using dropping pipettes and stored at -20 ºC until needed for analysis.

3.4.3 Serum Biochemical Analysis

Sera stored at -20 ºC were thawed at room temperature and the following biochemical parameters were determined: Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Albumin, Cholesterol, Glucose, Blood Urea Nitrogen, Creatinine, Total Protein, Calcium and Phosphorus. These biochemical parameters were analyzed using commercial assay kits, and the results were read using colorimetry with wave length of 546nm (Colorimeter 257, CIBA CORNING®). Globulin was derived from the difference between Total Protein and Albumin.

3.4.4 Harvesting of Spleen and Shell Gland

A of the procedure of Natalia and Roser (2011) were employed for dissection and harvesting of organs. Before slaughter, the birds were weighed using a weighing scale (HANA®, Model J1603444602, China). After slaughter, the abdominal cavity was opened and the spleen and shell gland were carefully harvested.

The harvested organs were fixed in 10% Neutral Buffered Formalin and transported to the Pathology Department of the Aminu Kano Teaching Hospital for histological processing according to Bancroft and Gamble (2008).

3.4.5 Histological Assessment of the Shell Gland

Prepared shell gland slides were sent to the Pathology Department, Ahmadu Bello University Teaching Hospital for assessment of qualitative changes across treatment groups.

3.4.6 Histomorphometric Evaluation of Spleen

The histological slides of the spleen were mounted on microscope and the structural components were assessed as described by Maas and Orthel (1976) with modifications. Each slide was observed across 5 random fields using an Olympus CX21 microscope at a total magnification of x100 (eyepiece + nosepiece). Observer-dependent percent composition of each component (Vascular skeleton, Red pulp, Ellipsoids, Peri-arteriolar lymphatic sheath (PALS) and B Follicles) visible on hematoxylin and eosin stained slides were noted and averages were recorded as percentages. Vascular skeleton was identified as medium- and small-sized arterioles with their branches visible at x100 magnification. The red pulp was identified as all red pulp areas including capillary and sinusoids. Ellipsoids were evaluated as merged with peri-arteriolar lymphatic sheath (PALS) without the use of reticulin stain which delineates them. B follicles were identified as circular aggregates of small-sized lymphocytes.

3.5 DATA ANALYSIS

The data generated were subjected to analysis using GraphPad InStat Package (GraphPad InStat®, version 3.05, 32 bit for Win 95/NT, GraphPad Software Inc., 2000). Kruskal-Wallis test was used to determine the effect of Estradiol Benzoate on serum levels of glucose, alanine and aspartate aminotransferases, alkaline phosphatase, blood urea nitrogen, creatinine, calcium, phosphorus, cholesterol, total protein, globulin and albumin, and proportion of red pulp, ellipsoids and peri-arteriolar lymphatic sheath (PALS). Significant mean rank differences were separated using Dunn's Multiple Comparisons test. One-Way Analysis of Variance (ANOVA) was used to determine the effect of Estradiol Benzoate on proportions of splenic vascular skeleton and B follicles. Significant mean proportions across treatments were separated using Tukey's test.

CHAPTER FOUR

4. 0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Serum Biochemistry

Serum glucose

The effect of Estradiol Benzoate on serum concentration of glucose in Lohmann Brown layers is presented in Table 1. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 9.414) differences in median serum glucose concentrations of 8.0, 11.1, 11.7, 11.7, 11.1 and 9.9 mmol/L across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum serum glucose concentrations were 6.6, 10.1, 10.3, 11.3, 10.6 and 7.9 mmol/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively. Maximum serum glucose concentrations were 8.1, 12.1, 14.8, 12.1, 11.2 and 11.2 mmol/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively.

Serum alanine aminotransferase

The effect of Estradiol Benzoate on serum concentration of alanine aminotransferase in Lohmann Brown layers is shown in Table 2. There were no statistically significant (P>0.05, Kruskal-Wallis statistics = 2.714) differences in median serum alanine aminotransferase concentrations (3, 3, 3, 3, 3 and 3 U/L) across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum *vs.* maximum serum alanine aminotransferase concentrations were 3 U/L *vs*. 6 U/L, 3 U/L *vs*. 3 U/L, 3 U/L *vs*. 3 U/L, 3 U/L *vs*. 3 U/L, 3 U/L *vs*. 15 U/L and 3 U/L *vs*. 6 U/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively.

Table 1: Summary Statistics (including KW Statistic) of Serum Glucose Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (mmol/L)	Minimum (mmol/L)	Maximum (mmol/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	8.0	6.6	8.1	8.0	2.667	
0.2	2	11.1	10.1	12.1	20.5	10.250	
0.4	3	11.7	10.3	14.8	36.0	12.000	
							9.414^{ns}
0.6	2	11.7	11.3	12.1	26.5	13.250	
0.8	3	11.1	10.6	11.2	27.5	9.167	
1	3	9.9	7.9	11.2	17.5	5.833	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Table 2: Summary Statistics (including KW Statistic) of Serum Alanine Aminotransferase Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (U/L)	Minimum (U/L)	Maximum (U/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	3	3	6	28.5	9.5	
0.2	2	3	3	3	14.0	7.0	
0.4	3	3	3	3	21.0	7.0	
							2.714^{ns}
0.6	2	3	3	3	14.0	7.0	
0.8	3	3	3	15	30.0	10.0	
1	3	3	3	6	28.5	9.5	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Serum aspartate aminotransferase

The effect of Estradiol Benzoate on serum concentration of aspartate aminotransferase in Lohmann Brown layers is highlighted in Table 3. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 9.624) differences in median serum aspartate aminotransferase concentrations of 23, 27, 19, 37, 36 and 36 U/L across respective Estradiol Benzoate treatment groups (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). The minimum serum aspartate aminotransferase concentrations were 19, 27, 13, 27, 31 and 31 U/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively. Maximum serum aspartate aminotransferase concentrations were 27, 27, 31, 47, 36, and 37 U/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively.

Serum alkaline phosphatase

The effect of Estradiol Benzoate levels on serum alkaline phosphatase concentration in Lohmann Brown layers is shown in Table 4. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 0.8569) differences in median serum alkaline phosphatase concentrations (43.0, 42.0, 47.0, 45.5, 43.0 and 46.0 U/L) across Estradiol Benzoate treatment levels. Minimum *vs*. maximum alkaline phosphatase concentrations were 42 U/L *vs.* 45 U/L, 37 U/L *vs*. 48 U/L, 38 U/L *vs.* 48 U/L, 43 U/L *vs.* 48 U/L, 40 U/L *vs.* 50 U/L and 38 U/L *vs.* 52 U/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively.

Table 3: Summary Statistics (including KW Statistic) of Serum Aspartate Aminotransferase Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (U/L)	Minimum (U/L)	Maximum (U/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	23	19	27	13.0	4.333	
0.2	2	27	27	27	13.0	6.500	
0.4	3	19	13	31	13.5	4.500	
							9.624^{ns}
0.6	2	37	27	47	22.5	11.250	
0.8	3	36	31	36	36.0	12.000	
1	3	36	31	37	38.0	12.667	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Table 4: Summary Statistics (including KW Statistic) of Serum Alkaline Phosphatase Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (U/L)	Minimum (U/L)	Maximum (U/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	43.0	42	45	21.0	7.000	
0.2	2	42.5	37	48	14.0	7.000	
0.4	3	47.0	38	48	26.5	8.833	
							0.8569^{ns}
0.6	2	45.5	43	48	20.0	10.000	
0.8	3	43.0	40	50	26.0	8.667	
1	3	46.0	38	52	28.5	9.500	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Blood urea nitrogen

The effect of Estradiol Benzoate on blood urea nitrogen concentration in Lohmann Brown layers is presented in Table 5. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 10.072) differences in median blood urea nitrogen concentrations (1.70, 1.75, 1.30, 1.75, 1.30 and 1.80 mmol/L) across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum *vs*. maximum blood urea nitrogen concentration were 1.4 mmol/L *vs*. 1.9 mmol/L, 1.7 mmol/L *vs*. 1.8 mmol/L, 1.2 mmol/L *vs*. 1.6 mmol/L, 1.7 mmol/L *vs*. 1.8 mmol/L, 1.2 mmol/L *vs*. 1.6 mmol/L and 1.7 mmol/L *vs*. 2.2 mmol/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate levels, respectively.

Serum creatinine

The effect of Estradiol Benzoate on serum concentration of creatinine in Lohmann Brown layers is highlighted in Table 6. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 6.748) differences in median serum creatinine concentrations (155.0, 128.0, 123.0, 153.5, 137.0 and 169.0 μmol/L) across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum *vs*. maximum creatinine concentrations were 150 μmol/L *vs*. 206 μmol/L, 128 μmol/L *vs*. 128 μmol/L, 110 μmol/L *vs*. 187 μmol/L, 142 μmol/L *vs*. 165 μmol/L, 114 μmol/L *vs*. 146 μmol/L and 146 μmol/L *vs*. 174 μmol/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively.

Table 5: Summary Statistics (including KW Statistic) of Blood Urea Nitrogen Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (mmol/L)	Minimum (mmol/L)	Maximum (mmol/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	1.70	1.4	1.9	29.5	9.833	
0.2	2	1.75	1.7	1.8	22.5	11.250	
0.4	3	1.30	1.2	1.6	11.5	3.833	
							10.072^{ns}
0.6	2	1.75	1.7	1.8	22.5	11.250	
0.8	3	1.30	1.2	1.6	11.5	3.833	
1	3	1. 80	1.7	2.2	38.5	12.833	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Table 6: Summary Statistics (including KW Statistic) of Serum Creatinine Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (μmol/L)	Minimum (μmol/L)	Maximum (μmol/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	155.0	150	206	37.0	12.333	
0.2	2	128.0	128	128	9.0	4.500	
0.4	3	123.0	110	187	19.0	6.333	
							6.748^{ns}
0.6	2	153.5	142	165	19.0	9.500	
0.8	3	137.0	114	146	16.5	5.500	
1	3	169.0	146	174	35.5	11.833	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Serum calcium

The effect of Estradiol Benzoate on serum calcium concentration in Lohmann Brown layers is highlighted in Table 7. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 1.937) differences in median serum calcium concentrations (2.5, 2.6, 2.5, 2.6, 2.5 and 2.6 mmol/L) across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum *vs*. maximum serum calcium concentrations were 2.5 mmol/L *vs*. 2.6 mmol/L, 2.5 mmol/L *vs*. 2.7 mmol/L, 2.4 mmol/L *vs*. 2.7 mmol/L, 2.6 mmol/L *vs*. 2.6 mmol/L, 2.3 mmol/L *vs*. 2.6 mmol/L and 2.3 mmol/L *vs*. 2.7 mmol/L across 0, 0.2, 0.4, 0.6, 0.8 and 1mg Estradiol Benzoate treatment levels, respectively.

Serum phosphorus

The effect of Estradiol Benzoate on serum concentration of phosphorus in Lohmann Brown layers is shown in Table 8. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 11.057) differences in median serum phosphorus concentrations (1.70, 0.90, 1.60, 1.75, 1.80 and 2.50 mmol/L) across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum serum phosphorus concentrations were 1.6, 0.3, 1.5, 1.5, 1.7 and 2.3 mmol/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate levels, respectively. Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg) gave maximum serum phosphorus concentrations of 1.80, 1.50 1.70, 2.00, 1.80 and 2.60 mmol/L, respectively.

Table 7: Summary Statistics (including KW Statistic) of Serum Calcium Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (mmol/L)	Minimum (mmol/L)	Maximum (mmol/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	2.5	2.5	2.6	23.0	7.667	
0.2	2	2.6	2.5	2.7	21.0	10.500	
0.4	3	2.5	2.4	2.7	24.0	8.000	
							1.937^{ns}
0.6	2	2.6	2.6	2.6	22.0	11.000	
0.8	3	2.5	2.3	2.6	18.5	6.167	
1	3	2. 6	2.3	2.7	27.5	9.167	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Table 8: Summary Statistics (including KW Statistic) of Serum Phosphorus Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (mmol/L)	Minimum (mmol/L)	Maximum (mmol/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	1.70	1.6	1.8	24.5	8.167	
0.2	2	0.90	0.3	1.5	4.0	2.000	
0.4	3	1.60	1.5	1.7	16.5	5.500	
							11.057^{ns}
0.6	2	1.75	1.5	2.0	16.0	8.000	
0.8	3	1.80	1.7	1.8	30.0	10.000	
1	3	2.50	2.3	2.6	45.0	15.000	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Serum cholesterol

The effect of Estradiol Benzoate on serum concentration of cholesterol in Lohmann Brown layers is presented in Table 9. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 10.594) differences in median serum cholesterol concentrations (1.30, 1.35, 1.80, 1.20, 1.20 and 2.40 mmol/L) across respective Estradiol Benzoate treatment levels of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg. Minimum *vs*. maximum serum cholesterol concentrations were 1.2 mmol/L *vs*. 1.7 mmol/L, 0.9 mmol/L *vs*. 1.8 mmol/L, 1.7 mmol/L *vs*. 2.1 mmol/L, 0.9 mmol/L *vs*. 1.5 mmol/L, 1.0 mmol/L *vs*. 1.6 mmol/L and 2.4 mmol/L *vs*. 2.6 mmol/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate levels, respectively.

Serum total protein

The effect of Estradiol Benzoate on serum concentration of total protein in Lohmann Brown layers is shown in Table 10. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 7.216) differences in median serum total protein concentrations of 60, 56, 56, 49, 49 and 53 g/L across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum serum total protein concentrations were 51, 49, 53, 47, 39 and 49 g/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively. Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg) gave respective maximum serum total protein concentrations of 61, 63, 57, 51, 50 and 57 g/L, respectively.

Table 9: Summary Statistics (including KW Statistic) of Serum Cholesterol Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (mmol/L)	Minimum (mmol/L)	Maximum (mmol/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	1.30	1.2	1.7	20.0	6.667	
0.2	2	1.35	0.9	1.8	13.0	6.500	
0.4	3	1.80	1.7	2.1	34.0	11.333	
							10.594^{ns}
0.6	2	1.20	0.9	1.5	8.5	4.250	
0.8	3	1.20	1.0	1.6	15.5	5.167	
1	3	2.40	2.4	2.6	45.0	15.000	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Table 10: Summary Statistics (including KW Statistic) of Serum Total Protein Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (g/L)	Minimum (g/L)	Maximum (g/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	60	51	61	36.5	12.167	
0.2	2	56	49	63	20.0	10.000	
0.4	3	56	53	57	33.0	11.000	
							7.216^{ns}
0.6	2	49	47	51	9.5	4.750	
0.8	3	49	39	50	11.0	3.667	
1	3	53	49	57	26.0	8.667	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Serum albumin

The effect of Estradiol Benzoate levels on serum concentration of albumin in Lohmann Brown layers is highlighted in Table 11. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 0.7317) differences in median serum albumin concentrations of 25.0, 25.5, 26.0, 26.0, 25.0 and 25.0 g/L across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg). Minimum serum albumin concentrations were 25, 25, 24, 25, 25 and 24 g/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively. Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg) gave respective maximum serum albumin concentrations of 27, 26, 28, 27, 26 and 27 g/L, respectively.

Serum globulin

The effect of Estradiol Benzoate levels on serum globulin concentrations in Lohmann Brown layers is presented in Table 12. There were no statistically significant (P>0.05, Kruskal-Wallis statistic = 7.713) differences in median serum globulin concentrations (33.0, 30.5, 29.0, 23.0, 24.0 and 29.0 g/L) across respective Estradiol Benzoate treatment levels. Minimum *vs*. maximum globulin concentrations were 26 g/L *vs*. 36 g/L, 24 g/L *vs*. 37 g/L, 28 g/L *vs*. 31 g/L, 20 g/L *vs*. 26 g/L, 14 g/L *vs*. 24 g/L and 24 g/L *vs*. 30 g/L across 0, 0.2, 0.4, 0.6, 0.8 and 1 mg Estradiol Benzoate treatment levels, respectively.

Table 11: Summary Statistics (including KW Statistic) of Serum Albumin Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (g/L)	Minimum (g/L)	Maximum (g/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	25.0	25	27	26.0	8.667	
0.2	2	25.5	25	26	17.0	8.500	
0.4	3	26.0	24	28	28.5	9.500	
							0.7317^{ns}
0.6	2	26.0	25	27	20.0	10.000	
0.8	3	25.0	25	26	23.0	7.667	
1	3	25.0	24	27	21.5	7.167	

^{ns} = not significant, P>0.05; N= Number of experimental birds

Table 12: Summary Statistics (including KW Statistic) of Serum Globulin Concentration across Estradiol Benzoate Treatment Levels in Lohmann Brown Hens

Estradiol Benzoate (mg)	N	Median (g/L)	Minimum (g/L)	Maximum (g/L)	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) Statistic, corrected for ties
0	3	33.0	26	36	36.5	12.167	
0.2	2	30.5	24	37	20.5	10.250	
0.4	3	29.0	28	31	32.5	10.833	
							7.713^{ns}
0.6	2	23.0	20	26	9.5	4.750	
0.8	3	24.0	14	24	10.0	3.333	
1	3	29.0	24	30	27.0	9.000	

^{ns} = not significant, P>0.05; N= Number of experimental birds

4.1.2 Spleen Histomorphometry

Vascular skeleton

Effect of Estradiol Benzoate on the proportion of vascular skeleton in the spleen of Lohmann Brown Hens is shown in Table 13. There were no statistically significant (P>0.05) differences in mean proportion (16, 11, 12, 15, 11 and 11 %) of splenic vascular skeleton across Estradiol Benzoate treatment levels.

Red pulp

Effect of Estradiol Benzoate on proportion of red pulp in the spleen of Lohmann Brown hens is presented in Table 14. There was a statistically significant (P<0.01; mean difference = -25.00 %) difference in spleen red pulp proportion between control birds and those administered 0.8 mg Estradiol Benzoate. All other comparisons among Estradiol Benzoate groups gave statistically similar (P>0.05) splenic red pulp proportion.

Ellipsoids and PALS

Effect of Estradiol Benzoate on the proportion of ellipsoids and PALS in the spleen of Lohmann Brown hens is highlighted in Table 16. There was a statistically significant (P<0.05; mean difference = 18.20 %) difference in mean proportion of splenic ellipsoids and PALS across Estradiol Benzoate treatment groups. All other comparisons among Estradiol Benzoate treatment levels gave statistically similar (P>0.05) mean proportion of splenic ellipsoids and PALS.

Table 13: Effect of Estradiol Benzoate on Proportion of Vascular Skeleton in the Spleen of Lohmann Brown Hens

Estradiol Benzoate (mg)	Mean Proportion (%)	Standard Error of Mean
0	16	1.871
0.2	11	1.871
0.4	12	1.225
0.6	15	3.162
0.8	11	1.000
1	11	1.000

P>0.05

Table 14: Effect of Estradiol Benzoate on Proportion of Red Pulp in the Spleen of Lohmann Brown Hens

Comparison	Mean Rank Difference (%)	Levels of Significance
0 mg *vs*. 0.2 mg	-13	ns
0 mg *vs*. 0.4 mg	-12	ns
0 mg *vs*. 0.6 mg	-14	ns
0 mg *vs*. 0.8 mg	-25	**
0 mg *vs*. 1 mg	-16	ns
0.2 mg *vs*. 0.4 mg	1	ns
0.2 mg *vs*. 0.6 mg	-1	ns
0.2 mg *vs*. 0.8 mg	-12	ns
0.2 mg *vs*. 1 mg	-3	ns
0.4 mg *vs*. 0.6 mg	-2	ns
0.4 mg *vs*. 0.8 mg	-13	ns
0.4 mg *vs*. 1 mg	-4	ns
0.6 mg *vs*. 0.8 mg	-11	ns
0.6 mg *vs*. 1 mg	-2	ns
0.8 mg *vs*. 1 mg	9	ns

ns = not significant; **P<0.01

Table 15: Effect of Estradiol Benzoate on Proportion of Ellipsoids and PALS in the Spleen of Lohmann Brown Hens

Comparisons	Mean Rank Difference (%)	Levels of Significance
0 mg *vs*. 0.2 mg	9.0	ns
0 mg vs. 0.4 mg	7.0	ns
0 mg *vs*. 0.6 mg	12.0	ns
0 mg *vs*. 0.8 mg	18.2	*
0 mg *vs*. 1 mg	10.0	ns
0.2 mg *vs*. 0.4 mg	-2.0	ns
0.2 mg *vs*. 0.6 mg	3.0	ns
0.2 mg *vs*. 0.8 mg	9.2	ns
0.2 mg *vs*. 1 mg	1.0	ns
0.4 mg *vs*. 0.6 mg	5.0	ns
0.4 mg *vs*. 0.8 mg	11.2	ns
0.4 mg *vs*. 1 mg	3.0	ns
0.6 mg *vs*. 0.8 mg	6.2	ns
0.6 mg *vs*. 1 mg	-2.0	ns
0.8 mg *vs*. 1 mg	-8.2	ns

ns = not significant; *P<0.05; PALS = Peri-arteriolar Lymphatic Sheath

B Follicles

Effect of Estradiol Benzoate on the proportion of splenic B follicles in Lohmann Brown hens is presented in Table 16. There were no statistically significant (P>0.05) differences in mean proportion (1.0, 0.0, 0.0, 0.0, 1.2 and 0.0 %) of splenic B follicles across respective Estradiol Benzoate treatment levels (0, 0.2, 0.4, 0.6, 0.8 and 1 mg).

Table 16: Effect of Estradiol Benzoate on Proportion of B Follicles in the Spleen of Lohmann Brown Hens

Estradiol Benzoate (mg)	Mean Proportion (%)	Standard Error of Mean
0	1. 0	1. 0000
0.2	0. 0	0. 0000
0.4	0. 0	0. 0000
0.6	0. 0	0. 0000
0.8	1. 2	0.4899
1	0. 0	0. 0000

P>0.05

4.1.3 Histological Responses of the Shell Gland

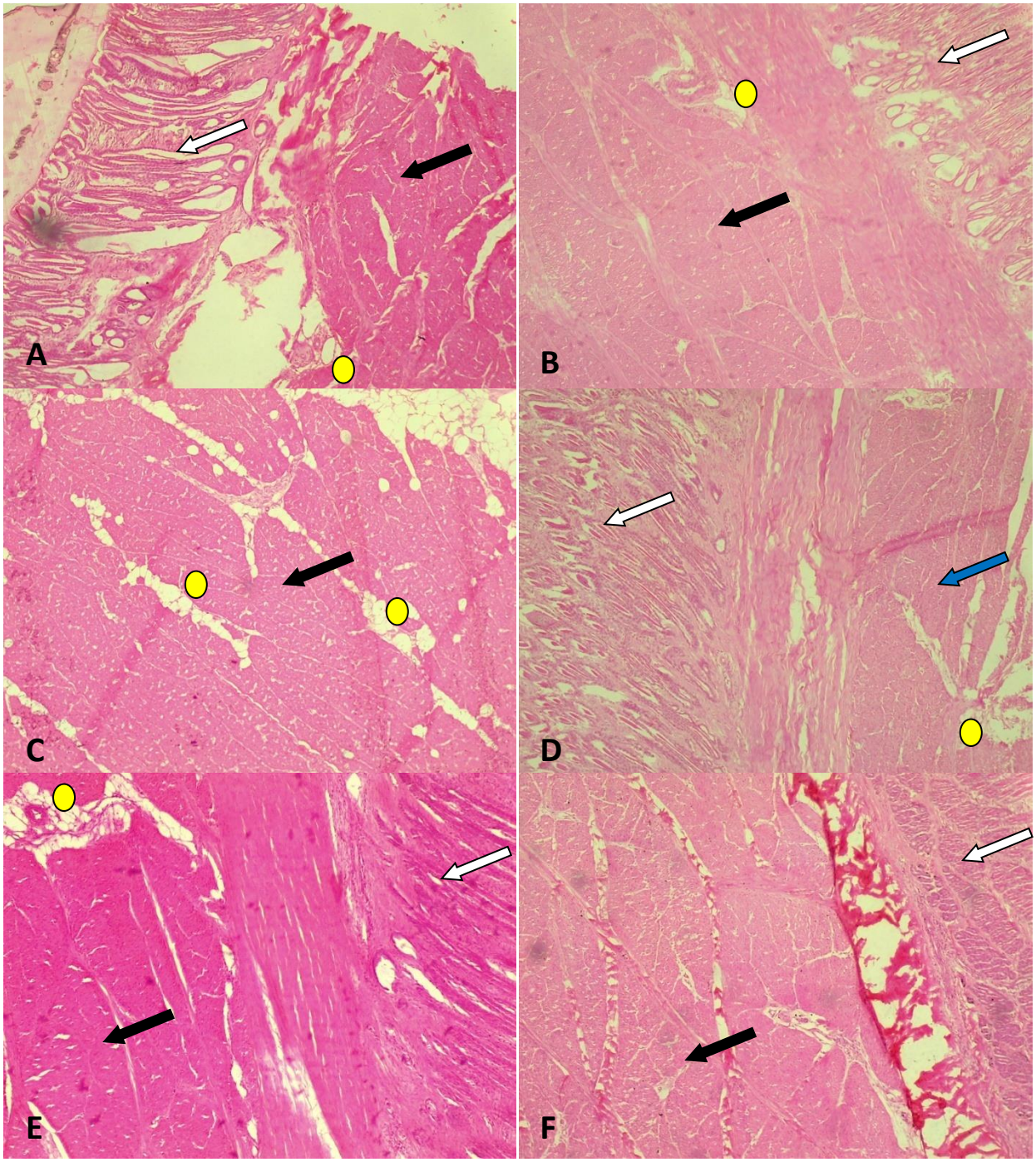

Plate (A-F) x100: Photomicrography of Shell Gland of Lohmann Brown Hens treated with Different Concentrations (A = 0 mg, B = 0.2 mg, C = 0.4 mg, D = 0.6 mg, E = 0.8 mg and F = 1 mg) of Estradiol Benzoate. White arrows show mucosal glands containing secretions; black arrows show hypertrophied muscularis; blue arrows show thinner muscularis; yellow dots show fatty infiltrates. **A -** Mucosa: straight tubular glands in scanty loose fibrous stroma. Glands are lined by low columnar to cuboidal cells and contain secretions. Thickness - 0.75 mm. Muscularis: no atrophic changes, fibres are separated by thickened fibrous septae. Fat infiltrates ++[1], thickness – 12 mm. **B -** Mucosa: straight tubular glands lined by cuboidal cells and containing secretions. Stroma is loose, fibrous with occasional lymphoid aggregate. Thickness - 0.75 mm. Muscularis: mild hypertrophy, fat infiltrates +++, Thickness – 12 mm. **C -** Mucosa: no mucosa on slide. Muscularis: fibres are mildly atrophic with cytoplasmic vacuoles, fat infiltrates ++++, Thickness - not measured[2]. **D -** Mucosa: straight tubular glands lined by cuboidal cells with luminal secretions. Scanty vascular stroma. Thickness - 0.5 mm. Muscularis: mild hypertrophy, fat infiltrates +++, Thickness - 10.5 mm. **E -** Mucosa: straight tubular glands lined by low cuboidal cells with luminal secretions, stroma is moderate and fibrous, Thickness - 0.5 mm. Muscularis: mild hypertrophy, fat infiltrates +++, Thickness – 16 mm. **F -** Mucosa: straight tubular glands lined by cuboidal cells with luminal secretions. Stroma is moderate and fibrous. Thickness - 0.5 mm. Muscularis: moderate hypertrophy, fat infiltrates +++, thickened septae, Thickness- 16 mm.

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[1] Fat infiltrate + - no fat cells present/5 (x100) fields, ++ - 1-3 fat cell aggregates/5 (x100) fields, +++ - 6-10 fat cell aggregates/5 (x100) fields, and ++++ - >10 fat cell aggregates/5 (x100) fields.

[2] No mucosa and no serosa identified on slide hence thickness is not measurable.

4.2 DISCUSSION

Median serum glucose levels recorded in the present study were within the range of values (7.0-13.2 mmol/L) reported by Nanbol *et al*. (2016) for layers in the 16- to 52-week age bracket in Plateau State, Nigeria. Different effects of estrogen have been reported in relation to glucose (Nematbakhsh *et al*., 2009 as cited in Elkhier *et al.*, 2017). While Verma *et al.* (2005) showed a lowered glucose level in ovariectomized rats following treatment with estradiol-17β, Nagira *et al*. (2006) found an increase in glucose level. Reports by Barros *et al.* (2009) and Foryst-Ludwig and Kintscher (2010) suggest that estrogens had profound modulating effects on systemic glucose homeostasis. However, in the current study similar glucose levels were recorded across Estradiol Benzoate groups which points to the possibility that the levels used were not enough to inhibit or raise endogenous insulin levels which will in turn increase or decrease glucose levels as the case may be (Verma *et al*., 2005; Nagira *et al*., 2006).

Activities of serum transaminases were not affected by exogenous estradiol-17β injection in Japanese quails during different stages of production (El-Ghalid, 2009). This is in agreement with the result of the current study as hens were actively laying during the present study which may confer a confounding effect on serum transaminase activities under Estradiol Benzoate injection. Median serum alanine aminotransferase (ALT) levels were one unit higher than the upper ALT range (1-2 IU/L) in layers reported by Nanbol *et al*. (2016). However, they were similar across Estradiol Benzoate groups. ALT is mainly found in the liver and kidney in pigeons (Lumeij *et al.*, 1988). It has been reported to exhibit low activity in plasma, high activity in erythrocytes and seasonal variation with limitation in terms of its use for diagnostic purposes (Lewandowski *et al.*, 1986; Lumeij & Westerhof, 1987).

Median aspartate aminotransferase (AST) levels in the present study were 6 to 12 times higher than the upper AST range (1-3 IU/L) reported in the work of Nanbol *et al*. (2016) and below the range (66.5-365 IU/L) reported for the Common Buzzard by Hernandez, Martin and Fores (1990). AST is widely distributed in avian tissues and its relative distribution varies from one genus to another (Lumeij & Westerhof, 1987). Alanine (ALT) and aspartate (AST) aminotransferases belong to a group of enzymes that catalyze inter conversion of amino acids and oxoacids by the transfer of amino groups and also in many cases, patients with severe liver damage had normal ALT activities, reflecting a low level of enzyme activity in liver cells from certain species (Hochleithner, 1994). This implies that specific diagnostic value of this enzyme in birds is poor. Elevated AST activity provides the best information on liver or muscle damage when combined with other more specific tests (Lumeij & Westerhof, 1987). Creatine kinase (CK) activity can be used to exclude muscle damage as a cause of increased AST activity (Hochleithner, 1994).

Median serum alkaline phosphatase (ALP) levels in the current study were within the reference range Uko and Ataja (1996) and Nanbol *et al*. (2016). ALP is a well established marker of bone diseases, but it is not specific for bone only (Pardhe *et al*., 2017). Bone-specific ALP measurement is, therefore, indicated in order to differentiate liver disease from osteoporotic activity (Bikle, 1997). The Estradiol Benzoate levels administered in the present study did not provoke any alterations in ALP activity in the liver and bone as enzyme level was similar to the control value. In poultry, ALP is elevated prior to egg laying (Hartsfield & McGrath, 1986). Birds have significantly higher alkaline phosphatase (ALP) activities from bone growth and development than adults and also seasonal changes in ALP activities have been described in

birds and also most enzyme assays are used to document damage to cells resulting in enzyme release (Hochleithner, 1994).

Median blood urea nitrogen (BUN) concentration in the current study was similar among Estradiol Benzoate treatments. Urea is present in very small amounts in avian plasma, and determining urea level has generally been considered of little value (Hochleithner, 1994). However, investigations have shown good correlation between increased plasma concentrations and renal disease in pigeons (Lumeij, 1987 as cited in Hochleithner, 1994). In other avian species, urea may have little value in detecting renal disease but can be used as a sensitive indicator of dehydration.

In the present study, median serum creatinine concentration did not differ across Estradiol Benzoate treatment groups. There is a slim margin between the physiologic and pathologic levels of creatinine (Hochleithner, 1994). Excretion of creatinine is solely via the kidneys (Hochleithner, 1994). It is freely filtered and reabsorbed in the tubules (Gylstorff & Grimm (1987) as cited in Hochleithner, 1994). In birds, creatine is excreted in urine before it's conversion to creatinine (Bell & Freeman, 1971). The urinary excretion of creatine may be one of the reasons that creatinine level does not provide an accurate assessment of avian renal function. Despite this, the similarity in median serum levels of creatinine in the current study implies that the injected Estradiol Benzoate doses did not affect renal function.

In the present study, Estradiol Benzoate treatments gave similar serum levels of calcium, total protein, albumin and globulin. Total calcium should always be interpreted along with albumin concentration (Hochleithner, 1994). Hypoalbuminemia will reduce the quantity of bound calcium and result in a decreased total calcium concentration without reducing biologically active calcium (ionized fraction) (Hochleithner, 1989; Lumeij, 1990; Hochleithner,

1991 as cited in Hochleithner, 1994). The hypoproteinemia that occurs with dehydration may result in an increased total calcium concentration (Hochleithner, 1994). Advancing age has been associated with increases in total protein in several bird species (Gylstorff & Grimm, 1987, Clubb *et al*., 1990, Clubb *et al*., 1991a and Clubb *et al*., 1991b as cited in Hochleithner, 1994). Hormones can have either an anabolic or catabolic effect on total protein (Hochleithner, 1994). In general, hormonal effects on total protein are minimal. However, testosterone, estrogen and growth hormone were found to increase total protein in chickens; thyroxine decreased concentrations (Kaneko, 1989).

Median serum phosphorus levels were similar across Estradiol Benzoate groups in the current study. Changes in inorganic phosphorus concentration can occur with several diseases, but not on a consistent basis (Hochleithner, 1994). No changes in inorganic phosphorus levels were noted in laying hens (Lewandowski *et al*., 1986).

Similar median serum cholesterol levels were recorded across Estradiol Benzoate treatments in the present study. Elevated and decreased cholesterol concentrations may occur from a number of physiologic influences and different diseases; however, the diagnostic value of this test in birds appears to be poor (Hochleithner, 1994). Cholesterol concentrations will vary with a birds' diet. Carnivorous birds have higher concentrations, whereas fruit- or grain-eating birds have lower concentrations (Lewandowski *et al*., 1986).

In the current study, proportion of vascular skeleton and B follicles were similar across Estradiol Benzoate treatments which disagree with the work of Razia *et al.* (2005), they reported an increase in splenic trabecular arteries and veins and disappearance of lymphoid follicles and lymphocytes, respectively, in the bursa and thymus in Japanese quails injected with estradiol-17β during the growth phase. The increase in splenic red pulp in the 0.8 mg Estradiol Benzoate group

in the present study could be as a result of a remarkable increase in the volume of red pulp due largely to a marked increase of erythroid cells (Sasaki & Ito, 1981). The decrease in splenic white pulp in the 0.8 mg Estradiol Benzoate group in the current study could be due to reduction of transitional B cells (Grimaldi, Michael & Diamond, 2001) which is mediated by both subtypes of the estrogen receptor, ERα and ERβ (Komm *et al*., 2005; Gennari, 2006).

A non-significant decrease in thickness of tunica muscularis with significant decrease in thickness of tunica mucosa in hen administered high dose diethylstilbesterol (DES) and bisphenol A (BPA) has been reported by Yigit and Daglioglu (2010). In the present study, the mucosal thickness was constant across EB groups except the 1 mg EB group which was twice the thickness in other groups. This finding should be carefully interpreted as the assessment was at best qualitative due to insufficient replications.

Estrogen regulates the amount of white adipose tissue in females (Wade & Gray, 1978 and Pederson, Borghum, Eriksen & Richelsen, 1992 as cited in Heine *et al.,* 2000). Lack of ERα produces large increases in white adipose tissue in both male and female mice and was accompanied by insulin resistance and glucose intolerance in both sexes (Heine *et al*., 2000). In the current study, shell gland fat infiltration decreased beyond a dose of 0.6 mg Estradiol Benzoate (EB) and remains constant thereafter which could imply that ERα is highly expressed at an EB dose of 0.6 mg after which expression decreases. However, a major drawback of this work was that expression studies were not done alongside other investigations.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 SUMMARY

The present experiment was carried out to determine the effect of Estradiol Benzoate injection at 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/bird on serum biochemistry, spleen histomorphometry and shell gland histology in Lohmann Brown hens. At the end of the six-week experimental period, the blood was collected in plain tubes, After clotting, blood was centrifuged at 4000 revolutions per minute for 30 minutes and sera were transferred into clean labeled plain tubes using dropping pipette, then stored at -20 ºC and subsequently used for biochemical analysis, and the spleen and shell gland were harvested and fixed in Neutral Buffered Formalin. Fixed spleen and shell gland tissues were processed using standard techniques. Slides produced from spleen tissues were subjected to histomorphometric analyses while slides from shell glands were interpreted qualitatively.

Data were analyzed using GraphPad InStat statistical package. Results showed non-significant difference ($P>0.05$) in serum glucose, blood urea nitrogen, creatinine, cholesterol, total protein, albumin, globulin, calcium, phosphorus, aspartate and alanine aminotransferases, and alkaline phosphatase across different levels of Estradiol Benzoate (EB) treatments. Similarly, there were no significant differences in the proportion of spleen vascular skeleton and that of B follicles following treatment with varying levels of EB. There was an increase in fat infiltration (1-3, 6-10 and >10 fat cell aggregates) of the shell gland across 0, 0.2 and 0.4 mg EB. Beyond 0.4 mg, the same magnitude (6-10 fat cell aggregates) of shell gland fat infiltration was recorded. Mean proportion of splenic red pulp in control birds was significantly ($P<0.01$) less than the corresponding proportion in birds administered 0.8 mg Estradiol Benzoate. Conversely,

mean proportion of ellipsoids and peri-arteriolar lymphatic sheaths (PALS) in control birds was significantly ($P<0.05$) greater than the corresponding proportion in birds administered 0.8 mg Estradiol Benzoate.

5.2 CONCLUSION

In conclusion, administration of Estradiol Benzoate had no effect on serum biochemistry and the proportion of spleen vascular skeleton and B follicles. Shell gland fat infiltration and the proportion of splenic red pulp and ellipsoids and PALS were affected by administration of Estradiol Benzoate.

5.3 RECOMMENDATIONS

Further studies should be carried out on the effect of Estradiol Benzoate on the following:

i. Extramedullary hematopoiesis in the spleen of laying hens.
ii. Systemic immune responses in the spleen of laying hens.
iii. Recommend expression study especially on estrogen receptor α

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