EVALUATION OF REPRODUCTIVE RESPONSES AND EGG QUALITY TRAITS IN LOHMANN BROWN LAYERS TREATED WITH CLOMIPHENE CITRATE

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DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. A.M. 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 thesis and the subsequent write-up (Amina Yunusa Raji, SPS/13/PAS/00004) were carried out under my supervision.

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Abstract

The aim of the study was to evaluate in Lohmann Brown (LB) layers the effect of Clomiphene Citrate (CC) administration on ovulation rate, oviposition pattern, egg quality, serum biochemistry, relative weight of internal organs, ovarian, hormonal as well as oviductal changes and egg bacteriology. The study was carried out in three phases (Phase I, II and III). In Phase I (Non-mating Phase), eighteen (18) LB hens were administered CC orally at 15 mg/kg body weight for six weeks (stimulatory stage) followed by withdrawal for another six weeks (withdrawal stage) in a repeated measures completely randomized design. In the Mating Phase (Phase II), 18 LB hens (6 per group) were bred by three ISA Brown cocks (1 cock per six hens). The hens in Phase II were subjected to oral CC treatment at three levels (CC-free, orally-administered CC, CC withdrawal) for six weeks in a single factor completely randomized design. In the Incubation Phase (Phase III), fertile eggs across the different experimental groups from the Mating Phase formed the experimental materials in a cross sectional design. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 16.0 and GraphPad InStat software. In Phase I, ovulation rate, total number of eggs laid, egg weight, width and length, yolk and albumen volumes, shell weight, and oviposition pattern parameters (total egg sequence, total egg sequence length, total laying pause and total inter sequence pause) during CC administration were significantly different ($P<0.05$) from corresponding parameters after CC withdrawal. With the exception of total egg sequence, all other parameters were higher in LB hens undergoing CC withdrawal when compared with their corresponding values during CC treatment. With respect to Phase II, ovulation rate, large white follicle count and serum levels of aspartate aminotransferase and progesterone were significantly ($P<0.05$) different across CC treatment levels, however, birds in both stimulatory and withdrawal groups recorded higher ovulation rates when compared to the control group (CC-free). In Phase III, significant differences ($P<0.05$) were recorded in fertility while the dead-in-shell embryo have the highest percentage of bacterial growth and highest total frequency of isolates followed by the hatchery environment and then other commercial eggs inside the incubator; Gram negative bacteria gave the highest frequency after Gram staining. The bacteria isolated were *Escherichia coli*, *Klebsiella* spp. *Enterobacter* spp. *Pseudomonas* spp. and *Salmonella* spp. *Escherichia coli* was resistant to Penicillin, *Klebsiella* spp. was resistant to Amoxicillin, Gentamicin and Penicillin while isolates of *Enterobacter* and *Pseudomonas* spp were resistant to Cefoxitin, Amoxicillin, Gentamicin, Penicillin, Neomycin and Streptomycin. In conclusion, CC administration during the Non-mating Phase influenced all reproductive and egg quality parameters except shell thickness. During the Mating Phase, ovulation rate, serum levels of aspartate aminotransferase and progesterone and large white follicle count were affected by CC treatment. In Phase III, only fertility was affected. Therefore, administration of CC orally at 15 mg/kg is

recommended for the improvement of ovulation rate, egg quality and oviposition pattern parameters in LB hens.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION

Poultry is described as group of birds with economic value to man, examples include chickens, pigeon, duck, pheasant, quail, guinea fowl and recently ostrich all of which belong to the zoological class *aves* (Atteh, 2004). Poultry offers a range of uses to humans which include provision of meat and egg, research and medicinal purpose, production of manure which helped to improve soil fertility and feathers from poultry birds which provide humans with aesthetic value (Banerjee, 2008).

The contribution of the poultry sector to the Nigerian economy is about 25% of its agricultural domestic products (FAO, 2010). Nigeria is currently rated as the leading country in Africa with respect to eggs production (USDA, 2013). The Nigerian poultry population is estimated at 140 million (Adeleke *et al.* 2010), with about 268,000 metric tons of poultry products produced annually (FAO. 2013).

The word ‘layer chicken’ refers to a female chicken that is more than 20-22 weeks of age (Kekeocha, 1984) and is expected to produce less tender but quality meat and can produce up to 300 eggs per year depending on the feed and management (Kabir, Oni, Akpa & Adeyinka, 2006). Maximum egg production rather than meat yield have been obtained from egg producing chickens (Oluyemi & Roberts, 2000). Chickens have short generation and incubation interval, high prolificacy, fast growth rate and are easy to raise (Oluyemi & Roberts, 2000; Kabir & Olufemi, 2013). Poultry meat and egg production have been reported to show an increasing trend with the introduction of improved breeds/strains of poultry (Haleem, 2009).

The Lohmann Brown hen is an egg laying strain of chicken. It is of hybrid origin and selectively bred from New Hampshires and other brown egg laying strains. They are observed to start laying at about 18 weeks, laying about one egg per day and laying up to 300 brown eggs a year (Feltwell, 2011). Eggs are laid daily, normally, at dawn or dusk. They are very inquisitive strains of chicken. Most Lohmann browns have a caramel/brown shade of feathers in a pattern round their necks, and white feathers at the tips of their tail feathers (Feltwell, 2011; Riggs, Willis & Ludlow, 2011).

The egg is a biological structure intended by nature for reproduction. The yolk present in the egg protects and provides nourishment for the developing embryo and serves as the principal source of food for the first few days of the chick's life (Mudhar, 2011). Egg is considered to be fertile if it contains living cells that can become viable embryo and subsequently a chick. Eggs are fragile and a successful hatch begins with undamaged eggs that are fresh, clean and fertile (Archer & Cartwright, 2013). The fertile egg is found to contain the necessary nutrients for the development of the embryo to hatching; however, lower or no hatchability at all have been attributed to certain physical and chemical conditions of the egg which could result due to the hen or environmental factors (Kalita, Pathak, Ahmed & Saikia, 2013). Fertility of egg is achieved when cock is intoduced to the hen within four days.

The most important parameters of reproductive performance are fertility and hatchability. Fertility refers to the percentage of incubated eggs that are fertile while hatchability is the percentage of fertile eggs that hatch (King'ori, 2011). The most influential egg parameters that influence hatchability are: weight, shell thickness and

porosity, shape index (described as maximum breadth to length ratio) and the consistency of the contents (Oluyemi & Roberts, 2000).

The reproductive system of animals is driven by hormones. These hormones are very small chemicals that are produced and released in one organ, but are transported by the blood stream to exert their effects in another organ (Robinson & Renema, 2003). Reproductive function in the female is controlled by the interaction of the sex steroids (progesterone, P_4 and oestradiol, E_2) from the ovary. Steroid hormones (P_4 and E_2) are important for chicken reproduction (Kang, Yun, Seo, Hong & Ko, 2001).

Clomiphene Citrate (CC) is a selective oestrogen-receptor modulator which is presumed to work by inducing ovulation through inhibiting negative, endogenous, oestrogen-feedback on the hypothalamic-pituitary gonadal axis, resulting in increased follicle stimulating hormone (FSH) secretion which brings about follicular growth, development and ovulation (Emily, Jungheim, Anthony & Odibo, 2010). An increased level of FSH hormones improves the chances of growing an ovarian follicle that can then trigger ovulation (Yilmaz, Sezer, Gonenc, Ilhan & Yilmaz, 2018). It is the most prescribed drug for women with weak estrogen properties and is used to reverse anovulation or oligoovulation (WHO, 2015).

1.2 PROBLEM STATEMENT

Low productivity has been identified as the major problem of poultry production in Nigeria (Onyenweaku & Effiong, 2006). Unraveling the cause of a sudden drop in egg production requires a thorough investigation into the history of the flock. Several factors that affect egg quality include egg size (Butcher & Miles, 2003), age of the bird (Coutts

& Wilson, 1990), genetics and diseases (McFerran & Adair, 2003; Jones, 2006) and stress (Igbokwe, 2018)

Prolonged egg storage, abnormal egg storage conditions, age of hens and incubation problems result in failure of fertile eggs to hatch (embryonic mortality) (Fasenko, Robinson, Hardin & Wilson, 1992; Wilson, 1997; Fairchild, Christensen, Grimes, Wineland & Bagley, 2002). Kabir and Muhammad (2011) further reported that poor egg shell quality depressed hatchability and results in weakening of the embryo.

The primary factor resulting in fewer chicks hatched per hen housed is the fertility of eggs because infertile eggs cannot produce chicks; also, losses in hatchability due to early dead embryos are reported to occur as a consequence of reduction in fertility (Bramwell, 2002). The hatchery is one of the most important target areas of disease risk in poultry operations due to heavy mortality of embryos (Elhariry, 2012). Osman, Almahdi and El Sanousi (2013) reported that bacteria represent the primary or secondary agent of dead-in-shell embryos. The dead-in-shell embryos portray the management status of the hatcheries which further leads to early chick mortality (Kalita *et al.*, 2013).

Hatchability was reported to be lower for small eggs as compared to that of medium and large eggs. Failure of a fertile egg to hatch is a serious problem which could arise due to lethal genes, insufficient nutrients in the egg and exposure to conditions that do not meet the needs of the developing embryo (King'ori, 2011).

The efficiency of reproduction of hens has been reported to decrease with age which is related to the internal egg composition or ratio, larger egg weight, poor shell quality, increased early and late embryo mortality (Leeson & Summers, 2005) and albumen quality deterioration (Tona, Onagbesan, Ketelaere, Decuypere, & Bruggeman,

2004) and increase in yolk cholesterol content (Dickmen & Sahan, 2007). Reproductive problems associated with egg production are cloacal prolapse (Keshavarz, 1990) and heavy egg production which is a factor predisposing hens to salpingitis or tumors of the oviduct (Barnes, Vaillancourt & Gross, 2003). The ovulation of two or more ova in one day results in soft, thin or poorly shelled and double-yolked eggs which could result due to increased sensitivity to pituitary hormones; thus, leading to increased rate of recruitment of follicles into the hierarchy of yellow follicles that eventually ovulate (Hocking & McMormack, 1995).

An unusually high basal level of circulating oestradiol (E_2) and an unusually low level of progesterone (P_4) is associated with failure to lay eggs in hens (Leszczynski, Pikul & Kummerow, 1983). Cessation of egg production induced by stress was associated with low circulating levels of both P_4 and E_2 in ducks (Bluhm, Philips & Burke, 1983). Low levels of circulating P_4 have been observed in turkey hens that are non-laying and in those hens with low egg production (Kang *et al.*, 2001).

Reduction of hatchability have been attributed to many causes and generally included bacterial infection (Ahmed, Salim & Mansour, 1981; Osman, 1996), adhesion of embryos to egg shell, temperature variation, incubator faults (Das, Mishra & Mishra, 1994; Fairchild, Christensen, Grimes, Wineland & Bagley, 2002), and mycoplasmal and viral infections (Sripkovits, Bove, Rousselot, Larrue, Labat & Vuillaume, 1985). Also about 20% of hatching eggs are wasted during incubation, mainly due to infertility and embryonic death, leading to an overall average hatchability of 80% of eggs under such conditions (Abdalla, 1991; Fairchild *et al.*, 2002).

Clomiphene Citrate (CC) has been reported to have many adverse effects in humans, such as ovarian enlargement, vasomotor flashes, abnormal vaginal bleeding, weight gain, shortness of breath etc. Acute pancreatitis has also been reported to be induced by taking CC (Siedentopf, Horstkamp, Stief & Kentenich, 1997; Keskin, Songür & Işler, 2007), so also myocardial infarction (Duran & Raja, 2007), hypertriglyceridemia (Yasar & Ertugrul, 2009), deep vein thrombosis (Benshushan, Shushan, Paltiel, Mordel & Laufer, 1995) and pulmonary embolism (Chamberlain & Cumming, 1986). CC has also been shown to cause ovarian and uterine abnormalities (Nagao & Yoshimura, 2001). In addition, CC has been used to boost the percentage livability and concentration of sperm cells of an infertile male Wista rat (Oyeyemi, Ola-Davies, Oke & Idehen, 2000). Higher doses of CC inhibit the activities of FSH and LH while lower doses play an important role in regulating the levels of LH and FSH (Schally *et al.*, 1970).

1.3 JUSTIFICATION FOR THE STUDY

One of the cheapest, most affordable and acceptable animal product is poultry egg because it is highly nutritious and serve important role in many food products due to its functional properties (Scott & Silversides, 2001). With a human population of about 174 million (PRB, 2013) in Nigeria and with over 70% of the population living on less than a dollar a day (Watts, 2006), the increase is not proportional to the efforts of poultry production due to various drawbacks like infectious diseases, managemental problems, hatchery problems etc (Kalita *et al.*, 2013). Poultry production thus offers an excellent means through which the animal protein of the populace can be met (Boland *et al.*, 2013; Oleforuh-okoleh, Ibom, Eze & Ideozu, 2016) because they have short generation and incubation interval, high prolificacy, fast growth rate and are easy to raise (Oluyemi &

Roberts, 2000; Kabir & Olufemi, 2013). The need thus arise for increased poultry egg production.

Egg quality is a factor which contributes to better profitability in terms of fertile and table eggs (Hanusova, Hrncar, Hanus & Oravcova, 2015). The quantitative traits of economic importance that show continuous variability include egg morphometric variables such as weight, size, albumen and yolk contents (Islam & Dutta, 2010). It is also an established fact that the shell, albumen and yolk of an egg are in direct proportion to its weight and these vary significantly between breeds or strains of the bird species (Jones, Musgrove, Anderson & Thesmar, 2010; Momoh, Ani & Ugwuowo, 2010). The external and internal quality traits of eggs in hens are reported to have significant effects on the hatchability of incubated and fertile eggs (Kabir & Muhammad, 2011) as well as the weight and development of the hatched chicks (Kabir, Oni & Akpa 2007; Mudhar, 2011).

Apart from management system and other improved husbandry techniques, the reproductive performance of other livestock has been improved using fertility drugs like CC (Clomid®) to enhance semen and egg production (Adebambo, 2005). A lot of these treatments have evidently produced positive results in female goats, sheep and rabbits as reported by Iheukwumere, Iloeje, Herbert and Umesiobi (2003) who reported works on the Nigerian local chicken.

The degree of agonistic and antagonistic activity of CC observed depends on the species, organ, tissues or cell type that is being examined and on the end point assay chosen (Iheukwumere, 2005). The effectiveness of Clomid® in superovulation of West African Dwarf (WAD) goats was demonstrated by Iheukwumere (2005). He was able to

conclude that Clomid® treatments were effective in making superovulation in WAD goats in Nigeria but a higher dose of 0.40 ml /kg body weight of Clomid® showed excellent result than the other dosage levels. Other studies have shown CC to be correlated with enhancement of embryo generation in human invivo and treatment of several clinical complications involving endometrial hyperplasia and precocious puberty in the human female (Zubair & Sajid, 2015).

The reproductive fitness of the hen and cock is reported to have significant impact on egg quality and according to Bennett (1992), lower quality of 3-9% was observed in thin shelled eggs compared to thicker shelled eggs and ascerts that a great amount of moisture loss occurs during incubation especially with thin shelled eggs. There is, therefore, the need to observe the effects of egg traits on incubation, fertility and subsequent hatchability of eggs. The physical characteristics of the egg play an important role in the processes of embryo development and successful hatching (Narushin & Romanov, 2002). The rates of embryo mortality need to be reduced to improve hatching rates. It is imperative to determine its causes and to solve the problems which may occur from laying to hatching.

The results reported for investigations on incubating eggs whose weights were not within the average values are contradictory. Eggs within 45-56 g weight were reported to hatch better than lighter eggs (Brotherstone, White & Meyer, 2000). Asuquo and Okon (1993) reported that medium sized eggs (45-50 g) hatch better than large eggs (51-56 g). These values were higher than the hatchability of small eggs (37.5-44 g) reported by Asuquo and Okon (1993). Abiola, Meshioye, Yerinde and Bamgbose (2008)

reported best hatchability value for medium sized eggs (50 g) of Anak broiler eggs. They reported that large eggs (60 g) have the lowest hatchability.

The ovulatory levels of progesterone ($P_{4)}$ has been observed to be correlated directly to the ovulation of mature follicles (Johnson, 2007) and preovulatory rise of plasma P_4 precedes and stimulates luteinizing hormone (LH) rise. A positive feedback mechanism exists between P_4 and LH for ovulation induction which resulted in positive correlations between circulating levels of P_4 and egg production in layers (Kang *et al.,* 2001).

Oestradiol on the other hand is not observed to be correlated to egg production but is reported to be associated with the regulation of yolk formation and egg weight (Palmer & Bahr, 1992). Effective ovulation by the animal body can be hampered by a number of conditions. One of the primary ways to address this challenge is with medications that stimulate the ovarian follicles to produce multiple eggs in one cycle. One of the most common fertility medications used to promote ovulation is Clomiphene Citrate (CC) (Clomid® or Serophene®) (Imasueh, Osa & Atanda, 2016).

CC is a non-steroidal anti-estrogen which also possesses weak estrogen properties. CC significantly increased early egg production in pullets when fed for approximately 8 weeks prior to the beginning of production (WHO, 2015). Allanah and Bratte (2015) reported that oral administration of 15 mg/kg CC body weight improves fertility in cocks. CC is reported to be absorbed in gastrointestinal tract, metabolized in the liver and generally well tolerated in the body. Drug and its metabolites are not changed by liver and are excreted in the feces and the biological half-life is 5–6 days (Ara & Asmatullah, 2011).

High dietary levels administered for 1 week produced a severe reduction in egg production and a heavy molt in hens (McGinnis &Wallace, 1973). CC appeared capable of either stimulating or inhibiting gonadotropic activity in chickens depending on the level administered (McGinnis & Wallace, 1973).

Gonadotropins have been used to boost reproduction in female goats, sheep and rabbits as reported in literature and these treatments have evidently produced positive results (Ikeobi, 2003). The relevance of hormones in reproductive process has been shown to affect egg production and quality. Most of the hormonal drugs possess mixed actions, for instance; Pregnant Mare Serum Gonadotropin (PMSG) preparations possess mixed activities of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Also, CC has been used to boost the percentage livability and concentration of sperm cells of an infertile male Wista rat (Oyeyemi, Ola-Davies, Oke & Idehen, 2000). Higher doses of CC inhibit the activities of FSH and LH while lower doses play an important role in regulating the levels of LH and FSH (Schally *et al.*, 1970). Many other studies have proved that low doses of CC not only increase gonadotropin secretion but also cause ovulation in the intact rat (Coppola & Perrine, 1965; Koch, Dikstein, Superstine & Sulman, 1971). The effect of CC is very clear and it can induce ovulation and increase blood levels of gonadotropins which is also correlated with follicular growth and elevation of estrogen secretion (Ross *et al.*, 1970; Rebar *et al.*, 1976).

Most of the studies on ovulation stimulating effect of CC were done in rats or humans, but, unfortunately the effects are just opposite to each other (Greenblatt, Barfield, Jungck & Ray, 1961). The mechanisms of action of CC on ovulation are not yet understood and seem to be more complex than the normal mechanisms by which

endogenous hormones function. The effect of CC in mice was shown to be mainly estrogenic by binding to estrogen receptors to stimulate gonadotropin secretion (Emmens, 1965; Pollard & Martin, 1968).

Liver and kidney functions have been shown to be improved by CC administration (WHO, 2015). CC was reported to be safe for women with polycystic ovarian syndrome (PCOS) and also improved renal function in patients with significant decreases in serum blood urea nitrogen levels, as well as creatinine with similar decreases in levels of liver transaminases (AST and ALT) (Aubuchon *et al.,* 2011). Serum cholesterol reduced in women that were treated with CC (Enk, Crona, Olsson & Hillensjo, 1986).

Increase in small and medium sized follicles was observed in CC treated animals than control animals (El-Sherry, Derar, Hussein, Shahin & Fahmy, 2011). Indian researchers consistently reported the efficacy of CC in induction of oestrus in cattle and buffaloes when CC was administered at the dose of 300 mg/ animal orally (Kankal, Khillare, Pargaonkar & Bakshi, 2008). Al-Amoudi (2012) found that treating rats with Clomid® induces many histopathological alterations and improved serum biochemical profiles in indigenous chickens (Urom, Ukpabi, Alum, Akanele & Oko, 2016). It was also observed to positively influence the weight of the reproductive organs of Nigerian indigenous cocks (Urom, 2016).

Studies of blood parameters have been shown to be correlated with factors such as nutrition, gender and rearing temperature (Anita, Moortry & Vishwanathan, 2007), and hematological and biochemical clinical status of the animal (Keskin *et al.,* 2007). Moreover, these blood profiles are most commonly used in nutritional studies for

chickens (Abu Hashim, 2012) and other birds (Mori, 1986; Chaudhuri, Mukherjea & Chakraborty, 1990; Nagao & Yoshimura, 2001) but have been rarely applied in reproductive studies with respect to chickens.

1.4 OBJECTIVES OF THE STUDY

The objectives of the study are:

i. To compare ovulation rate, oviposition pattern and egg quality parameters during and after Clomiphene Citrate (CC) administration in Lohmann Brown hens in the non-mating phase.

ii. To assess the effect of administration of CC on egg laying and oviposition pattern in Lohmann Brown hens during the mating phase.

iii. To determine changes in serum biochemical profile and hormones following administration of CC to Lohmann Brown layers.

iv. To determine changes in ovarian parameters and internal organ weights as affected by administration of CC in Lohmann Brown layers.

v. To assess the effect of administration of CC on fertility and hatchability of eggs laid by Lohmann Brown hens.

vi. To determine the type of isolates and the number of dead-in-shell chicks in the incubation phase.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 POULTRY

One of the most valuable sources of animal protein recommended for human consumption is the poultry egg, it offer a means of solving the problems of animal protein shortage especially in the rural areas in many African countries (Olawoyin, 2006). Fertility, hatchability, egg production and growth are economically important traits in chicken production systems and chickens are kept for both meat and egg production. (Abou El-Ghar, 2014).

An aspect of livestock production, poultry production, is important to the biological needs, economic and social development of the people in any nation (Oladeebo & Ambe-Lamidi, 2007). Poultry industry in Nigeria occupies a prominent position as a major source of animal protein supply to the citizens. Over the years, the growth of poultry industry has followed a pattern closely dictated by the economic fortunes of China, the Middle East and Africa (USDA, 2013).

2.1.1 Lohmann Brown Hen

Al-Nasser *et al.* (2006) reported that the Lohmann Brown hen is an egg laying strain of chicken which is of hybrid origin and selectively bred from New Hampshires and other brown egg laying strains (Riggs, Willis & Ludlow, 2011); these birds are observed to start laying at about 18 weeks, laying about one egg per day and laying up to 300 brown eggs a year (Feltwell, 2011). Eggs are laid daily, normally, at dawn or dusk (Riggs, Willis & Ludlow, 2011). They are very inquisitive strains of chicken (Feltwell, 2011). Most Lohmann browns have a caramel/brown shade of feathers in a pattern round

their necks, and white feathers at the tips of their tail feathers (Feltwell, 2011; Riggs, Willis & Ludlow, 2011).

2.1.2 Reproductive Characteristics of Chicken

One of the most important aspects of poultry breeding is reproduction (Abou-Elewa, Enab & Abdou, 2017) and it is characterized by parameters, such as, age at sexual maturity, fertility, hatchability, clutch size and clutch length (Addisu, 2013). Sexual maturity is paramount in terms of progress in poultry breeding among reproduction traits (Chiemela *et al.,* 2018). The reproductive system achieves its complete development age at sexual maturity and it has long been considered as an important factor that determines fecundity trait and affects subsequent performance (Forment *et al.,* 2009).

In females, age at sexual maturity can be easily determined externally as age at which hens lay their first egg (Tadondjou *et al.,* 2014). Fertility, hatchability, egg production and growth are economically important traits in local poultry production systems, since the developed chickens are kept for both meat and egg production (Abou El-Ghar, 2014). High fertility and hatchability of eggs of breeder stock and survivability of the chicks are necessary to produce large numbers of birds. The supply of day-old chicks is very important for the success of the poultry production chain (King'ori, 2011). Fertility and hatchability are two major parameters that highly influence the demand for day-old chicks.

Usually eggs are laid normally but sometimes there are some abnormalities and these could be resulted from various causes. Occasionally, a hen can produce double-yolked eggs which are not suitable for hatching. Yolkless eggs are usually formed when a bit of tissue is sloughed off the ovary or oviduct. This tissue stimulates the secreting

glands of the different parts of the oviduct and a yolkless egg results (Jacob & Pesfafore, 2013). Blood spots are normally found on or around the yolk. The main cause is a small break in one of the tiny blood vessels around the yolk when it is ovulated. Occasionally an egg will be laid without a shell. The shell membranes are formed on the yolk and albumen, sometime somehow missing the "shell mechanism" and the shell was not deposited and it is resulting from a deficiency of calcium, phosphorus and/or vitamin D (Burley & Vadehra, 1989).

2.1.3 Egg Shell Protective Mechanism

When faced with physical and/or microbial aggression, the egg has two major defensive mechanisms; a chemical protection system composed of yolk, albumen and egg shell matrix proteins that provide antimicrobial protection and the intact egg shell that acts as a physical barrier to protect against bacterial invasion (Mine & Kovacs-Nolan, 2006).

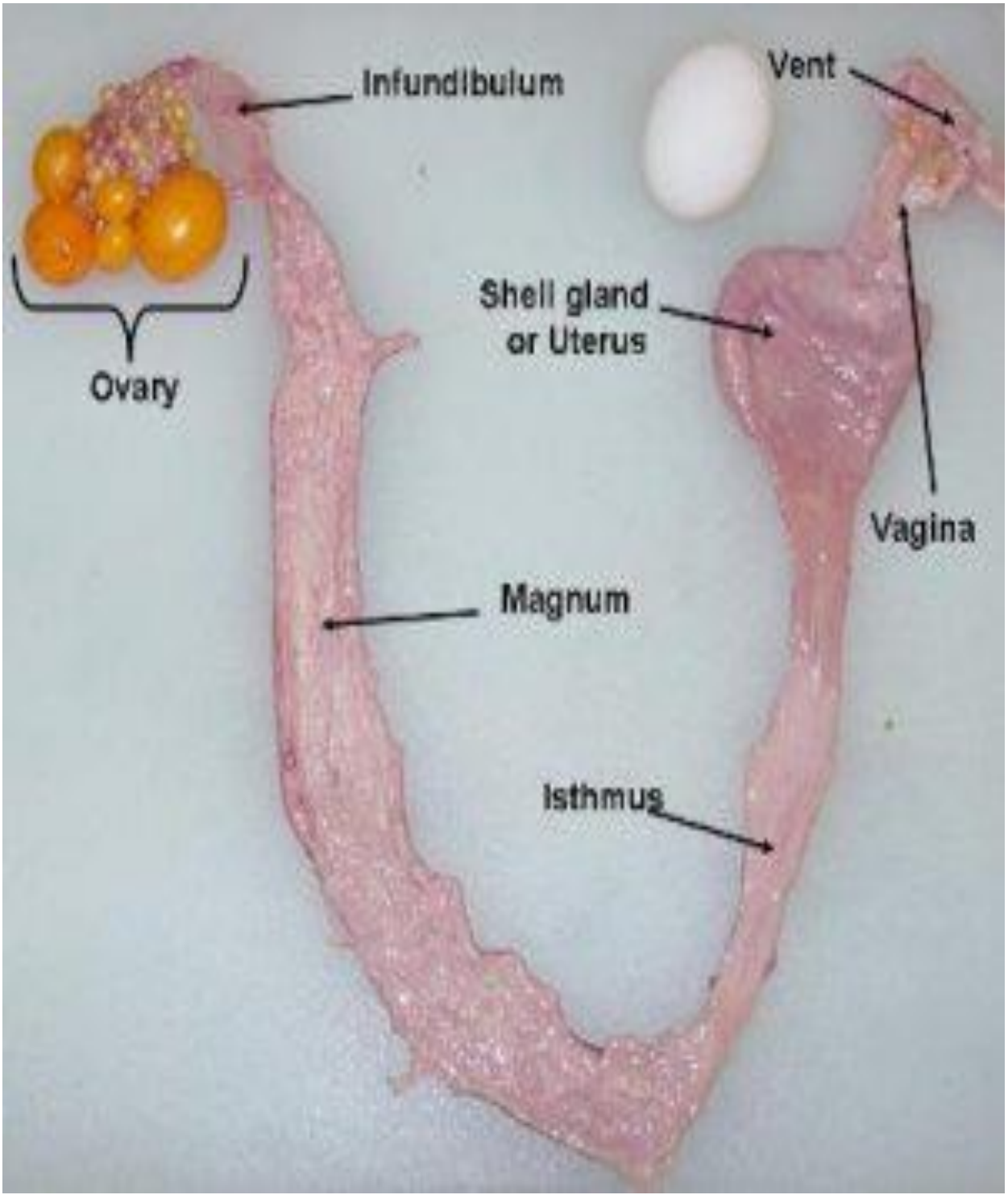
2.2 Ovarian Morphology

In chicken, ovary follicular development is a continuous process from the activation of small cortical follicles to the ovulation of hierarchical follicles. Small white follicles are recruited into a hierarchy of large pre-ovulatory follicles and become yellow due to yolk deposition (Johnson, 2015). Johnson also reported that the largest follicle (F1) ovulates almost on a daily basis and is replaced by a new pre-hierarchal follicle that is selected to enter the hierarchy each day. However, no new follicle is selected when an ovulation does not occur. Gilbert, Perry, Waddington and Hardie (1983) assumed that the large yellow follicles (LYF) are recruited from the pool of small yellow and large white follicles in the ovary. Yellow follicles are those with size of >10 mm in diameter, yellow

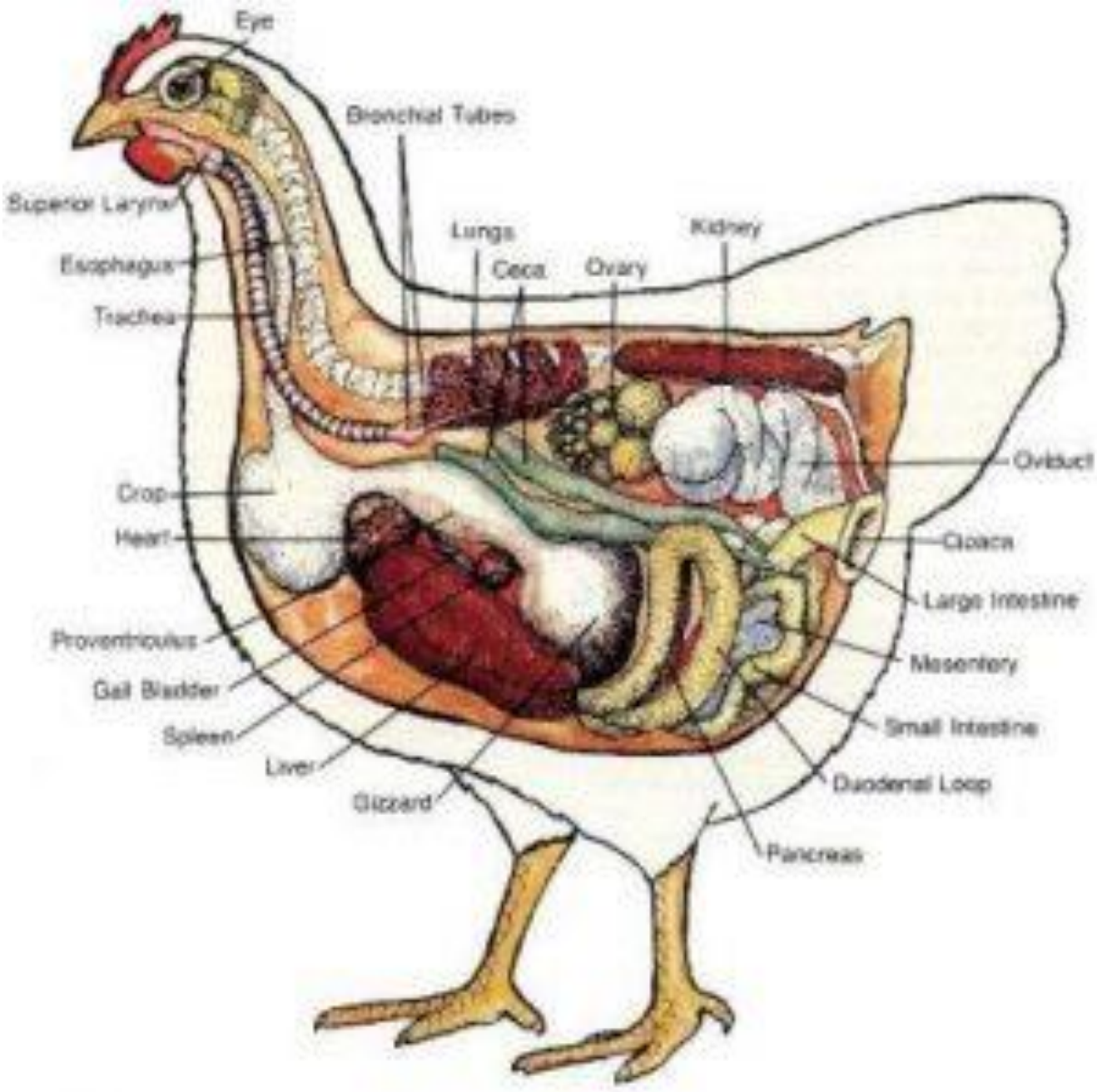
follicles with size of about 5-10 mm in diameter, white follicles with size range of about 3-5 mm in diameter and white follicles measuring about 1-3 mm in diameter are classified as large yellow follicles (LYF), small yellow follicles (SYF), large white follicles (LWF) and medium white follicles (MWF). The stroma is the ovarian tissue remaining after the removal of LYF (Ebeid, Eid, Saleh & El-hamid, 2008).

2.3 Avian Female Reproductive Organs

The reproductive system of a female chicken is made up of the ovary and the oviduct (Oyebanji & Atoki, 2018). Plate 1 shows the female chicken reproductive system, and Plate 2 shows the location of the reproductive system in the body (Pollock & Orosz, 2002). In almost all species of birds, including poultry, only the left ovary and oviduct are functional. Although the female embryo has two ovaries, only the left one develops. The right one typically regresses during development and is nonfunctional in the adult bird; there have been cases in which the left ovary has been damaged and the right one has developed to replace it (Pollock & Orosz, 2002).



Source: Jacob and Pescafore (2013)
Plate 1. Female Chicken Reproductive System



Source: Jacob and Pescafore (2013)
Plate 2. Anatomy of the Female Chicken

The second major part of the hen's reproductive system is the oviduct (Plate 1) which is around 68 cm in length. The oviduct is attached to the dorsal body wall by the mesotubarium ligament (Rahman, 2013). In the immature or non-breeding hen, the oviduct is an inconspicuous, narrow tube, straight and uniform in diameter. In the reproductively active female bird, the oviduct undergoes tremendous enlargement and occupies a large part of the abdominal cavity (Pollock & Orosz, 2002). The increase in length causes a folding of the oviduct upon itself. Oviduct is divided into five distinct regions associated with egg formation: (1) infundibulum, (2) magnum, (3) isthmus, (4) uterus or shell gland, and (5) vagina.

Infundibulum is the first section of the oviduct (Plate 1), is 10 cm in length and has two portions; a thin walled funnel which opens into a thick walled tubular region which constitutes the second portion of the infundibulum

(Rabbani *et al*., 2006, 2007). The funnel opening is an elongated slit which faces towards the ovary. The funnel is brownish or whitish in colour, the next portion is reddish due to the presence of tubular glands and enriched vascularization. The infundibulum and magnum portions are separated from each other by leaving a demarcation region which seems to be an oblique line (Plate 1) after which comparatively less reddish magnum starts (Rahman, 2013).

The magnum, 33 cm long and is the largest section of the oviduct. It is readily distinguished from the infundibulum by its coiling nature, greater external diameter and markedly thicker wall caused by the presence of numerous glands packed into massive mucosal folds (Pollock & Orsosz, 2002).

The third section of the oviduct, the isthmus is 15 cm long. The isthmus, as its name implies, is slightly constricted ('isthmus' refers to a narrow band of tissue connecting two larger parts of an anatomical structure), and has two parts i.e., upper white isthmus and lower red isthmus from where the inner and outer shell membranes are added, respectively. The constriction separates the magnum and isthmus and marked by a thin, translucent line (Plate 1) which can be seen on the mucosal surface with the unaided eye. The segment of the oviduct immediately succeeding the isthmus is of similar diameter but after a short course, expands to form a uterus in which the egg is retained during the entire period of shell formation (Pollock & Orosz, 2002; Rahman, 2013).

The next section of the oviduct is the shell gland or uterus. The shell gland is 7cm long, reddish in colour and pouch like in structure. The pouch always expands and contracts for which when egg comes in the uterus, the egg rotates. The egg remains in uterus for 19-20 hours of laying cycle (Banerjee, 2008).

The vagina is the short and terminal portion of the oviduct measuring 3 cm long proximal to its opening into the urodeum. This part does not really play role in egg formation but is important in laying of the egg. Powerful muscles of the vaginal wall and a well-developed muscular sphincter at the uterine-vaginal junction serve to expel the egg out of the hen's body (Rahman, 2013).

The cloaca is the terminal chamber of the gastrointestinal and urogenital systems, opening at the vent. The vent in chicken is the common opening of cloaca through which intestinal, urinary and reproductive tracts become empty (Pollock & Orosz, 2002: Banerjee, 2008).

2.4 Anatomy of Egg

Chicken has highly complex reproductive cell (the ovum), which is essentially a tiny center for life. Development of the embryo initially takes place in the blastoderm in case of fertile egg. The albumen enclosed by the shell membranes surrounds the egg yolk and protects this potential life. It is an elastic, shock-absorbing viscous material with higher percentage of water content. In case of chicken, the yolk and albumen are prepared to support the life of a growing embryo for three weeks. This entire mass is surrounded by two shell membranes (internally) and an external hard covering called the shell. The shell provides for an exchange of gases and mechanical (physical) as well as physiological (antimicrobial) means of conserving the food and water supply within the egg. Except the blastodisc, egg-yolk and inner vitelline membrane, all parts of the egg are formed in the oviduct (Bentley, Tsutsui & Wingfield, 2007). An egg is a single cell until it is fertilized. A chicken egg has the following anatomical structures:

Egg shell: The hard outer surface of egg is the shell. It provides protection to the inner portion of the egg and to the structure of embryo during incubation. The egg shell is composed of approximately 95% calcium carbonate crystals, which are stabilized by protein matrix. Normally, the shape of egg is oval but sometimes might be different. Microscopic studies show that the hen's egg shell is a highly ordered structure throughout its entire thickness. The egg shell is composed with three parts; cuticle, matrix and shell membrane. The cuticle is brownish as it contains the pigment protoporphyrin and the matrix is whitish located between the cuticle and shell membrane. The shell matrix is a semi transparent proteineous vesicular structure after decalcification of eggshell and is found between the cuticle and shell membranes. Layers of vesicles were interconnected

with each other by fibers or fiber sheets and they all are hidden by different types of calcites during eggshell formation in the uterus (Hernández *et al*., 2008; Hincke *et al*., 2000). Lastly, the shell membranes lie between the eggshell and the egg white. Two white proteinous membranes termed as shell membranes, protect the embryo and all the inner structures supporting it. Microscopic view apparently shows the two membranes differ in their structure: smooth one is towards the egg white (inner) and comparatively rough layer is towards the egg shell.

Air cell: The air cell is the space between the outer and inner shell membranes at the egg's blunt end, where the shell is most porous, and air can easily enter. When the egg is laid, the inner portion of the egg contracts and forms an air cell between the two shell membranes, and gradually expands as the embryo matures. It is also found in hard cooked egg (Hernández *et al*., 2008).

Chalazae: Inside the egg, yolk is suspended in the egg white, hanged by two spiral bands of tissue called chalazae. The chalazae hold the yolk securely in the center of the egg, while allowing play to absorb shock. These are twisted ropes of protein fibers attached with vitelline membrane of egg inside and with egg shell outside (Burley & Vadehra, 1989).

Vitelline membrane: The casing that encloses the yolk is called vitelline membranes.This membrane has inner and outer layers. The inner layer is transparent, encircles the blastodisc with yolk while the outer layer is whitish, has been described as inner portion of chalaziferous layer (Rahman *et al*., 2007, 2009) rather than outer vitelline membrane (Rahman, 2013).

Germinal disc: On the yolk surface, there is a germinal disc enclosed by inner vitelline membrane. This is a small disc of cytoplasm containing the DNA molecules of the ovum. The germinal disc or blastodisc is visible with the naked eye, appearing on the surface of the yolk as a white dot. In case of fertile egg, the germinal disc is called blastoderm which has a concentric circle around the nucleus and there is centrally a transparent zone called area pellucida and peripherally non transparent zone the area opaca. The primordial germ cells (PGCs) originate from the epiblast, the central part of the area pellucida which are the progenitor cells of ova and spermatozoa of adult. The infertile egg, there is only a dot like structure called blastodisc (Rahman *et al.,* 2007).

Egg white (albumen): Egg white or albumen is the common name for the transparent cytoplasmic liquid contained within an egg. Its function is to provide an efficient defense against bacterial invasion and a base for shell formation (Johnston and Gous, 2007). It also plays an important role during embryonic development (Willems, Decuypere, Buyse and Evaraert, 2014). Not only for formation of sub-embryonic fluid, but albumen proteins are used as the the main source of proteins for tissue synthesis and are known to flow into the amniotic cavity, the yolk sac and finally the digestive tract of the embryo (Willems *et al.,* 2014)

Egg yolk: The egg yolk is the part of an egg which serves as the food source for the developing embryo. Prior to fertilization the yolk together with the germinal disc is a single cell. It is one of the few single-cells that can be seen by the naked eye. The egg yolk is suspended in the egg white by chalazae. The shape of the egg yolk is round and contains germinal disc, and the entire structure is covered by transparent thin vitelline membrane (Rahman *et al.,* 2007).

2.5 Egg and Oviposition Parameters

Hen-day production is the total number of eggs laid by the flock in a given period divided by the product of the number of days and the number of hens alive on each of these days. It is calculated as HDEP = (Number of eggs produced on daily basis ÷ Number of birds available in the flock on that day) x 100 (North, 1984). Chicken with high egg production is the goal of maintaining laying hens. The higher the HDEP value shows the better the egg production of a group of chickens (Rahayu & Widjastuti, 2019).

Egg quality can be measured by both the internal and external characteristics. External quality focuses on egg weight, shell cleanliness, soundness of shell, texture, color and shape. These features are important to the processor as eggs with superior external qualities arrive in a better condition for the consumer (Sabbir, Zulfiqar, Mohammad & Taseer, 2013). The internal quality, on the other hand, is determined by the quality of the egg white (albumen), relative viscosity of albumen, shape and firmness of yolk, strength of yolk, size of air cell and presence and absent of blood or meat spot.

Egg weight is the most important egg quality trait in both table and hatching eggs, as the nutrient content of eggs and the weight of day-old chicks depend on it (Khan, Khatun & Kibria, 2004; Saatci, Kirmizibayrak, Aksoy & Tilki, 2005). The egg weight of a hen is affected by many factors such as heredity (Crowford, 1990), breed, strain, age of hen (Cook & Briggs, 1997), body weight, feed and water consumption, ambient temperature, and diseases (Fowler, 1972). It is an important trait that influences egg quality as well as egg grading, hence economic needs and value (Farooq *et al*., 2001). Egg weight is calculated based on the average weight of eggs produced during a

maintenance period (Rahayu & Widjastuti, 2018) and is influenced by the weight of its components as well (Zhang, Ning, Xu, Chou & Yang, 2005).

The shell thickness is the average of the thicknesses of blunt, middle and sharp points of the egg and is measured using a micrometer screw gauge (Abdurehman & Urge, 2016) Shell quality is one of the most important factors that influence hatchability (Roque & Soares, 1994). The productivity and quality of breeding eggs have an overall significance for the continuity of the flocks and for an economic breeding (Ojedapo, Adedeji, Ameen, Olayeni & Amao, 2009). (Peebles, 1986) reported that reduction in egg shell quality depressed hatchability and result in weakening of embryos.

Egg shell quality has significant impact on the reproductive fitness of the parent (Bennett, 1992; Abanikannda, Leigh, Ojedapo & Kpossu, 2007; Chukwuka *et al.*, 2011). The shell thickness and porosity help to regulate the exchange of carbon dioxide and oxygen between the developing embryo and the air during incubation (Roque & Soares, 1994; Chukwuka *et al.*, 2011)). Shell thickness also has a very significant effect on moisture loss during incubation (Bennett, 1992; Chukwuka *et al.*, 2011). Thin-shelled eggs loose more moisture than do thick-shelled eggs, causing the chick to have difficulty hatching (Roque & Soares, 1994; Chukwuka *et al.*, 2011).

The albumen of the broken eggs was carefully separated from the yolk. Rahayu and Widjastuti (2019) reported that the proportions of egg yolk, albumen and shell vary respectively from 25 to 35%, 50 to 65% and 8 to 14%. The shape of the egg is usually oval and can be characterized by its shape index. This index varies mostly between 0.7 and 0.75; the extreme values are ranging from 0.65 to 0.85. A young hen's egg, which is

rounder than an egg at the end of production cycle, is more resistant to static pressure (Rahayu & Widjastuti, 2019).

Egg laying is a complex process in which oviposition time, oviposition interval and clutch lengths are inter-related to each other in an intricate way (Roy, Kataria & Roy, 2014). In the chicken, oviposition is characteristic in a way that the poor layers have frequent pause days and therefore short sequences whereas the best hens have the ability to lay one egg at roughly the same time every day. This entails that reproductive fitness can be assessed by examining egg-laying characteristics such as sequence length and inter-sequence pause lengths (Wentworth *et al.*, 1983; Reddy, David, Sharma & Singh, 2002; David, Reddy& Singh, 2003). Genotype and age affects egg production as well as external and internal egg quality which are considered natural influences. Poultry lay eggs in clutches. A clutch is a group of eggs laid by a hen on consecutive days. After laying a clutch, a hen has a rest period of about a day or more and then lays another clutch. Clutch sizes are species and breed-specific. For commercial egg layers, clutch size is typically large. Clutch size, as well as the number of clutches laid in a hen's laying cycle, varies by species, but the principle is the same across species. In laying hens, egg production starts at approximately 18 weeks of age (Samiullah, Omar, Roberts & Chousalkar, 2017), peaks at approximately 26 weeks of age, and then it gradually decreases.

Total egg production is positively correlated with the length of laying sequence. The laying sequence refers to the number of eggs laid between two consecutive interval periods (Samiullah *et al.*, 2017). Its length affects the time of oviposition because if the first egg in the sequence is laid early in the morning, then successive oviposition occurs

later each day (Johnston & Gous 2007). Egg weight and egg shell quality are mostly affected by time of oviposition (Pavlovski, Vitorovic, Skrbic & Vracar, 2000; Zakaria *et al*., 2005; Tumova & Ledvinka 2009; Svobodova and Tumova, 2014) and egg shell quality is more affected by the delay in ovulation than by the length of egg formation (Tumova, Vlckova & Chodova, 2017). There is a vital role of oviposition in determining the egg shell quality. Shell deposition is a linear function of time spent in the shell gland (Ebeid & Tumova, 2004). The proportion of egg components and internal quality is affected by oviposition time and Ebeid and Tumova (2004) observed yolk percentage decreasing with oviposition time in cages but no effect on litter.

2.6 Incubation

In the last few years, artificial egg incubation systems have experienced a technological, economic, and social revolution. Remarkable technological and scientific developments allowed the transition from manual incubation to large incubation machines and hatcheries, which incubate a much greater number of eggs using less labor, increasing chick production throughout the year (Paniago, 2005). An incubator should be able to regulate factors, such as temperature and humidity, and to allow air renewal and egg turning, providing the perfect environmental conditions for embryonic development, aiming at achieving high hatchability of healthy chicks, which is directly correlated with the survival and performance of individual chicks in the field (North & Bell, 1990). Currently, incubators capable of incubating different numbers of eggs of different species of birds are commercially available, with more or less sophisticated temperature, humidity, ventilation, and egg turning control systems. Modern state-of-the-art commercial hatcheries are provided with automatic systems controlling all the physical

factors of incubation: egg turning; environmental temperature set according to eggshell temperature determined by thermo sensors; air relative humidity and egg water loss determined by egg tray weight using weight sensors; and air quality (O_2 and CO_2 levels). However, despite the technological advances of the modern incubation machines, the success of incubation still depends on the quality of labor both inside and outside the hatcheries, which requires training (Paniago, 2005).

2.6.1 Candling

Not all incubated eggs will hatch. Most probably, only 80 to 90 percent of incubated eggs are fertile. Removing eggs that can be identified as infertile or dead will reduce disappointment and remove possible sources of contamination from the incubator. Some of these eggs can be identified by candling (Oluyemi & Robert, 2000). To examine the infertile eggs and dead embryos in the shell (embryonic mortality), eggs are candled on the 7^{th} and 14^{th} day of incubation with a Candler box made of wood and torch light, making the house dark by closing the door and other openings. Infertile and other eggs with dead embryos were removed on the 14th day (Paniago, 2005).

Percent fertility is calculated as (Total number of fertile eggs/Total number of eggs set) ×100. The fertile eggs remained in the incubator until hatching. Fertile eggs are turned hourly and exposed to temperature of 37.5 ºC and relative humidity of 60% at the initial stage. They were further exposed to temperature of 36.5 ºC and relative humidity of 65% towards hatching time from day 18 of incubation (Banerjee, 2008).

2.6.2 Hatching

Hatching is to produce young one from an egg (Van De Van *et al.,* 2011). Chicken eggs hatch after approximately 21 days of incubation, but within a single batch

there may be a gap of 24-48 hours from the first to the last hatching, corresponding to 5–10% of embryonic development (Tong, Romanini & Exadaktylos, 2013; Van de Ven, van Wagenberg, Debonne, Decuypere & Kemp, 2011). This spread of hatch is often referred to as the hatch window (Molenaar, Reijrink, Meijerhof & Van den Brand, 2010). Percent hatchability were calculated as (Total number of chicks hatched/Total number of fertile eggs) × 100 (Banerjee, 2008).

Few studies have looked at length of incubation in a natural setting, and detailed knowledge of factors affecting hatching time of chickens is therefore mainly known from artificial incubation. It has been shown that intrinsic characteristics of the egg itself may have an effect, including age (Ruiz & Lunam, 2002; Ulmer-Franco, Fasenko & Christopher, 2010; Vieira, Almeida & Lima, 2005) and breed (Ruiz & Lunam, 2002) of the mother, egg size (Vieira, Almeida & Lima, 2005; Careghi, Tona, Onagbesan, Buyse & Decuypere, 2005) and sex of the chick (Van de Ven, van Wagenberg, Debonne, Decuypere, Kemp, 2011; Reis, Gama, Soares, 1997).

Interestingly, Hamburger and Hamilton (1951) found that differences in timing of incubational stage arise even in the very first days of embryonic development. Furthermore, incubation practices may also influence the length of incubation. For example, length (Reis, Gama & Soares, 1997; Tona, Bamelis, De Ketelaere, Bruggeman & Moraes, 2003a) and temperature (Ruiz & Lunam, 2002 ; Tona, Malheiros, Bamelis, Careghi & Moraes, 2003b) in storage before incubation, as well as incubator temperature (Yildirim & Yetisir 2004), egg position (Van de Ven, Baller, van Wagenberg, Kemp & van den Brand, 2011) and turning conditions (Tona, Onagbesan, Bruggeman, Mertens & Decuypere, 2005) have been found to be important. Hatching may also be stimulated by

sound, such as clicking sounds from other eggs (Vince, Green & Chinn, 1970), and gaseous environment, more specifically, an increase in CO_2 levels during incubation (Buys, Dewil, Gonzales & Decuypere, 1998; Everaert, Kamers, Witters, De Smit & Debonne, 2007).

2.6.3 Dead in shell

Dead-in-shell is when the embryo in an egg develops half way but dies without hatching. The cause of death could be from bacteria or non bacteria organisms. The death of chick embryos that showed no bacterial or mycoplasmal growth may be attributed to other factors, or the infective agent may disintegrate after the death of the embryo (Ahmed *et al.,* 1981). Embryonic mortality may be due to nutritional deficiencies. Abdalla (1991) reported that vitamin deficiencies cause poor hatchability. Egg shape and air cell position during incubation of the egg influence the frequency of death of embryos. Malposition of embryos results also in death (Benoff, 1980). Temperature influence egg penetration and survival within the egg (Al Aboudi, *et al.,* 1988). Reduction of hatching percentage may be due to adhesion and temperature variation as a result of incubator faults (Das *et al.,* 1994). Eggs with specific gravities lower than 1.080 have the highest embryonic mortalities and the lowest hatchability (Abdalla, 1991). It was reported that early embryonic death is associated with too low pore concentration at the large end of the egg, and excessively thick shell. Late death and piped embryos had a lower pore concentration in all regions relative to hatched shells (Peebles, 1986).

Bacteria isolated from dead-in-shell chick embryo: A larger number of aerobic Gram-positive and Gram-negative bacteria were isolated from dead-in-shell chicks (Abdalla, 1991). The Gram positive bacteria included *Bacillus*, *Staphylococcus*,

Enterococcus, *Corynebacterium*, *Micrococcus* and *Aerococcus*. The Gram negative bacteria included *Klebsiella*, *Hafina*, *Salmonella*, *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Alcaligenes*, *Erwinia*, *Escherichia*, *Proteus*, *Chromobacterium*, *Cardiobacterium*, *Morganella*, *Yersinia*, *Flavobacterium*, *Serratia*, *Plesiomonas*, *Acinetobacter*, *Aeromonas*, *Providencia* and *Brodetella*.

Gram-positive bacteria isolated from dead-in-shell chicks: *Staphylococcus*: *Staphylococcus spp* are ubiquitous, normal flora of skin and mucous membranes of animal. *Staphylococcus* septicemia reported to causing acute death in laying bird (Abdalla, 1991). In Sudan *Staphylococcus spp*. were isolated from dead-in-shell chick embryo by Ahmed *et al.* (1981) and by Osman (1996). Alaboudi, Hammed, Basher & Hassen (1992) in Mosul (Iraq) isolated *Staphylococcus* from 4-local hatcheries with other 7 genera. In Anambra State (Nigeria) 79 pooled samples from 632 dead-in-shell chick embryos in 2 hatcheries were cultured for aerobic bacteria, Staphylococcus represented 25 of these 79 pooled sample isolates (Orajaka & Mohan, 1985). Wang (1992) isolated *Staphylococcus* from batch of dead embryos.

Gram-negative bacteria isolated from dead-in-shell chick embryo: *Salmonella* are short rods usually motile by peritrichous flagella except *S. gallanirum* and *S. Pullorum* (Abdalla, 1991). *Salmonella* is an intercellular pathogen (Tierney, Mcphee & Papadakis, 2004). Cason, Cox & Bailey (1994) observed that nearly 44% of chicks from uncontaminated egg become infected with *S. typhimurium* when hatched along with surface contaminated eggs. In Saudi Arabia, Barbour and Nabbut (1982) recovered *Salmonella* from in shelled chicken embryos. In Poland, Mazukiewicz, Latala, Wieliczko, Zalesinski and Gielel (1988) isolated *Salmonella* from dead chick embryos. Alaboudi *et*

al. (1992) isolated *Salmonella* from dead chick embryo in Mousl (Iraq). *Salmonellosis* represented 35% of the 100 developing embryos isolated in Bhutanese (India) where reduced hatching percentage of eggs was observed (Das *et al.,* 1994). Ahmed *et al.* (1981) and Osman (1996) isolated *Salmonella* from dead-in-shell chick embryo in Sudan. *Salmonella enteritidis* was isolated by Hiba (2007) from cases of enteritis in (ACOLID) Company. Fumigation with formaldehyde has been found to be useful for controlling *salmonellae* on hatching eggs (Williams, Davies, Wilson, Marsh, Leach & Humphrey, 1988). Bailey *et al.* (1994) found *Salmonella* on 17% of egg shell samples and 21% of chick sample obtained from commercial broiler hatcheries in the United States. Miyamoto, Baba, Sasai, Fukata and Arakawa (1998) reported that *Salmonella* penetration was significantly decreased by cooling the egg at 4 ^{o}C and Williams and Benson (1978) observed the survival of *Salmonella typhimurium* for 16 months in feed and 18 month in litter stored at 25 ^{o}C.

Escherichia coli is a natural inhabitant of the larger intestine and lower small intestine of all mammals. *E. coli* is the type of the species of the genus *Escherichia* (Banerjee, 2008). Pathogenic strains of *E. coli* accounted for 43 of 245 isolates from dead embryos (Abdalla, 1991). In Nigeria, Orajaka and Mohan (1985) isolated 23 *Escherichia coli* from 632 dead-in-shell embryos from 2 hatcheries. In Iraqi, Alaboudi *et al.* (1992) isolated 8 *Escherichia coli* from 36 isolates. In Sudan, Ahmed, Salim & Mansour (1981) and Osman (1996) reported isolation of *Escherichia coli* from many farms in the country. In Mexico, Rosario *et al.* (2004) isolate *E. coli* from dead-in-shell embryo and chicken with yolk sac infection. Most of the embryos die at the various stage of incubation is due to Escherichia coli infection. So, *Escherichia coli* can be detected at the rate of 1.8 -

32.5% of the embryos died within 8 days, 16.6 - 41.1% of embryo died within 14 days and 67.3 - 94% of the embryos died during the last days of incubation (Sripkovits *et al.,* 1985). The most important source of egg infection seems to be fecal contamination of the egg surface with subsequent penetration of the shell and membranes resulting from rapid spread after hatching. *Coliform* bacteria can be found in litter and fecal matter and is found in poultry houses in dust particles of 10^5 - 10^6 /g entailing that *Coliform* bacteria can persist for long periods, especially under dry conditions (Harry, 1964). *E. coli* is the most isolated bacterial contaminant in both dead in shell and one day old chicks which was 9.4% and 6.5% respectively when compared with other contaminating microorganism (Yaguchi *et al.,* 2007).

It has been reported that eggs with intact or damaged shells having low proportion were found to be infected with *Citrobacter spp*., *Klebsiella spp*., *Proteus spp*., *Campylobacter spp*, and *Pseudomonas spp*., and *Salmonella spp* (Stepień-Pyśniak, 2010). Antimicrobial testing of 31 isolates from diseased or dead turkey pollutes between 1 – 35 days of age indicated sensitivity only to enroflatacin (Salman & Watts, 2000).

Klebsiella has been recovered occasionally from dead embryo causing embryo mortality, yolk sac infection and mortality in young chicken, turkeys and ostriches (Kabilika & Sharma, 1999). *Klebsiella* was isolated from dead-in-shell embryos from local hatcheries in Mousl (Iraq) (Alaboudi *et al.,* 1992) and Sudan (Ahmed *et al.* 1981 and Osman, 1996) while it was isolated infrequently from reproductive diseases including salpingitis and oophoritis in hens (Sharma & Bati, 1980).

Enterobacter is a normal inhabitant of the avian digestive tract (Binek *et al.,* 2000). As with other Gram-negative bacteria in the Enterobacteriaceae family, it is found

to infect eggs and young birds causing embryo loss, omphalitis, yolk sac infections and mortality in young birds (Texeira *et al.,* 2013).

Predisposing factors

Shell and shell membranes of the eggs of medium and poor shell quality have been linked to infection by several species of the genus *Salmonella* and also found to penetrate significant percentage of eggs of excellent shell quality with freshly laid eggs penetrated by *Salmonella* was found to be suppressed by cooling the eggs at 4 °C (Svobodova & Tumova, 2014). *Salmonella typhimurium* penetrated significantly fewer incubating fertile eggs than infertile eggs held under the same conditions (Musgrove, Jones, Northcutt, Harrison, & Cox, 2005) was able to identify 84 out of the 105 isolates of *Enterobactriaceae* affecting shell surfaces to be *Salmonella*.

The Egg shell membranes from eggs of different shell porosities were similar in their ability to resist penetration by *Pseudomonas fluorescence* (Svobodova & Tumova, 2014) and the inner shell membrane was the most important physical barrier to penetration by *Pseudomonas* fluorescence followed by the egg shell and outer shell membrane (Svobodova & Tumova, 2014) and they can digest egg shell cuticle if the humidity is high. The most important source of egg infection seems to be fecal contamination of the egg surface with subsequent penetration of the shell and membranes. Population of *E. coli* in reused chicken litter can reach up to 9.7×10^4 CFU/g while in single use litter, it has been found to be 4.2×10^5 CFU/g (Chinivasagam, Redding, Runge & Blackall, 2010) and Coliform bacteria can be found in litter and fecal matter (Hofacre, Cotret, Maurer, Garritty & Thayer, 2000).

Possible sources of infection

The contents of the great majority of new-laid eggs are sterile (Oluyemi & Roberts, 2000). Microorganisms that contaminate eggs play a significant role in poultry production pathology and in the spreading of diseases. These microorganisms cause increased mortality of embryos, lower hatchability, and increased early chick mortality. Infections of humans are also common (Milakovic – Novak & Prukner, 1990). Bacteria such as *Salmonella*, *Escherchia coli*, *Pseudomonas* and *Proteus* and Moulds (such as *Aspergillus fumigatus*) can contaminate both egg and chick. Gram-positive bacteria dominate rotten and tainted eggs, (Ledouy, 2002).

Prevention and control measures of bacterial infection

Hatchery building must be checked by swabs for bacterial contamination. Setter and hatcheries are checked by the settle plate methods for bacterial load. Fumigation of hatching eggs must be done in the farm immediately after laying. - Storage room of fertile eggs must be cleaned and disinfected daily (North and Bell, 1990).

2.7 Serum Biochemical Parameters

Biochemical parameters may be used as physiological indicators in birds. These values are influenced by species, age, sex, season, geographic region, nutrition, and physiological condition (Frandson, 2002). Serum biochemical parameters provide valuable information on the health status of animals (Iheukwumere *et al.*, 2006) and also reflect an animal's responsiveness to its internal and external environment (Esonu *et al*., 2001; Anyaehie & Madubuike, 2004). The evaluation of blood biochemistry in birds allows for the identification of metabolic alterations due to many endo- and exogenous factors including the genetic type, husbandry conditions, season, sex and age. Moreover,

the biochemical blood parameters provide valuable information on the health state and are often helpful in revealing health disorders already in the preclinical stage. Hematological and biochemical parameters which are important as diagnostic tools and physiological indicators in birds has been documented (Ibrahim, 2012).

2.7.1 Total Protein, Albumin and Globulin

Albumin and globulin are the major proteins found in the blood. Albumin concentration in the blood can fluctuate depending on the nutrition of the animal and the presence of infection/disease. There is often a drop in cases of malnutrition and infection. Globulin proteins consist of antibodies, enzymes and other types of proteins (Wasan, Najafi, Wong & Pritchard, 2001). They are essential in fighting infections and enhancing the blood clotting process and serve as hormone carrier, transporting the hormones to different parts of the body. Breed and nutritional status could bring about differences in serum total protein (Esonu *et al.* 2001). The range of 0.076 + 0.0027 – 0.082 + 0.0030 g/L was reported by Iheukwumere *et al.* (2006) for Nigerian chickens while the values for the total protein content in the majority of birds fall within the range of 30–50 g/L (Coles & Campbell, 1986; Coleman, Fraser & Scanlon, 1988; Kaneko, Harvey & Bruss, 1997; Khazraiinia, Saei, Mohri, Haddadzadeh, Darvisihha & Khaki, 2006; Suchý, Straková, Kroupa, Steinhauser & Herzig, 2010). Significant differences in serum total protein and albumin among males and females during breeding season could be due to the egg production that may affect the concentration of the blood proteins (Schmidt, Paulillo & Caron, 2008).

At the end of the laying period lower values were recorded for plasma total protein in comparison with the values of the given indicators at the beginning of the

laying period (Hrabcakova, Volasrova, Bedanova, Pistekova, Chloupek & Vecerek, 2014). Low albumin suggests poor clotting ability of blood and hence poor prevention of hemorrhage (Robert *et al.,* 2000), the range of 0.031 + 0.0027 – 0.035 + 0.022 g/L was reported by Iheukwumere *et al.* (2006) for Nigerian chickens. Babatunde and Oluyemi (2006) opined that the higher the value of globulin, the better the ability to fight against disease. The serum globulin values of 21 – 37 g/L were reported for birds by Banerjee (2008) and it was reported that in female birds, a considerable increase in plasma total protein concentration occurs just prior to egg laying, which could be attributed to an estrogen-induced increase in globulins. The proteins were the yolk precursors (vitellogenin and lipoproteins), which were synthesized in the liver and transported *via* the plasma to the ovary where they were incorporated in the oocytes (Ritchie, Harrison & Harrison, 1994; Ibrahim, 2012).

2.7.2 Serum Urea and Creatinine

Urea is the first acute renal marker upon renal injury and creatinine is the most trustable renal marker and increases only when the majority of renal function is lost (Borges, Bruno & Frankignoulle, 2005). Creatinine is similar to urea in terms of origin and diagnostics purposes because plasma/serum levels of both reflect glomerular filtration rate (GFR) (Wasan *et al.*, 2001). It has been observed that serum urea content depends on both the quantity and quality of protein supplied in the diet (Iheukwumere *et al*., 2006). In birds, uric acid is a major product of the catabolism of nitrogen. Age and diet may influence the concentration of blood uric in birds. In addition, high level of uric acid was reported during ovulatory activity (Ritchie *et al.,* 1994;

Ibrahim, 2012). Simaraks, Chinrasri & Aengwanich (2004) demonstrated increased serum uric acid of female Thai indigenous chickens as they started laying.

2.7.3 Serum Alkaline Phosphatase (ALP), Alanine transaminase (ALT) and Aspartate Transaminase (AST)

Usually, a blood test that measures certain enzyme levels is done to assess how well the body's systems are functioning and probable damage to tissues. Alanine transaminase (ALT) and aspartate aminotransferase (AST) are the two main liver function blood serum tests (Greaves, 2007). The AST and ALT are found primarily in the liver but also in the heart, kidney, pancreas, muscles and in red blood cells. It is normally elevated in cases of tissue damage, especially heart and liver. The magnitude of AST and ALT elevations vary depending on the cause of the hepatocellular injury. A very high ALT level suggests viral or severe drug-induced hepatitis, or other hepatic disease with extensive death of liver cells (Nelson & Cox, 2005). The AST levels fluctuate depending on the extent of cell death. According to Schmidt *et al.* (2010), who studied biochemical parameters in turkeys, certain physiological changes in the metabolism of female birds may occur as a consequence of the laying of eggs. Alkaline phosphatase assay is useful in the diagnosis of obstructive liver disease (Murray *et al.*, 2003). The normal value reported by Kaneko *et al.* (1997) for chicken is 482.5u/L. At the end of the laying period, an increase was measured in the concentrations of aspartate aminotransferase (Hrabcakova, Volasrova, Bedanova, Pistekova, Chloupek & Vecerek, 2014).

2.7.4 Serum Minerals

The greater release of calcium into the blood of laying hens is closely related to its accumulation in the shell (Suchy, Strakova, Vecerek and Sterc, 2001; Hrabcakova *et*

al., 2014). Ovulating hens have significantly higher calcium levels than non-reproductive females (Ritchie *et al*., 1994; Ibrahin, 2012). At the time when the laying capacity approached a maximum, a fall was recorded in phosphorus and an increase in calcium content but towards the end of laying, increase in both phosphorus and calcium was observed (Hrabcakova *et al.,* 2014).

2.7.5 Serum Hormones

Investigations of the hormonal control of growth and/or metabolism in poultry have predominantly been performed using chickens (Scanes, 2009). Not only have such studies focused on the chicken as an important agricultural animal but also the chick embryo has long been a model for developmental biology. Moreover, the chicken is the model species for birds. There are numerically many fewer studies in turkeys, ducks and ostriches together with a substantial body of research in Japanese quail as another avian model species (Scanes, 2014). Hormones have been defined for long as organic chemical messengers to perform intercellular signals, many of which are secreted by ductless glands (Banerjee, 2008). They reach their target via the bloodstream or tissue fluids, eliciting changes in their specific target cells which may be the same cell that produced them or situated anywhere in the body in minute amounts (Banrjee, 2008).

The situation in the egg producing female is more complex and is concerned with the interaction of two asynchronous rhythms namely the luteinizing hormone (LH) release mechanism and the process of follicular maturation. Extensive review of the role of ovarian hormones revealed that estrogens promote the synthesis of the yolk precursors in the liver and with progesterone, they induce the growth and differentiation of the oviduct and the synthesis of egg white proteins, and with the androgens they cause the

formation of medullary bone and influence Ca metabolism (Banerjee, 2008; Elnagar., Khalil, & El-Sheikh, 2014).The production of the ovarian steroid hormones in the chicken is intricately controlled. This involves thecal and granulosa cells having different sterodogenic capabilities or sensitivities to gonadotrophins and these changes during follicular maturation (Tilly, Kowalski & Johnson, 1991; Elnagar *et al*., 2014)

Elnagar *et al*. (2014) reported that when egg production is low, it indicates that the ovary of the hens has a large portion of small follicles which mature at slower rate than the high producer ovary and further supports the fact of Yu, Robinson & Etches (1992) who suggested that small follicles rather than large ones, are the main sources of estrogen in domestic fowl. In addition, Etches (1996) who stated that steroid secretion from small follicles is limited to androgens and oestrogens and as the follicles begin to sequester yolk, their production of oestrogens declines and at the time of ovulation, very little, if any oestrogens is secreted by the follicle.

With respect to progesterone when the egg production is low, it indicates that the ovary of the hens has a large portion of small follicles which mature at slower rate than the high producer ovary (Elnagar *et al*., 2014). It is well established that the largest preovulatory follicle is the main source of progesterone secretion (Elnagar *et al*., 2014). Etches (1996) stated that as oestrogens and androgen production are diminished during the recruitment of yolk into hierarchical follicles, the production of progesterone by the granulosa cells increases and at the time of ovulation, the granulosa cells of the hierarchical follicle have attained their maximum capacity to produce progesterone. It was reported by Tetel, Getzinger and Blaustein (1994) that estradiol together with progesterone, are required for priming the hypothalamus and pituitary in order that

progesterone can induce LH release, in other words regulating ovulation in the laying hen. On the other coast, oestradiol increases the binding capacity of progesterone receptors of the hypothalamus and pituitary for the gonadotrophin secretion during the hens' ovulatory cycle (Kawashima, Ukai, Kamiyoshi & Tanaka, 1992).

Thyroid hormones (THs) have multiple effects on vertebrate metabolism and development. In homeothermic animals, THs regulate basal metabolic rate and are essential for the maintenance of high and constant body temperature (Darras, van der Geyten & Kuhn, 2000). The effect of THs on protein and lipid metabolism is of a biphasic nature: in low physiological concentrations they are anabolic while at higher concentrations they are catabolic. During development THs stimulate both growth and differentiation (or maturation). Their action can be direct, indirect or permissive. Most of the actions of THs seem to be dependent on the binding to a nuclear thyroid hormone receptor (TR) (Darras, van der Geyten, & Kuhn, 2000). Two major isoforms of these receptors are known (TRαand T Rβ) and both of them preferentially bind 3,5,3'-triiodothyronine (T3). The main secretory product of the thyroid gland, 3,5,3',5'-tetraiodothyronine or thyroxine (T4), is considered to be a relatively inactive prohormone due to its low binding affinity to TRs. Consequently, the peripheral metabolism of T4by activating and inactivating pathways is very important in the regulation of the availability of receptor-active T3and hence of thyroid activity. (Prati, Calvo & Morreale de Escobar, 1992).

Studies to date indicate that thyroid hormones are present in the eggs of chickens and quail and that those hormones are primarily in the yolk. Quail hens deposit thyroid hormones into eggs in proportion to their own thyroid status, but appear to show some

regulation of this process (MCnabb & Wilso, 1997). Indirect studies suggest that thyroid hormones are transferred into oocytes bound to lipoproteins and trans-thyretin, both of which are taken up by receptor-mediated processes (MCnabb & Wilso, 1997). Thyroid hormones bound to yolk lipoproteins may enter embryos with yolk that is taken up by non-specific endocytosis or they may be transported into the embryo by specific carriers. In quail, very high T4 content of eggs is associated with accelerated differentiation and growth of embryonic pelvic cartilage, a thyroid hormone-responsive tissue (MCnabb & Wilson, 1997).

2.8 Clomiphene Citrate

Recently, studies have been conducted on various anti estrogen compounds used for the control of physiological processes. Clomiphen (CLM) and Tamoxifen (TAM), selective estrogens receptor modulators-SERMs, have been used in poultry most frequently (Lisowski & Bednarczyk, 2005). Clomiphene Citrate (CC) is a non-steroidal anti oestrogen, is a selective oestrogen receptor modulator (SERM) of the triphenylethylene group that has been the mostly prescribed drug for ovulation induction to reverse anovulation or oligoovulation (WHO, 2015), it also possesses weak oestrogen properties, For iinstance; pregnant mare serum gonadotropin preparations possess mixed activities of LH and FSH activities. Also Clomiphene citrate has been used to boost the percentage livability and concentration of sperm cells of an infertile male Wista rat (Oyeyemi *et al.,* 2000). Furthermore, Clomiphene citrate administration improved sperm production in ram without any deleterious effect (Iheukwumere *et al.* 2008). An increased level of FSH hormone improves the chances of growing an ovarian follicle that can then trigger ovulation (Yilmaz, Sezer, Gonenc, Ilhan &Yilmaz, 2018).

Clomiphene citrate is also the traditional first-line treatment for chronic anovulation that characterizes polycystic ovary syndrome (PCOS) with half-life of two weeks increasing ovulation rate to a 60-85% and pregnancy rate to 10-20% per cycle (Lidor *et al.,* 2000). Polycystic ovaries (PCO) are endowed with an abnormally rich pool of growing follicles from classes 1–5 (until 5 mm), probably due to intraovarian hyperandrogenism that promotes excessive early follicular growth. Second, the selection of one follicle from the increased pool of selectable follicles and its further maturation to a dominant follicle does not occur, an abnormality that is called ―follicular arrest (FA) due to discontinuous growth in a series of follicles from one bird (Hocking, Walker, Waddington & Gilbert, 1987).

In addition to its many positive roles, some adverse effects have also been reported in humans, such as ovarian enlargement, vasomotor flashes, nausea, vomiting, breast discomfort, headache, abnormal vaginal bleeding, visual symptoms, weight gain and shortness of breath. Acute pancreatitis has also been reported to be induced by taking CC (Siedentopf, Horstkamp, Stief & Kentenich, 1997; Keskin, Songür & Işler, 2007), so also myocardial infarction (Duran & Raja, 2007), hypertriglyceridemia (Yasar & Ertugrul, 2009), deep vein thrombosis (Benshushan, Shushan, Paltiel, Mordel, & Laufer, 1995) and pulmonary embolism (Chamberlain & Cumming, 1986). CC has also been shown to cause ovarian and uterine abnormalities (Nagao & Yoshimura, 2001).

Approximately 85% of an administered dose is eliminated after approximately 6 days, although traces may remain in the circulation for much longer (Mikkelson *et al.,* 1986; Alkushi *et al.,* 2015). Available evidence indicates that enclomiphene is the more potent isomer and the one primarily responsible for the ovulation-inducing actions of CC

(Van, Borreman, Wyman, & Antaki, 1973). Enclomiphene levels rise rapidly after administration and fall to undetectable concentrations soon thereafter. Zuclomiphene is a less active isomer; it is cleared far more slowly (Young, Opsahl & Fritz, 1999). The biological half-life in mice is 5–6 days; however, its metabolites have been found in feces up to 6 weeks (Ara & Asmatullah 2011).

2.8.1 Mechanism of Action of Clomiphene Citrate

Clomiphene citrate exerts its effects in the body by competitively binding to hypothalamic estrogen receptors thereby blocking the negative feedback of estrogen on gonadotropin production. Consequently, the hypothalamus produces its gonadotropin-releasing hormones (GnRH) which, in turn, cause the pituitary to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Taylor & Levine 2010; Allanah & Bratte, 2015). It comprises of two isomeric forms, cis and trans, which in the current nomenclature corresponds to zuclomiphene and enclomiphene, respectively (Sovino, Sir-Petermann & Devoto, 2002). The accumulation action of zuclomiphene due to its long half-life was the main force in the ovulation inducing action by clomiphene citrate, and thus that the clomiphene citrate's action was mainly caused by zuclomiphene (Young, Opsahl & Fritz, 1999).

2.8.2 Effect of Clomiphene Citrate on Ovarian Morphology

Birkenfeld, *et al.* (1985) studied the effect of clomiphene citrate on the rat and Alkushi (2015) studied its effect on the rabbit ovary and both found that the folliculogenesis in those animals appeared normal and the morphology of follicles was within normal ranges. It was observed by Alkushi (2015) that low dose of clomiphene citrate increased the number of atretic follicles ranging from 30 to 400 μm in diameter,

while it decreased the number of atretic follicles ranging from 401 to 600 μm in diameter. In contrast, the high dose of clomiphene citrate increased the number of atretic follicles ranging from 51 to 600 μm in diameter and cause the cessation of estrous cycle. Bostrom, Becedas, and Depierre (2000) further explain that the clomiphene citrate has both oestrogenic and anti-oestrogenic effects. Oestrogen reduces the number of ovulated oocytes in rats and induces atresia of the preovulatory follicles. This action is produced by clomiphene citrate, so it has oestrogenic effect. Oestrogen also is responsible for repeated cell division of follicular cells and so stimulate the growth of follicles along with FSH and LH. This action is against the action of clomiphene citrate which inhibits the follicular growth, so it has antiestrogenic effect. Increase in small and medium sized follicles were observed in clomiphene citrate treated animals than control animals (El-Sherry *et al.*, 2011).

2.8.3 Effect of Clomiphene Citrate on Egg Production and Quality Parameters

Urom (2016) observed that the number of eggs laid in the Nigerian indigenous hen increased with 10mg Clomid inclusion but decreased with 30mg Clomiphene citrate treatment administration. In a study by Biswas, Mohan and Sastry (2010), non significant effect of Tamoxifen (an anti estrogen) treatment in Indian native Kadaknath fowl when administered at different doses was observed. The findings of Jaccoby *et al.* (1992) also observed non significant effect of Tamoxifen on egg weight, the reason attributed was that anti estrogen such as Clomiphene Citrate or Tamoxifen could lead to increase in egg number and egg production due to the physiological effect of estrogen on the ovary and oviduct making them to become activated and thus the ovulatory process is enhanced (Jaccoby *et al.*, 1992; Biswas *et al.*, 2010).

In a study by Biswas *et al.* (2010) using Tamoxifen, they observed no significant differences between the various egg production traits except for the egg number. In a report by Williams (2015), they observed that eggs laid after Tamoxifen treatment (7 days after) were significantly larger than sham controls and that in the laid clutches, the smallest eggs were obtained 1-3 days during stimulation with Tamoxifen while largest eggs was obtained 5-9 days after injection. Capony and Williams (1981) explained that the binding of Tamoxifen to oestrogen receptors is described as a passive process in birds and doesn't compromise the subsequent response to oestrogen receptor, if the inhibition is short term, up regulation of the number of receptors is observed and there may be a net increase in active oestrogen receptors, transient increase in synthesis of yolk precursors and increase in the uptake rates at the ovary (Williams, 2015).

Rsaza *et al.* (2009) reported a decrease in the weight of the ovary in Tamoxifen treated birds which was attributed to the reduction in the weight of yellow hierarchical follicles without affecting the weight of the stromal tissue and the white ovarian follicles; blockage of estrogen receptors in hens treated with antiestrogen such as Clomiphene Citrate or Tamoxifen leading to reduction in ovary weight or egg weight could arise due to inhibition of the hypothalamo-pituitary gonadal axis activity leading to follicular atresia and suppression of the estrogen-stimulated synthesis and secretion of precursors of egg yolk in the liver and their deposition in the oocyte (Sakimura *et al.*, 2001). In a report by Zhang *et al.* (2005), he noted that the weight of the egg influences the weight of its components as well.

It has also been reported that Tamoxifen (an antiestrogen) completely blocks oestradiol induced oviduct growth and yolk precursor production (Sutherland, Mester &

Baulieu, 1977), this is because both oviduct growth and yolk precursor development are oestradiol dependent (Burley & Vadehra, 1989; Etches, 1996 and Johnson, 1999). Emily, Tony and Williams (2007) reported that Tamoxifen had no effect on albumen content of eggs. Biswas *et al.* (2010) did not observe any difference in yolk weight and albumen weight when administered Tamoxifen at 1, 0.5 and 2.5 mg per kg body weight.

Egg shell percentage and shell thickness (mm) were observed to be significantly affected in a study by Hanafy (2011) with highest records obtained in birds administered 1 mg per kg body weight in local chickens. It has been estimated to be necessary for the eggs to have at least a 50% chance to withstand normal handling condition without breakage, it should at least have an egg shell thickness of 0.33 mm (Stadelman, 1995).

2.8.4. Effect of Clomiphene Citrate on Incubation, Fertility and Hatchability

Increased value of fertility and hatchability was recorded in birds administered with 10mg of clomiphene citrate while reduced value in these parameters was obtained in those offered Clomiphene Citrate at 30mg in the Nigerian indigenous chickens (Urom, 2016) which also agrees with the work of Adedeji *et al.* (2016) who recorded similar findings in favour of Fulani ecotype. The birds offered Clomphene Citrate at 20mg also recorded the highest value (15.8%) in dead-in-shell while the lowest value (2.0%) in dead-in-shell was recorded in those offered clomiphene citrate at 10mg per kg body weight (Urom, 2016). The value of percentage dead-in-shell recorded by Urom (2016) was lower than values (10.4-38.1%) reported by Adedeji *et al.* (2016) except in those offered Clomiphene Citrate at 30mg per kg body weight that recorded 15.8% in dead-in-shell. In another study, the highest fertilization (87.5%) was noted in eggs from hens inseminated with semen produced by cocks administered with Tamoxifen at 10mg per kg

body weight, whereas the lowest (66.6%) was from those offered Tamoxifen at 1mg per kg body weight.

Hatchability from egg set was found to be highest (63.6%) in group TAM 10mg while the lowest (11.1%) was in those given Tamoxifen at 1mg per kg body weight. On the other hand, the highest hatchability from the fertilized egg was observed in group with 5mg Tamoxifen (87.5%) and the lowest (66.6%) was in group administered Tamoxifen at 1mg per kg body weight (Lisowski & Bednarczyk, 2005). Urom (2016) observed that very high dosage of Clomiphene Citrate does not encourage hatchability and fertility, and weakened the embryo; ascerting that a very high dosage of Clomiphene Citrate encouraged low density lipoprotein level in blood of embryo since Yafei and Nobel (1990) reported that low density lipoprotein in the plasma does not support hatchability and that this is found mostly in juvenile parents as a result of low lipid absorption from the parent. It is possible that high level (30mg) of Clomiphene Citrate caused reduction in lipid absorption from parents because reduced lipid absorption from parents to embryo can lead to increase embryonic mortality (Dalton, 2000).

2.8.5. Effect of Clomiphene Citrate on Oviposition Parameters

Robinson, Shafir, Perek and Snapir (1984) reported that administration of Clomiphene Citrate to broody turkey hens increased egg production due to the fact that Clomiphene Citrate is a competitive inhibitor of gonadal steroids for binding to their receptors. Egg production was suppressed when high dose of Tamoxifen was administered with low doses enhancing gonadotropic activity and egg laying (Jaccoby *et al.,* 1992). The number of egg sequence is the number of times the hen laid eggs in a

sequence and the egg sequence length is the number of eggs laid by individual hen on successive days without pause.

Clomiphene Citrate when taken orally, it binds to oestrogen receptors in the brain and causes pituitary FSH levels to rise and this action stimulates the growth of the ovarian follicle and thus initiates ovulation (Greenbatt, Barfield, Jungck & Ray, 1961). Poor layers have been reported to have short sequences whereas the best hens have the ability to lay one egg at roughly the same time everyday (Reddy, David & Khub Singh, 2005). Number of egg sequence was highest in the control group and shortest in those administered bromocriptine through subcutaneous route (32.50) and through oral feeding (39.50) (Reddy, David & Khub Singh, 2005). White Leghorn layers were observed to have number of egg sequence of 11.10 (Roy, Kataria & Roy, 2014). Administration of bromocriptine treatment to layers lead to a decrease in total pause days through subcutaneous injection (38.90) and through oral feeding (49.50) with the highest value recorded in the control group (93.30) and Reddy, David and Raju (2006) confirmed that shorter laying pause in hen leads to increased egg production.

It has been reported that higher doses of Clomiphene Citrate inhibit the activities of follicle stimulating hormone (FSH) and luteinizing hormone (LH) while, lower doses play an important role in regulating the level of LH and FSH (Schally *et al.*, 1970), many other studies have proved that low doses of Clomiphene Citrate not only increase gonadotropins secretion but also cause ovulation in the intact rat (Coppola & Perrine, 1965; Koch *et al.*, 1971).

The physiological mechanism that is responsible for taking pause days between the sequences of egg lay, inter sequence pause length, clutch length in hen may be the

consequence of reduced rate of follicular maturation and its subsequent recruitment into the hierarchy following ovulation which is partly regulated by FSH (Etches & Cheng, 1981). Even if a slight increase in clutch length is achieved, it will result into increase in egg production.

2.8.6 Effect of Clomiphene Citrate on Serum Chemistry

Alanine amino transferase (ALT) is thought to be more specific for hepatic injury because it is present mainly in the cytosol of the liver and in low concentrations elsewhere (Paul & Giboney, 2005). Whereas the AST is a mitochondrial enzyme found in the heart, liver, skeletal muscle, and kidney and is normally present in plasma (Zilva, Pannall & Mayne, 1988). The response to the dose of (0.5 mg / kg) of CC was fluctuating between having positive impacts by reducing significantly the AST and ALT and all the lipids lipids profile and serum creatinine were similar (Al-Attabi & Al-Diwan, 2012). Treatment of rats with clomiphene citrate result in significant decrease in serum AST and ALT enzymes (Aubuchon *et al.,* 2011; Al-Attabi & Al-Diwan, 2012) and was found to be ineffective in all lipids profile and serum creatinine (Al-Attabi & Al-Diwan, 2012), ALP also improved with administration of Clomiphene Citrate (Urom *et al.,* 2016). Urom *et al.* (2016) observed local chickens treated with 10 mg of Clomiphene citrate per kg body weight having highest value for urea in the local chickens; though the values were lower than the reported values by Iheukwumere, Abu & Ameh (2006) who reported that sharp increase in serum urea level could result in gonadal degeneration and infertility. With respect to creatinine, the lowest value (70.00mmol/L) was observed on birds treated with Clomiphene citrate at 30 mg per kg bdy weight while the highest value (135.55mmol/L) was recorded in the control (Urom *et al.,* 2016). A major source of creatinine in the blood

is from the muscle when wasting occurs and creatinine phosphate catabolized (Iheukwumere *et al.,* 2006). On serum total protein, Urom *et al.* (2016) rcorded the least value in the control (O mg Clomiphene citrate) and the highest value in those administered Clomiphene citrate. It has also been reported that serum, urea, creatinine and total protein contents depend on the quality of protein supplied in the diet.

2.8.7 Effect of Clomiphene Citrate on Serum Minerals

Minerals are inorganic substances present in all body tissues and fluids and they are necessary for the maintenance of certain physicochemical processes which are important to life (Eruvbetine, 2003). Afaf, Mahmoud and Amel (2018) observed significant differences in all the serum minerals studied. Tamoxifen supplemented groups were observed to show slight increase in the serum mineral of Ca except for P in groups fed 50 and 100 mg Tamoxifen supplemented groups for 60 days which showed slight decrease in its levels. Filipovic *et al.* (2015) studied the effects of Tamoxifen at a dose of 0.3mg per kg body weight daily for 3 weeks on biochemical markers of bone metabolism by using middle-aged orchidectomized (Orx) rats and found that blood serum levels of calcium (Ca^{2+}) and phosphorus (P) were significantly decreased.

2.8.8 Effect of Clomiphene Citrate on Relative Internal Organ Weights

Clomiphene Citrate was reported to positively influence the weight of the reproductive organ of the Nigerian indigenous cocks (Urom, 2016). Non-significant effect of Tamoxifen treatments (29-33g) in the white leghorn chickens of 14 weeks old pullets was recorded by Jaccoby *et al.* (1992), abdominal adipose tissue were not affected in their study (9-14g) while ovary and oviduct weight were affected with those on Tamoxifen 1 mg per kg body weight having higher values than controls: the increase in

the oviduct weight was manifested due to it been an oestrogen target tissue as a result of increase in the plasma oestrogens. Moon *et al.* (1989) observed that in hypophysectomised rats, oestradiol induced ovarian growth with consequent sensitizing of the ovary to the ovulation inducing effect of gonadotropins, he opined that when anti-oestrogens were administered simultaneously, there is reduction in the stimulatory effects of oestradiol on the ovary.

In another work by Jaccoby *et al.* (1994), liver, ovary and oviduct weights were affected by Tamoxifen and oestradiol treatments. Hanafy (2011) observed non-significant differences in relative weights of liver, abdominal fat in two local chicken strains treated with Tamoxifen while relative weights of ovary and oviduct were affected by the treatments with Tamoxifen which was also in line with a report by Biswas *et al.* (2010) who discovered that administration of Tamoxifen might have been the reason for the precocious maturation of gonadal pituitary axis in Indian native Kadaknath hen. Johnson (2007) and Bacon *et al.* (1980) reported that rapid development of the oviduct is regulated by gonadal hormones and this occurs before and during sexual maturation.

2.8.9 Effect of Clomiphene Citrate on Serum Hormones

Clomiphene citrate was found to enhance the hormonal profile and maintain the weight of Nigerian indigenous Cocks without any deleterious effect (Urom, 2016).The higher progesterone concentration was seen after clomiphene citrate treatment in infertile women which is due to developed CL or combined production of more than one CL (Downs and Gibbson, 1983; Guzick & Zeleznik, 1990; Zubair & Sajid, 2015). Clomiphene was reported to inhibit progesterone synthesis in rat follicles (Laufer *et al.*, 1982) and granulosa cells (Welsh *et al.,* 1984), monkey luteal cells (Westfahl & Resko,

1983) and hen granulosa cells (Sgarlata *et al.*, 1984). In contrast, the effects of clomiphene on oestrogen biosynthesis *in vitro* have been less consistent. Some investigators report a stimulation (Zhuang *et al.*, 1982; Welsh *et al.*, 1984), and others an inhibition (Laufer, Bra, Shenker and Safriri, 1982).

The inhibitory effects of clomiphene on ovarian steroidogenesis may have two consequences. First, the reduced oestrogen production may enhance LH secretion. It is believed that clomiphene acts at the level of the hypothalamus to displace oestrogen from its receptors, thereby preventing the negative feedback of oestrogen on gonadotrophin release; as a result, LH secretion is stimulated (Watasuki and Sagara, 2002).

The hypothalamic action of clomiphene could be enhanced by a direct inhibitory effect of clomiphene on oestrogen production. On the other hand, impaired folliculogenesis may be another consequence of a direct inhibitory effect of clomiphene on ovarian steroidogenesis. Appropriate quantities of oestrogen are needed, with gonadotrophins, to stimulate follicular maturation (Goodman & Hodgen, 1983). Therefore, an inhibitory effect of clomiphene on oestrogen biosynthesis could impair the process of folliculogenesis. In another sense, the increase in estrogen levels (as a result to the increase in number of mature follicle) is possible to cause the increment in the triglyceride serum concentration that could lead to hypertriglyceridemia that may cause later the development of cardiovascular disease in women as confirmed by some studies (Wakatsuki & Sagara, 2002; Ettinger, Friedman, Bush & Quesenberry, 2002). In their studies, the use of induction leads to more mature follicle that means extraordinary levels of estrogen and the use of induction for three month lead to increase of triglyceride levels especially in the third month.

In vitro animal studies in ovarian physiology have suggested that thyroid hormones play an important direct role (Wakim, Marrero, Polizoto, Burholt & Buffo, 1993). In hypothyroxinemia patients, CC treatment with thyroid hormone replacement therapy is of a great value for luteal phase defect and ovulation induction. It was reported that Clomiphene Citrate directly influence thyroid hormones and function (Feldt-Rasmussen., Lange., Date & Hansen, 1979). Thyroxine level (T_4) in infertile women increased within one month after ovarian stimulation while Thyrotropin level (T_3) did not change after CC treatment (Poppe, Glinoer, Tournaye, Schiettecatte & Devroey, 2004; Poppe, Glinoer, Tournaye, Schiettecatte & Heantjens, 2005). It was also reported that thyroid stimulating hormone (TSH), T_3 and T_4 levels remained unchanged in women after ovarian stimulation (Monteleone, Parrini, Faviana, Carletti & Casarosai, 2011).

A similar study indicates that TSH level was increased, while T_4 level was not changed in patients during ovarian stimulation (Muller, Verhoff, Mantel, De Jong, & Berghout, 2000). In addition, non-significant difference in TSH level wasfound in CC administration group and CC plus L-thyroxin group in women (De Leo, IaMarca, Lanzetta & Morgante, 2000). Some finding of a previous study on patients with primary testicular failure recorded an increase in TSH level and no changes in both T_3 and T_4 levels after CC administration (Spitz, Kerem, Zylber-Haran, Shilo & Laufer, 1982).

Another study was done on healthy men, they were given 100 mg /day of CC for 5 and 12 consecutive days, the finding of the study recorded small but statistically significant decrease in T_3 and T_4 levels on day 4 of CC and a slight increase in TSH level was found in day 5 of CC administration (Feldt-Rasmussen *et al.*, 1979). In a recent study on albino rats, it was indicated that Treated animals showed a significant decrease in

TSH hormone in both groups of animals administered CC at 50 mg for 30 days CC + Humegon (H) injected at 0.23 IU for 10 days. CC group showed significant increase in T3, while (CC + H) group showed significant decrease in T3 while T4 value drop significantly in treated animals (CC; CC + H) in contrast with control group (Awad & Hamad, 2017). Plasma T3 and T4 hormones were reported to be highest in Oestradiol-Tamoxifen treatments (Jaccoby *et al.,* 1994) and Culbert *et al.* (1977) observed that the administration of Clomiphene Citrate might have resulted in an increase in the level of hormone.

CHAPTER THREE

3.0 MATERIALS AND METHODS

The entire work was carried out in three phases, that is, non-mating (Phase I), mating (Phase II) and incubation phases (Phase III). The general experimental layout for the phases is shown below:

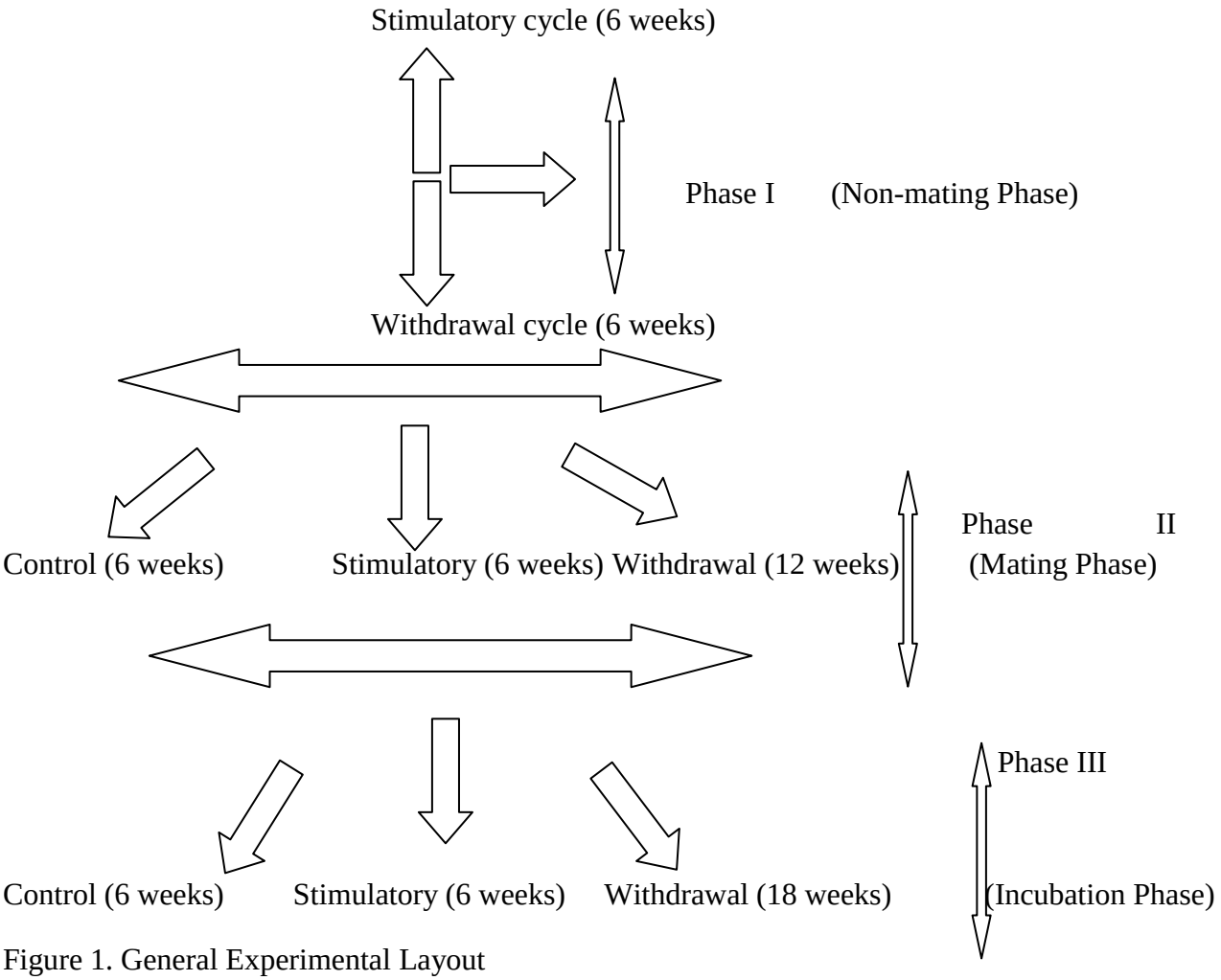


Figure 1. General Experimental Layout

3.1 STIMULATORY-CUM-WITHDRAWAL PHASE

3.1.1 Experimental Site

The Experiment was carried out at the Poultry Unit of the Department of Animal Science (GPS Coordinates: N11.97643º, E008.42995º), located within the Faculty of Agriculture Teaching and Research Farm, Bayero University, Kano.

3.1.2 Sample Size Determination

The resource equation of Mead, Gilmore and Mead (2012) was used in determining the sample size. The equation is as follows:

N-1 = T + E

Where,

N = number of experimental units, T = number of degrees of freedom (number of test groups – 1) and E = number that estimates the error of the remainder.

Mead and colleagues proposed that E should be between 10 and 20. The sample size was calculated as follows:

18 - 1 = (2 - 1) + E

N - 1 = T + E

E = 18 - 2 = 16

Therefore, the sample size used was 18 because it gives an estimate of error (E) that is in the range of 10 to 20.

3.1.3 Experimental Design and Treatment

The study was laid out in a repeated measures design with two treatments (Clomiphene Citrate stimulation and Clomiphene Citrate withdrawal). Clomiphene Citrate (CC) (Global Napi Pharmaceuticals, Sanofi Aventis, France) was administered to

eighteen (18) Lohmann Brown hens at 15 mg/kg body weight per *os* for six weeks (stimulatory stage) and withdrawn for another six weeks (withdrawal stage). Therefore, the two stages of this phase lasted for a total period of twelve weeks.

3.1.4 Management of Experimental Birds

A total of 36 Lohmann Brown layers weighing between 1.2 – 2.0 kg aged 37 weeks were used for the study. The birds were purchased at point of lay at Sovet International limited, housed in battery cages. Layers mash was purchased at Sovet International and fed to the hens in the morning and afternoon hours with a commercial layer ration (Super Layer®) containing 16.0 % crude protein, 5.0 % fat, 6.0 % fibre, 3.5 % calcium, 0.4 % phosphorus and 2600 kcal/kg metabolizeable energy . Clean water was regularly served *ad libitum*. The birds were given multivitamin as anti-stress (Anupco Vitalyte Extra®, Anglian Nutrition Products Company, UK) orally via drinking water at 0.5 g per litter during the stabilization period of two weeks to reduce the stress of transportation.

Egg production, oviposition pattern and egg quality parameters during the stimulatory and withdrawal stages of the Phase I were recorded as follows:

Egg production parameters

1. Ovulation rate was determined using the Hen Day Egg Production (HDEP) formula as follows: HDEP = (Number of eggs produced everyday ÷ Number of birds available in the flock on that day) x 100 (North, 1984).
2. Total number of eggs laid per bird per day and their weights.

Oviposition pattern parameters

These were determined according to the procedure described by Blake, Balander, Flegal and Ringer (1987). Oviposition pattern in terms of number of egg sequences (the number of times the hen laid eggs in sequence during the experimental period), egg sequence length (the number of eggs laid on successive days by a particular hen without pause), pause days (the number of days with no record of egg production by each hen) and inter-sequence pause days (the number of pause days between an egg sequence and another).

Egg quality parameters

1. Egg length (along the longitudinal axis) was measured using a vernier caliper.
2. Egg width (along the equatorial axis) was measured with a vernier caliper.
3. The yolk and albumen volume were determined by gently making a hole in the egg to expel the albumen content into a container and then gently openend the egg shell to expel the yolk carefully into a measuring cylinder. The already separated albumen was poured into a measuring cylinder and measured in ml.
4. Egg shells were dried by cleaning the remaining albumen. Following Anderson's (2004) procedure, shell weight (with membrane) was measured using a laboratory scale by placing the dried shell on the scale and recording its weight.
5. Shell thickness (with membrane) was measured at the sharp poles, blunt poles and equatorial parts of each egg with a vernier calliper. Shell thickness was obtained from the average values of these three parts.

3.1.5 Statistical Analysis

To compare ovulation rate, oviposition pattern and egg quality parameters during and after Clomiphene Citrate administration in Lohmann Brown hens, paired samples t-test was used as implemented in the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL, USA).

3.2 MATING PHASE

3.2.1 Experimental Site

The experiment was carried out in the same location as the non-mating phase.

3.2.2 Experimental Design and Treatment

The study was laid out in a single factor completely randomized design with three treatments (CC administered, CC withdrawn and control groups) each replicated six times. The CC administered group included a group of hens that were administered CC for six weeks at 15 mg/kg body weight for the first time. The CC withdrawn group comprises hens from Phase I earlier stimulated for six weeks with CC at 15 mg/kg body weight withdrawn from CC for six weeks and now undergoing further withdrawal for six weeks. The control group include hens that have not been administered CC at all. The experiment involved 18 Lohmann Brown hens. To facilitate natural mating, each group of hens (6 hens per group) had one cock of the ISA Brown strain, making a total of three cocks used in this phase.

3.2.3 Experimental Birds and their Management

Hens were managed in a deep litter system and each tagged with a masking tape for identification purpose. The hens receiving CC were weighed in order to calculate the total dose per hen. Afterwards, sound eggs without cracks were collected for the study.

Layers mash were fed to the hens *ad libitum* with a commercial layer ration (Super Layer®) containing 16.0 % crude protein, 5.0 % fat, 6.0 % fibre, 3.5 % calcium, 0.4 % phosphorus and 2600 kcal/kg metabolizable energy. Clean water was regularly served. The hens and cocks were given multivitamin as anti-stress (Anupco Vitalyte Extra®, Anglian Nutrition Products Company, UK) orally via drinking water during the stabilization period for the stress of weighing and transfer from battery cage to deep litter. This phase lasted for six weeks.

3.2.4 Data Collection

Parameters of egg production and oviposition pattern

Egg collection commenced a week into the experiment for a total period of six weeks. Eggs laid in each treatment group were collected, counted, weighed and kept in egg crates. Crates were placed weekly in cartons kept at room temperature for six weeks. Ovulation rate (Hen Day Egg Production, HDEP) and parameters that define oviposition pattern (such as number of egg sequences, egg sequence length, pause days and inter-sequence pause days) were determined as earlier described.

3.2.5 Blood Collection and Serum Harvesting

At the end of the experimental period, all the hens'weight were recorded and then slaughtered (six hens per group). Blood from each treatment group was collected into plain bottles at the point of slaughter. It was then placed in Styrofoam box with ice pack and transported to Aminu Kano Teaching Hospital, Kano, for laboratory evaluation. On arrival at the laboratory, blood was allowed to stay at room temperature for two hours. The clot formed was dislodged with the aid of a blunt wooden stick. The remaining content in the tube was centrifuged at 4000 revolutions per minute for 30 minutes in a

centrifuge (Centrifuge 800®, Techmel, USA). Transfer/dropping pipette was used to dispense the supernatant (serum) into a clean-labelled tube and stored at -20 ºC until further analysis.

3.2.6 Internal Organ Harvesting

The carcass of birds slaughtered above were dissected using standard technique according to Brown (2012). Each bird was placed on its back with its feet facing the dissector and the wings reflected back. The legs were put flat aside by making a cut through the skin and then the breast. A cut was made at the caudal edge of the keel in order to remove the skin from the ventral surface and then pulling the skin cranially. The skin was peeled away thus exposing the peritoneum and breast muscle. Scissors or a scalpel blade was used to cut through the peritoneal wall and then the cut extended just behind the breast bone to expose the abdominal viscera cranially. The harvested organ was collected weighed individually using digital measuring scale (China).

3.2.7. Serum Digestion

Nitric-perchloric acid digestion method was used to wet-digest the serum samples using AOAC (1990) with some modifications. Briefly, plastic containers (50 ml) were washed with deionised water and labelled properly. The serum earlier stored was removed from freezer and thawed within 2 hours at room temperature and 1 ml was pipetted into 50 ml glass beaker. Afterwards, 10 ml nitric acid (HNO_3) was added. For each serum sample the procedure was repeated. The hot plate was utilized to heat the beaker gently thereby bringing the content to a boiling point in order to enhance oxidation of all easily digestible matter. After cooling, 5 ml of 70% perchloric acid was added and the content was again boiled until dense white fumes appear. The content was

then cooled after which 20 ml distilled water was incorporated. This mixture was then boiled gently to release any fume. When the cooling was achieved, the solution was then filtered using Whatmann chromatographic paper and distilled water (30 ml) was added finally to the filtrate.

3.2.8. Relative Weight of Internal Organs, Ovarian Morphology and Fixation

The ovary was stored in 10% Neutral Buffered Formalin for three days in order to harden the follicles to facilitate easy measurement. Three days after storage in the fixative, the ovary was removed. Each follicle was measured with a digital scale (China made), classified based on the method described by Ebeid *et al.* (2008) and counted. Yellow follicles with size >10 mm in diameter, yellow follicles with size of about 5-10 mm in diameter, white follicles with size range of about 3-5 mm in diameter and white follicles measuring about 1-3 mm in diameter were classified as large yellow follicles (LYF), small yellow follicles (SYF), large white follicles (LWF) and medium white follicles (MWF). The stroma comprising the ovarian tissue remaining after the removal of LYF was weighed.

3.2.9 Determination of Mineral Concentration

Zinc (213nm), cobalt (240.7nm), chromium (357.9nm), magnesium (285.2nm), manganese (279.5nm), copper (324.8nm) and iron (248.3nm) levels were determined from the digested sera described above using atomic absorption spectrophotometry (Atomic Absorption Spectrophotometer, Model Agilent Technologies, 240SS, UK). Flame photometry (Flame Photometer, Model PF97, Jenway Ltd, England) was used to determine sodium (589nm) and potassium (762nm) serum levels. Calcium (580nm) and

phosphorus (450nm) levels were determined by the colorimetric method (Ciba-Corning® Colorimeter, Model 257, Sherwood Scientific Ltd, UK).

Quantitative determination of serum hormone levels

The serum frozen above was thawed at room temperature. Accubind® Enzyme-linked Immunosorbent Assay (ELISA) kits (Monobind Inc., Lake Forest, USA) were used to quantify serum levels of progesterone, oestradiol, triidothyronine and thyroxine according to manufacturer's instruction. Results (absorbance and associated concentration) were obtained from a microplate reader (Microplate Reader®, Model RT-2100C, Rayto Life and Analytical Science, Germany) with wavelength of 450nm.

Serum chemistry

Serum bicarbonate, urea, creatinine, total protein, albumin, aspartate aminotransferase (AST, 546nm), alkaline phosphatase (ALP, 580nm) and alanine aminotransferase (ALT, 546nm) levels were determined by the colorimetric method using commercial kits according to manufacturer's instruction.

Statistical Analysis

To determine the effect of Clomiphene Citrate on egg production and oviposition pattern parameters in Lohmann Brown hens during the mating phase, One-Way Analysis of Variance (ANOVA) was used. Where there was significant difference in means, Tukey's test was used as a mean separation method. One-way analysis of variance (ANOVA) was used to determine the effect of Clomiphene Citrate (CC) on serum chemistry, hormones (oestrogen, progesterone, thyroxine, and triiodothyronine) and minerals, follicular counts, follicular, stroma and oviductal weights in Lohmann Brown hens was analyzed using Kruskal-Wallis Test with mean rank differences compared using

Dunn's multiple comparison test. All statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL, USA) and Graphpad InStat®, version 3.05, 32 bit for Win 95/NT, Graphpad Software Inc., 2000.

3.3 INCUBATION PHASE (PHASE III)

3.3.1 Experimental Site

The experiment was conducted in four locations, the Faculty of Agriculture Teaching and Research Farm, Department of Animal Science (GPS Coordinates: N11.97º, E008.42995º), Hatchery I (Amsad) located at Yahaya Gusau Road opposite AP filling station (GPS Coordinates: N11.9534732º, E8.457495º) and Hatchery II (Amsad) located off Kuntau Secondary School, Kano (GPS Coordinates: N11.9749º, E8.46638º) and also the Department of Microbiology at the Aminu Kano Teaching Hospital, Kano (GPS Coordinates: N11.94).

3.3.2 Study Design

Cross sectional design was employed in this study. Fertile eggs across the different experimental groups from the Mating Phase constituted the experimental materials.

3.3.3 Incubation

Eggs collected during the Mating Phase from the Teaching and Research Farm were placed in a single-stage incubator at Amsad Hatchery I and II a week after collection and candled a week later by passing them through light. Eggs that appeared clear were removed while those that appeared dark with developing embryo were retained. Clear eggs are those that were not fertilized after candling and were discarded after a week of incubation. Fertile eggs are those that have been fertilized showing a

visible network of blood vessels spreading from the centre of the egg outwards or showing the dark outline of the embryo at the centre of the network of blood vessels. Data on fertility was recorded for each group. Percent fertility was calculated as (Total number of fertile eggs/Total number of eggs set) ×100. The fertile eggs remained in the incubator until hatching. Fertile eggs were turned hourly and exposed to temperature of 37.5 ºC and relative humidity of 60% at the initial stage. They were further exposed to temperature of 36.5 ºC and relative humidity of 65% towards hatching time from day 18 of incubation.

After hatching, the number of hatched chicks were recorded. Percent hatchability and percent dead-in-shell were calculated as (Total number of chicks hatched/Total number of fertile eggs) × 100 and (Total number of dead in shell /Total number of fertile eggs) x 100, respectively. The hatched chicks were identified according to experimental groups using permanent markers of different colours and weighed thereafter. They were vaccinated against diseases such as Newcastle and Marek's.

3.3.4 Egg and Hatchery Microbial Analysis

Total aerobic bacterial count

The pour plate method (Harrigan & McCance, 1976) was used to determine the total aerobic bacterial count in the Lohmann Brown eggs from the farm, commercial eggs inside the incubator, hatchery surroundings and also the dead-in-shell eggs (from Lohmann Brown eggs) in the incubator. A sterile swab stick was made wet with few drops of normal saline and swabs were taken on the surfaces of Lohmann Brown eggs from the farm, commercial eggs inside the incubator, hatchery surroundings and inside dead-in-shell eggs. Different swab sticks were used for the different categories and the

swabs were transferred aseptically into sterile Petri dishes containing 10 ml of molten nutrient agar. The Petri dishes were gently swirled and allowed to cool and solidify. The plates were incubated for 24 to 48 hours at 37 ºC. After incubation, growth were observed on the plate and if found, were selected and counted using electronic colony counter.

Bacterial Identification

The growth on the plates from the Lohmann Brown eggs from the farm, commercial eggs inside the incubator, hatchery surroundings and also the dead-in-shell eggs (from Lohmann Brown eggs) in the incubator were cultured for aerobic bacteria on chocolate and McConkey agar. These plates were incubated for 24 hours at 37 ºC. Sabouraud's Dextrose Agar and Mycoplasma medium were used to rule out fungi and Mycoplasma. Pure culture of the microbes were obtained from the colonies which appeared after 24 hours of incubation at 37 ºC by purifying it through repeated sub-culturing before microscopic examination for Gram reaction (Claus, 1992) as well as cell morphology using 24 hour old cultures, motility, pigmentation and sporulation (Harrigan & McCance, 1976). Bacterial characterization of the isolates was done using nitrate reduction, oxidase, catalase, triple sugar iron, citrate, indole and urease tests (Cheesbrough, 2002).

Antibiotic Susceptibility Test

The organisms identified were subjected to testing of antibiotic susceptibility using single discs containing Gentamicin, Penicillin, Streptomycin, Amoxicillin, Cefoxitin and Neomycin by the method of Kirby-Bauer disc diffusion (Bauer, Kirby, Sherris & Turck, 1966). Briefly, the pure colonies of bacterial growth were suspended in tubes containing infusion broth of 5 ml and was later adjusted to 0.5 McFarland turbidity

standards. Sterile cotton swab indicator sticks were used to take 10 µl each of diluted bacterial suspensions for inoculation into Mueller Hinton agar plates by uniformly rubbing against the entire agar surfaces and allowed to dry. The surfaces of the inoculated plates were impregnated with antimicrobial discs using sterile forceps 1.5 cm away from the edges of the plates and 3 cm away from each other with guide of a template which were placed under the petri dish.

Forceps were used to gently press the discs to ensure complete contact with the agar surface. Invasion of the plates took place followed by aerobic incubation at 37 ºC for 24 hours. A caliper was used on the underside of the plates to measure (in millimeter) the zones of inhibition of bacteria by the antimicrobial discs. The bacteria that are susceptible were determined based on the breakpoints recommended by the Clinical Laboratory Standards Institute (CLSI, 2011) (Table 1).

Table 1. Antimicrobial Susceptibility pattern of the Bacterial Isolates

Antibiotic	Code	Disc Potency	Diameter of Zone of Inhibition (mm)	
			Susceptible	Resistant
Streptomycin	S	10 µg	≥ 26	≤ 22
Gentamicin	CN	10 µg	≥ 15	≤ 12
Neomycin	N	30 µg	≥ 26	≤ 22
Cefoxitin	FOX	30 µg	≥ 23	≤ 14
Penicillin	P	10 IU	≥ 29	≤ 28
Amoxicillin	AML	20 µg	≥ 18	≤ 13

Cheesbrough (2002) and CLSI (2011)

3.3.5 Data Analysis

All statistical analysis were done using Graphpad InStat package (Grapghpad InStat, version 3.05, 32 bit for Win 95/NT, Graphpad Software Inc., 2000). Descriptive statistics was used to analyze data on the effect of administration of CC on fertility, hatchability and dead-in-shell eggs laid by Lohmann Brown hens, differences were tested with one way analysis of variance ANOVA (if data was normally distributed with or without transformation) or Kruskal-Wallis test (if data was not normally distributed). To isolate and identify organisms associated with the Lohmann Brown eggs from the farm, commercial eggs inside the incubator, hatchery surroundings and also the dead-in-shell eggs (from Lohmann Brown eggs) in the incubator, descriptive statistics was used in analyzing the data.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Non-mating Phase (Phase I)

Egg production parameters

Ovulation rate in Lohmann Brown hens during and after oral Clomiphene Citrate administration is presented in Table 2. There was statistically significant (t-value = 5.039; P<0.0001) difference in ovulation rate between the stimulatory and withdrawal groups. Ovulation rate (61.45%) in hens during CC treatment (CC) was lower than ovulation rate (78.29%) in hens after Clomiphene Citrate.

The total number of eggs laid by Lohmann Brown hens during and after oral Clomiphene Citrate (CC) administration is presented in Figure 1. A statistically significant (t-value = -7.072; P<0.001) difference was recorded in number of eggs laid during and after CC administration (sum = 472 eggs vs. 600 eggs; mean = 0.62 vs. 0.77 eggs, respectively).

Oviposition pattern parameters

Oviposition pattern parameters in Lohmann Brown hens during and after oral CC administration are presented in Table 3. Number of eggs laid (32.88 eggs) and egg sequence length (22.44 eggs) after CC withdrawal were significantly higher than number of eggs laid (25.83 eggs) and egg sequence length (16.83 eggs) during CC stimulation (P<0.001 *vs*. P<0.01; t-value = -6.074 *vs*. -2.621). The reverse was the case for number of egg sequence (5.61 vs. 4.22 eggs; t-value = 2.314; P<0.01), number of laying pause (15.88 vs. 8.38 days; t-value = 6.280; P<0.001) and number of inter-sequence pause (7.11

vs. 3.55 days; t-value = 3.140; P<0.001) where parameters during CC stimulation were significantly higher than corresponding values after CC withdrawal.

Table 2: Ovulation Rate in Lohmann Brown Hens during and after Clomiphene Citrate Administration.

Bird Identification	Observation (n)	Ovulation Rate (%)	
		Stimulatory Group	Withdrawal Group
1	42	23	34
2	42	27	29
3	42	31	38
4	42	32	39
5	42	27	37
6	42	22	28
7	42	34	37
8	42	33	40
9	42	28	36
10	42	15	31
11	42	31	37
12	42	37	36
13	42	24	36
14	42	03	09
15	42	22	35
16	42	25	32
17	42	27	23
18	42	24	35
Sum	774	465	592
Mean		61.45	78.29
t-value		5.039****	

****=P<0.0001

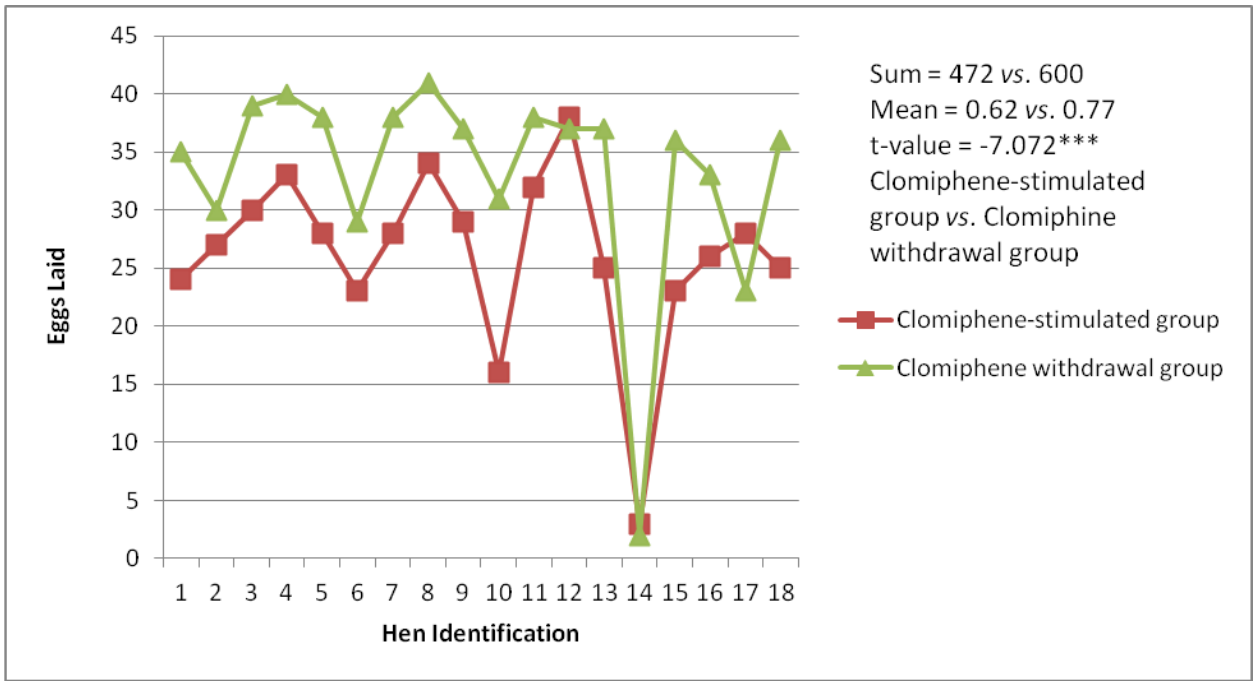


Figure 1. Eggs Laid by Lohmann Brown Hens during and after Oral Clomiphene Citrate Administration

Table 3. Oviposition Pattern Parameters of Lohmann Brown Hens during and after Oral Clomiphene Citrate Administration

Parameter	N	Group		t-value	Significance
		Stimulatory	Withdrawal		
Eggs laid	18	25.83±1.831	32.88±1.729	-6.074	***
No. of egg sequence	18	5.61±0.486	4.22±0.501	2.314	**
Egg sequence length	18	16.83±1.592	22.44±1.875	-2.621	**
No. of laying pause	18	15.88±1.791	8.38±1.584	6.280	***
No. of inter-sequence pause days	18	7.11±1.131	3.55±0.543	3.140	***

All values are expressed as mean ± SEM, ***= P<0.001 **=P<0.05

Egg quality parameters

Changes in egg weight during and after Clomiphene Citrate (CC) administration in Lohmann Brown hens are presented in Figure 2. The mean egg weight (47.10 g) after CC administration was significantly (t-value = -8.364; P<0.001) higher than the mean egg weight (36.27 g) during CC administration.

Changes in width of Lohmann Brown eggs during and after oral CC administration are presented in Figure 3. The mean egg width (33.23 mm) after CC treatment was significantly (t-value = -7.76; P<0.001) higher than its corresponding mean value (25.47 mm) during CC administration.

Changes in egg length during and after oral CC administration in Lohmann Brown hens are presented in Figure 4. The egg length (44.27 mm) after CC withdrawal increased significantly (t-value = -6.43; P<0.001) by 25.6% of its corresponding value (35.26 mm) during CC treatment.

Changes in yolk volume of Lohmann Brown eggs during and after oral CC administration are presented in Figure 5. The mean yolk volume (12.27 ml) after CC administration was significantly (t-value = -8.434; P<0.001) higher than mean yolk volume (9.42 ml) during CC administration.

Changes in albumen volume of Lohmann Brown eggs during and after oral CC administration are presented in Figure 6. The mean albumen volume (29.48 ml) after CC treatment was significantly (t-value = -8.40; P<0.001) higher than mean albumen volume (22.64 ml) during CC treatment.

Changes in shell thickness of Lohmann Brown eggs during and after oral CC administration are presented in Figure 7. The mean shell thickness (0.31 mm) after CC

administration was significantly (t-value = -1.6; P<0.001) higher than mean shell thickness (0.33 mm) during CC administration.

In all the egg quality parameters presented, there was a particular drop in the chart with reference to bird number 14 during and after CC administration.

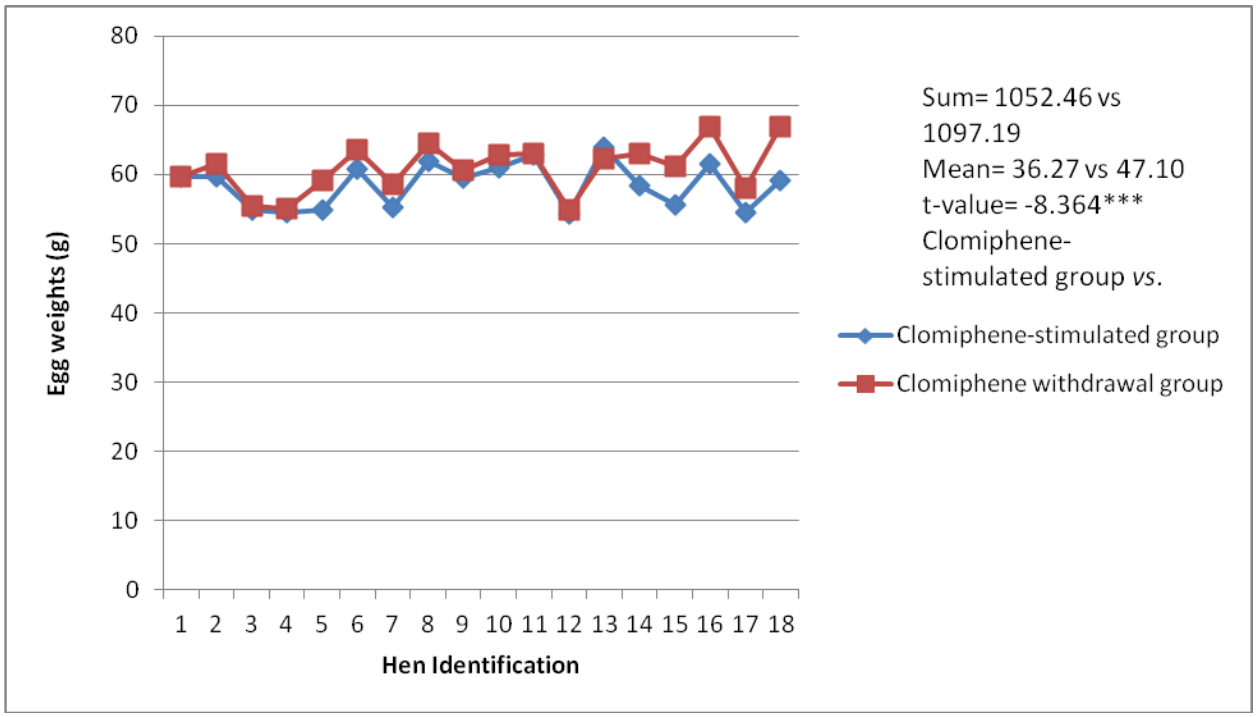


Figure 2. Egg Weight of Lohmann Brown Eggs during and after Oral Clomiphene Citrate Administration.

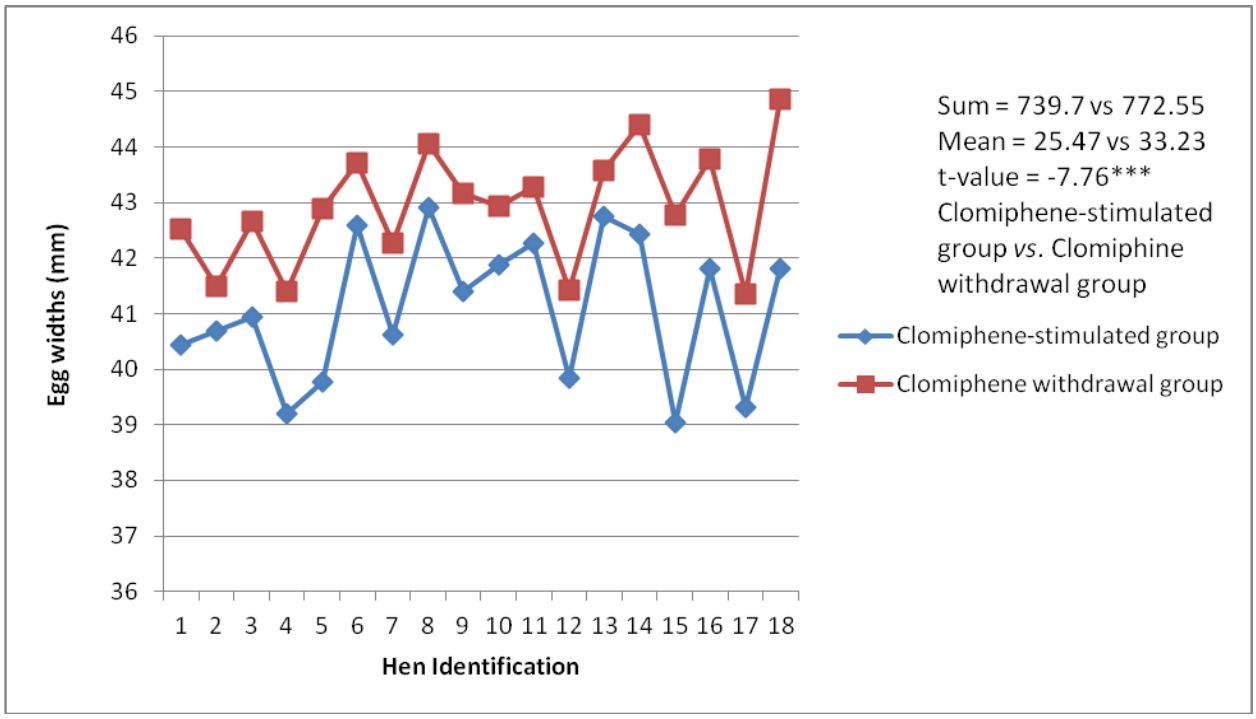


Figure 3. Egg Width of Lohmann Brown Eggs during and after Oral Clomiphene Citrate Administration.

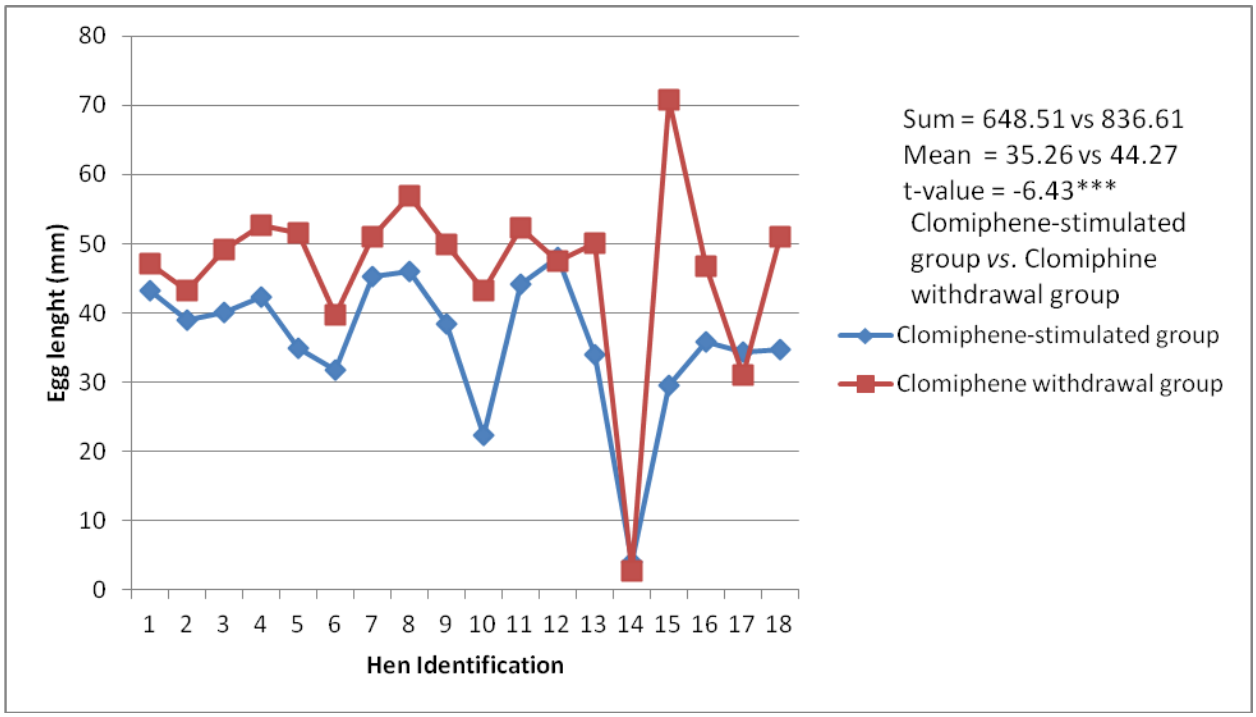


Figure 4. Egg Length of Lohmann Brown Eggs during and after Oral Clomiphene Citrate Administration.

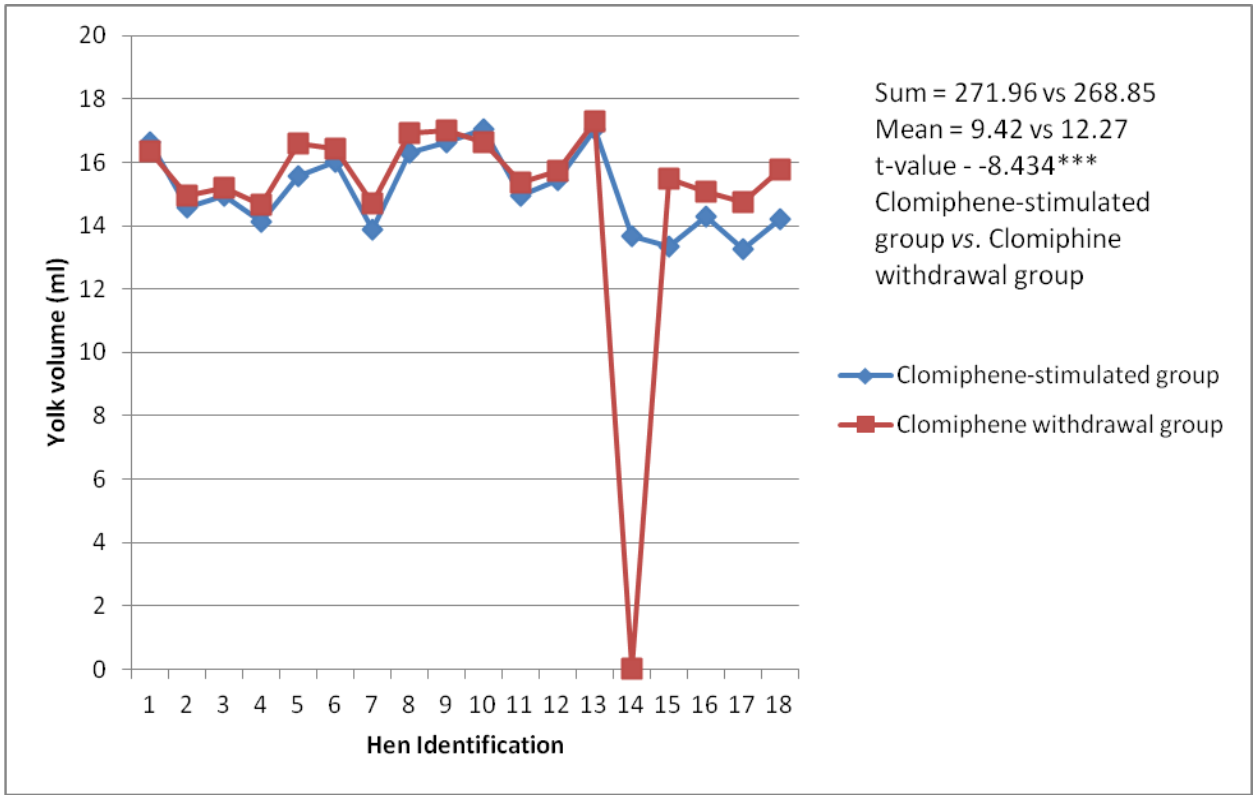


Figure 5. Yolk Volume of Lohmann Brown Eggs during and after Oral Clomiphene Citrate Administration.

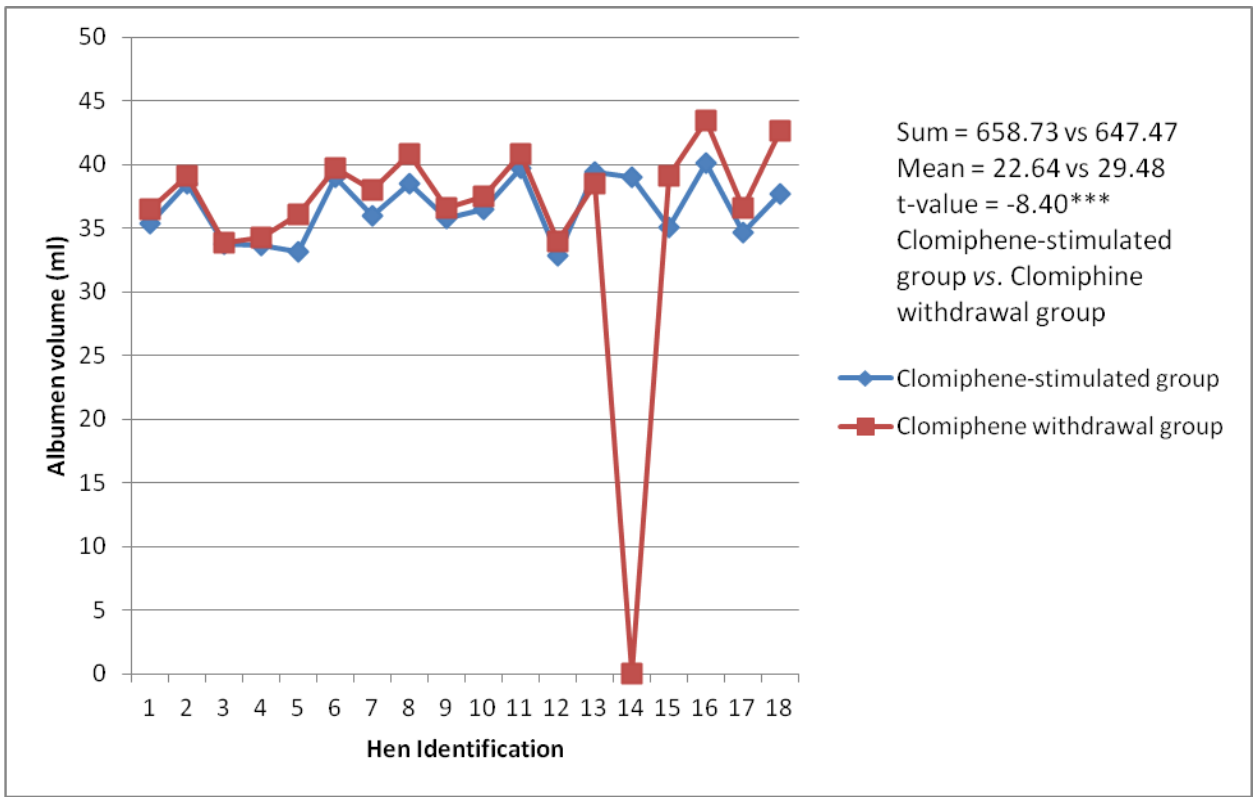


Figure 6. Albumen Volume of Lohmann Brown Eggs during and after Oral Clomiphene Citrate Administration.

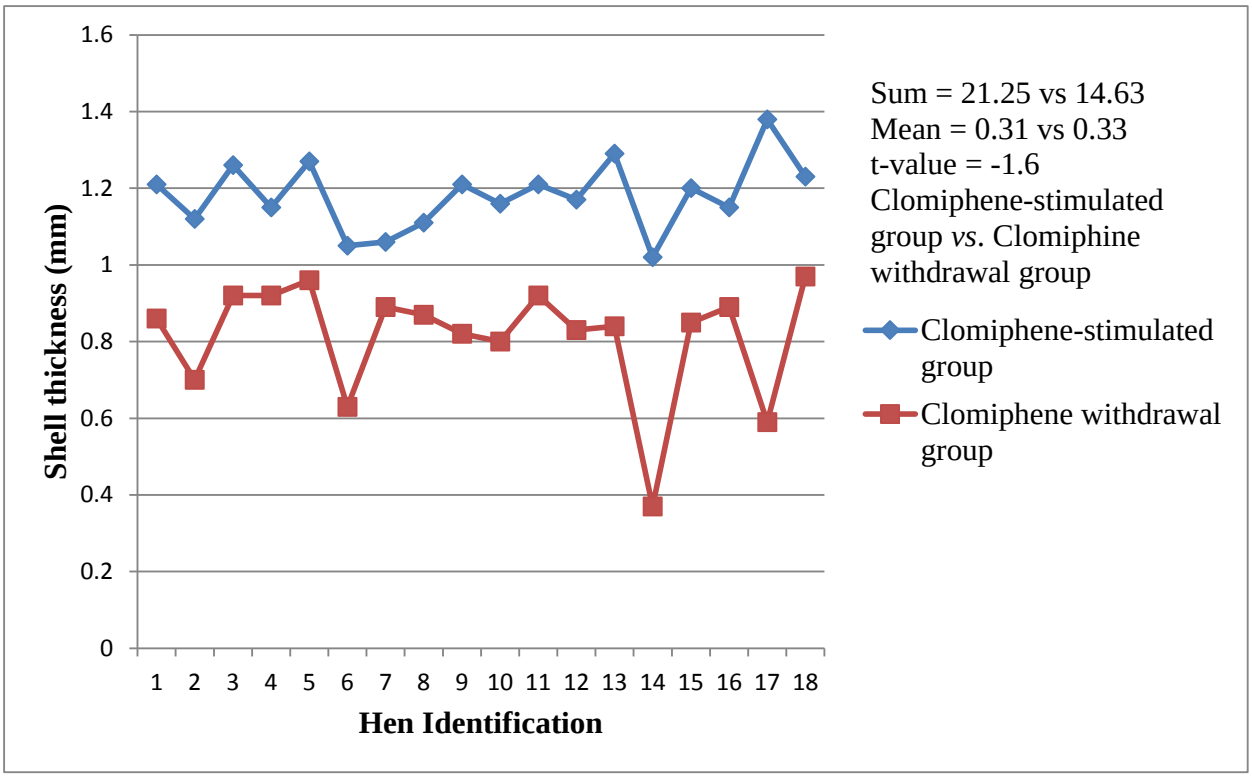


Figure 7. Shell Thickness of Lohmann Brown Eggs during and after Oral Clomiphene Citrate Administration.

Changes in shell weight of Lohmann Brown eggs during and after oral Clomiphene Citrate (CC) administration are presented in Figure 8. Mean shell weight (6.18 g) after CC administration was significantly (t-value = -11.52; $P<0.001$) higher than mean shell weight (4.44 g) during CC administration.

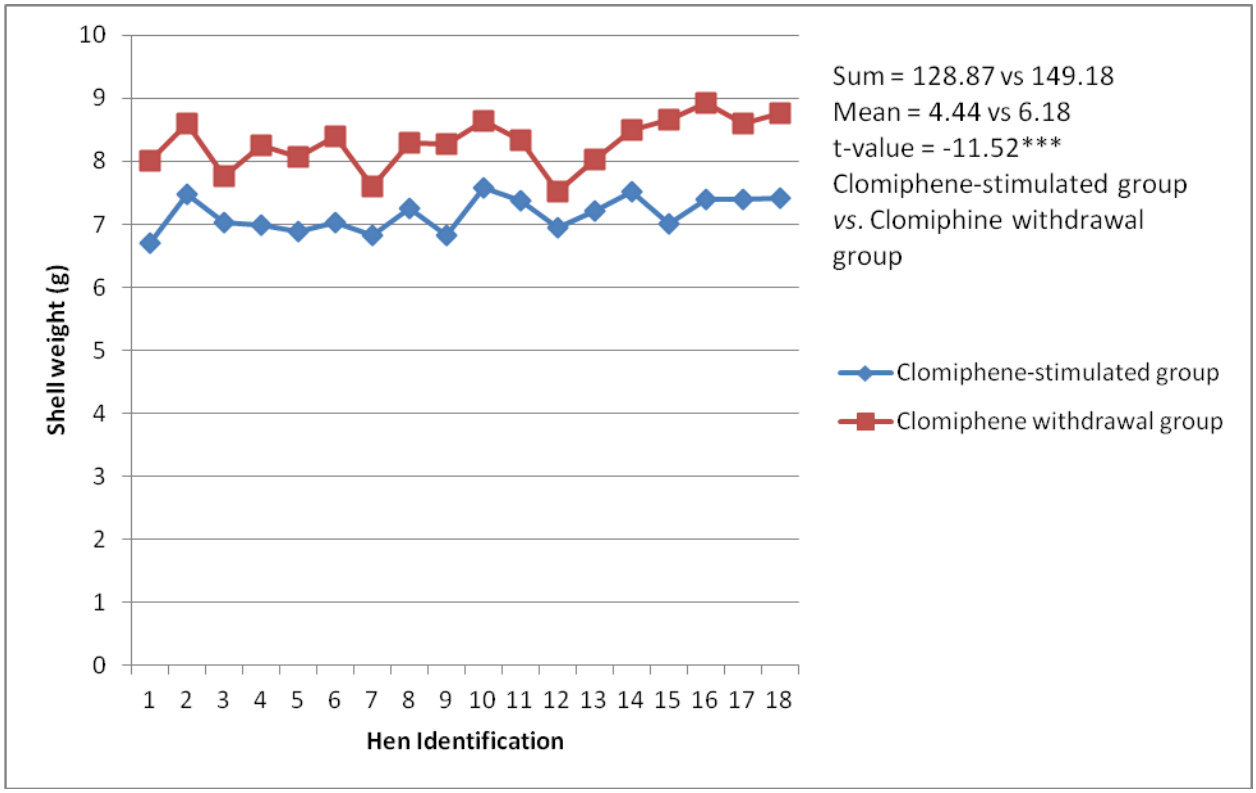


Figure 8. Shell weight of Lohmann Brown Eggs during and after Oral Clomiphene Citrate Administration.

4.1.2 Mating Phase (Phase II)

Egg production parameters

The effect of administration of CC on ovulation rate in Lohmann Brown hens is presented in Table 3. Ovulation rate across control, stimulatory and withdrawal groups was statistically significant ($P<0.0001$; Kruskal-Wallis statistic = 32.411). Median ovulation rate (83%) in stimulatory group was significantly ($P<0.001$; mean rank difference = -41.17) higher than median ovulation rate (50%) in the control group. Similarly, median ovulation rate (83%) in the withdrawal group was significantly ($P<0.001$; mean rank difference = -34.98) higher than median ovulation rate (50%) in the control group. However, no significant ($P>0.05$) difference was recorded in ovulation rate between the stimulatory and withdrawal groups.

The effect of CC administration on number of eggs laid by Lohmann Brown hens is presented in Figure 9. Mean number of eggs laid across treatments group was not statistically ($P>0.05$) different.

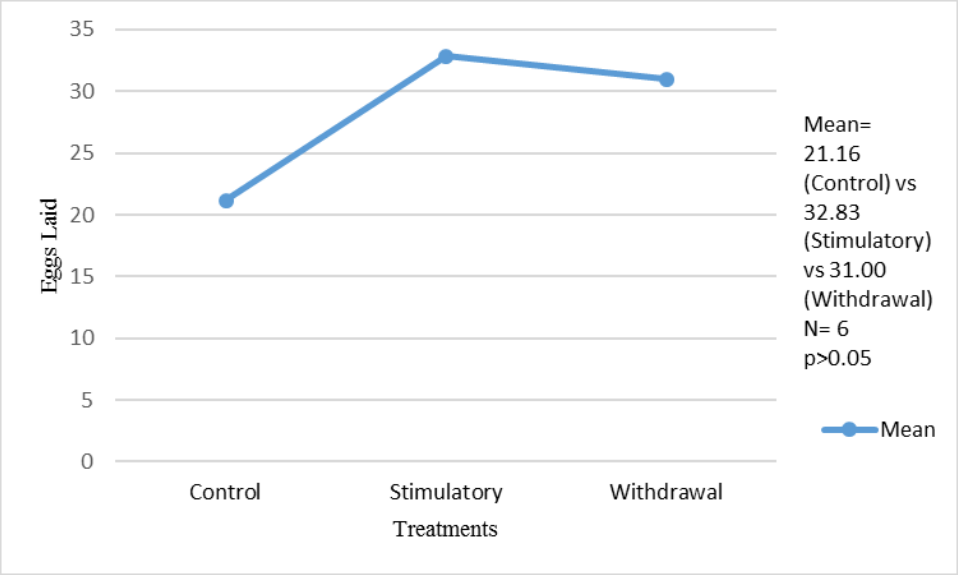


Figure 9. Effect of CC Administration on Number of Eggs Laid by Lohmann Brown hens

Oviposition pattern parameters

The effect of CC administration on oviposition pattern parameters in Lohmann Brown hens is presented in Table 4. Results revealed no significant (P>0.05) differences in number of egg sequence, egg sequence length, number of laying pause and number of inter-sequence pause days across treatment groups.

Table 4. Summary statistics, Kruskal-Wallis Statistic and Selected Pairs Dunn's Multiple Comparisons Test for Paired Hen-Day Egg Production across Clomiphene Citrate Treatments in Lohmann Brown Hens

Treatments	Observation (n)	Median	Min	Max	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) statistic, corrected for ties
Control	42	50.00	17.00	83.00	1601.0	38.12	
Stimulatory	42	83.00	33.00	100.00	3330.0	79.29	32.411*
Withdrawal	42	83.00	17.00	100.00	3070.0	73.09	

Group Comparison	Mean Rank Difference	Level of Significance
Control vs. Stimulatory	-41.17	***
Control vs. Withdrawal	-34.98	***
Stimulatory vs. Withdrawal	6.19	Ns

*=P<0.0001, ns= Not Significant

Table 5. Oviposition Pattern Parameters of Lohmann Brown Hens Treated with Clomiphene Citrate

Parameter	N	Control	Stimulatory	Withdrawal	Significance
No. of egg sequence	6	3.00±1.265	3.50±1.478	4.50 ±1.745	ns
Egg sequence length	6	8.83±4.269	16.33±5.948	14.00 ±5.080	ns
No. of laying pause	6	11.83±5.180	7.50±4.023	9.33 ±4.724	ns
No. of inter sequence pause days	6	4.83±2.737	2.16±1.077	3.83 ±1.740	ns

ns= not significant, P>0.05

4.1.3 Serum Biochemical Profile

Serum biochemical parameters

The effect of Clomiphene Citrate on serum total protein, serum albumin, serum globulin, serum bicarbonate, serum urea, serum creatinine, serum alkaline phosphatase (ALP) and serum alanine aminotransferase (ALT) in LB hens is presented in Table 5. Result revealed a non-significant effect ($p>0.05$) in the serum biochemical parameters with respect to total protein (control (79.00 g/L), stimulatory (78.33 g/L) and withdrawal (72.83 g/L)), albumin (control (44.16 mg/L), stimulatory (44.16 mg/L) and withdrawal (41.50 mg/L)), globulin (control (31.50), stimulatory (34.66) and withdrawal (31.33), bicarbonate (control (18.33 mmol/l), stimulatory (21 mmol/l), withdrawal (19.66 mmol/l)), urea (control (2.31 mmol/l), stimulatory (3.00 mmol/l), withdrawal (3.21 mmol/l)), creatinine (control (23.50 µmol/l), stimulatory (28.33 µmol/l), withdrawal (30.66 µmol/l)), ALP (control (48.00 U/L), stimulatory (50.66 U/L) and withdrawal (47.50 U/L)) and ALT (control (3.33 U/L), stimulatory (4.50 U/L) and withdrawal (4.33 U/L) wherein all the results across treatment groups were similar.

The effect of Clomiphene Citrate on serum aspartate aminotransferase is presented in Table 6. There were a high statistically significant difference ($P<0.05$; mean rank difference= 8.167, 7.333 and -0.830; KW statistic= 8.803) in Clomiphene Citrate treatment across the different groups The minimum and maximum values recorded for the control is 8 and 11 U/L with a median of 10 U/L, the stimulatory group recorded 6 U/L for the minimum and 9 U/L for the m aximum and 7 U/L for its median while in the

withdrawal group, 1.0 U/L was recorded for the minimum and 9 U/L for the maximum with a median of 8 U/L recorded.

Table 6. Serum Biochemical Parameters of Lohmann Brown Hens Treated with Clomiphene Citrate

Parameters	Treatment			
	Control	Stimulatory	Withdrawal	P value
Total protein (g/L)	79.00±5.750	78.33±4.786	72.83±2.006	0.576
Albumin (g/L)	44.16±1.077	44.16±0.945	41.50±0.885	0.115
Globulin (g/L)	31.50±4.064	34.66±4.136	31.33±1.475	0.748
Bicarbonate (mmol/L)	18.33±1.202	21.00±1.238	19.66±0.614	0.236
Urea (mmol/L)	2.31±0.228	3.00±0.376	3.21±0.331	0.148
Creatinine (mmol/L)	23.50±1.607	28.33±2.789	30.66±2.765	0.142
ALP (U/L)	48.00±7.384	50.66±3.480	47.50±4.201	0.903
ALT (U/L)	3.33±0.421	4.50±0.223	4.33±0.333	0.056

*=P<0.05 ALP=alkaline phosphatase, ALT=alkaline transaminase. Tables are presented as means ± SEM

Table 7. Summary Statistic and Selected Pairs Dunn's Multiple Comparisons Test for Effect of Clomiphene Citrate Treatments on Serum Aspartate Aminotransferase (U/L) in Lohmann Brown Hens

Treatments	Observation (N)	Median	Minimum	Maximum	Sum of Ranks	Mean of Ranks	KW statistic, corrected for ties
Control	6	0.006	0.005	0.007	39.000	6.50	
Stimulatory	6	0.026	0.013	0.055	88.000	14.66	8.512
Withdrawal	6	0.105	0.002	0.018	44.000	7.33	

Group Comparison	Mean Rank Difference	Level of Significance
Control vs. Stimulatory	-8.167	*
Control vs. Withdrawal	-0.833	ns
Stimulatory vs. Withdrawal	7.333	ns

***=**P<0.05, ns=not significant

4.1.4 Serum Mineral Profile

The effect of Clomiphene Citrate on serum mineral profile in LB hens are presented in Table 8. Non-significant result was obtained ($P>0.05$) with respect to sodium (Na), potassium (k), chloride (Cl), magnesium (Mg) and cobalt and a statistically significant result ($P<0.05$) with respect to calcium (Ca), phosphorus (P) and chromium (Cr). They have mean values of Na (139.17 for control, 137 for CC Stimulated and 134 for CC withdrawn) K (5.20 for control, 5.63 for CC Stimulated and 4.51 for CC Withdrawn), Cl (101.67 for control, 100.50 for CC Stimulated and CC Withdrawn each), Mg (0.51 for control, 0.49 for CC Stimulated and 0.56 for CC Withdrawn), Co (0.01 for control, 0.02 for CC Stimulated and 0.02 for CC Withdrawn) while the mean values for Ca, P and Cr are 2.51 for control, 2.99 for CC Stimulated and 3.09 for CC Withdrawn for Ca, 0.76 for control, 1.16 for CC Stimulated and 1.35 for CC Withdrawn for P and 0.02 for control, 0.06 for CC Stimulated and 0.04 for CC Withdrawn for Cr respectively.

Table 8. Effect of Clomiphene Citrate on Serum Mineral Concentration in Lohmann Brown Hens

Parameters	Treatments				
	Control	CC Stimulated	CC Withdrawn	P-value	Levels of Significance
Sodium (mmol/L)	139.17±3.458	137.00±2.145	134.00±1.880	0.389	ns
Potassium (mmol/L)	5.20±0.357	5.63±0.239	4.51±0.368	0.082	ns
Chloride (mmol/L)	101.67±2.499	100.50±0.885	100.50±1.586	0.868	ns
Calcium (mmol/L)	2.51±0.195^{b}	2.99±0.078^{ab}	3.09±0.104^{a}	0.019	*
Phosphorus (mmol/L)	0.76±0.149^{b}	1.16±0.080^{a}	1.35±0.080^{a}	0.005	**
Magnesium (mmol/L)	0.51±0.027	0.49±0.028	0.56±0.040	0.303	ns
Chromium (mg/L)	0.02±0.01^{b}	0.06±0.01^{a}	0.04±0.01^{ab}	0.005	**
Cobalt (mg/L)	0.01±0.007	0.02±0.007	0.02±0.005	0.787	ns

*= P<0.05, ns=not significant, CC=Clomiphene Citrate, Tables are presented as means ± SEM

Table 9. Summary Statistic and Selected Pairs Dunn's Multiple Comparisons Test for Effect of Clomiphene Citrate Treatments on Serum Zinc (mg/L) in Lohmann Brown Hens

Treatments	Observation (N)	Median	Min	Max	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) statistic, corrected for ties
Control	6	0.006	0.005	0.007	39.000	6.50	
Stimulatory	6	0.026	0.013	0.055	88.000	14.66	8.512
Withdrawal	6	0.105	0.002	0.018	44.000	7.33	

Group Comparison	Mean Rank Difference	Level of Significance
Control vs. Stimulatory	-8.167	*
Control vs. Withdrawal	-0.833	ns
Stimulatory vs. Withdrawal	7.333	ns

* = P<0.05, ns=not signicant, KW=Kruskal Wallis

Table 10. Summary Statistic and Selected Pairs Dunn's Multiple Comparisons Test for Effect of Clomiphene Citrate Treatments on Serum Copper Concentration (mg/L) in Lohmann Brown Hens

Treatments	Observation (N)	Median	Min	Max	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) statistic, corrected for ties
Control	6	0.033	0.021	0.042	66.000	11.00	
Stimulatory	6	0.022	0.019	0.047	46.500	7.750	1.135
Withdrawal	6	0.029	0.025	0.033	58.500	9.750	

Group Comparison	Mean Rank Difference	Level of Significance
Control vs. Stimulatory	3.25	ns
Control vs. Withdrawal	1.25	ns
Stimulatory vs. Withdrawal	1.25	ns

P>0.05, ns=not significant

The effect of Clomiphene Citrate on serum zinc in LB hens is presented in Table 9. There were extremely significant (P< 0.01; mean rank differences= -8.167,-0.833 and 7.333; KW Statistic= 8.512) differences in Clomiphene Citrate treatment across the different treatment groups with respect to zinc. Mean rank of 6.50 for control, 14.66 for stimulatory and 7.33 for withdrawal groups were recorded respectively. The minimum and maximum values recorded for the control is 0.005 and 0.007 with a median of 0.006, the stimulatory group recorded 0.013 for the minimum and 0.055 for the maximum and 0.026 for its median while in the withdrawal group, 0.002 was recorded for the minimum and 0.018 for the maximum with a median of 0.105 recorded.

The effect of Clomiphene Citrate on serum mineral copper is presented in Table 10. There were no statistically significant (P>0.05, Kruskal-Wallis Statistic = 1.135) differences in Clomiphene Citrate across the different treatment groups. The minimum and maximum values recorded for the control is 0.021 and 0.042 with a median of 0.033, the stimulatory group recorded 0.019 for the minimum and 0.047 for the maximum and 0.022 for its median while in the withdrawal group, 0.025 was recorded for the minimum and 0.033 for the maximum with a median of 0.029 respectively.

4.1.5 Serum Hormonal Levels

Serum oestrogen, progesterone and serum thyroxine

The result on the effect of Clomiphene Citrate on serum oestrogen, serum progesterone and serum thyroxine level in Lohmann Brown hens is presented in Table 11. Clomiphene Citrate affected none of the measured parameters except serum progesterone. Serum oestradiol was not affected by Clomiphene Citrate administration ($p>0.05$) and values recorded for the control, stimulatory and withdrawal group were 4.13, 4.84 and 4.5 ng/mL respectively, $P=0.233$. The serum progesterone was highly affected ($P<0.05$) across treatments, mean values during the stimulatory and withdrawal periods were 103.14 and 102.54 ng/mL and lowest in the control group (93.23 ng/mL), $P=0.008$. With respect to the serum thyroxine, non significant effect ($P>0.05$) was recorded with the highest value recorded in the control group (16.35 ng/mL), followed by withdrawal group (9.92 ng/mL) and then the stimulatory group (8.73 ng/mL) respectively, $P=0.08$.

Serum Triiodothyronine

The effect of Clomiphene Citrate on serum triiodothyronine in LB hens is presented in Table 12. There was no statistically significant ($P>0.05$, Kruskal-Wallis Statistic = 0.2924) difference in Clomiphene Citrate treatment across the different treatment groups. Mean rank of 10.33 for control, 8.66 for stimulatory and 9.50 for withdrawal groups were recorded respectively. The minimum and maximum values recorded for the control is 1.47 and 10.03 (ng/mL) with a median of 2.49, the stimulatory group recorded 1.62 for the minimum and 4.24 (ng/mL) for the maximum and 2.30 for its

median while in the withdrawal group, 1.77 was recorded for the minimum and 4.85 (ng/mL) for the maximum with a median of 2.35 recorded.

Table 11. Effect of Clomiphene Citrate on Serum Estradiol, Progesterone and Thyroxine Levels (ng/mL) in Lohmann Brown Hens

Parameters	Treatments			Significance
	Control	Stimulatory	Withdrawal	
Serum Estradiol	4.13±0.454	4.84±0.145	4.50±0.096	ns
Serum Progesterone	93.23±2.001[b]	103.14±1.623[a]	102.54±2.673[a]	*
T4	16.35±2.929	8.73±2.364	9.92±1.718	ns

T4= Serum Thyroxine, ns=not significant, *=P<0.05. Tables are presented as means ± SEM

Table 12. Summary Statistic and Selected Pairs Dunn's Multiple Comparisons Test for Effect of Clomiphene Citrate Treatments on Serum Tri-iodothyronine (T3) Level (ng/mL) in Lohmann Brown Hens

Treatments	Observation (N)	Median	Min	Max	Sum of Ranks	Mean of Ranks	Kruskal-Wallis (KW) statistic, corrected for ties
Control	6	2.496	1.470	10.032	62.000	10.333	
Stimulatory	6	2.309	1.629	4.224	52.000	8.667	0.2924
Withdrawal	6	2.395	1.775	4.857	57.000	9.500	

Group Comparison	Mean Rank Difference	Level of Significance
Control vs. Stimulatory	1.667	ns
Control vs. Withdrawal	0.8333	ns
Stimulatory vs. Withdrawal	-0.8333	ns

P>0.05, ns= not significant

4.1.6 Relative Internal Organ Weights

The effect of Clomiphene Citrate on the relative internal organ weights in LB hens is presented in Table 13. Non-significant result (P>0.05) was recorded on the effect of Clomiphene Citrate on relative internal organ weights in Lohmann Brown hens.

The average lung weight in the control, stimulatory and withdrawal groups were 0.42, 0.37 and 0.39g respectively. The average kidney weight was 0.53, 0.31 and 0.41g for the control, stimulatory and withdrawal groups respectively. Relative heart weight recorded was 0.42, 0.43 and 0.42g respectively for the control, stimulatory and withdrawal groups.

For the full gizzard, relative weights for the three treatments were 2.84, 2.98 and 3.15g respectively. With respect to the empty gizzard, the mean relative weights for the control, stimulatory and withdrawal includes 1.80, 1.83 and 2.04g respectively.

The liver weights recorded were 1.85, 1.83 and 2.04g for the control, stimulatory and withdrawal groups respectively. The proventriculus had relative mean weights of 0.48, 0.41 and 0.46g respectively for the control, stimulatory and withdrawal groups.

The shell gland had relative mean weights of 2.5, 1.76 and 2.43g respectively for the control, stimulatory and withdrawal groups respectively. The caecal tonsil recorded relative mean weights of 0.09, 0.10 and 0.11g for the control, stimulatory and withdrawal groups respectively.

With respect to the oviduct, 1.95, 2.89 and 1.86g were the values recorded for the control, stimulatory and withdrawal groups respectively. The ovarian stroma too was

similar and had recorded values of 1.53, 1.26 and 2.09g respectively for the control, stimulatory and withdrawal groups respectively.

The abdominal fat and the pancreas were also not statistically different ($P>0.05$) and had relative mean values of 1.70, 1.11 and 1.31g and 0.22, 0.20 and 0.20g for the abdominal fat and the pancreas respectively.

Table 13. Effect of Clomiphene Citrate on Relative Internal Organ Weights (g) in Lohmann Brown Hens

		Treatments		
Organs (g)	N	Control	Stimulatory	Withdrawal
Average lung	18	0.43±0.050	0.38±0.018	0.39±0.045
Average kidney	18	0.53±0.074	0.32±0.049	0.418±0.074
Heart	18	0.42±0.054	0.44±0.064	0.42±0.066
Full gizzard	18	2.84±0.469	2.98±0.286	3.15±0.339
Empty gizzard	18	1.80±0.319	1.84±0.197	2.04±0.260
Liver	18	1.85 ±0.312	1.84±0.193	2.04 ±0.263
Proventriculus	18	0.48±0.042	0.42±0.049	0.46±0.059
Shell gland	18	2.54± 1.124	1.77±0.428	2.43±0.369
Caecal Tonsil	18	0.09±0.021	0.11±0.011	0.12±0.012
Oviduct	18	1.95±0.393	2.89±0.540	1.86±0.480
Ovarian Stroma	18	1.54±0.403	1.26± 0.291	2.09±0.487
Abdominal Fat	18	1.71±0.616	1.11±0.449	1.31±0.343
Pancreas	18	0.23±0.017	0.20±0.014	0.21±0.017

N= observations, All values are expressed as means ± SEM

4.1.7 Ovarian Parameters

The effect of Clomiphene Citrate on ovarian parameters in Lohmann Brown hens is presented in Table 9. The result on the effect of Clomiphene Citrate on ovarian parameters (Oviduct weight, Large yellow follicle, Small yellow follicle, Large white follicle, Medium white follicle and Ovarian stroma weight) in Lohmann Brown hens reveals a non-significant ($P>0.05$) result in the oviduct weight, large yellow follicle (LYF), small yellow follicle (SYF), medium white follicle (MWF), ovarian stroma (OS) except for the large white follicle (LWF) that recorded a significant ($P<0.05$) result. With respect to the oviduct weight, 33.93g, 52.44g and 33.15g was obtained in the control, stimulatory and withdrawal groups respectively. The LYF was 3.83g in the withdrawal group, 3.17g in the stimulatory group and 2.33 g in the control. SYF recorded 0.83, 0.67 and 0.5 g with respect to the control, stimulatory and withdrawal respectively. LWF on the other hand were highest ($P<0.05$) in the stimulatory (11.17g) and withdrawal groups (10.67 g) and lowest in control (4.33 g); the trend was such that birds administered Clomiphene at the Stimulatory stage and those on withdrawal group recorded the highest values while the control had the least recorded values. The medium white follicles (MWF) recorded a non-significant result ($P>0.05$) with those on withdrawal recording the highest value of 30.67g followed by those on the stimulatory group (29.33g) and lastly the control group with a recorded mean value of 25.33g. the ovarian stroma weight was also not significant ($p>0.05$) with the highest values obtained in the withdrawal and control group (6.91g and 6.52g) and the least in the stimulatory group (5.69g).

Table 14. Effect of Clomiphene Citrate on Ovarian Parameters (g) in Lohmann Brown Hens

		Treatments		
Organs	N	Control	Stimulatory	Withdrawal
Oviduct weight (g)	18	33.93±5.496	52.44±10.365	33.15±8.441
Large Yellow Follicle (g)	18	2.33±0.760	3.17±0.792	3.833±0.401
Small Yellow Follicle (g)	18	0.83±0.166	0.67±0.210	0.50±0.223
Large White Follicle (g)	18	4.33 ± 2.290^{b}	11.17 ± 1.138^{a}	10.67 ± 1.022^{a}
Medium White Follicle (g)	18	25.33±4.006	29.33±3.313	30.67±4.333
Ovarian Stroma Weight (g)	18	6.52±0.764	5.69±0.897	6.91±1.151

Means with different superscripts across treatments are significantly different, $P<0.05$, N=observation

4.1.8 Fertility, Hatchability and Dead-in-Shell

Fertility

The effect of Clomiphene Citrate (CC) on percent fertility of incubated Lohmann Brown (LB) eggs is presented in Table 15. There was a highly significant result ($P<0.0001$) across treatments. Birds on control treatment had the highest fertility (87%) followed by the birds on stimulatory group (60%) and those on withdrawal group (25%). The minimum and maximum for the control group was between 63.5% to 100%, while the stimulatory and withdrawal groups recorded 44.8% to 70.9% and 0.00% to 52.0% respectively.

Hatchability

The effect of Clomiphene Citrate (CC) on percent hatchability of incubated LB eggs is presented in Table 15. There was no statistically significant ($P>0.5$) differences across treatments. Mean values were all similar with the withdrawal group recording the highest figure of 2.783% followed by the control group (2.38%) and stimulatory group (1.75%). The control group recorded a minimum and maximum hatchability value of 0.00% and 14.30% while the stimulatory and withdrawal group have hatchability values as 0.00% to 10.50% and 0.00% to 16.70% respectively.

Dead-in-Shell

The effect of Clomiphene Citrate (CC) on percent dead-in-shell of incubated LB eggs is presented in Table 15. There was no statistically significant ($P>0.05$) differences across treatment groups. The recorded mean values was 98.24% for the CC stimulation which is the highest value followed by the control group which recorded a mean value of 97.62% and then CC withdrawal group which recorded the least value of 97.22%. The

minimum and maximum values recorded for the study include 85.71 and 100%, 89.47 and 100%, and 83.33 and 100% for the control, stimulatory and withdrawal groups respectively.

Table 15. Effect of Clomiphene Citrate on Percent Fertility, Hatchability and Dead-In-Shell of Incubated Lohmann Brown Eggs

Parameter(s)	Treatments	Observation (N)	Mean	SEM	Minimum	Maximum
Fertility	Control	6	86.89[a]	5.64	63.48	100.0
	Stimulatory	6	60.23[b]	3.72	44.80	70.96
	Withdrawal	6	25.41[c]	8.83	0.00	52.00
Hatchability	Control	6	2.383	2.38	0.00	14.30
	Stimulatory	6	1.750	1.75	0.00	10.50
	Withdrawal	6	2.783	2.78	0.00	16.70
Dead-in-Shell	Control	6	97.6	2.38	85.71	100
	Stimulatory	6	98.2	1.75	89.47	100
	Withdrawal	6	97.2	2.77	83.3	100

All vales are expressed as mean ± SEM, SEM= Standard Error of mean, Means with different superscripts in a column are significantly different ($P<0.0001$)

4.1.9 Isolation Pattern of Bacteria from Different Sources

The isolation pattern of bacteria from the eggs of Lohmann Browns collected from the farm, commercial eggs inside the incubator, hatchery surroundings and dead-in-shell eggs is presented in Table 16. Bacteria were isolated from twelve swabs (6%) of all the swabs in the study. The prevalence of bacteria was highest in the dead-in-shell where 14% (n=8) of the eggs had isolates, followed by the commercial eggs in the incubator and hatchery surroundings where each recoded 2% (n=4) isolates and there were no any record from the Lohmann Brown eggs collected from the farm (0%, n=6) respectively. The frequency of isolation was twelve (12) where the highest frequency was isolated in the dead-in-shell with 8 (31.8%) isolates, followed by the commercial eggs inside the incubator and the hatchery surroundings whereby 2 (9.1%) of the isolates where recorded in each while the eggs from the farm recorded no bacterial isolate.

Table 16. Isolation Pattern of Bacteria from Different Sources

Source of isolates	Frequency of isolates	Percentage frequency of isolates (%)
A	0	0.00
B	2	16.67
C	2	16.67
D	8	66.66
Total	12	100

A=Lohmann Brown eggs from the farm, B=Commercial eggs inside the incubator, C=Hatchery surroundings, D=Dead-in-Shell eggs

The distribution of bacterial isolates according to Gram stain reaction is shown in Table 17. Out of the 12 bacterial isolates, Gram negative bacteria was the most abundant as observed within 11 (91.7%) isolates while Gram positive bacteria had the remaining 1 (8.3%). the Lohmann Brown eggs from the farm, commercial eggs inside incubator, hatchery surroundings and dead-in-shell eggs recorded 0, 2, 1, and 8 Gram negative bacterial isolates, respectively. The hatchery surroundings is the only source recording 1 Gram positive bacterial isolates with the Lohmann Brown eggs from the farm, commercial eggs inside the incubator and dead-in-shell eggs having no any isolates recorded.

Table 17. Distribution of Bacteria Detected based on Gram Stain Reaction from the Different Sources

Sources	Gram +ve	Gram –ve	Total
A	0(0.0%)	0 (0.0%)	0
B	0(0.0%)	2 (100.0%)	2
C	1(50%)	1 (50.0%)	2
D	0(0.0%)	8 (100%)	8
Total	1(8.3%)	11 (91.7%)	12

A=Lohmann Brown eggs from the farm, B=Commercial eggs inside the incubator, C=Hatchery surroundings, D=Dead-in-Shell eggs

The biochemical identification of bacterial isolates from the different sources is presented in Table 18. Five (5) types of bacteria were identified among the 12 bacterial isolates recorded. The identified types of bacteria were *Escherichia coli, Klebisiella aeuruginosa*, *Enterobacter spp, Salmonella spp* and *Pseudomonas spp*. *Escherichia coli* was urease and citrate negative but indole positive and does not produce hydrogen sulphide gas with a yellow slope and butt in Kigler's iron agar. *Kleibsiella spp* was urease and citrate positive having yellow slope and butt with no gas production, *Enterobacter spp* were urease and indole negative with no hydrogen sulphide production. *Salmonella spp* has all urease, citrate and indole recorded negative with hydrogen sulphide production but no gas and having a yellow slope with red butt. *Pseudomonas aeuruginosa* was urease and indole negative having no hydrogen sulphide gas production.

Table 18. Biochemical Identification of Bacterial Isolates of eggs from different sources

Bacterial Isolates	Biochemical test						
					Kliger's Iron Agar		
	Urease	Citrate	Indole	Slope	Butt	H_2S	Gas
Escherichia coli	_	_	+	Yellow	Yellow	_	+
Klebsiella spp.	+	+	_	Yellow	Yellow	_	+
Enterobacter spp.	_	+	_	Nil	Nil	_	+
Salmonella spp.	_	_	_	Yellow	Red	+	_
Pseudomonas spp.	_	+	_	Nil	Nil	_	_

H_2S=Hydrogen sulphide (blackening), R=Red-pink (alkaline reaction), Yellow (acid reaction)

The susceptibility of the bacterial isolates to various antibiotic discs is presented in Table 19. The result shows that *Enterobacter spp* and *Pseudomonas spp* were resistant to all the antibiotics while *Escherichia coli* and *Kleibsiella spp* were resistant to all except Cefoxitin for which they were found to be susceptible. On the other hand Salmonella spp were found to be resistant to all the antibiotics except Penicillin.

Table 19. Susceptibility of the Isolates to Various Antibiotic Discs

S/N	Bacterial Isolates	Antibiotics Susceptibility Disc					
		S	P	CN	AML	NV	FOX
1	*Escherichia coli*	R	R	R	R	R	S*
2	*Klebsiella pneumoniae*	R	R	R	R	R	S*
3	*Enterobacter* spp.	R	R	R	R	R	R
4	*Salmonella* spp.	R	S*	R	R	R	R
5	*Pseudomonas aeruginosa*	R	R	R	R	R	R

R=Resistant, S*=Sensitive, S=Streptomycin, P=Penicillin, CN=Gentamycin, AML=Amoxicillin, NV=Neomycin, FOX=Cefoxitene

4.2 DISCUSSION

The study was conducted to evaluate the effect of Clomiphene Citrate (CC) administration on ovulation rate, oviposition pattern, egg quality and serum biochemistry, relative weight of internal organs, ovarian, oviductal, hormonal changes and egg bacteriology.

The ovulation rate in hens was higher after CC treatment in the non-mating Phase than during CC treatment. A similar trend was observed by Togun, Okwusidi, Amao and Onyiaoha (2004) who used different crude protein levels and follicular stimulation (using CC) at 1.5 mg/ kg body weight on recovery and performance of post moulted aged Nera Black hens. They observed enhanced attainment of higher ovulation rate in groups on 16% crude protein (CP) plus follicle stimulation with CC after 49 days of treatment compared to untreated groups or groups with higher CP percentage. In the current study the CP content used was also 16% but not varied, the CC administered was at 15 mg/kg body weight with six weeks (42 days) of withdrawal period and the hens used were not aged but were approaching the peak of their production which could account for sources of variation to that of Togun *et al.* (2004) despite recording similar results. This indicates that Clomiphene Citrate (CC) induces high rate of ovulation (Lisowski & Bednarczyk, 2015). CC therefore enhanced ovulate rate of the Lohmann Brown layers at withdrawal period.

Total number of eggs laid by hens in the current study under the non-mating phase was higher after CC administration. The result agrees with the findings by Jaccoby *et al.* (1992) on the effect of Tamoxifen an anti-estrogen similar to Clomiphene Citrate on white leghorn hen on egg production; they attributed the increase in egg number and egg

production traits to the physiological effect of oestrogen on the ovary and oviduct making them to become activated and thus enhancing the ovulatory process. In another report by Robinzon, Shafir, Perek and Snapir (1984), they reported that administration of Clomiphene Citrate to broody turkey hens increased egg production. In another study by Biswas, Mohan and Sastry (2010) using Tamoxifen, they observed non-significant differences between the various egg production traits except for the egg number contrary to what was observed in the current study. In a report by Williams (2015), they observed that eggs laid after Tamoxifen treatment (7 days after) were significantly larger than sham controls and that in the laid clutches, the smallest eggs were obtained 1-3 days during stimulation with Tamoxifen while largest eggs was obtained 5-9 days after injection which corresponds to what was observed in the current study using Clomiphene Citrate even though the withdrawal period was higher. CC has influence on the egg production traits by increasing the total number of eggs laid after six weeks of withdrawal eventhough other studies recorded shorter periods, differences could be due species differences and drug administration routes.

Williams (2015) reported that Tamoxifen had no effect on albumen content of eggs. Similarly, Biswas *et al.* (2010) did not observe any differences in yolk weight and albumen weight when they administered Tamoxifen at 1, 0.5 and 2.5 mg per kg body weight. Their observation goes contrary to the recorded results in the current study because Clomiphene Citrate (CC) during withdrawal led to increase in weights in both the yolk and albumen respectively. This agrees with earlier findings that as long as there is increase in the component of the egg, it also lead to increases in the internal

components. Differences could be in the species of poultry used and in the dosage of administration of the CC used in the current study.

The hens after CC stimulation had increases in shell weight compared to hen groups during stimulation. Williams (2015) reported that Tamoxifen treatment had no effect on the relative shell content of eggs while egg shell percentage and shell thickness (mm) in another study were observed to be significantly affected with highest records obtained in birds administered 1 mg per kg body weight in local chickens (Hanafy, 2011). It has been estimated to be necessary for the eggs to have at least a 50% chance to withstand normal handling condition without breakage; it should at least have an egg shell thickness of 0.33 mm (Stadelman, 1995).

With reference to the bird number 14 that initially started laying but later on dropped and later ceased production was later found to be due to egg binding because the egg was palpable in the abdomen, this situation results where an egg is physically suck inside the hen. The situation was further confirmed after dissecting the bird and observing it.

During the non-mating phase, the oviposition pattern parameters of the Lohmann Brown hens after CC administration had highest number of eggs laid, shorter egg sequence, higher egg sequence length with the shortest laying pause and also shortest number of intersequence pause days. According to Samiullah, Omar, Roberts and Chousalkar (2017), total egg production is positively correlated with the length of laying sequence.

In the chicken, oviposition is characteristic in such a way that the poor layers have frequent pause days and therefore short sequences whereas the best hens have the ability

to lay one egg at roughly the same time every day (Wentworth *et al.*, 1983; Reddy, David, Sharma & Raju, 2006; David, Reddy & Singh, 2003). Samiullah *et al.* (2017) asserts that the longer the length of the laying sequence, the greater the number of days with recorded egg production. Even if a slight increase in clutch length is achieved, it will result into increase in egg production. Tamoxifen administration to egg laying females was found not to have any effect on the timing or pattern of egg laying to Sham controls (Williams, 2015) an observation which goes contrary to recorded result. The physiological mechanism that is responsible for taking pause days between the sequences of egg lay, inter sequence pause length, clutch length in hen may be the consequence of reduced rate of follicular maturation and its subsequent recruitment into the hierarchy following ovulation which is partly regulated by FSH (Etches & Cheng, 1981).

The aspartate aminotransferase (AST) was highest in the control group and least after CC administration. This result agrees with findings by Urom *et al.* (2016) who worked with indigenous chicken (cocks) reported that those receiving CC at 10 mg/kg body weight had the least value in AST (84.5g/L) eventhough the values they reported were higher than that reported in the current study, differences observed were Urom *et al*. (2016) used cocks and not hens as Fudge (2000) observed that this parameter may be influenced by sex, probably why hens recorede lower values in the current study; another factor is that Urom *et al*. (2016) administered the CC for a week in contrast to the six weeks used to administer to the hens in this study. Alanine aminotransferase (ALT) and AST were reported to be significantly high in rats administered Clomiphene Citrate at 50 and 100 mg/kg body weight (Al-Amoudi, 2012) in contrast to the present study were there was an increasing trend of ALT level from the control to the withdrawal stages

while for AST the trend was decreasing from control to Withdrawal. Nelson and Cox (2005) reported that an increase in an alkaline phosphatase (ALP), ALT and AST values signified necrosis or myocardial infarction which is an indicator of drug toxicity or harmful chemical in the body; side effects of Tamoxifen administration in humans could arise from increased concentrations of AST (Lupu, 2000), this Tamoxifen is a non-steroidal selective oestrogen receptor modulator similar to CC (Steiner, Tarplan & Paulson, 2005). A randomized controlled trial found that Tamoxifen was as effective as CC in ovulation induction (Boostanfar *et al.*, 2001) and that's why in the literature, some discussions used Tamoxifen to interpret and discuss some of the results where that of CC were unavailable.

CC used in the current study affected minerals such as calcium (Ca), phosphorus (P), chromium (Cr) and zinc (Zn). The results obtained were in partial agreement with the report by Afaf, Mahmoud and Amel (2018) who observed significant differences in Ca, P, Cr and Zn. In the serum Ca and P concentrations, they observed that Tamoxifen supplemented groups show slight increase or were nearly stable in results except for P in groups fed 50 and 100 mg Tamoxifen supplemented group for 60 days which showed slight decrease in its levels, in the present study however, their concentrations seem to follow an increasing pattern from the control to the Stimulated group down to the Withdrawal group in both the Ca and P respectively; differences could accrue from the fact that Afaf *et al.* (2018) used a fish species in their research with different treatments of Tamoxifen at different duration of 30, 60 and 90 days continuously unlike what obtains in the current study. The similarities in value of sodium, potassium, chloride, magnesium and cobalt as observed indicated efficiency of electrolyte balance in the

Lohmann Brown hens administered CC. Filipovic *et al.* (2015) studied the effects of Tamoxifen at a dose of 0.3 mg per kg body weight daily for 3 weeks on biochemical markers of bone metabolism by using middle-aged orchidectomized (Orx) rats found that blood serum levels of calcium (Ca^{2+}) and phosphorus (P) were significantly decreased. The variation in the mineral results reported could be as a result of the methodology applied, type of animal and genetic line which may also influence these parameters (Harr, 2002; Gyenis, Suto, Romvari & Horn, 2006).

An increased level of FSH hormone following Clomiphene Citrate administration improves the chances of growing an ovarian follicle that can then trigger ovulation (Yilmaz *et al.,* 2018). Progesterone concentration was found to be highest during and after oral clomiphene citrate. Zubair and Sajid (2015) supports this result from their finding that higher progesterone concentration was seen after clomiphene citrate treatment in infertile women which is due to developed corpus luteum (CL) or combined production of more than one CL. It was observed that in women, Clomiphene Citrate (CC) may act directly on the ovary, enhancing the secretion of progesterone from the corpus luteum (Miyake *et al.,* 1987). However, even though a similar trend was reported with respect to the progesterone increase, reproductive process in humans and birds differed as birds do not form corpus luteum, hence no variation in or alternation between the follicular and luteal phase but rather go through ovulation, oviposition and incubation (Banerjee, 2008). Rsaza *et al.* (2009) reported a similar result to oestradiol concentration in the hen ovarian follicles with the lowest progesterone concentration in the stroma of cortical follicles (3.2ng/g tissue) while the highest in the F1 follicles (47.1ng/g), values which are quite lower than that obtained in the current study; differences could be in the

treatments, environments and the tissue used. In addition to this, it could also be that the consequent reduction of oestradiol in the blood serum led to significant increase in the progesterone concentration and the opposite occurs in the ovarian tissue implying that the activation of oestrogen is necessary for the expression of $P450_{SCC}$ P (responsible for the pregnenolone synthesis) or 3β-hydroxysteroid dehydrogenase (3β-HSD), the enzyme converting pregnenolone to progesterone, in the hen ovary. Also, noting that one of the primary effect of CC is generally believed to be at the hypothalamic-pituitary level with secondary effects at the level of the gonads; in the chicken, the steroid producing cells of the thecal gland in the ovary undergo morphological changes after CC administration (McGinnis and Wallace, 1973) leading to increased level of FSH and thereby improving the chances of growing an ovarian follicle that can then trigger ovulation (Hsueh *et al*., 1978).

The major parameters of reproductive performance in the birds are the fertility and hatchability which are most sensitive to environmental and genetic influences (Stromberg, 1975) with the non-genetic factors having a higher influence on these traits (King'Ori, 2011).The quality of the newly hatched chick is a major factor in determining its liveability, growth, and health (Wiley, 1950). McGinnis and Wallace (1973) reported that Clomiphene Citrate (CC) improves the fertility of the female bird. The results of fertility in the current study shows that the control group had the highest fertility rates with the least value at withdrawal. This did not agree with the findings of Urom (2016) whereby birds on 10 mg/kg body weight of CC administration obtained the highest fertility figure of 79%. High CC administration have been reported to be associated with low fertility and this encouraged low-density lipoprotein level in blood of embryo since

low-density lipoprotein in the plasma does not support fertility (Urom, 2016). Reduction in fertility could be because of availability of few sperms to fertilize the hen's egg as this is reported to be associated with reduction in fertility as well as an increase in early embryonic mortality (Bramwell, 2002). In another study by Lisowski and Bednarczyk (2005) who used Tamoxifen, they obtained highest percentage of 77% at 10 mg dosage and obtained the lowest value in those administered Tamoxifen at 1 mg/kg body weight in cocks (66%), Hanafy (2011) did not observe differences among experimental groups or between chicken strains (fertility, 95%), Herbert *et al.* (2002) also revealed that CC improved fertility rates in rabbits while Oyebanji and Atoki (2018) reported lowest values in birds treated with CC similar to the current findings; differences could be due to specie, treatments and feed type.

In the current investigation, *Escherichia coli*, *Klebsiella aeuruginosa*, *Enterobacter spp*, *Salmonella spp* and *Pseudomonas spp* constituted the bacteria isolated from different sources in the reproduction cycle of the hen. The current study revealed that *Escherichia coli* and *Klebsiella spp* were among the isolates predominant in other commercial eggs inside the incubator and also the dead-in-shell embryo. The *E-coli* is reported to be highly pathogenic and could lead to lower reproductive efficiency (Sheldon & Dobson, 2004) while *Klebsiella spp* were reported to be associated with reproductive diseases including salpingitis and oophoritis in hens (Sharma, Joshi and Bati, 1980). *E-coli* was found to be resistant to penicillin and sensitive to Cefoxitin, Gentamicin, Amoxicillin, Neomycin and Streptomycin while *Klebsiella spp* were resistant to Amoxicillin, Gentamicin and Penicillin in the current study.

Salmonella spp were isolated from the hatchery environment and the dead-in-shell embryo in the present study. This bacteria is a well-known contaminant of chicken eggs (Buchanan *and* Gibbons, 1974) and has resulted in many chicks becoming infected after hatching (Cason *et al.* 1994). *Salmonella spp* was sensitive to penicillin and resistant to the other antibiotics.

Pseudomonas spp were only isolated form the dead-in-shell embryo in the current study a finding similar to that by Alaboudi *et al.* (1992) and Ahmed *et al.* (1981) and Osman (1996). The *Pseudomonas spp* were resistant to all the antibiotics used.

The only Gram positive bacteria isolated in the current study was *Enterobacter spp* isolated from the hatchery environment alone. It was found to infect eggs and cause embryo loss (Lin, Shyu & Shyu, 1996). From the current study, it was observed to be resistant to all the antibiotics.

At the end of the research, a checklist in form of a questionnaire was produced to find out the problems associated with the hatchery where the eggs were incubated and it was discovered that improper management in terms of temperature, relative humidity, egg turning, mixing of different eggs and overcrowding of the eggs where the major highlights leading to so many embryo infection and deaths recorded.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 SUMMARY

The study was conducted to evaluate the effect of Clomiphene Citrate (CC) administration on egg production, oviposition pattern, egg quality parameters, serum chemistry, reproductive physiology and egg bacteriology in Lohmann Brown (LB) layers. The experiment was carried out at the Poultry Unit of the Department of Animal Science located within the Faculty of Agriculture Teaching and Research Farm, Bayero University, Kano.

The study was carried out in three phases. Phase I (Non-mating Phase) involved eighteen (18) LB hens that were administered CC at 15 mg/kg body weight for six weeks (stimulatory stage) and withdrawn for another six weeks (withdrawal stage) in a repeated measures completely randomized design. Phase II (Mating Phase) involved 18 LB hens with three ISA Brown cocks in a single factor completely randomized design with three treatments (CC stimulatory, CC withdrawal and control groups) each replicated six times. The CC stimulatory group include a group of hens that were administered CC for six weeks at 15 mg/kg body weight for the first time. The CC withdrawal group comprises hens from Phase I earlier stimulated for six weeks with CC at 15 mg/kg body weight and withdrawn from CC for six weeks and undergoing further withdrawal for six weeks. The control group include hens that have not been administered CC at all. In the incubation phase (Phase III), fertile eggs across the different experimental groups from the mating phase formed the experimental materials in a cross sectional design.

In Phase I, paired samples t-test was used to compare ovulation rate, number of eggs laid, oviposition pattern parameters and egg quality during and after Clomiphene Citrate (CC) administration in LB hens. In Phase II, One-Way Analysis of Variance (ANOVA) was used to determine the effect of CC on ovulation rate, number of eggs laid, oviposition pattern parameters, serum hormonal levels, serum chemistry and serum mineral concentration in LB hens. Significantly different means were separated using Tukey's test. All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL, USA). In Phase III, cross sectional design was used. Parameters considered include fertility, hatchability, dead-in-shell, total aerobic bacterial count, bacterial identification and antibiotic susceptibility test, Data were analysed using Graphpad InStat software.

Phase I results revealed statistically significant ($P<0.05$) differences in ovulation rate, total number of egg laid, egg weight, width and length, yolk and albumen volumes, shell weight, and oviposition pattern parameters (total egg sequence, total egg sequence length, total laying pause and total inter-sequence pause) during and after CC administration. All parameters except number of egg sequence were higher in LB hens undergoing CC withdrawal when compared with their corresponding values during CC treatment.

Phase II results recorded non-significant ($P>0.05$) changes in total eggs laid, total egg sequence, total egg sequence length, total laying pause, total inter-sequence pause, serum oestradiol, tri-iodothyronine, thyroxine, total protein, albumin, globulin, bicarbonate, urea, creatinine, levels of serum alkaline phosphatase and alanine aminotransferase, serum sodium, potassium, chloride, magnesium, cobalt, copper,

relative weight of internal organs, oviductal and ovarian stroma weights as well as counts of large and small yellow and medium white follicles. However, ovulation rate, large white follicle count and serum levels of calcium, phosphorus, chromium, zinc, aspartate aminotransferase and progesterone was significantly ($P<0.05$) different across CC treatment levels.

Phase III results reveals non-significant ($P>0.05$) in hatchability and dead-in-shell embryo while fertility was significantly ($P<0.05$) affected across the treatment groups. In addition, the dead-in-shell embryo from LB eggs have the highest percentage of bacterial growth and highest total frequency of isolates followed by the hatchery environment and then other commercial eggs inside the incubator; Gram negative bacteria gave the highest frequency after Gram staining with a single Gram positive bacteria. The bacteria isolated were *Escherichia coli*, *Klebsiella spp*, *Enterobacter spp*, *Pseudomonas spp* and *Salmonella spp*. *Escherichia coli* was resistant to penicillin, *Klebsiella spp* was resistant to amoxicillin, gentamicin and penicillin while isolates of *Enterobacter* and *Pseudomonas spp* were resistant to Cefoxitin, Amoxicillin, Gentamicin, Penicillin, Neomycin and Streptomycin. In conclusion, during the Mating Phase, serum levels of aspartate aminotransferase and progesterone, serum calcium, phosphorus, chromium, zinc and large white follicle count were affected by CC treatment, while during the Incubation Phase, fertility alone was affected by CC treatment.

5.2 CONCLUSION

The current study demonstrated that CC administration at 15 mg/kg influenced ovulation rate, oviposition pattern parameters, egg quality, fertility, hormonal, serum biochemical and oviductal changes in Lohmann Brown hens leading to increased number

of eggs laid, shortened egg sequence with increased days of egg laying and reduced pause in lay and inter-sequence pause days, increased fertility rate, increased progesterone secretion, reduced serum aspartate aminotransferase and increased weight of the large white follicles.

5.3 RECOMMENDATIONS

Based on the findings and drawbacks of the current study, the following recommendations emerged:

i. CC should be given to laying LB hens during the Non-mating Phase at a dose of 15 mg/kg per os in order to improve ovulation rate, number of eggs laid, oviposition pattern parameters, egg quality, fertility, hormonal, serum biochemical and oviductal changes..

ii. Future research should consider a repeated measures design for the Mating and Incubating Phase instead of an independent design.

iii. The pharmacokinetics and pharmacodynamics of CC administered to laying hens via the oral and other routes should be carried out during the non-mating and mating phases.

iv. Methodology in the current study that focuses on the effect of CC administration on fertility, hatchability of eggs and brooding stage from LB hens should form part of future studies.

v. The effect of CC administration to cocks on fertility and hatchability of eggs should be studied.

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