

**Studies on the Relationship between Nutrition and
Reproduction in the Japanese Quail**

ウズラにおける栄養と生殖の関係に関する研究

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LIST OF ABBRIVIATIONS

Abbreviation	Meaning
PS	Phytosterols
CVD	Cardiovascular diseases
LDL	Low-density lipoprotein
TC	Total cholesterol
HDL	High-density lipoprotein
NPC1L1	Niemann-Pick C1-Like1
ABCG5	ATP-Binding Cassette Sub-Family G Member 5
ABCG8	ATP-Binding Cassette Sub-Family G Member 8
ABCA1	ATP-Binding Cassette transporter A1
ACAT2	Acetyl-CoA Acetyltransferase 2
HMG-CoA	Hydroxymethylglutaryl-CoA
CH	Cholesterol
FCH	Free cholesterol
CHM	Chylomicron
CHE	Esterified cholesterol
MCT	Medium Chain Triglyceride
FBS	Fetal-Bovine-serum
DMSO	Dimethylsafuxide
SC	Subcutaneous
BW	Body weight
FOV	Fields of view

LIST OF ABBRIVIATIONS

Abbreviation	Meaning
EFN	Eyepiece field number
SQF	Semen Quality Factor
ACTH	Adrenocorticotrophic hormone
LH	Luteinizing hormone
GnRH	Gonadotropin releasing hormone
GnIH	Gonadotropin inhibitory hormone
LXR	Liver X receptor
stAR	Steroidogenic acute regulatory protein
3 β -HSD	3 β -Hydroxysteroid dehydrogenase
17 β -HSD	17 β -Hydroxysteroid dehydrogenase
BBB	Blood-Brain-Barrier
HPG-axis	Hypothalamic- pituitary-gonadal axis

CHAPTER ONE

Introduction

The relationship between nutrition and reproduction is an important topic for poultry farmers and animal nutrition experts. Among the several factors, nutritional factors have crucial roles on humans and animals' health and reproduction. Evidence from the literatures and experiences suggest that the role of nutritional factors is vital in terms of direct effects on reproduction in animals (Smith and Akinbamijo, 2000). Moreover, food costs accounted 50 – 70% of the total cost of animal production. Therefore, price change of animal feed and dietary ingredients has major impact on profitability of livestock farming (Veldkamp and Bosch, 2015). On the other hand, undernutrition and overnutrition have effects on animals' health and reproduction and impose costs for the farmers. Proper dietary ingredients are fundamental for animals' growth, maintenance and reproductive activities.

1.1 Role of nutrition on reproduction

Reproductive performances in livestock are controlled by four factors such as genetic merit, environments, nutrition and management. Proper nutrition and dietary ingredients encourage livestock to reach their genetic potential, reduce the negative effects of a harsh physical environment and minimize the influence of poor management techniques (Smith and Akinbamijo, 2000). The physiologic processes that underlie the reproductive cycles impose considerable nutrients demand on a female. In mammals, incongruous dietary intake (inadequate, excess or imbalanced nutrient) has adverse effects on different stages of animals' reproduction such as delayed puberty, reduced ovulation, lower conception

rate; high embryonic and fetal losses, long postpartum anestrus, poor lactation and high mortality (Robinson, 1996; Houdijk *et al.*, 2001; Robinson *et al.*, 2006). In general, dietary nutrients (nutritional factors) affect directly on the reproductive organs, or indirectly effects *via* the hypothalamus–pituitary–gonadal axis (HPG-axis) (Houdijk *et al.*, 2001).

The endocrine system is one of the key mediators between changes in the external environment and internal responses, with proven impacts not only in reproductive anatomy and physiology, but also on both reproductive behavior, and efficiency (Meza-Herrera and Tena-Sempere, 2012). Gonadotropin-releasing hormone (GnRH) is responsible to link between the brain and the reproductive system, by the release of GnRH into the pituitary-portal blood system, which triggers the release of the gonadotropins; luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary into the circulatory system. In turn, these pituitary gonadotropins cause gonadal growth and increase plasma sex steroid concentrations (Scanes and Griminger, 1990; Meza-Herrera and Tena-Sempere, 2012).

1.2 Phytoadditives

Increasing demands for foods led to find additional dietary supplements to improve animals' productions both quantitatively and qualitatively. Currently, consumers are increasingly interested in healthy foods which are rich in protein and low in cholesterol and lipid contents.

Phytosterols or plant sterols naturally founds in many foods originating from plants. Phytosterols are increasingly added as food additives in humans, and animals' dietary regimens due to the beneficial effects against cholesterol. The cholesterol lowering

activity of phytosterols was firstly reported in chickens (Peterson *et al.*, 1951). Phytosterols are bioactive components of all vegetable foods with higher amounts in nuts, seeds and legumes (Lagarda *et al.*, 2006). The structural difference between phytosterols and cholesterol are mainly on the extra ethyl (β -sitosterol) or methyl (campesterol) groups in the side chain C24 or an additionally double bond in C22 (stigmasterol) (Weststrate *et al.*, 1999; Calpe-Berdiel *et al.*, 2009). More than 200 types of phytosterols reported in plants, but the most abundant phytosterols are β -sitosterol (24- α -ethylcholesterol), campesterol (24- α -methylcholesterol), and stigmasterol (Δ^{22} , 24- α -ethylcholesterol) (Ling and Jones, 1995; Kritchevsky and Chen, 2005; Lagarda *et al.*, 2006) (Fig. 1-1).

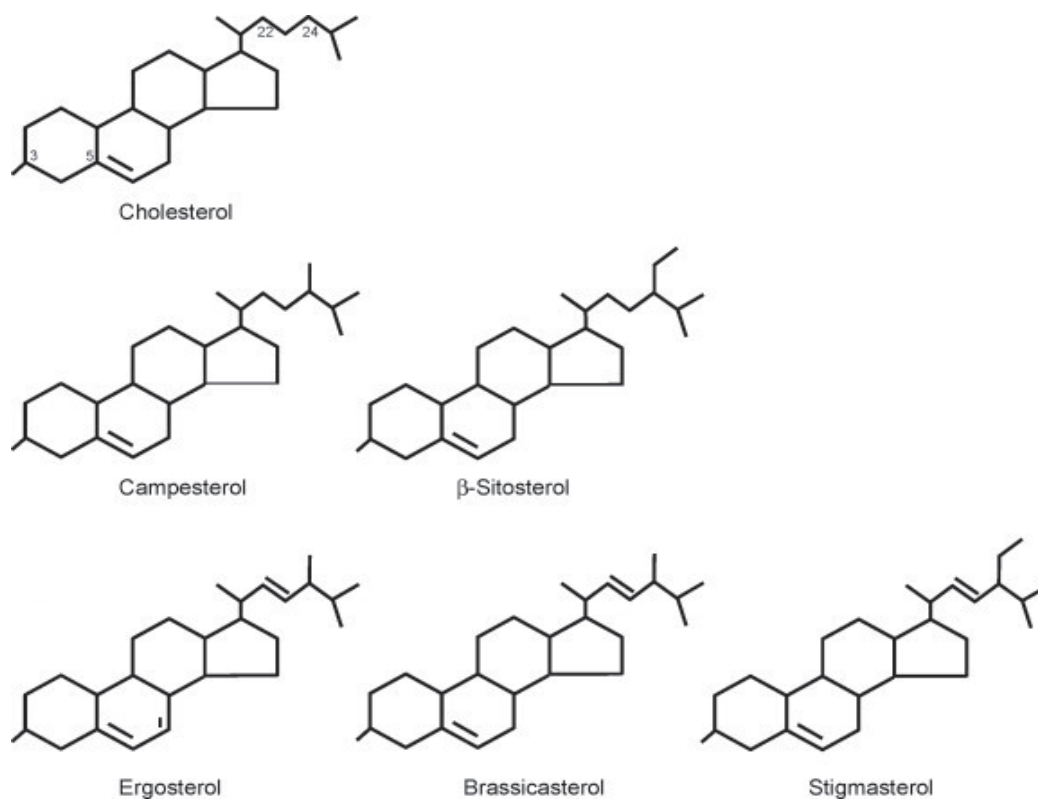


Fig. 1-1. Chemical structures of cholesterol and phytosterols (Calpe-Berdiel *et al.*, 2009).

1.2.1 Phytosterols and cholesterol metabolism

1.2.1.1 Absorption

Figure 1-2 shows the intestinal cholesterol and phytosterols absorption and metabolism. Compared to cholesterol, phytosterols are poorly absorbed in the intestinal lumen. In human, the intestinal absorption rates of phytosterols are about 5%, whereas more than 40% of dietary and biliary cholesterol is absorbed (Salen *et al.*, 1985; Tilvis and Miettinen, 1986). Furthermore, intestinal rates of phytosterols absorption are highly depends on their chemical structures and physical states (size of phytosterols crystals) (Ling and Jones, 1995; von Bonsdorff-Nikander *et al.*, 2005; Marangoni and Poli, 2010). In addition, the intestinal absorption of phytosterols are divers; campesterol is greatly absorbed in the intestinal lumen compared to β -sitosterol and stigmasterol respectively (Ikeda *et al.*, 1988). Moreover, various animal species showed vary in phytosterols absorption rate ranging from 0% (rabbit) to 4% (rat), and 6% (human being) (Kritchevsky and Chen, 2005).

Phytosterols are primarily act on the intestinal cholesterol absorption, and compete for the absorptive mixed micelles. Indeed, non-cholesterol sterols are easily associated with the mixed micelles, and thereby limit the micellar solubilization of cholesterol. Niemann-Pick C1-Like 1 (NPC1L1) protein is considered to be responsible for the majority of cholesterol and phytosterols uptake into enterocytes and the biliary cholesterol reabsorption. (Davis *et al.*, 2004; Plösch *et al.*, 2006). Moreover, the annexin2/caveolin1 (ANXA2/CAV1) complexes may also play a role on phytosterols cholesterol-lowering activity. Calpe-Berdiel *et al.* (2007) reported that ANXA2 could be down regulated by plant sterols, thereby reducing cholesterol processing and transport. Furthermore, the cellular cholesterol and phytosterols uptakes can be regulated by secretion back in the

intestinal lumen by heterodimers of the ATP-binding cassette transporter (ABCG5 and ABCG8) (Davis *et al.*, 2004; Plösch *et al.*, 2006; Calpe-Berdiel *et al.*, 2009). ABCG5 and ABCG8 expressed on the apical surface of enterocytes and the canalicular membrane of hepatocytes, and their functions are required to export the unesterified cholesterol and phytosterols back into the intestinal lumen and bile respectively (Davis *et al.*, 2004; Plösch *et al.*, 2006; Yu *et al.*, 2014). Phytosterols as ligands of liver X receptor (LXR) induces the expression of ABCG5/G8, and thereby reduces the intestinal cholesterol absorption (Kaneko *et al.*, 2003). Within the enterocyte, 70 – 90% of the absorbed cholesterol is esterified by acyl-cholesterol acyl transferase-2 (ACAT-2), packaged into chylomicrons at the basolateral membrane, and secreted into the lymphatic system. In contrast, phytosterols are poor substrates for ACAT-2, and after absorption, only a small part is esterified (Field and Mathur, 1983). Free form of phytosterols mobilized *via* ATP-binding cassette transporter A1 (ABCA1) (located at the basolateral membrane of enterocytes) and become parts of apolipoprotein A-I (apo A-I), containing high-density lipoprotein (HDL) particles and transported to hepatocyte (Calpe-Berdiel *et al.*, 2009; Othman *et al.*, 2013).

Phytosterols taken up by the liver through lipoproteins, incorporated into very low-density lipoprotein or secreted *via* the bile. ABCG5 and ABCG8 are also responsible for biliary excretion of phytosterols. Because of the low affinity of ACAT for the β -sitosterol in liver, β -sitosterol has a higher secretion rate and higher hepatic clearance than campesterol (Brufau *et al.*, 2008).

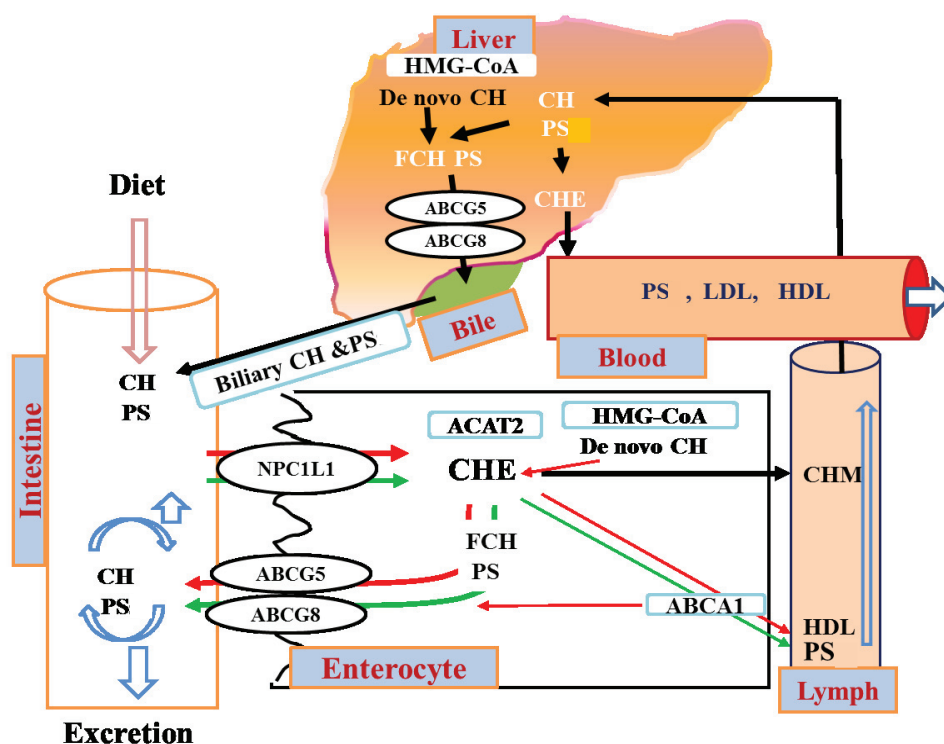


Fig. 1-2. Schematic overview of intestinal absorption of cholesterol (red arrows) and phytosterols (green arrows), and further processing in the enterocyte and hepatocyte. Intestinal cholesterol and phytosterols transported by mixed micelles and internalized *via* the apical NPC1L1 membrane protein. Within the enterocyte, cholesterol is mostly esterified by ACAT-2. The esterified-cholesterol incorporated with chylomicrons (CHM) and transported to liver by lymphatic capillaries. Phytosterols as poor substrate for ACAT-2 mobilized *via* the ABCA1, together with HDL cholesterol and enters to lymphatic capillaries. It has also been suggested that phytosterols may interfere with the activity of ACAT-2 within the enterocyte and inhibit chylomicron formation by making cholesterol less available in intestinal cells. Free cholesterol (FCH) and phytosterols (PS) extruded to the intestinal lumen through the apical ABCG5/ABCG8 heterodimer. Cholesterol (CH) and PS under go to similar process in the liver. CH, cholesterol; PS, phytosterol; FCH, free cholesterol; CHM, chylomicron.

1.2.1.2 Distribution and excretion

Similar to cholesterol, absorbed phytosterols transported *via* the chylomicrons to liver. In experimental animals, such as rats approximately 12% of esterified form of phytosterols transported by chylomicrons compared to 70 – 80% in cholesterol. phytosterols in esterified or free forms carried *via* the lipoprotein particles in the circulation as cholesterol, and HDL particles majorly transport phytosterols, while LDL mostly transport cholesterol (Plat and Mensink, 2005). In healthy adult men, plasma levels of phytosterols ranged from 7 to 24 $\mu\text{mol/L}$, which are accounted less than 1% of total plasma sterol concentrations (Salen *et al.*, 1985; Moghadasian, 2000).

Accordingly, the elimination of phytosterols occurs by excretion into bile and takes place primarily *via* the biliary route same as cholesterol (Ling and Jones, 1995). Bhattacharyya *et al.* (1983) reported that skin also has a role on the excretion of small fraction of phytosterols. Compared to cholesterol; the internal pool of phytosterols is low in the size due to the minimum intestinal absorption, and faster excretion by the bile (Trautwein *et al.*, 2003). After absorption, phytosterols are mainly accumulated in the liver, adrenal glands and gonads (Boberg *et al.*, 1985; Sanders *et al.*, 2000; Calpe-Berdiel *et al.*, 2009).

1.2.2 Cholesterol lowering activity of phytosterols

The initial mechanism of phytosterols actions is based on cholesterol lowering activity, and the phytosterols effects are greatly depends on their lipophilic structures. It is believed that mixed micelles in the intestinal lumen are the site for cholesterol lowering activity of phytosterols. The lipophilic structures of phytosterols facilitate to easily associate with mixed micelles and reduce cholesterol (hydrophilic structure) absorption in

the gut (Trautwein *et al.*, 2003; Calpe-Berdiel *et al.*, 2009). Effecting on ABCG5/G8 heterodimer proposed as another mechanism underlying the hypocholesterolemic effect of phytosterols. Phytosterols as natural ligands for LXR, reduce cholesterol levels by increasing the activity of ABCG5 and ABCG8 in the enterocyte and hepatocyte (Kaneko *et al.*, 2003; Calpe-Berdiel *et al.*, 2009). Moreover, phytosterols affects ACAT and reduces the esterification of cholesterol in the enterocyte and hepatocyte (Brufau *et al.*, 2008). In addition, it has been proposed that phytosterols feeding reduce biosynthesis of cholesterol by effecting on liver hydroxymethylglutaryl-CoA synthase (HMG-CoA) enzyme (Field *et al.*, 1997; Calpe-Berdiel *et al.*, 2009). Phytosterols significantly reduced plasma low-density lipoprotein cholesterol (LDL cholesterol) level, while the high-density lipoprotein (HDL cholesterol) and triglycerides (TG) were not significantly altered (Moghadasian and Frohlich, 1999; Brufau *et al.*, 2008). Despite the low absorption rate, phytosterols can greatly reduce plasma cholesterol level.

1.2.3 Endocrine-disrupting activity of phytosterols

The endocrine-disrupting activity of phytosterols was reported in the different laboratory animals such as rats, mice, and goldfish (Moghadasian, 2000). The concept of endocrine-disruption of phytosterols was firstly reported in aquatic animals. Pulp and paper mill effluents highly contain phytosterols. Intraperitoneal injection of β -sitosterol was associated with decrease in plasma levels of testosterone and 17β -estradiol in male and female goldfish, respectively (Maclatchy and Vanderkraak, 1995). Moreover, the endocrine-disrupting activity of phytosterols also reported in rodents. Administration of β -sitosterol to male albino rats caused a significant reduction in the testes weight and

sperm counts (Malini and Vanithakumari, 1991; Singh and Gupta, 2016). Feeding of phytosterols significantly reduced testosterone production and metabolism in rats (Awad *et al.*, 1998). Dietary phytosterols exhibited infertility and loss of abdominal fat both in male and female sterolin-deficient mice (Solca *et al.*, 2013). Sharpe *et al.*, (2007) reported that β -sitosterol significantly reduced gonadal steroidogenic acute regulatory protein (stAR) gene expression in goldfish. They proposed that low expression of stAR may cause low mitochondrial-cholesterol trafficking, and consequently low testosterone levels. In young male Japanese quails under phytosterols treatment significantly increased corticosterone production in respond to adrenocorticotrophic hormone (ACTH) challenge (liu *et al.*, 2012). In contrast, phytosterolemic patient had adrenal insufficiency and low corticosterone levels in human (Mushtaq *et al.*, 2007). Daily intakes of 2 g phytosterols for two weeks did not significantly effects on testosterone, FSH, and sex hormone-binding globulin levels in men, or estradiol, FSH and sex hormone-binding globulin levels in women (Volpe *et al.*, 2001).

1.2.4 Proposed mechanism of endocrine-disrupting effects

1.2.4.1 Accumulation of phytosterols on brain cell membrane

Phytosterols are known to affect hypothalamic-GnRH and reduce the inflammatory-related aging (Shi *et al.*, 2015). In contrast to cholesterol, circulating phytosterols levels effectively pass the blood-brain-barrier (BBB) and accumulate in brain cell membranes, and disturb cholesterol homeostasis (Vanmierlo *et al.*, 2012; Saeed *et al.*, 2015). Shi *et al.* (2015) reported that membrane β -sitosterol was able to prevent TNF- α -induced GnRH decline. They suggested that the membrane β -sitosterol impose the inhibitory effects through the estrogen receptors.

1.2.4.2 Effect of phytosterols on the adrenal glands

Adrenal glands and gonads are considering as preferential organs for accumulation of phytosterols (Boberg *et al.*, 1985; Calpe-Berdiel *et al.*, 2009). Yang *et al.* (2004) reported that plant sterol disrupts the adrenal cholesterol homeostasis in sterolin-deficient mice. Mushtaq *et al.* (2007) reported the adrenal insufficiency in phytosterolemic patient in human. In contrast, Liu *et al.* (2012) found that feeding of phytosterols enhanced corticosterone productions in immature male Japanese quail in responses to ACTH challenge.

1.2.4.3 Effects of phytosterols on reproductive function

Awad *et al.* (1998) found that phytosterols feeding significantly reduced the plasma testosterone concentrations in Sprague-Dawley male rats. They proposed that phytosterols may alter testosterone metabolism by inhibiting 5- α -reductase and aromatase activity in rats. Moreover, phytosterols feeding disrupted the endocrine function with infertility in males and females sterolin-deficient mice (Solca *et al.*, 2013). They suggested that phytosterols accumulation might interrupt the fertility by effecting on the sperm and oval membrane. Singh and Gupta (2016) was also found that given β -sitosterol reduced testosterone, LH and FSH concentrations and sperm count in male albino rats. Moreover, testicular weight was also reduced compared to control. They proposed that low gonadal weight and testosterone concentrations reduced the spermatogenesis and led to infertility in rats. In addition, phytosterols had capacity to reduce the mitochondrial-cholesterol pool by affecting transcriptional changes of stAR and consequently the testosterone production in goldfish (Sharpe *et al.*, 2007). Previous report showed that phytosterols had estrogenic activity in aquatic animals (Davis and Bortone, 1992; Mellanen *et al.*, 1996).

1.2.5 Other proposed activities of phytosterols

Previous *in vivo* and *in vitro* studies showed that phytosterols exposure had some unexpected effects in the laboratory animals. Dietary phytosterols modulate the immune function associated with the reduction of pro-inflammatory cytokines, including IL-6 and TNF- α , and increased levels of anti-inflammatory IL-10 in spleen cells in the lipopolysaccharide stimulated Apo-E knockout mice (Nashed *et al.*, 2005). *In vitro* study found that membrane β -sitosterol prevents aging by decreasing the hypothalamic inflammation-induced GnRH decline (Shi *et al.*, 2015).

Moreover, previous researches suggested that phytosterols feeding possess anti-cancer effects against lung, stomach; ovaries, prostate and estrogen-dependent breast cancer (Awad *et al.*, 1998; Awad and Fink, 2000; Woyengo *et al.*, 2009; Bradford and Awad, 2010). However, significant decreases in plasma levels of total protein, calcium, vitamins E, K, and D were observed in rats treated with high dietary doses phytosterols (Moghadasian, 2000). In addition, the antioxidant activity of phytosterols had been previously reported (Naji *et al.*, 2014).

1.3 Maternal condition and offspring

In the avian species, nutrition and the dietary ingredients are probably the most crucial factors affecting reproductive successiveness. Unlike mammals, avian lay eggs, and parts of reproductive activities and embryonic development occur outside of mothers' body in the egg. Maternal body condition has a role on egg quality which itself directly influences chicks' survival (Grindstaff *et al.*, 2005). Environmental experienced mothers, make manipulation of their eggs to survive the offspring during early post hatching. Before

laying, mothers manipulate laying date, clutch size and the yolk contents (nutrients and hormones) (Karell *et al.*, 2008).

Like mammals, steroid hormones are important mediators of prenatal effects, and early exposure to the hormones can results long-lasting organizational effects on morphology, and physiological brain and behaviors traits (Groothuis *et al.*, 2005; Tobler *et al.*, 2007). Indeed, steroid hormones translate the environmental conditions experienced by mother and her partly heritable physiological state into adaptive phenotypic variation of the offspring. Avian egg yolk contains substantial amounts of steroids such as testosterone, androstenedione; dihydrotestosterone, estradiol, and corticosterone have attracted attention, because of numerous phenotypic traits influences on the offspring. For example, maternally allocated androgens in the egg yolk affect offspring phenotype, behaviors, growth and immunity (Groothuis *et al.*, 2005; von Engelhardt *et al.*, 2009). Moreover, prenatal corticosterone exposure effects the offspring development, begging behavior and immunity in birds (Rubolini *et al.*, 2005).

1.4 Objective of the present studies

Phytosterols as novel feed additive recently attracted attention due to beneficial effects on plasma cholesterol level and cardiovascular disease treatment (CVD) for human. Nowadays phytosterols are increasingly added to human and animals' dietary regimens and widely used as supplementary nutrient. Although, phytosterols are known to act as endocrine-disrupting chemicals in many animals' species, but the mechanism(s) are poorly understood. To further investigate about the underlying mechanism(s) on the adrenal reproductive endocrine functions I used Japanese quails as laboratory animal models.

Japanese quail offer an excellent laboratory model for studying the reproduction (short lifespan) and cholesterol metabolism (Yuan *et al.*, 1997; Murakami *et al.*, 2010; Li *et al.*, 2012). The first part of the current study is mainly focused on the effects of phytosterols as food additives on the adrenal gland and reproductive endocrine functions in the Japanese quail during growing and adult periods. I tried to express the exact mechanism(s) which phytosterols acts on the animal reproductive functions. To elucidate the objectives, experiments were conducted as follow;

Chapter 2. Effects of phytosterol as food additive on the adrenal and reproductive endocrine function during sexual maturation in male and female Japanese quail (*Coturnix coturnix japonica*)

To investigate the acute and chronic phytosterols effects on the adrenal and reproductive functions in the growing male and female Japanese quail.

Chapter 3. The effects of phytosterol on the sexual behavior and reproductive function in the Japanese quail

To evaluated the direct acting mechanism of phytosterols on the sexual behaviors and the testicular functions in the Japanese quail as avian model.

Chapter 4. Effects of phytosterol on the hypothalamic endocrine function in the adult male Japanese quail

To investigate the phytosterols effects on the brain regulation of gonadal endocrine function in male quail.

The second part of this study is related on the physiologic variations in the steroid hormones concentrations in the egg concomitant with embryonic development and hatchling periods. In this part I tried to evaluate the hormonal relationship between mother and the offspring during the different embryonic developmental sages by measuring the eggshell hormonal contents. Thus, the main objective in this part was:

Chapter 5. Accumulation of the steroid hormones in the eggshells of Japanese quail

- a) quantifying steroid accumulation in the eggshells of Japanese quail
- b) Finding the variation steroids of the eggshell at three important time-periods (after laying, 15 days of incubation and post hatching) between the sexes.

CHAPTER TWO

Effects of phytosterol as food additive on the adrenal and reproductive endocrine function during sexual maturation in male and female Japanese quail (*Coturnix coturnix japonica*)

2.1 Background

Phytosterols are plant-derived chemicals, naturally founds in nuts, legumes, and seeds (Lagarda *et al.*, 2006; Liu *et al.*, 2012). Since 1951, phytosterols widely used into human and animals' dietary regimens due to their cholesterol-lowering activity, and treatment of cardiovascular disease (CVD) (Peterson *et al.*, 1951; Brufau *et al.*, 2008). Feeding of phytosterols effectively reduced LDL cholesterol without significantly altering HDL cholesterol and triglycerides levels (Calpe-Berdiel *et al.*, 2009).

Phytosterols are plant-based chemicals, and humans and animals cannot endogenously synthesize these substances. Instead, cholesterol plays central role for cellular membrane function and steroidogenesis (Yang *et al.*, 2004). Phytosterols are known to act as endocrine-disrupting chemicals in different laboratory and aquatic animals (Vanderkraak, 1995; MacLatchy *et al.*, 1997; Maclatchy and Moghadasian, 2000). Awad *et al.* (1998) reported that feeding of phytosterols significantly reduced testosterone and dihydrotestosterone levels in Sprague-Dawley male rats. Moreover, β -sitosterol feeding significantly decreased testicular weights and sperm count in male rats (Malini and Vanithakumari, 1991; Singh and Gupta, 2016). Goldfishes (*Carassius auratus*) exposed to β -sitosterol decreased the sex steroid levels and gonadal capacity for steroidogenesis (Maclatchy and Vanderkraak, 1995). In immature male Japanese quails, feeding of

phytosterols increased adrenal corticosterone production after ACTH stimulation (Liu *et al.*, 2012). However, no information about the phytosterols effects on the reproductive endocrine function in the avian species during the sexual maturation had been previously reported.

The main objective in this chapter was to investigate the chronic and acute effects of phytosterols on the adrenal and reproductive endocrine function of growing male and female Japanese quails. I tried to find the mechanism(s) of phytosterols effects on the adrenal and gonadal function in this animal.

2.2 Materials and methods

2.2.1 Chemicals

Phytosterol was provided by Tama Biochemical Co., Ltd, Tokyo, Japan, with 97.2% purity (β -sitosterol 42.9%, stigmasterol 23.8%, campesterol 25.6% and brassicasterol 7.7%). Medium chain triglyceride (Miglyol 812N) is provided by Mitsuba Trading Co., Ltd, Tokyo, Japan. ACTH (CORTROSYN Z) was purchased from the Daiichi Sankyo Co. Ltd., Tokyo, Japan.

2.2.2 Phytosterols solution preparation

To minimize the crystallization of phytosterols and enhance intestinal absorption, the suspension was prepared as previously described with minor modifications (von Bonsdorff-Nikander *et al.*, 2005). In brief, phytosterols dissolved in medium-chain triglyceride (MCT) by heating in a vessel at 100°C until a clear solution was formed. After cooling the solution to 90°C, the vessel was immediately immersed in ice and the suspension and stirred until it reached room temperature (25°C). The suspension was then

stored in an airtight glass container at 4°C. Before use, the container was kept in warm water (37°C) for a time to create a solid for easy gavage into the crop sac or subcutaneous injection (SC).

2.2.3 Experimental animals and housing conditions

Male and female Japanese quails were used in both experiments. The quail chicks were obtained by incubating fertilized eggs from our laboratory quail stock in a humidifier egg incubator (Showa Furanki, Saitam, Japan). Both, adult quails and the chicks were housed in controlled environment (lighting from 5:00–19:00 hours, temperature [$25 \pm 2^\circ\text{C}$] and humidity [$50 \pm 10\%$]). An additional heater was put into the cage for the first two weeks in order to maintain the temperature at 37°C. Quails had free access to standard commercial feed (Cosmos Company, Aichi, Japan) and water.

2.2.4 Experimental design

For the chronic experiment, total 100 male and female quail chicks were randomly divided into 5 groups; control, MCT control and phytosterols in the doses of 8 mg, 80 mg and 800 mg/kg body weight (BW) from 6 days post hatching. Daily single doses of phytosterols and MCT were gavaged by syringe having plastic needle into the crop sac from 7 to 50 days of ages. At the age of 44 day, half of each group (5 males and 5 females in each group) was challenged with daily intramuscular injection (IM) of a single dose of ACTH (1IU/100g of BW) for consecutive 6 days (every morning at 7:00am) as previously reported (Liu *et al.*, 2012). Synchronously, the same volume of the ACTH-vehicle was IM injected for the normal quails (non-stimulated by ACTH). After 2 and 6 days of ACTH challenge, blood was taken by jugular venipuncture (one ml tuberculin

syringe and 27-gauge needle) and cervical dislocation of the quails under sedation of diethyl ether respectively. Blood samples were then centrifuged at 2700g for 15 min and the plasma stored at -20°C for hormonal assay. At the end of the experiment, quails were sacrificed for the internal organs sampling. The harvested internal organs were isolated from the extra tissues and weight by electronic digital balance (ASONE, Corporation, China).

In the acute experiment, sixty adult male and female quails at the age of 10 weeks were randomly divided into four groups (control and phytosterols [8 mg, 80 mg, and 800 mg/kg BW]). Before using the quails for the experiment, animals were kept under observation in order to check the sexual activities both in male and female. On the day of experiment, a single dose of MCT and phytosterols (three different doses) was SC injected at 7:00 am. After injection, blood sample was collected at 3, 6 and 24 hours by the jugular venipuncture in heparinized plastic tubes and processed as above batch. All experimental procedures were performed in accordance with the guidelines approved by Tokyo University of Agriculture and technology committee for Animal Welfare and Ethics.

2.2.5 Testicular histology

Testicular histology was examined using hematoxylin-eosin (H&E) staining. After weighting of the testes, left testis of each animal was quickly fixed in 4% paraformaldehyde (Wako Co., Osaka, Japan). The fixed testes were dehydrated in an ethanol series and embedded in paraffin wax. Serial sections (6µm) were mounted on poly-L-lysine-coated slides (Matsunami Glass Ind., Ltd., Osaka, Japan) and stained with H&E staining (for details refer to Li *et al.*, 2015). Testicular structures then evaluated

histologically using an Olympus microscope (BX50F, Olympus Optical Co., Ltd., Tokyo Japan) with 40 x objective lens.

2.2.6 Hormones assay

Plasma testosterone, progesterone (Taya *et al.*, 1985) and corticosterone (Kanesaka *et al.*, 1992) were measured using double antibody radioimmunoassay (RIA) with ¹²⁵I-labeled radioligand. Anti-sera against testosterone (GDN250) were provided by Dr. Niswender GD (Colorado State University, Fort Collins, CO. USA), anti-serum against progesterone was #KZ-SH-P13 (Cosmo Bio Co., Ltd., Tokyo, Japan) and anti-sera against corticosterone was goat anti-corticosterone. LH concentrations were measured with RIA (Beltsville, MD, USA) using USDA-cLH-1-3 for iodination and USDA-cLH-K-3 for the standard of the chicken LH. The anti-avian LH (HAC-CH27-01 RBP75, Gunma University, Gunma, Japan) was used as antisera for LH as previously described (Li *et al.*, 2006).

2.2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (San Diego, CA, USA). Data in each time-point were analyzed by one-way ANOVA to determine whether there was significant difference among the groups, and Tukey and Dunnett multiple comparison tests were used for chronic and acute experiments respectively. A three-way ANOVA (trial version of GraphPad Prism 7) was used to examine the corticosterone levels under the effects of phytosterols, ACTH challenge, and the days (2 and 6) after injection among the control, MCT and phytosterols treated quails in each half of the groups in chronic experiment. Moreover, the hormonal concentrations in acute

experiment were analyzed by two-way ANOVA to examine the changes at 3, 6 and 24 hours after SC injection of phytosterols. All results are represented as the mean \pm standard error of the mean (SEM). $P < 0.05$ was considered as statistically significant.

2.3 Results

2.3.1 Chronic feeding of phytosterols

As illustrated in the Tables 2-1 and 2-2, chronic phytosterols gavage not significantly altered body weight, adrenal glands and liver weights both in male and female quails respectively. None of the quails died during the entire experiment. However, the testicular weights were significantly decreased in the phytosterols-treated male quails as compared with the intact and MCT controls. In contrast, ovary weights were not significantly different among the groups of female quails. Moreover, no significant differences were found in cholesterol levels among the groups both in male (control $[211 \pm 65 \text{ mg/dL}]$, MCT control $[201 \pm 33 \text{ mg/dL}]$, and phytosterols-gavaged in the doses of 8 mg $[208 \pm 97 \text{ mg/dL}]$, 80 mg $[193 \pm 41 \text{ mg/dL}]$, and 800 mg/kg BW $[188 \pm 99 \text{ mg/dL}]$) and female (control $[180 \pm 18 \text{ mg/dL}]$, MCT control $[183 \pm 32 \text{ mg/dL}]$, and phytosterols-gavaged in the doses of 8 mg $[179 \pm 44 \text{ mg/dL}]$, 80 mg $[173 \pm 11 \text{ mg/dL}]$, and 800 mg/kg BW $[174 \pm 88 \text{ mg/dL}]$ quails respectively.

2.3.2 Hormonal levels

2.3.2.1 In male quails

As shown in Fig. 2-1, plasma corticosterone concentrations in male Japanese quails were not significantly affected by phytosterols treatment among the normal groups (non-ACTH challenge). However, after 6 days ACTH challenge corticosterone levels were

significantly increased in male quails treated with 80 mg and 800 mg/kg BW of phytosterols as compared with MCT and control groups ($P < 0.01$ and $P < 0.05$ respectively). In order to determine whether corticosterone levels varied with any of three factors; phytosterols treatment, ACTH challenge and days of injection, a three-way ANOVA was employed and the result indicated that ACTH injection significantly increased corticosterone levels as compared to normal groups (non-ACTH challenge). However, no difference was found in corticosterone levels in relation to the time and phytosterols treatment.

In contrast, testosterone levels were significantly low in the groups of 80 ($P < 0.05$) and 800 mg/kg BW ($P < 0.01$) of phytosterols-gavaged as compared to MCT and control. Moreover, the testosterone levels were significantly low in 80 and 800 mg/kg BW of PS after a 6 days ACTH injection as compared to non-ACTH challenged animals (Fig. 2-3). In addition, no significant difference was found in LH levels among the groups without (control $[4.99 \pm 0.48 \text{ ng/ml}]$, MCT $[5.22 \pm 0.53 \text{ ng/ml}]$, and phytosterols in the doses of 8 $[5.50 \pm 0.75 \text{ ng/ml}]$, 80 $[6.84 \pm 0.55 \text{ ng/ml}]$, and 800 mg/kg BW $[4.52 \pm 0.65 \text{ ng/ml}]$) or with ACTH injection (control $[5.12 \pm 1.09 \text{ ng/ml}]$, MCT $[5.74 \pm 1.00 \text{ ng/ml}]$, and phytosterols in the doses of 8 $[4.60 \pm 0.82 \text{ ng/ml}]$, 80 $[4.76 \pm 1.46 \text{ ng/ml}]$, and 800 mg/kg BW $[4.37 \pm 0.14 \text{ ng/ml}]$).

2.3.2.2 In female quails

A similar pattern was found in corticosterone levels in the female Japanese quail (Fig. 2-2). However, no differences were found in corticosterone concentration after long-term phytosterols feeding among the groups both in normal (non-ACTH), and ACTH

challenged female quails. In addition, ACTH injection significantly increased corticosterone production as compared with normal groups (non-ACTH) ($P < 0.001$).

Moreover, slightly decrease was also found in testosterone concentrations in female Japanese quails by feeding of phytosterols in the doses of 80 mg and 800 mg compared with the control and MCT groups. Testosterone concentrations were not significant difference among the normal (non-ACTH) and ACTH-challenged groups in female Japanese quails (Fig. 2-4).

Progesterone levels in female Japanese quails were dose-dependently reduced in the groups fed phytosterols. Progesterone concentration was significantly low in female quails fed 800 mg/kg BW ($P < 0.05$). A two-way ANOVA results indicated that progesterone levels was significantly low ($P < 0.001$) in the groups challenged with ACTH compared with the normal quails (non-ACTH) (Fig. 2-5).

In addition, no significant difference was found in LH levels among the groups without (control [6.94 ± 1.3 ng/ml], MCT [5.27 ± 0.71 ng/ml], and phytosterols in the doses of 8 [4.76 ± 0.86 ng/ml], 80 [8.83 ± 0.91 ng/ml], and 800 mg/kg BW [8.29 ± 0.41 ng/ml]), and with ACTH injection (control [5.4 ± 1.35 ng/ml], MCT [5.41 ± 0.79 ng/ml], and phytosterols in the doses of 8 [4.14 ± 0.49 ng/ml], 80 [6.79 ± 1.86 ng/ml], and 800 mg/kg BW [6.39 ± 2.05 ng/ml]).

2.3.3 Testicular histology

Histological examination of the testes of quails in the different groups revealed the difference in the structures. Testes of the animals in control and MCT groups showed the enlargement of the seminiferous tubules with the lumen (Fig. 2-6A and 2-6B respectively). Moreover, the regular spermatogenesis and multi-nuclei spermatocytes had

shown the functional maturity of the testes. However, testes of the quails under the treatment with different doses of phytosterols (8, 80, 800 mg/kg BW) showed the smaller size of seminiferous tubules (Fig. 2-6C, 2-6D, and 2-6E respectively). In addition, the seminiferous tubules lumen and the active spermatozoa were not formed.

2.3.4 Acute effects of phytosterols

2.3.4.1 Effects in male quail

Male quails in the groups of 8 and 80 mg/kg phytosterols had significantly high ($P < 0.05$) corticosterone level at 3h after phytosterols injection. However, the corticosterone level was reduced and maintained to the basal level, and no difference was found among the groups in the different time-point (Fig. 2-7). Slightly reduction of testosterone level was found in each time point after phytosterols injection in comparison with control (Fig. 2-9). Moreover, LH levels were not significantly changed among the groups in each time-point; 3 hours (control [6.58 ± 1.28 ng/ml] and phytosterols in the doses of 8 [7.16 ± 1.17 ng/ml], 80 [6.95 ± 1.79 ng/ml] and 800 mg/kg BW [6.52 ± 0.95 ng/ml]), 6 hours (control [6.58 ± 1.33 ng/ml] and phytosterols in the doses of 8 [4.63 ± 0.97 ng/ml], 80 [5.69 ± 1.23 ng/ml] and 800 mg/kg BW [5.37 ± 0.88 ng/ml]), and 24 hours (control [5.80 ± 0.46 ng/ml] and phytosterols in the doses of 8 [4.41 ± 0.60 ng/ml], 80 [5.27 ± 0.73 ng/ml] and 800 mg/kg BW [4.61 ± 0.75 ng/ml]) respectively. No significant difference was found in cholesterol levels among control (188 ± 92 mg/dL) and phytosterols treatment in the doses of 8 mg (180 ± 64 mg/dL), 80 mg (179 ± 13 mg/dL) and 800 mg/kg BW (175 ± 81 mg/dL).

2.3.4.2 Effects in female quails

Corticosterone concentrations did not significantly alter after 3 and 6 hours phytosterols injection among the groups in female Japanese quail. However, corticosterone levels were significantly high after 24 hours in the quails injected with 80 mg and 800 mg/kg BW of phytosterols as compared with control ($P < 0.05$; Fig. 2-8).

Slightly decrease was also found in testosterone concentration after 6 and 24 hours phytosterols injection in the groups of 80 mg and 800 mg/kg BW of phytosterols compared with control. Concomitantly, no significant difference was found among the groups in order to different time-point in testosterone level (Fig. 2-10).

Similarly, progesterone level was low in female quails fed 80 mg and 800 mg/kg BW after 3, 6, and 24 hours post injection compared with control. Moreover, progesterone level was significantly low after 24-hour phytosterols injection in the dose of 800 mg /kg BW compared to control (Fig. 2-11).

In addition, LH level was not significantly different among the group after 3 hours (control $[6.67 \pm 1.13 \text{ ng/ml}]$ and phytosterols in the doses of 8 $[5.02 \pm 0.2 \text{ ng/ml}]$, 80 $[5.19 \pm 0.78 \text{ ng/ml}]$ and 800 mg/kg BW $[6.12 \pm 0.8 \text{ ng/ml}]$), 6 hours (control $[7.99 \pm 2.17 \text{ ng/ml}]$ and phytosterols in the doses of 8 $[6.51 \pm 0.63 \text{ ng/ml}]$, 80 $[6.67 \pm 1.08 \text{ ng/ml}]$ and 800 mg/kg BW $[7.84 \pm 1.07 \text{ ng/ml}]$), and 24 hours (control $[8.86 \pm 1.87 \text{ ng/ml}]$ and phytosterols in the doses of 8 $[5.91 \pm 2.13 \text{ ng/ml}]$, 80 $[6.18 \pm 1.34 \text{ ng/ml}]$ and 800 mg/kg BW $[7.69 \pm 1.35 \text{ ng/ml}]$) respectively. No significant difference was found in cholesterol levels among control ($147 \pm 16 \text{ mg/dL}$) and phytosterols treatment in the doses of 8 mg ($148 \pm 12 \text{ mg/dL}$), 80 mg ($145 \pm 10 \text{ mg/dL}$) and 800 mg/kg BW ($142 \pm 16 \text{ mg/dL}$).

2.4 Discussion

This study is the first to investigate the phytosterols effects on the reproductive endocrine function in the avian species. Except of the testicular weights in male quails, no significant differences were found in the body weight, adrenal glands and liver weights among the groups, and no signs of toxicity and death observed during the experiments, both in male and female quails. The testicular weights were significantly low in the treated animals after chronic feeding of phytosterols. Corticosterone concentrations were significantly elevated in the groups exposed to 80 mg, and 800 mg/kg BW, but testosterone levels showed a tendency to decrease both in male and female Japanese quails. In similar fashion, progesterone concentrations were significantly low in female Japanese quails after chronic feeding of phytosterols in the dose of 800 mg/kg BW.

In this study, the testicular weights and histology were significantly different among controls and long-term phytosterols-treated male quails. Transverse section of control and MCT testes showed developed conditions with enlargement of seminiferous tubules containing big lumen, and all the stages of spermatogenesis with bunch of spermatozoa. On the other hand, in phytosterols-treated male quails' testis showed less gonadal development (smaller seminiferous tubules without lumen and any active spermatozoa inside). Singh and Gupta (2016), and Malini and Vanithakumari (1991) reported that phytosterols exposure significantly decreased testicular weight and sperm count rats. Furthermore, in fish, plasma sex steroid levels and gonad size are reduced (Van Der Kraak *et al.*, 1992). In male Japanese quails, testicular weights concomitant with testosterone concentrations and cloacal gland size rapidly increase between 25 to 60 days of age (Ottinger and Brinkley, 1979). Thus, exposing the young animals to chronic high doses of phytosterols might delay the testicular growing.

The current results indicated that quails exposed to phytosterols significantly increased corticosterone levels in both sexes by stimulation of ACTH. In human, expect one study no previous data indicating that phytosterols feeding cause low production of corticosterone. Mushtaq *et al.* (2007) reported that in human phytosterolemic-patient corticosterone productions was significantly low. However, corticosterone levels were not significantly changed with phytosterols treatment in sterolin-deficient mice (Yang *et al.*, 2004). In contrast, phytosterols feeding enhanced the corticosterone production in a dose dependent manner in male Japanese quails (Liu *et al.*, 2012). They suggested that phytosterols may use as substrate for corticosterone production, or endogenous cholesterol is still enough to produce corticosterone and maintain a normal response to a stressor.

In this study, testosterone concentrations were significantly reduced after long term phytosterols feeding in the doses of 80 mg and 800 mg/kg BW in male quails. Moreover, moderately decreased were also found in testosterone levels both in female and male quails after acute exposure to phytosterols. Awad *et al.* (1998) reported that phytosterols feeding significantly reduce testosterone concentrations in plasma of male Sprague-Dawley rats. Additionally, inter-peritoneal injection of β -sitosterol significantly reduced testosterone, and 11-ketotestosterone levels in male and testosterone and estradiol in the female goldfish respectively (Van Der Kraak *et al.*, 1992; Sharpe *et al.*, 2007). The conversely responses to phytosterols exposure by gonads and adrenal glands in Japanese quails seems to be resulting from the usage of the different sources of lipoprotein cholesterol for steroidogenesis as previously reported in some species. In rats, lipoprotein-cholesterol, rather than de novo cholesterol is the major substrate for the production of steroid hormone by adrenal glands and gonads (Andersen and Dietschy,

1978). In humans, rats and mice HDL cholesterol is the major source for adrenal steroid productions (Yang *et al.*, 2004; Bochem *et al.*, 2013). In contrast, gonadal steroidogenesis is mostly depending on continuous supply of LDL cholesterol levels. On the other, phytosterols are well known for their efficiently reducing the total cholesterol, and LDL cholesterol as well in the circulation (Calpe-Berdiel *et al.*, 2009). These results suggested that low testosterone concentration may be due to low testicular weight and low cholesterol levels (LDL cholesterol) caused by phytosterols feeding in Japanese quails.

Chronic and acute exposure to phytosterols dose dependently reduced progesterone concentrations in plasma of female quails. Progesterone levels were significantly low in the dose of 800 mg/kg BW of phytosterols both in chronic and acute (after 24 hour) experiments. Ayesha *et al.* (1999) reported that feeding of margarine enriched with phytosterols ester significantly reduced progesterone levels in women. However, they did not explain about the mechanism. In goldfishes, phytosterols administrations reduced the expression of steroidogenic acute regulatory protein (StAR, the rate-limiting mediator of steroid biosynthesis, delivering cholesterol to the inner mitochondrial membrane), and the activity of P450 side chain cleavage (P450scc) enzyme, the first step in the steroid biosynthetic pathway that converts cholesterol to pregnenolone (Gilman *et al.*, 2003; Sharpe *et al.*, 2007). Thus, phytosterols feeding in high doses may alter the expression of cholesterol converting enzymes responsible for steroidogenesis and reduced progesterone production in female quails.

In this study, phytosterols-treated male and female quails had a tendency to decrease LH levels both in chronic and acute experiment. In laying hens during late period of egg production, feeding of phytosterols significantly increase estradiol and decrease the LH levels (Yanmin and Tian, 2008). In female birds, LH stimulates the production of

progesterone and androstenedione by granulosa (largest follicle), and theca cells (second largest follicles). In male chickens and quails, LH has responsibility to increase the number of matures Leydig cells and testosterone productions (Scanen, 2014). The results of this study suggested that the directly impact of phytosterols on gonads and adrenal function is stronger than indirect effects through the hypothalamic-pituitary-gonadal axis. Because LH secretion and release by pituitary glands occur in an episodic manner in the avian, further studies will be necessary to stimulate LH levels and find the exact mechanism.

In summary, these results suggest that phytosterols feeding might induce locally effects on adrenal gland and on gonadal functions. The controversial effects of phytosterols on adrenal and gonadal steroidogenesis might cause preferential utilization of lipoprotein-cholesterol as steroid precursor. Moreover, chronic feeding of phytosterols might ultimately delay sexual maturation and reduce testosterone and progesterone production in male and female Japanese quails.

CHAPTER TWO

Table 2-1. Effects of gavage of phytosterols on body and organs weights in male Japanese quails

Groups	Body weight (g)	Testes weight (g)		Adrenal weight (mg)	Liver weight (g)
		Right	Left		
Control	107.86 ± 2.3	0.92 ± 0.12 ^a	1.00 ± 1.15 ^a	12.1 ± 1.7	3.58 ± 0.2
MCT	107.74 ± 1.9	0.99 ± 0.18 ^a	1.09 ± 0.17 ^a	15.6 ± 4.1	3.15 ± 0.12
8 mg/kg BW	107.00 ± 1.5	0.65 ± 0.12 ^b	0.65 ± 0.13 ^b	10.5 ± 0.8	3.59 ± 0.22
80 mg/kg BW	104.19 ± 2.9	0.69 ± 0.11 ^b	0.77 ± 0.13 ^b	9.23 ± 1.3	3.19 ± 0.23
800 mg/kg BW	107.79 ± 2.4	0.62 ± 0.12 ^b	0.53 ± 0.15 ^b	10.3 ± 1.8	3.62 ± 0.19

Values in the table represent means ± standard error of mean (SEM), and different superscript letters denote statistical significance ($P < 0.05$). Numbers without letters shown non-significant differences. Total number of quails in each group is 10 animals.

Table 2-2. Effects of gavage of phytosterols on body and organs weights in female Japanese quails

Groups	Animal No	Body weight (g)	Ovary weight (g)	Adrenal weight (mg)	Liver weight (g)
Control	10	122.05 ± 4.37	1.82 ± 0.22	15.20 ± 4.83	3.76 ± 0.32
MCT	10	125.27 ± 2.59	1.94 ± 0.51	14.60 ± 1.78	4.41 ± 0.21
8 mg/kg BW	10	115.20 ± 3.93	1.19 ± 0.16	16.30 ± 0.94	3.45 ± 0.25
80 mg/kg BW	10	122.35 ± 4.77	1.49 ± 0.75	17.67 ± 0.97	4.04 ± 0.26
800 mg/kg BW	10	118.46 ± 1.51	1.19 ± 0.12	16.50 ± 0.79	3.65 ± 0.15

Values in the table represent means ± SEM. Total number of quails in each group is 10 animals.

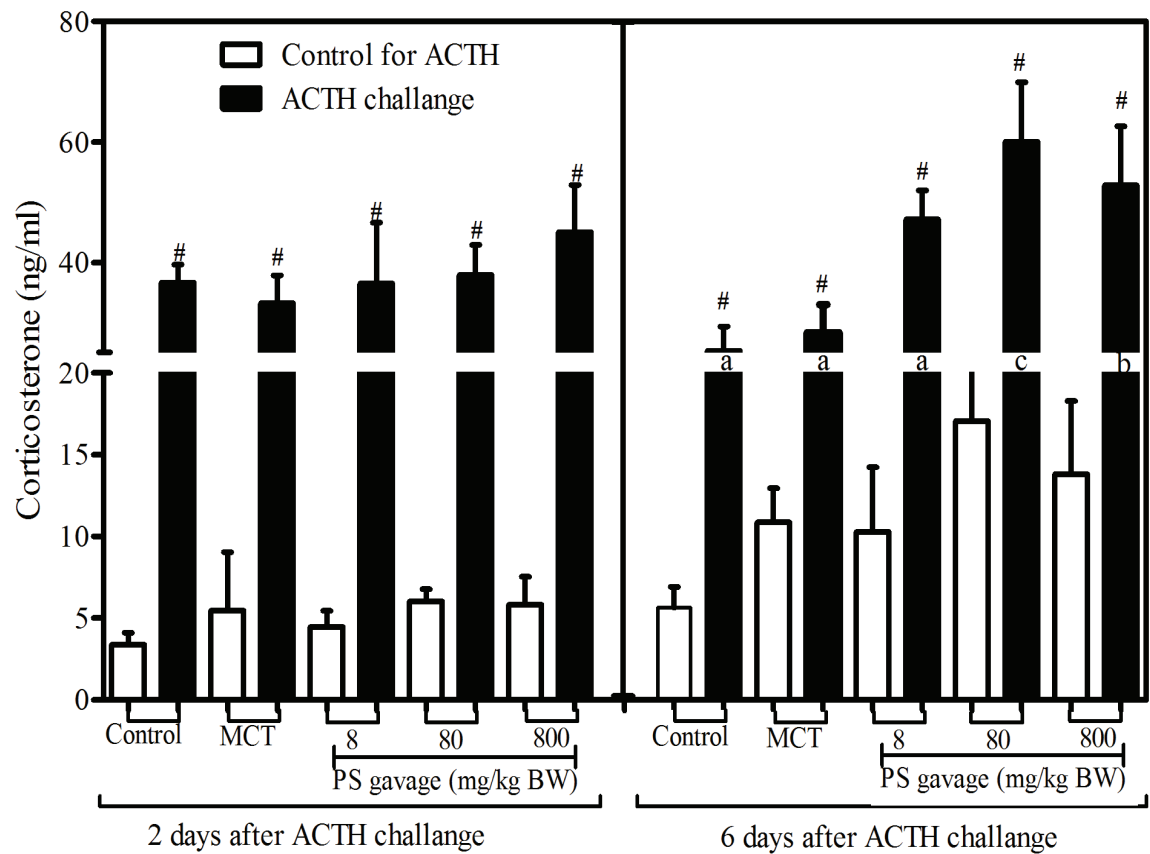


Fig. 2-1. Corticosterone concentrations in normal (non-ACTH, white bars) and ACTH-challenged (black bars) male quails after 2 and 6 days ACTH injection. Hash-marker indicated significant difference between normal and ACTH-challenged groups, and different letters denote significant difference among the groups ($P < 0.05$).

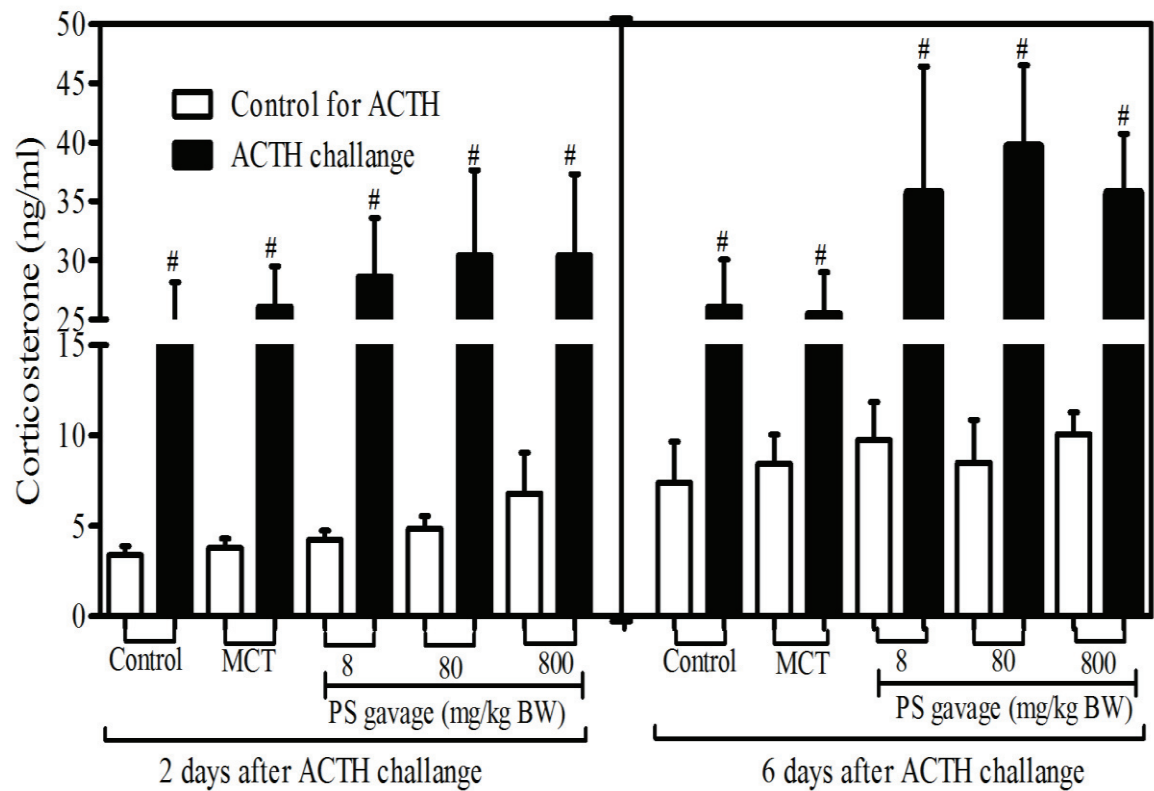


Fig. 2-2. Plasma corticosterone concentrations in normal (non-ACTH, white bars) and ACTH-challenged (black bars) female quails after 2 and 6 days ACTH injection. Hash-marker indicated significant difference between the normal and ACTH-challenged groups ($P < 0.05$).

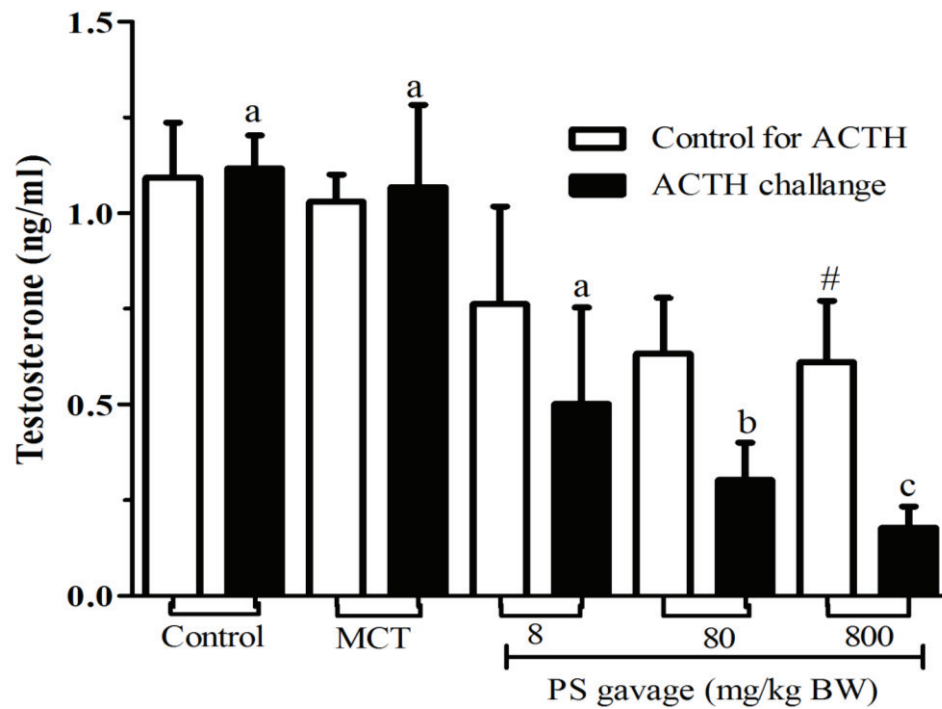


Fig. 2-3. Plasma testosterone concentrations in normal (non-ACTH, white bars), and ACTH-challenged (black bars) male quails after 6 days ACTH injection. Different letters denote significant difference among the groups ($P < 0.05$).

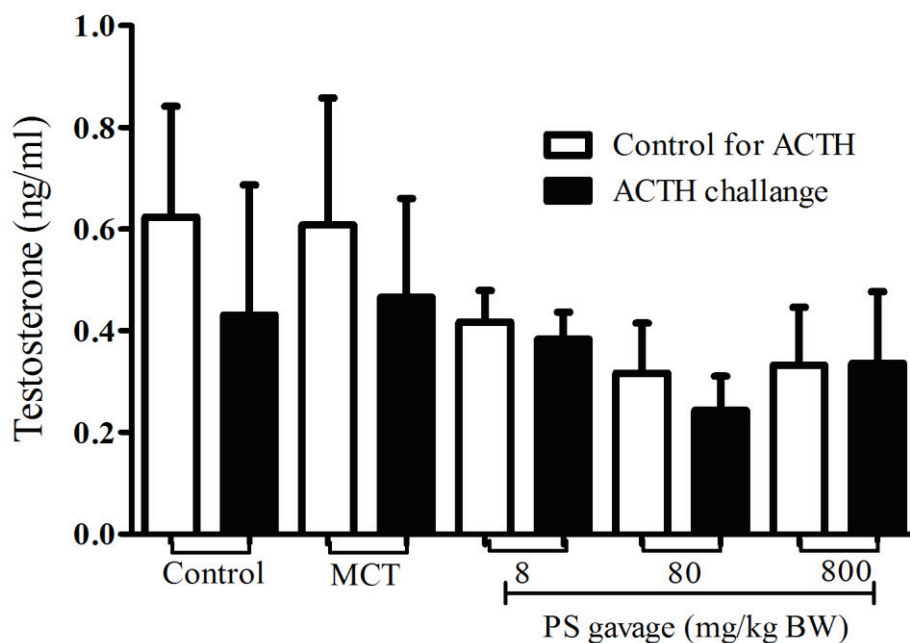


Fig. 2-4. Plasma testosterone concentrations in normal (non-ACTH, white bars), and ACTH-challenged (black bars) female quails after 6 days ACTH injection.

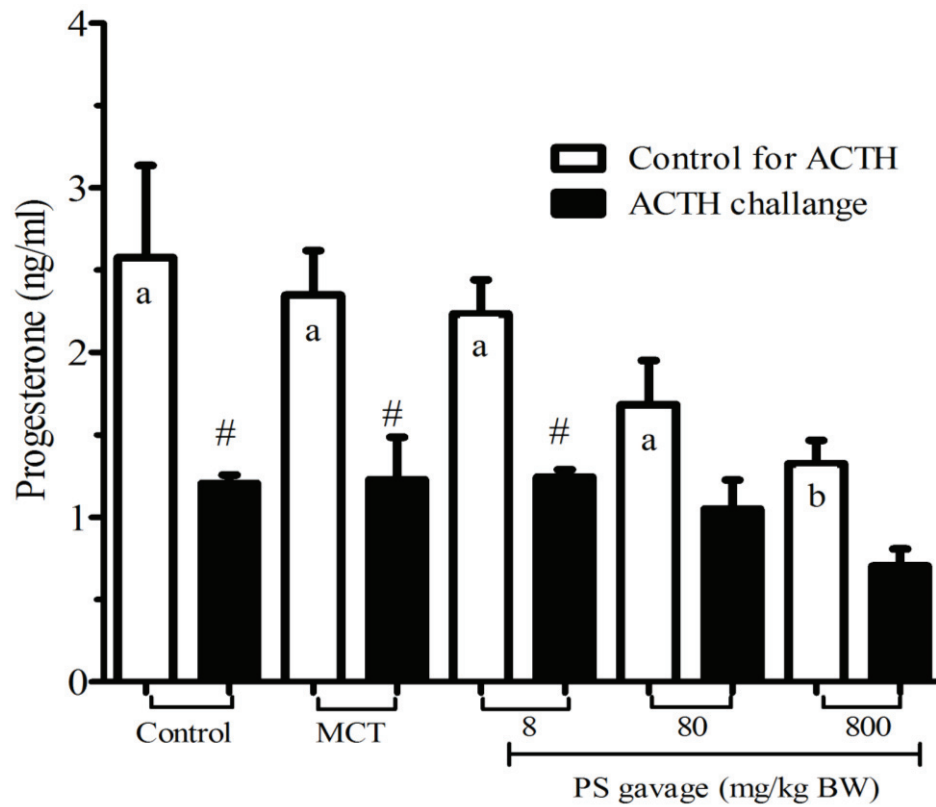


Fig. 2-5. Progesterone levels in normal (non-ACTH, white bars), and ACTH-challenged (black bars) female quails after 6 days ACTH injection. Different letters denote significant difference among the groups. Hash-marker indicates significant difference between normal and ACTH-challenged groups ($P < 0.05$).

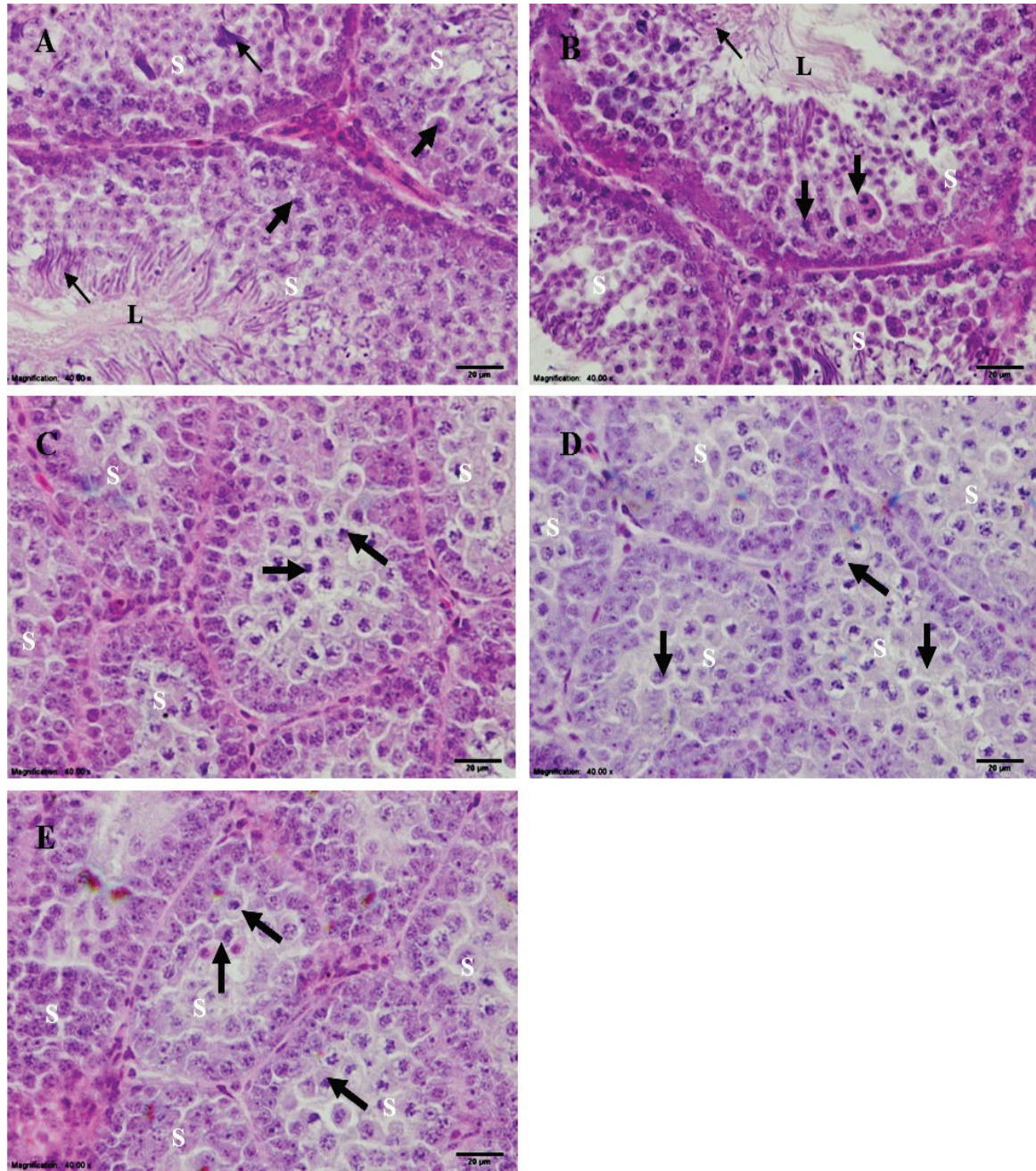


Fig. 2-6. Transvers section of testes of quails in the different groups; control (A), MCT (B), and phytosterols in the doses of 8 mg (C), 80 mg (D), and 800 mg/kg BW (E). The different letters represent the seminiferous tubule (S) and lumen (L). Arrows denote the spermatozoa (thin arrows) and spermatocytes (thick arrows).

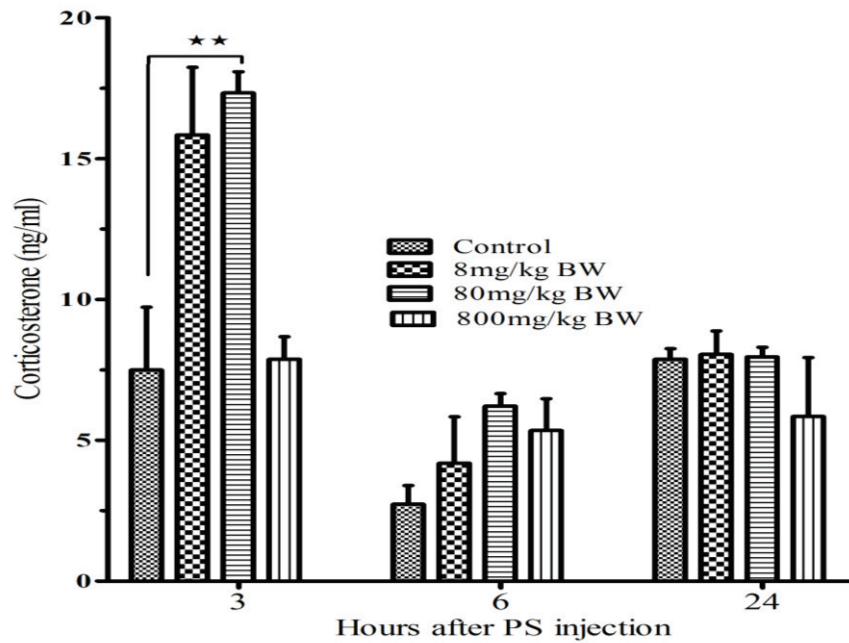


Fig. 2-7. Plasma corticosterone concentration in male Japanese quails after 3, 6, and 24 hours SC injection of phytosterols. Asterisk denote the significant of difference from control ($P < 0.05$).

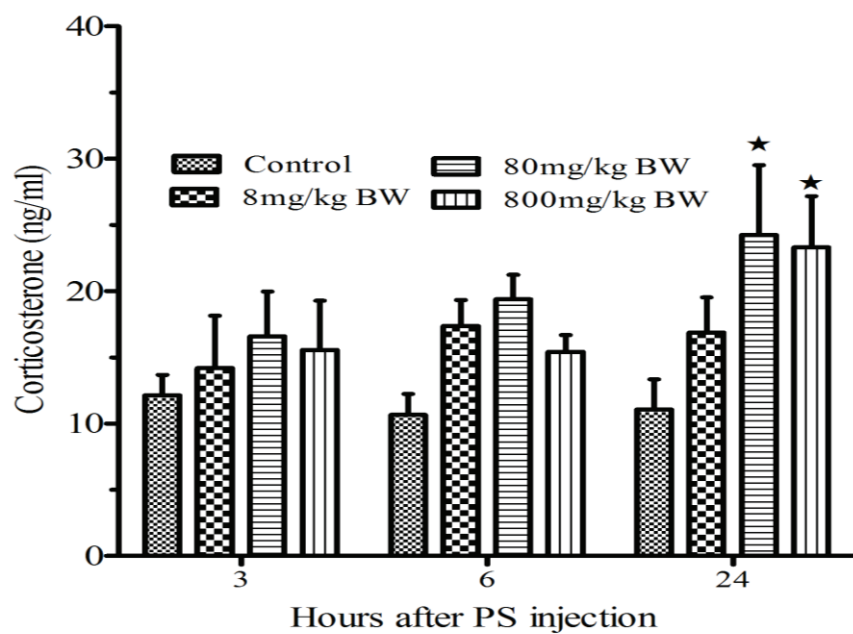


Fig. 2-8. Corticosterone levels in female Japanese quails after 3, 6, and 24 hours SC injection of phytosterols. Asterisks denote the significant difference from control ($P < 0.05$).

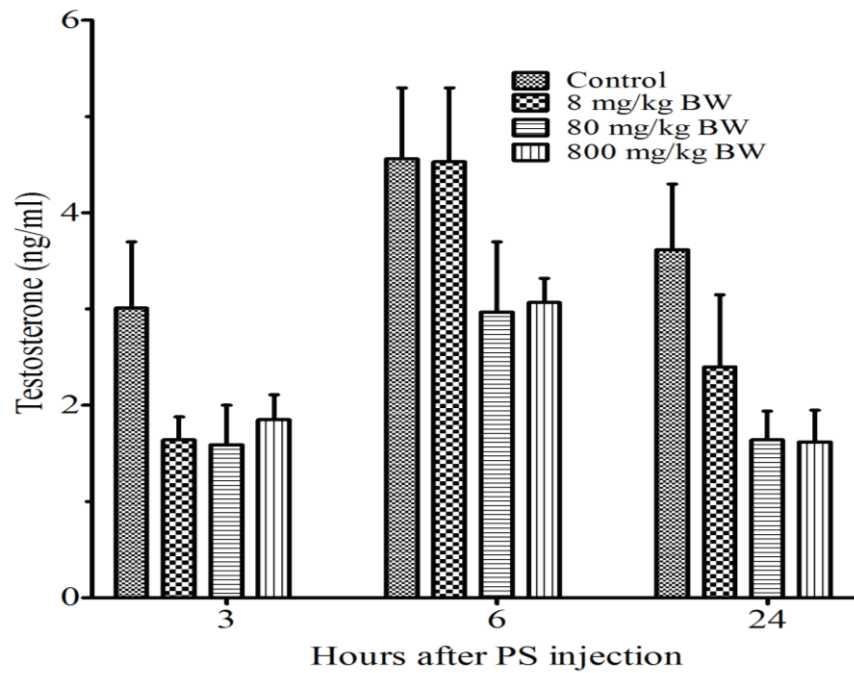


Fig. 2-9. Plasma testosterone concentrations in male Japanese quails after 3, 6, and 24 hours SC injection of phytosterols.

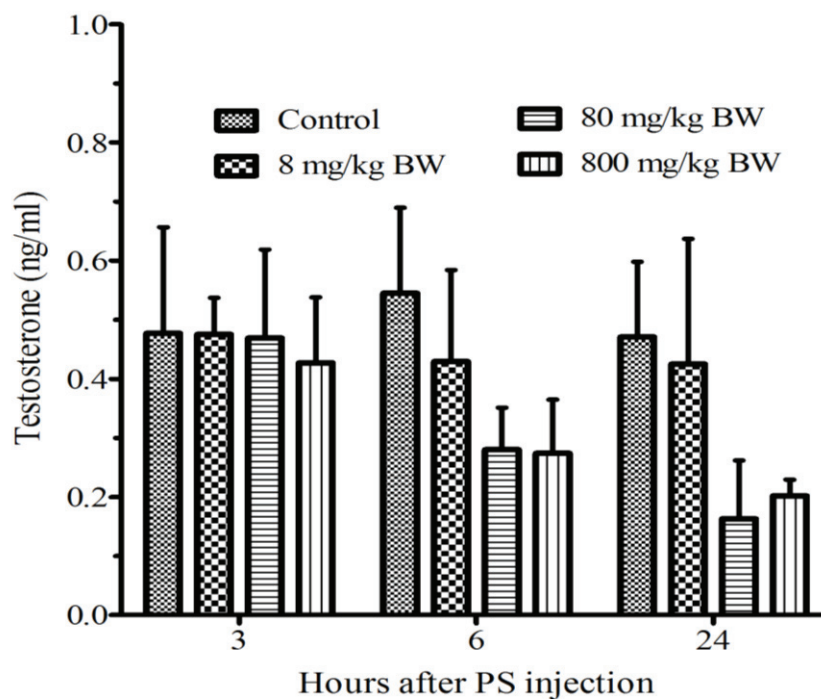


Fig. 2-10. Testosterone levels in female Japanese quails after 3, 6, and 24 hours SC injection of phytosterols.

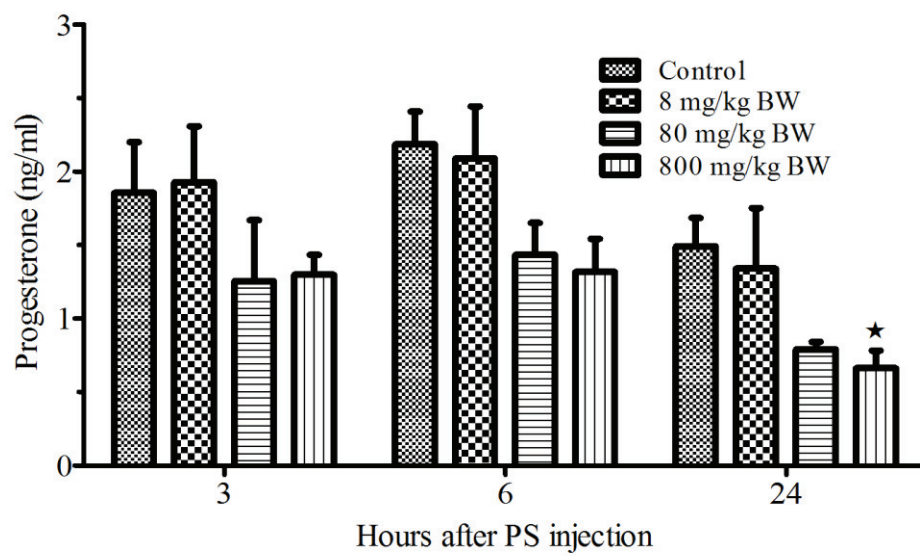


Fig. 2-11. Progesterone levels in female Japanese quails after 3, 6, and 24 hours SC injection of phytosterols. Asterisk denote the significance from control ($P < 0.05$).

CHPATER THREE

The effects of phytosterol on the sexual behavior and reproductive function in the Japanese quail

3.1 Background

Phytosterols or plant sterols are steroid compound which are naturally exist in many foods such as seeds, beans and legumes. Dietary phytosterols are structurally similar to cholesterol except of some extra ethyl and methyl groups on C24 or in combination with a double bond at C22 (Moghadasian and Frohlich, 1999). There are over 250 phytosterols and related compounds are reported in plants and marine materials, but β -sitosterol, campesterol and stigmasterol are abundantly found in nature (Ling and Jones, 1995; Piironen *et al.*, 2000).

Phytosterols is increasingly added into human and animals' dietary regimens because their beneficial effects against cholesterol and atherosclerosis (Moghadasian, 2000; Lagarda *et al.*, 2006). Dietary phytosterols feeding significantly decrease LDL cholesterol and cholesterol biosynthesis in the body (Tilvis and Miettinen, 1986; Vanstone *et al.*, 2001). Furthermore, the anti-inflammatory, anti-cancer and anti-oxidative effects of phytosterols have been well documented (Bouic, 2001; Yoshida and Niki, 2003; Lagarda *et al.*, 2006).

In addition, the acting mechanism of phytosterols on the reproductive endocrine function is not fully understood. Phytosterols are known to act as endocrine disrupting chemical on the reproductive function (Moghadasian, 2000; Calpe-Berdiel *et al.*, 2009). Awad *et al.* (1998) reported that feeding phytosterols significantly reduced the

testosterone production and metabolism in rats. Moreover, the antifertility activity of phytosterols in rat and the sterolin-deficient mice were confirmed (Solca *et al.*, 2013; Singh and Gupta, 2016). In Japanese quails, phytosterols induces the adrenal corticosterone production after ACTH challenge (Liu *et al.*, 2012). My previous results were shown that feeding of phytosterols significantly reduced the testosterone level in male and female Japanese quails without significant difference in the LH level.

To assess and better understanding the endocrine disrupting activity of phytosterols, I investigated the direct acting mechanism of phytosterols on the sexual behaviors and the testicular functions in the Japanese quails as avian model.

3.2 Materials and methods

3.2.1 Chemicals and preparation of phytosterols solution

Phytosterols and MCT was provided by same companies and identical method performed for making the solution for gavage into crop sac as detailed in chapter two.

3.2.2 Animal condition and experimental design

Male Japanese quail chicks were used in this study. Fertilized quail eggs of our stock animals were incubated in a humidifier egg incubator. The chicks were reared in a similar condition explained in chapter two. After 15 days of age the chicks were randomly divided into three groups (control and treatments). Phytosterols suspension in a single daily dosage of 80mg and 800mg/kg body weight were gavaged into the crop sac until 100 days of age. Blood was collected after cervical dislocation of animals in heparinized micro tube for hormonal assay and cholesterol measurement in plasma. All procedures

were done in accordance to the guiding principle of Tokyo University of Agriculture and Technology, for use of laboratory animals.

3.2.3 Sexual behaviors test

After maturation (50 days of age) animals were recruited for the sexual behavior test. All procedures for the sexual behavior test were performed as previously described method in chicken with minor modifications (Hirao *et al.*, 2009). Briefly, male quails were individually housed in metal cages (40cm x 20cm x 18cm) for two weeks. Thereafter, intact females (same age) were introduced to each male for five min and sexual activities of individual male quails were recorded by JVC video recorder (Everio model No: GZ-E225-T). The frequencies of three important males' sexual-respond to females were observed during five min as listed as follows:

3.2.3.1 Waltzing

A display was performed by male when the male approached the female in a sideways or circling path with his far wing lowered. His head was usually lowered and his feet made a rasping sound as they passed through the primary feathers of the wing (Millman *et al.*, 2000; Hirao *et al.*, 2009).

3.2.3.2 Mount

The male approached female and held the comb or neck feathers of a female while attempting to induce the female into a sitting or crouching position by placing a foot on her back. The female avoided the male, and no further elements of the copulatory sequence were observed (Millman *et al.*, 2000; Hirao *et al.*, 2009).

3.2.3.3 Copulation

The male mounted, gripped and trod a female, and appeared to achieve cloacal contact (Millman *et al.*, 2000).

3.2.3.4 Cloacal gland size and foam amounts

The sizes of cloacal gland were measured using a Vernier caliper (the longest length \times the greatest width) as previously reported (Li *et al.*, 2006). One hour following the removing foam from cloacal gland, the amount was collected by gentle squeezing of foam glands and indivedually measured using a digital balance (ASONE Corporation, China).

3.2.4 Semen collection

Following the sexual behavior test, male quails were trained two weeks to allow semen collection. The female teaser method was applied as previously described with minor modifications (Chelmonska *et al.*, 2008; Hanafy *et al.*, 2016). Briefly, to minimize the chance of semen contamination, feathers surrounding the cloacal vent were clipped. The procedure adopted for quails began by taking the male from the cage using the left hand and the cloacal foam was removed by gently squeezing the gland; the bird was then paced back in its cage, and the female was introduced into the male cage. In order to prevent unintentional copulation, the palm of the hand was placed under the male's cloaca (Fig. 3-1). At the moment of intense male excitation, manifested by the characteristic arching of the back and spreading of the wings, the male was quickly taken from the cage. The procedure was repeated for each quail during the days of training.

On the experimental day, after excitation of the male, the lateral wall of the cloaca was gently squeezed until the copulatory organ was everted. From the dense and viscous area, 5µl semen was collected by micropipette. Minimum Essential Medium (MEM) was used as semen extender as previously described (Dumpala, *et al.*, 2006; Adkins-Regan, 2014), and the sperm morphology was studied in nigrosin-eosin stained smears under a light microscope (BX50F, Olympus Optical Co., Ltd., Japan). The concentration of spermatozoa was calculated using hemocytometer. The semen quality factor (SQF) was evaluated according to the following formula: $SQF = \text{ejaculate volume (ml)} \times \text{sperm concentration (x106/ml)} \times \text{live normal spermatozoa (\%)} / 100\%$.

3.2.5 Fertilization test

Fertilization test was performed in the same procedure previously described with some modifications (Hanafy *et al.*, 2016). Briefly, a couple (consisting male and intact female) was placed in a separate cage for one week. During the week, eggs laid by intact females were not used for the test. After a single insemination in the Japanese quail, female birds typically store spermatozoa for several days (approximately 10 days) in the sperm storage tubule (SST) (Blesbois and Brillard, 2007). After one week, laid eggs were collected daily and stored for one week at 15°C. The fertilized eggs were then incubated in a humidified egg incubator (Showa Furanki, Co., Ltd., Saitam, Japan) for 8 days. The candling method was used for identification of fertilized and non-fertilized eggs.

3.2.6 Hormonal analysis

Plasma testosterone and LH levels was measured using double antibody RIA with 125I-labeled ligand (for detail refer to chapter two)

3.2.7 Histological examination and Leydig cell counting in the testis

The left testis was immediately fixed in 4% paraformaldehyde (Wako, CO. Osaka, Japan) after decapitation of the animals. The fixed testis was paraffin embedded, sectioned at 6 μ m, and the sections placed on poly-L-lysine-coated slides (Matsunami Glass Ind., Ltd., Osaka, Japan) for H&E staining (for details refer to Li *et al.*, 2006).

For histologic examination, three transverse sectioned slides were observed for each testis using an Olympus BX50 microscope. For the Leydig cell counts, 5 field on each slide was randomly selected based on the shape of the seminiferous tubules (pentagonal or hexagonal), under low magnification (10x). The Leydig cell numbers were counted under high magnification (40x) using the entire field of view (FOV). The total numbers of cells were calculated with the following formula: FOV= eyepiece field no. (EFN)/objective lens magnification (M), in μ m.

3.2.8 Interstitial cell isolation

All procedures for interstitial cell isolation were described previously with minor modifications (Li, *et al.*, 2006). Three intact adult male quails were killed by cervical dislocation, and the harvested testes were then immediately decapsulated and dispersed by pipetting in M199 (Sigma #M4530) medium containing 2.2 mg/ml HEPES, collagenase (0.25 mg/ml), and soybean trypsin inhibitor (0.025 mg/ml); and kept at 37°C for 30 min in a shaking water bath. The supernatants containing Leydig cells were then decanted through nylon mesh to remove debris. The cells were washed twice by centrifugation and resuspended in 10 ml M199 with 1% fetal bovine serum (FBS) and incubated at 37°C in a 95% air and 5% CO₂ atmosphere in a 96-well plate. Following a 20-min equilibration period, the cells were treated with phytosterols at doses of 87.5 or

0.875 µg/ml dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Company, Osaka, Japan). At the same time, for half of the 96-well plate (50% of cells in each group), the cells were artificially stimulated with 0.25 mg/ml ovine-LH (O-LH) (NIAMDD-oLH-24, 5 mg, NIAMDD-NIH, Bethesda, MD) for testosterone production. Following 24 hours incubation, media and cells were separately collected for testosterone measurements, and gene expressions were measured in the cells.

3.2.9 RNA isolation and real time PCR

Total RNA was extracted from the testis and the cultured cells using Isogen reagent kit according to the manufacturer's instructions (Nippon Gene, Tokyo, Japan). The concentration and purity of the isolated total RNA were determined spectrophotometrically using a Nanodrop lite (Thermo Fisher Scientific Inc. Wyman St., Waltham, MA, USA). cDNA was synthesized using PrimeScript reverse transcriptase (TaKaRa Bio, Shiga, Japan) according to the manufacturer's protocol. Oligonucleotide primers were chosen with assistance of Primer3, a Web-based online software system (Table 3-1). Amplification sizes for samples and housekeeping gene (β -actin) were firstly checked using 3% agarose gel electrophoresis run in standard TBE buffer and visualized by ethidium bromide staining of regular PCR products. Real-time PCR was performed using Ex TaqR Hot Start Version containing SYBR-Green I (TaKaRa Bio). The expression of each target mRNA relative to β -actin was determined using the $2^{-\Delta\Delta CT}$ method.

3.2.9.1 Statistical analysis

Statistical analysis was performed using GraphPad Prism5 (San Diego, CA, USA). One-way ANOVA followed by Dunnett's test was used to determine statistically significant of differences among the groups after log transformation of testosterone and LH data. Moreover, a Kruskal-Walis test for non-parametric data was used to analyze statistical significance among the groups for other data. Time-point evaluation of the sexual behaviors was analyzed by two-way ANOVA. All results are represented as means \pm SEM and a value of $P < 0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Chronic effects of phytosterols

Total body, testis and adrenal gland weights and daily feed intake were not statistically different among the groups. Throughout the study period, no treatment-related deaths were observed and no adverse effects were noted in clinical signs of toxicity. However, the total cholesterol level in plasma was slightly decreased in the groups' gavaged phytosterols as compared to control (Table 3-2).

3.3.2 Sexual behavior test

Phytosterols feeding decreased male quails' mating behaviors and courtship. Compared to control subjects, phytosterols-gavaged animals had low mount and copulation behaviors during the first min. Quails that were fed 80 mg/kg BW of phytosterols had low frequencies of mounting behavior (first min $P < 0.001$) and copulation (first, fourth and fifth min; $P < 0.01$) behaviors compared to controls. Quails in the group of 800 mg/kg BW of phytosterols showed low waltzing behavior ($P < 0.05$) in the first min and

relatively high behavior during the last min (Fig 3-2A). Moreover, the mount and copulation behaviors were low ($P < 0.05$) after the first min at the high dose of phytosterols compared to controls (Fig. 3-2BC). Over the entire 5-min period, no differences were found in the waltzing behaviors among groups (Fig. 3-2a). However, phytosterols feeding decreased mounting behavior both 80 and 800 mg/kg BW compared to controls ($P < 0.01$) (Fig. 3-2b). Moreover, the copulation behavior was also low at the high dose of phytosterols compared to controls ($P < 0.05$) (Fig. 3-2c).

3.3.3 Testosterone and LH concentration

As illustrated in Table 3-3, phytosterols dose-dependently decreased the plasma testosterone level. Testosterone levels were decreased after chronic feeding of phytosterols at 800 mg/kg BW (Log 3.11 pg/ml) compared with controls (Log 3.35 pg/ml) ($P < 0.05$). However, LH levels were not different among control (Log 1.16 pg/ml) and either phytosterols dose (80 mg [Log 1.12 pg/ml] or 800 mg/kg BW [Log 1.18 pg/ml]).

3.3.4 Testis morphology and histology

Testes weights and macroscopic feature were not different between the groups. However, microscopic differences were observed in the testicular structures of phytosterols-treated animals. In the phytosterols-treated male quails, Leydig cells surrounded by seminiferous tubules appeared smaller in the size, and contained small dark nuclei. In contrast, Leydig cells in the control testes had large bright nuclei and much more cytoplasm (Fig. 3-3A-C). Leydig cell number was also significantly low ($P <$

0.05) in the testis of phytosterols-treated quails (800 mg/kg BW compared with controls (Table 3-3).

3.3.5 Cloacal gland size and foam amount

The size of the cloacal gland was small ($P < 0.05$) in the phytosterols groups (80 mg and 800 mg/kg BW) compared with controls. However, the amount of cloacal foam was not different among control and phytosterols-treated quails (80 mg, and 800 mg/kg BW) (Table 3-3).

3.3.6 Semen collection and fertilization ability

As illustrated in Table 3-4, no significant difference was found in the semen quality including the semen quality factor (SQF), concentration of spermatozoa, and percentage of live and dead spermatozoa, or the percentage of the normal and deformities spermatozoa. However, the fertilization rate of the eggs fertilized by phytosterols-treated male Japanese quails was slightly reduced as compared to control (Table 3-5).

3.3.7 Real time PCR for gene expressions analysis

No difference was found in the expression of steroid acute regulatory protein (stAR) or 3β -hydroxysteroid dehydrogenase (3β -HSD) genes among the control and phytosterols-treated animals. Moreover, the expression of the 17β -HSD gene was low at 800 mg/kg BW of phytosterols compared with controls as shown in Fig. 3-4.

3.3.8 Interstitial cells testosterone production and gene expression

Compared to the intact cells, phytosterols dose-dependently decreased testosterone levels. Moreover, a similar pattern was observed in testosterone levels in the media from the O-LH stimulated cells and phytosterols-treated cell. Compared to control, testosterone levels were low in phytosterols at the dose of 87.5 $\mu\text{g/ml}$ ($P < 0.05$), (Fig. 3-5A). No difference was found in the expression of stAR, 3β -HSD, or 17β -HSD genes among the phytosterols-treated cell with or without o-LH stimulation and controls (Figs. 3-5B, 3-5C and 3-5D, respectively). Although, phytosterols treatment significantly reduced testosterone levels and steroidogenic enzymes gene expression, O-LH effectively induced testosterone and the expression of stAR, 3β -HSD and 17β -HSD genes in all groups.

3.4 Discussion

During this study, animals did not show the toxicity sign and no died quail were observed. In contrast to our preliminary finding, phytosterols had no effects on the body and organs (gonads and adrenal glands) weight. However, slightly lower feed intake was observed during growing periods in phytosterols-treated cohorts compared with control. Surprisingly, it is in contrast to our previous unpublished data which I found that feeding phytosterols in high doses had significantly effects on the testis weight during maturation. In addition, these results are in agreement with previous reports in the chicken (Elkin and Lorenz, 2009) and hamster (Vanstone *et al.*, 2001). I assume that phytosterols might be delayed the maturation time in the Japanese quail.

Indeed, the direct effects of phytosterols on the testis were the main question for this research. It was found that endocrine organs (gonads and adrenal glands) are the preferential organs for accumulation of plant sterol/stanol (Boberg *et al.*, 1986; Wolfreys

and Hepburn, 2002). Moreover, steroids syntheses in the testis are strongly depend on the LDL cholesterol level as precursor. On the other hand, the phytosterols efficacy on cholesterol lowering is mostly summarized on the LDL cholesterol level (Tilvis and Miettinen, 1986; Vanstone *et al.*, 2001). In previous research I found that feeding of phytosterols in higher doses significantly decreased the testosterone level in the male and female Japanese quail while the basal level of LH was constant (unpublished data). Similar results were previously reported in goldfishes (Maclatchy and Vanderkraak, 1995; Sharpe *et al.*, 2006). Awad *et al.* (1998) demonstrated that Feeding of phytosterols had significantly reduced the testosterone level in rats. Singh and Gupta (2016) confirmed that β -sitosterol has antifertility activity in rats. These results strongly support the supposition that phytosterols might be has direct effects on the gonadal androgen production *in vivo* and *in vitro* since the pulsatile secretion of LH was not changed and no pituitary responded to phytosterols. Thus, I speculate that testosterone reduction might be the cause of low cholesterol (LDL cholesterol) or may be due to accumulation side effects.

It is well known that the Leydig cell has an undeniable role on the testosterone production and spermatogenesis (Li *et al.*, 2006). In this study, histological staining of the testis was revealed that the Leydig cells in terms of morphology and size was different between control and phytosterols treatment. In control testis, the Leydig cells had bigger size having large amount of cytoplasm while the Leydig cells in the testis of phytosterols treated quails were smaller size and mostly had the shape similar to fibroblast like cell. The Leydig cells had irregular and relatively low amount of cytoplasm. Moreover, the Leydig cells number was significantly low in the group of 800mg/kg phytosterols treatment. Based to previous knowledge, two mechanisms might be involved in the

impairment of leydig cell function: 1) phytosterols as feed additives might delay the Leydig cell maturation. 2) phytosterols as ligands for the Liver X receptor α and β (LXR, obligate heterodimers belong to a subclass of nuclear receptors) which has responsibility for the lipid homeostasis in the cell, may apply their effects on the Leydig cells. LXR α which is highly expressed in the Leydig cells has apoptotic function while the LXR β is mostly expressed in the Sertoli cell has proliferative functions (Bradford and Awad, 2010; El-Hajjaji *et al.*, 2011; Maqdasy *et al.*, 2016).

Cloacal gland size and the amounts of foam production in male quails are closely related to the gonadal activity (Biswas *et al.*, 2007; Hanafy *et al.*, 2016). The remarkable testosterone dependent functionality of the gland makes a valuable indicator for the sexual activity (Li *et al.*, 2006; Biswas *et al.*, 2007; Hanafy *et al.*, 2016). The present study revealed that phytosterols administration in higher doses has significantly decreased the cloacal gland size. However, the cloacal amounts of foam production were not significant different between the groups. The smaller size of cloacal glands in phytosterols-treated group might be the cause of low testosterone level in the circulation.

In male quails, the sex-related behaviors are quite sensitive to the low androgen level, perhaps the role of testosterone is more crucial (Ball and Balthazart, 2010; Hanafy *et al.*, 2016). In this study, given phytosterols for the animals had significant effects on the animals' sex libido and courtship. Male quails especially in the high dose phytosterols feeding had significantly low mounting and copulation behaviors when compared with control. I assume that low sexual activities in the phytosterols groups might be the cause of low level of testosterone. Moreover, phytosterols may have central effect on the brain because phytosterols can easily pass the BBB (Saeed *et al.*, 2015).

Despite of poor knowledge about the basic molecular mechanism of phytosterols on testis, it is generally believed that phytosterols have regulatory effects on the two major cholesterol regulatory pathways, LXR and sterol response element binding protein (SREBP) (Calpe-Berdiel *et al.*, 2009). Phytosterols are known as naturally ligand for the LXR (Kaneko *et al.*, 2003; Volle and Lobaccaro, 2007; Chuu, 2011). In the steroidogenic tissues, stAR, 3 β -HSD and 17 β -HSD are the target protein for the LXR (Volle and Lobaccaro, 2007). On the other hand, stAR, 3 β -HSD and 17 β -HSD are the key enzymes involved both, in cholesterol transport and testosterone biosynthesis (Bello *et al.*, 2014). Phytosterols feeding didn't significant change the testicular expression of stAR and 3 β -HSD genes in this study. However, the expression of 17 β -HSD was slightly low in the dose of 800 mg/kg phytosterols compared with control. Consequently, lower expression of 17 β -HSD might also cause the low testosterone (active androgen) level due to the low conversion rates of androstenedione. Because, low expression of 17 β -HSD is the sign of immature testis in birds and immature birds can produce more androstenedione than testosterone (Deviche *et al.*, 2011). In addition, no significant changes were found on the expression of stAR, 3 β -HSD and 17 β -HSD in the *in vitro* experiment.

In conclusion, phytosterols as feed additive had direct effects on the reproductive endocrine function and the sex-related behaviors in the male Japanese quail. Indeed, the phytosterols effects are stronger in the specific Leydig cells then Sertoli cells and germ cells. Phytosterols might delay the animal maturation and presumably make unbalance the lipid metabolism in the testis.

Table 3-1. Primer sequences used for real time PCR

Gene	Primer pairs	Accession No.
Quail stAR	F, (TTTGCAGCAGTCACCAGAAC) R, (GACAACTTGCTGAGCTCCTG)	XM015883089.1
Quail 3 β -HSD	F, (GACTGCTGGACAAAGCCTTC) R, (CGATGATGGATGCTGTATGG)	XM015874122.1
Quail 17 β -HSD	F, (TCTTGGTGTGGGAATGTGAA) R, (CCGGAATAGAAGGAACACCA)	XM015849994.1
Quail β -Actin	F, (ACCCCAAAGCCAACAGAGA) R, (AGGCATACAGGGACAGCACA)	NM001199954.1

F, Forward primer; R, Revers primer; No. Number.

Table 3-2. Body and organs weights, feed intake and blood cholesterol level

Groups	Body weight (g)	Testis weight (g)		Adrenal gland weight (g)	Total feed intake (g)*	Plasma cholesterol level (mg/dL)
		Right	Left			
Control	118 \pm 2.92	1.92 \pm 0.1	2.08 \pm 0.13	0.009 \pm 0.001	4722.1	140.32 \pm 5.7
80 mg/kg BW	115 \pm 075	1.89 \pm 0.1	2.08 \pm 0.11	0.01 \pm 0.001	4228.2	133.59 \pm 6.2
800 mg/kg BW	115 \pm 2.41	1.79 \pm 0.1	2.07 \pm 0.05	0.01 \pm 0.001	4494.2	111.68 \pm 16.8

*Feed intakes represent the total daily intake by 10 animals for 35 days (15 to 50 d of age).

Total animals in each group = 10; BW, body weight.

Table 3-3. Testosterone, LH, Leydig cell number, cloacal glands size and foam amount in the male quail after phytosterols treatment

Groups	Testosterone (pg/mg)	LH (pg/mL)	Leydig cell No./550 μ m area	Cloacal gland size (cm)	Amount cloacal foam (mg)
Control	Log 3.35 ^a	Log 1.16	65.13 \pm 3.63 ^a	1.86 \pm 0.03 ^a	0.021 \pm 00
80 mg/kg BW	Log 3.28 ^a	Log 1.12	53.12 \pm 3.92 ^a	1.64 \pm 0.06 ^b	0.019 \pm 00
800 mg/kg BW	Log 3.11 ^b	Log 1.18	50.76 \pm 2.45 ^b	1.62 \pm 0.08 ^b	0.02 \pm 00

Values are representing as means \pm SEM, and different letters denote the significant difference from the controls. BW, body weight; No, number. Each group contained 10 animals.

Table 3-4. Semen volume, quality factor and percentages of different spermatozoa in control and phytosterols-treated male Japanese quail

Characteristics	Groups (10 animals each group)		
	Control	80 mg/ kg BW	800 mg/kg BW
Ejaculate velum [ml]	0.005	0.005	0.005
Sperm concentration [x106 ml]	394 \pm 31	388 \pm 65	384 \pm 39
SQF (%)	16.77	15.79	15.40
Live sperm (%)	77.08 \pm 1.11	77.11 \pm 0.52	74.44 \pm 0.78
Dead sperm (%)	22.92 \pm 1.11	22.89 \pm 0.52	25.56 \pm 0.78
Live normal sperm (%)	85.12 \pm 1.59	81.40 \pm 0.88	80.22 \pm 1.02
Live deformities sperm (%)	15.67 \pm 1.68	18.76 \pm 0.83	20.02 \pm 0.78

SQF, semen quality factor;

Table 3-5. Total numbers and percentages of fertilized and unfertilized eggs laid by intact females mated with control and phytosterols-gavaged male Japanese quails

Groups	Animals	Total eggs	Fertilized eggs		Unfertilized eggs	
			No	%	No	%
Control	10	67	51	76.12	16	23.88
80 mg/kg BW	10	62	42	67.74	20	32.26
800 mg/kg BW	10	65	45	69.23	20	30.77
P< 0.86						

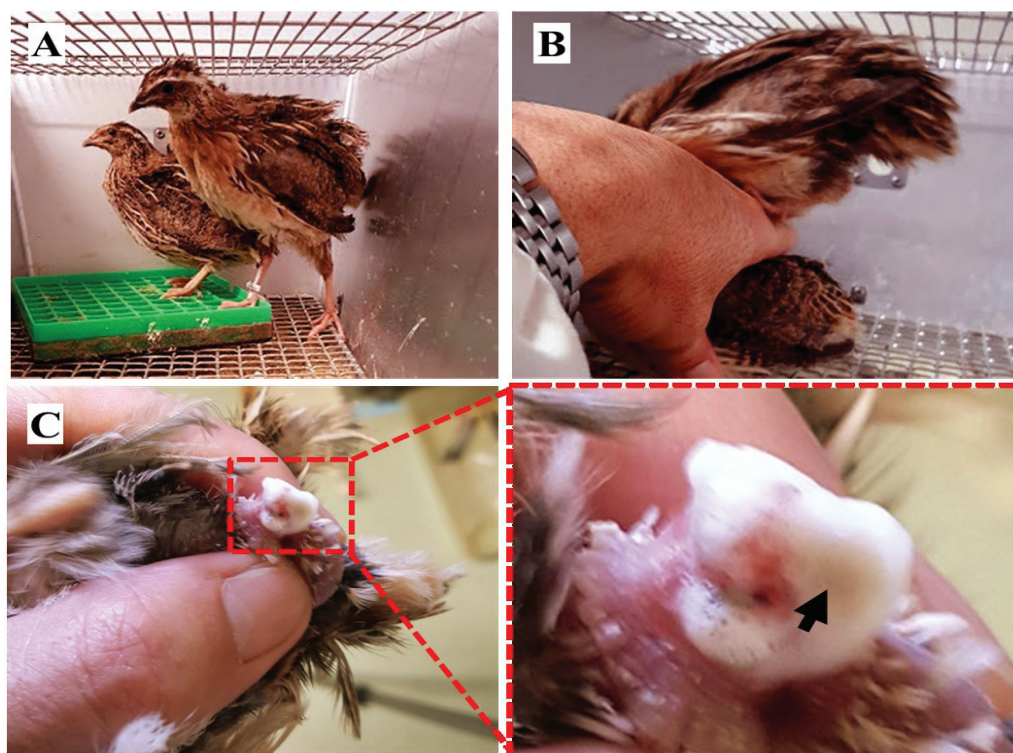


Fig. 3-1. Female teaser method for semen collection in male Japanese quails. A couple of (male and female) quails were placed in the individual cage (A), and the left hand was placed under the male cloacal vent to prevent unintentional insemination (B). Semen was collected from the dense and viscous area by gently squeezing of the cloacal vent of excited male (C).

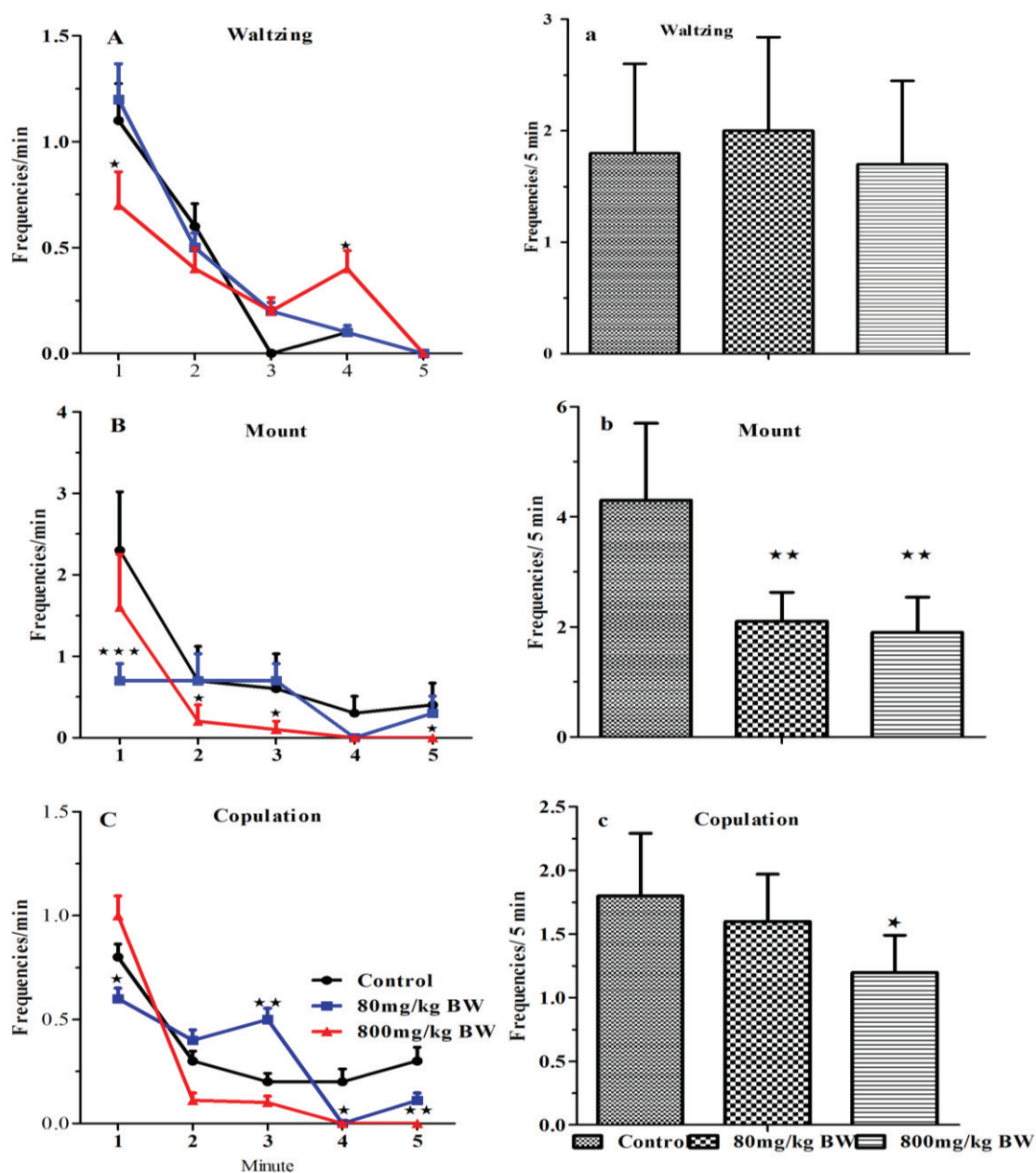


Fig. 3-2. Frequencies of waltzing, mount, and copulation behaviors in control and phytosterols-treated male Japanese quails. Line graphs represent the frequencies of waltzing (A), Mount (B) and copulation (C) behaviors in each min in control (black line), and phytosterols in the dose of 80 mg (blue line), and 800 mg/kg BW (red line). Bar graphs had shown the frequencies of waltzing (a), mount (b) and copulation (c) in total five min among the groups. Asterisk denote the significant from the control ($P < 0.05$).

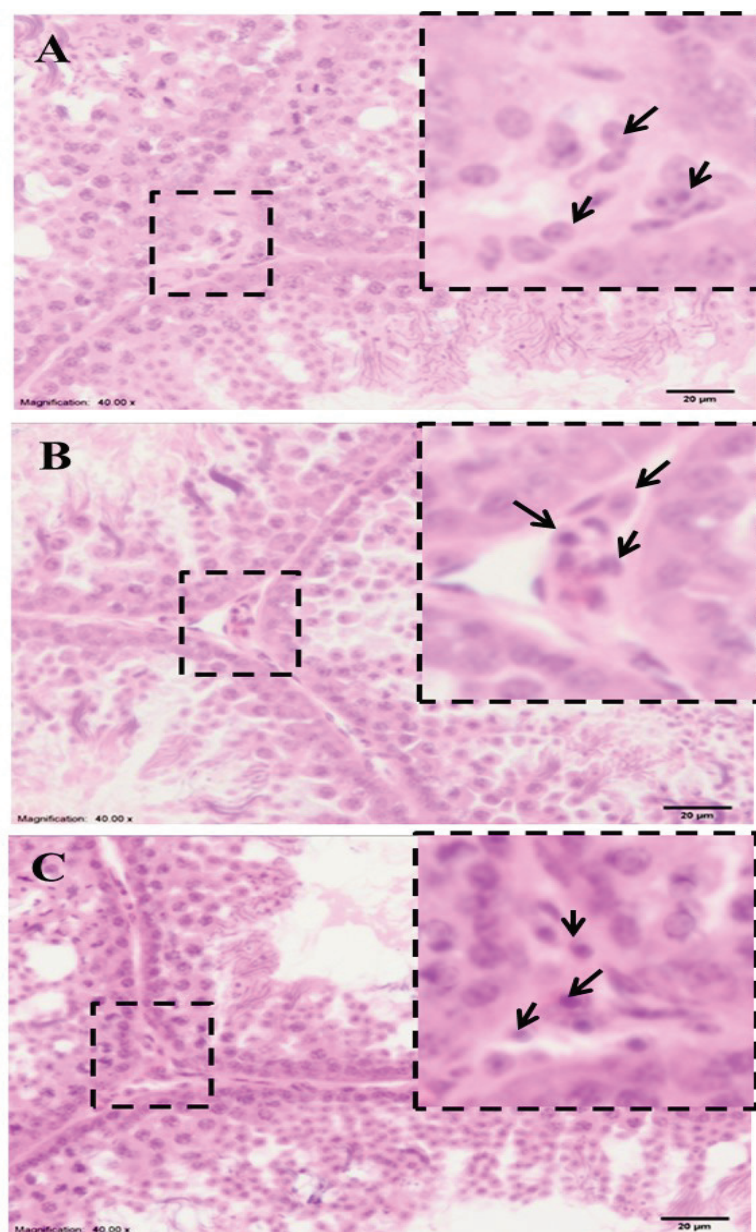


Fig. 3-3. Histological structure of testes in control and phytosterols-treated male Japanese quails. Leydig cells in the testes of control (A) animals were bigger in the size having bright nuclei surrounded by much cytoplasm. While in the testes of phytosterols-treated male quails (80 mg [B] and 800 mg/kg BW [C]) Leydig cells were smaller and low amounts of cytoplasm surrounded the dark nuclei of the cells. Horizontal smaller pictures (having black border) represent the magnification of black squares pointed in the sections (black arrows). Objective magnification, x40.

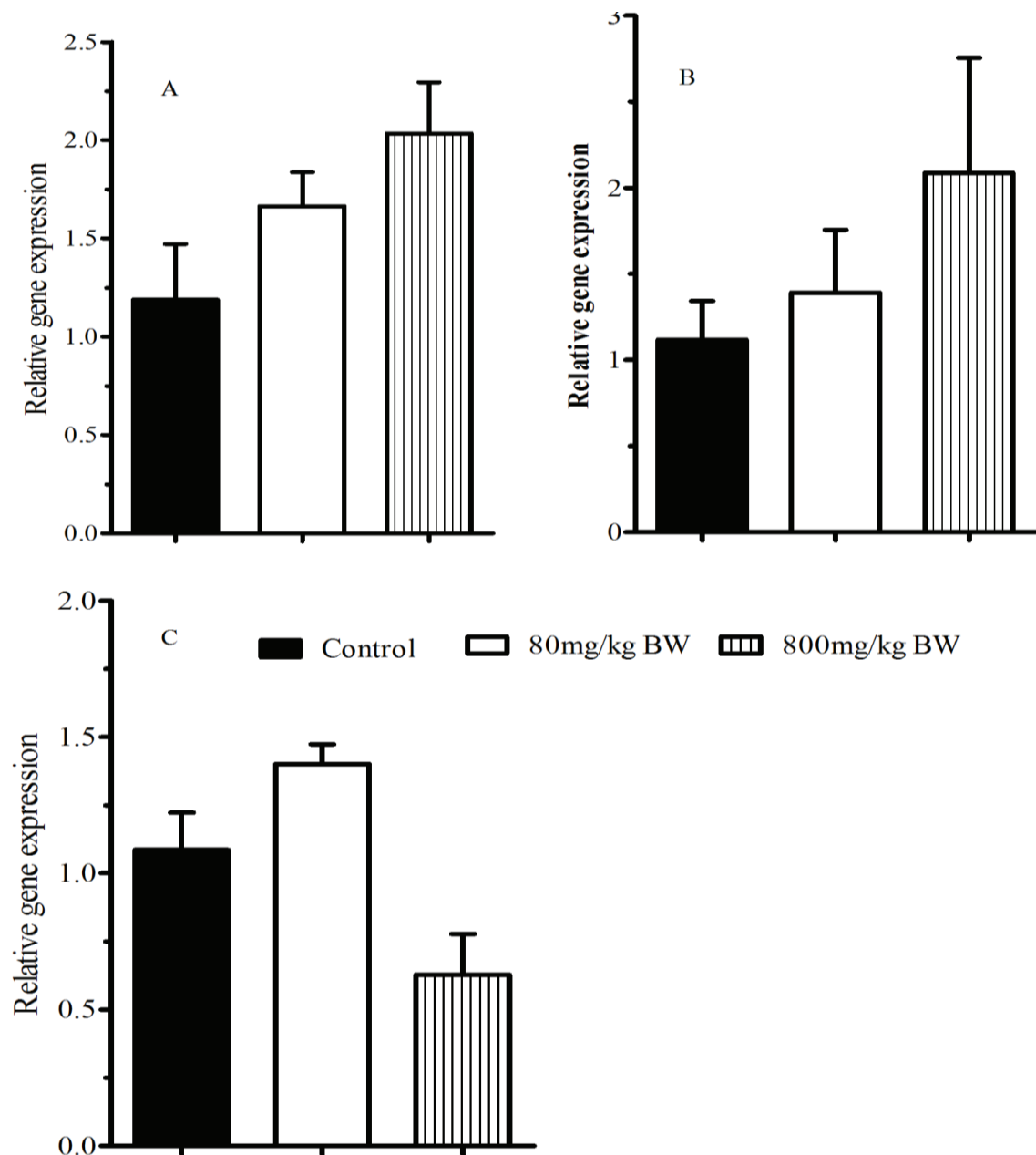


Fig. 3-4. Relative gene expression of cholesterol converting enzymes in the testis of Japanese quails. Bar graphs represent the expression of stAR (A), 3β-HSD (B) and 17β-HSD (C) genes in the testis of control and phytosterols-treated quail.

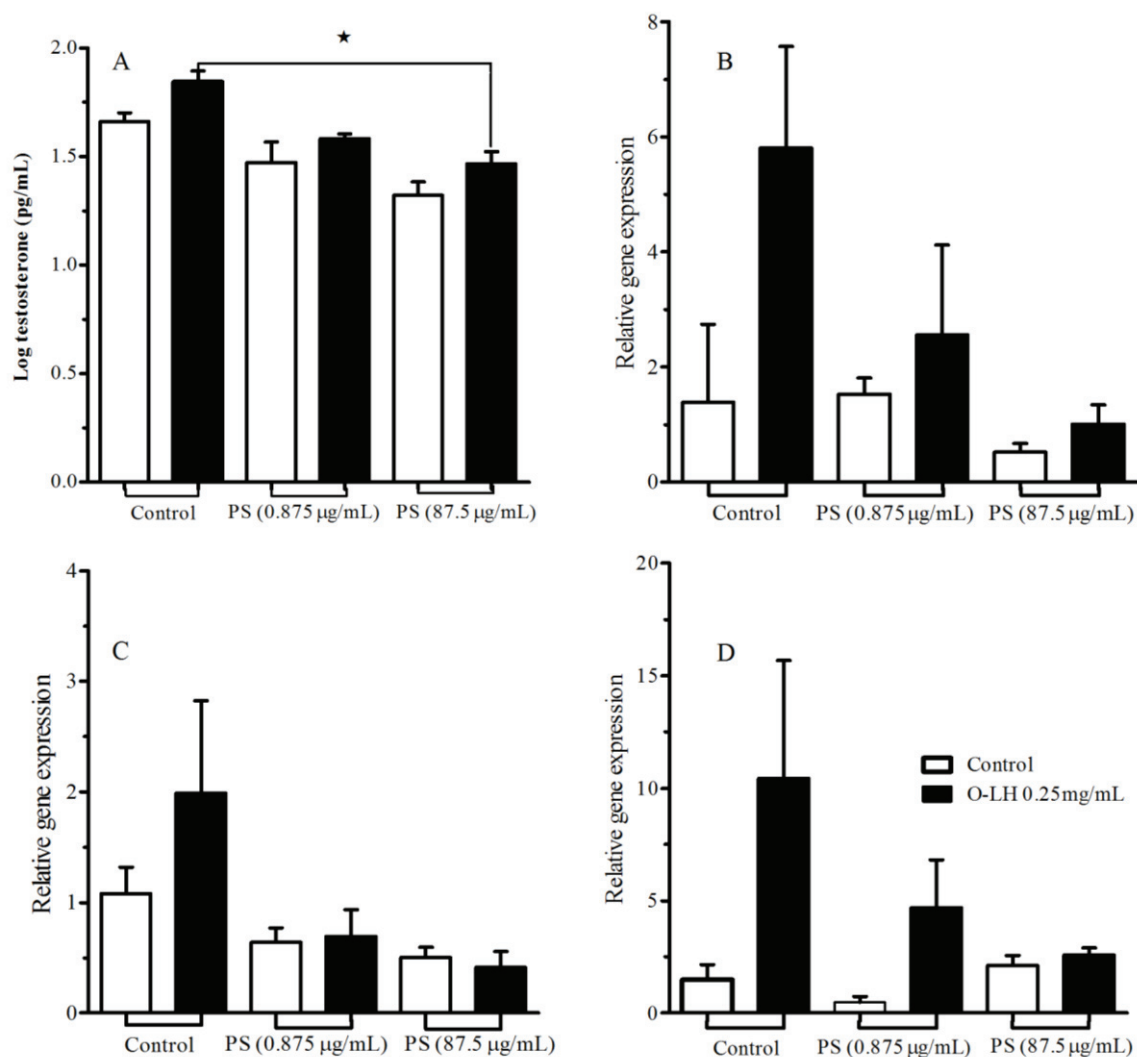


Fig. 3-5. Testosterone concentrations and steroidogenic enzymes expression in the interstitial cell isolated from the testes of male Japanese quails. Dose dependent effects of phytosterols on testosterone secretion by cultured interstitial cells from male quail testis (A), and relative gene expression of stAR (B), 3 β -HSD (C), and 17 β -HSD (D). White bars represent unstimulated intact controls and phytosterols-treated cells and the black bars designate groups stimulated by O-LH (0.25 mg/mL). Each bar graph represents mean \pm SEM, and the asterisks denote statistical significance ($P < 0.05$).

CHAPTER FOUR

Effects of phytosterols on the hypothalamic endocrine function in the adult male Japanese quail

4.1 Background

Phytosterols also called plant sterols, are natural compounds in plants, structurally similar to cholesterol except for some extra ethyl (β -sitosterol) or methyl (campesterol) groups in the side chain (Lagarda *et al.*, 2006; Brufau *et al.*, 2008; Ras *et al.*, 2013). Phytosterols significantly reduce intestinal cholesterol absorption and widely use as anti-atherosclerosis. Moreover, the anti-inflammatory, anti-cancer and anti-oxidative activity of phytosterols have been well documented (Moghadasian, 2000; Yoshida and Niki, 2003; Lagarda *et al.*, 2006).

In previous chapters, I found that gavage of high doses of phytosterols to Japanese quails reduced testosterone concentrations without significantly altering LH levels in Japanese quails. Although, testosterone reduction by phytosterols feeding has been previously reported in rats (Awad *et al.*, 1998) and goldfishes (Maclatchy and Vanderkraak, 1995). Recently, it was found that phytosterols, in contrast to cholesterol, can efficiently pass the BBB, and irreversibly accumulate in brain cellular membranes (Vanmierlo *et al.*, 2012). Shi *et al.* (2015) reported that membrane β -sitosterol in brain is able to prevent tumor necrosis factor- α (TNF- α)-induced gonadotropin-releasing hormone (GnRH) decline, and prevent aging. However, it is unknown whether phytosterols have functional roles on GnRH and gonadotropin-inhibitory hormone (GnIH) secretion, and the hypothalamic-pituitary-gonadal axis regulation or not.

In the current chapter, the evidence of the accumulation of phytosterols in brain cell membranes (Vanmierlo et al., 2012; Saeed et al., 2015) and gonads (Lagarda et al., 2006; Calpe-Berdiel et al., 2009) has motivated me to further investigate this aspect and expand my previous findings regarding phytosterols effects on reproduction. In the present study, I investigated the possibility that chronic feeding of high doses of phytosterols played regulatory roles in reproductive endocrine function through the hypothalamic-pituitary-gonadal axis (HPG axis) in male Japanese quails. I hypothesized that phytosterols would affect both GnRH and GnIH expression in brain or locally in testes, and subsequently reduce testosterone production in adult male quails

4.2 Materials and methods

4.2.1 Chemicals

Phytosterols and MCT were provided from the same companies and in the same qualities as referenced in chapter two. Chicken-gonadotropin-releasing hormone-1 (cGnRH-1) was purchased from LKT Laboratories, Inc. (Lot# 2596106).

4.2.2 Laboratory animals

Male quail chicks were obtained by incubating of the fertilized eggs of our laboratory stock. The quails were kept in the similar environmental conditions detailed in chapter two, and provided by the chicks and adults feed ad libitum (Cosmos Company, Aichi, Japan).

4.2.3 Experimental design

A total of 30 male quail chicks were randomly allotted equally to 3 groups (control, 80 mg/kg BW phytosterols and 800 mg/kg BW phytosterols) after segregation from the female chicks by feather color at the age of two weeks. Daily single doses of either phytosterols solution or MCT were gavaged into the crop sac of each individual quail from 15 to 100 days of age. On the last day of the experiment, each group was divided into 2 subgroups: 1 subgroup received 100 μ l of phosphate-buffer saline (PBS) solution/quail (“sham controls”), the other subgroup was challenged with single intramuscular injection of 10 μ g of chicken GnRH-1 (cGnRH-1) in 100 μ l PBS/quail (Davies and Collins, 1979). Blood was collected before and 30 min after injection of GnRH or vehicle from the jugular venipuncture and decapitation under sedation with diethyl ether, respectively. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared at the Tokyo University of Agriculture and Technology.

4.2.4 Tissues harvesting and preparations

Blood samples were collected into heparinized micro-tubes and the plasma was separated by centrifugation at 2700g for 15 min at 4°C, and stored at -20°C for hormonal measurements. Whole brain and left testes were immediately harvested after sacrifice and kept under -80 °C for further PCR analysis. Individual pituitary glands were kept in tubes containing ice-cold 1 ml of physiologic saline. The pituitary gland supernatant was obtained by homogenization and centrifugation at 20,000g for 30 min at 4°C, and stored at -20°C for LH assay as reported previously (Ahmed et al., 2015).

4.2.5 Hormonal analysis

Testosterone concentrations in plasma and LH levels in plasma and pituitary extraction were measured using double-antibody RIA with ^{125}I radioligand (for details refer to chapter two).

4.2.6 RNA isolation and real time PCR

Total RNA was extracted from the testes and the diencephalon area of brain using Isogen reagent kit according to the manufacturer's instructions (Nippon Gene, Tokyo, Japan). cDNA was synthesized using PrimeScript reverse transcriptase (TaKaRa Bio, Shiga, Japan) according to the manufacturer's protocol. Oligonucleotide primers were chosen with the assistance of Primer3, a Web-based online software system (Table 1). Real-time PCR was performed using Ex TaqR Hot Start Version containing SYBR-Green I (TaKaRa Bio). The expression of each target mRNA relative to β -actin was determined using the $2^{-\Delta\Delta\text{CT}}$ method.

4.2.7 Statistical analysis

All results are presented as means \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism5 (San Diego, CA, USA). A D'Agostino-Pearson normality test was used to assess the hormonal data. To determine statistical significance among groups at each time-point, one-way analysis of variance (ANOVA) and Dunnett's test were performed. Real-time PCR data were analyzed using a Kruskal-Wallis test for non-parametric data. A two-way ANOVA analysis for repeated measures was performed to compare the time-course changes in hormonal levels before and 30 min after cGnRH injection groups. $P < 0.05$ was considered to be statistically significant.

4.3 Results

4.3.1 Chronic effects of phytosterols

As illustrated in Table 4-2, phytosterols feeding not significantly altered the quails body, testis, and adrenal glands weights compared with control. However, slightly decrease was found in cloacal gland size in phytosterols-treated animals compared to control. The physiological condition of the quails fed phytosterols seems normal, and no signs of toxicity and dead were observed through the experiment period. However, a decreasing tendency was observed in total plasma cholesterol levels after long-term phytosterols gavage as compared to control quails.

4.3.2 Plasma testosterone and LH levels

Testosterone concentrations dose dependently decreased in the phytosterols-treated male Japanese quails. Testosterone concentrations were significantly low after phytosterols gavage at the dose of 800 mg/kg BW as compared to control before cGnRH-1 stimulation ($P < 0.05$). Moreover, testosterone levels were significantly increased after 30 min cGnRH-1 injection as compared with the sham control groups ($P < 0.001$). However, testosterone concentration was significantly low in the phytosterols-treated animals 30 min after stimulation with cGnRH-1 as compared to control ($P < 0.01$) (Fig. 4-1).

LH levels show a tendency of dose dependent decrease in phytosterols-treated male quails. However, LH concentrations were not statistically difference among the groups before cGnRH-1 stimulation. LH concentration was significantly increased 30 min after cGnRH-1 stimulation compared to sham control groups ($P < 0.001$). In addition, phytosterols-treated quails at the dose of 800 mg/kg BW field to increase LH levels as

compared to control ($P < 0.05$) (Fig. 4-2). Concomitantly, LH level was also low in the extract of pituitary glands of quails fed 800 mg/kg BW after stimulation with cGnRH-1 compared to control ($P < 0.05$) (Fig. 4-3).

4.3.3 GnRH-1 and GnIH gene expression in brain and testis

Non-stimulated male quails were fed phytosterols showed no significant decrease in the expression of hypothalamic GnRH-1 compared to controls. However, the expression of GnRH-1 was not significantly affected by cGnRH-1 stimulation (Fig. 4-4A). In contrast, GnIH expression was dose dependently increased with phytosterols treatment, significantly increase found in the dose of 800 mg/kg BW of phytosterols as compared to control ($P < 0.05$). However, GnIH expression was not different among the groups after stimulation of cGnRH-1 (Fig. 4-4B).

As illustrated in Fig. 4-5A, testicular GnRH-1 expression was relatively lower in the phytosterols-treated animals before GnRH-1 stimulation as compared to control. However, the expression of GnRH-1 was significantly low after 30 min injection of GnRH-1 in the testes of quails fed 800 mg phytosterols/kg BW compared to control ($P < 0.05$). No difference was found in the testicular expression of GnIH among the groups before GnRH-1 stimulation. In addition, GnIH expression was significantly

4.4 Discussion

This is the first report demonstrating the effects of phytosterols on the expression of hypothalamic and testicular GnIH and GnRH-1 in adult male Japanese quails, and subsequent effects on reproductive endocrine regulation. Male quails were injected with cGnRH-1 to stimulate release of LH from the anterior pituitary gland and thereby allow

me elucidate the regulatory role of phytosterols on HPG axis. In the current study, I found that long-term phytosterols feeding itself reduced testosterone concentrations without significantly altering LH levels in plasma and pituitary of adult male quails. Moreover, cGnRH-1 stimulation significantly enhanced LH release from pituitary and testosterone from testis as well. However, the quails fed phytosterols failed to increase LH and testosterone concentrations relative to control quails. Similar results I previously found the similar results discussed in chapter two and three. Moreover, a decrement in testosterone caused by feeding of phytosterols feeding has been previously reported in rats (Awad *et al.*, 1998), goldfishes (Maclatchy and Vanderkraak, 1995; Sharpe *et al.*, 2007); and infertility in male and female sterolin-deficient mice (Solca *et al.*, 2013). Singh and Gupta (2016) reported that treatment with β -sitosterol isolated from the roots of Porcupine flower (*Barleria prionitis*) dose-dependently reduced testosterone, FSH, LH, sperm quality in male albino rats. However, daily intakes of 2 g of phytosterols for two weeks did not significantly effects on testosterone, FSH and sex hormone-binding globulin levels in men; or estradiol, FSH and sex hormone-binding globulin levels in women (Volpe *et al.*, 2001). The authors suggested that, feeding of phytosterols in the lower doses may not have adverse effects on reproductive endocrine function. Previously I found that long-term phytosterols gavaging especially in the dose of 800 mg/kg BW significantly reduced testicular weights and testosterone level during the growing period in male Japanese quails. The result in chapter three showed low levels of testosterone and Leydig cells number in male Japanese quails. I concluded that long-term phytosterols feeding may reduce testosterone levels by directly affecting on gonadal maturation or interfering with the functions of the enzymes responsible for cholesterol trafficking for steroidogenesis mainly 17 β -HSD which convert androstenedione to testosterone. From

these results, I can clearly conclude that phytosterols has effects directly (on testicular function) and indirectly (through the HPG axis) on testosterone productions in male quails.

In this chapter, phytosterols-treated male quails exhibited lower expression of GnRH-1 in brain and testis; and although the injection of cGnRH-1 to male quails did not change the expression of GnRH-1 in brain, testicular expression was significantly reduced in phytosterols-treated animals. These results are inconsistent with a previous report that found that accumulated β -sitosterol in brain membrane prevented an inflammatory reduction of GnRH in vitro (Shi *et al.*, 2015). The lipophilic structure of phytosterols facilitates efficient passage through the BBB and allows it to accumulate in brain cell membranes, while circulating cholesterol cannot enter the brain (Vanmierlo *et al.*, 2012). Thus, it is possible that membrane phytosterols may alter GnRH response to feedback by low testosterone production from the testes, or hypothalamic GnRH-1 expression may be downregulated by local aromatization of testosterone to estrogen (Sun *et al.*, 2001) due to the estrogenic activity of phytosterols as previously reported (Awad and Fink, 2000). Furthermore, high expression of GnIH in phytosterols-treated animals might also reduce the expression of GnRH and LH as well.

In this study, long-term phytosterols feeding induced the expression of hypothalamic and especially testicular GnIH in male quails. The hypothalamic dodecapeptide GnIH was first reported in Japanese quails showing an inhibitory role on gonadotropin release from the anterior pituitary gland (Tsutsui *et al.*, 2000). In the testicles, GnIH and its receptors are primarily localized to Leydig cells and germ cells (spermatocytes and spermatids), and to the epididymis of birds indicate possible involvement of this peptide on the regulation of gonadal functions (Bentley *et al.*, 2008). Furthermore, the testicular high

expression of GnIH even after cGnRH-1 injection in our study support the previous in vitro finding indicated that GnIH and its receptor (GPR147) expression in the gonadotropin-stimulated testis culture significantly reduced testosterone production in house sparrow (*Passer domesticus*) (McGuire and Bentley, 2010). Additionally, high expression of GnIH both in brain and testis of phytosterols-treated male quails (800 mg/kg BW) may imply the autocrine/paracrine role of GnIH in testicular function. Local expression of GnIH in the testis may also reduce Leydig cell functions including testosterone production as discussed in chapter three.

Collectively, these results suggest that phytosterols possess HPG-axis regulatory role in the reproductive endocrine functions of male Japanese quails. phytosterols administered at a dose of 800 mg/kg BW to Japanese quail induced the expression of GnIH in brain and testis. Consequently, induction of GnIH may reduce GnRH gene expression and LH secretion, and subsequent attenuation of testosterone production by the testis. Moreover, phytosterols may induce GnIH and its receptor locally in the Leydig cells of quail testes, and thereby perturb testosterone production.

Table 4-1. Primers used for real-time PCR for analysis of the genes expression

Gene	Sequences	Accession No.
Quail GnRH-1	F, (CGCTGAAAATCTGGTGGAAT)	XM_015882894.1
	R, (TTGTTGGCGTTGTGGATTTA)	
Quail GnIH	F, (ATGGTGCGTGCCTAGATGAAC)	AB820136
	R, (AGCAACTGAATTTGGCACTTTG)	
Quail β -actin	R, (AGGCATACAGGGACAGCACA)	NM001199954.1
	F, (ACCCCAAAGCCAACAGAGA)	

The primer sequences marked as F for forward and s for revers primer.

Table 4-2. Total body, testes, and adrenal glands weights and blood cholesterol levels in control and phytosterols-gavaged male Japanese quails

Groups	Body weights (g)	Testes weight (g)		Adrenal weight (mg)	Cloacal gland (cm)	Blood size cholesterol (mg/dL)
		Right	Left			
Control	116.57 \pm 2.3	1.82 \pm 0.1	2.01 \pm 0.1	9.3 \pm 1	1.72 \pm 0.05 ^a	109.27 \pm 6.70
80 mg/kg BW	112.05 \pm 2.4	1.61 \pm 0.1	1.89 \pm 0.1	8.9 \pm 0.5	1.3 \pm 0.07 ^a	100.21 \pm 16.6
800 mg/kg BW	112.62 \pm 1.9	1.84 \pm 0.1	2.04 \pm 0.1	9.1 \pm 1.3	1.2 \pm 0.06 ^b	99.32 \pm 7.60

Values are representing as means \pm SEM. BW, body weight.

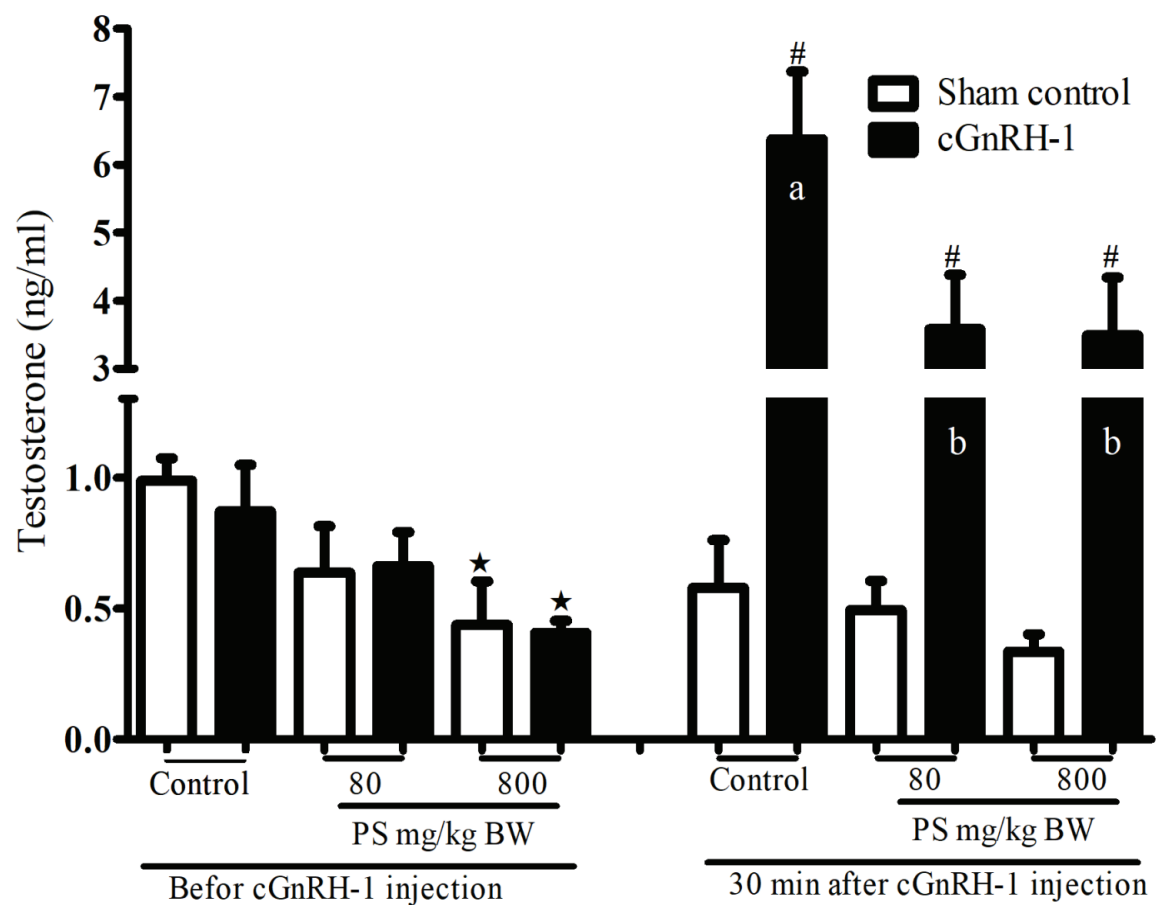


Fig. 4-1. Testosterone concentrations before and 30 min after cGnRH-1 injection in control and phytosterols-gavaged male Japanese quails. White bars represent testosterone levels in sham control and black bars shown levels in cGnRH-1 injected animals. Asterisks denote the significant difference from control individually in sham control and cGnRH-1 challenged male quails ($P < 0.05$). Different letters denote significant difference among the groups ($P < 0.01$), and the hash marks represent significant difference between sham control and cGnRH-1 injected male Japanese quails.

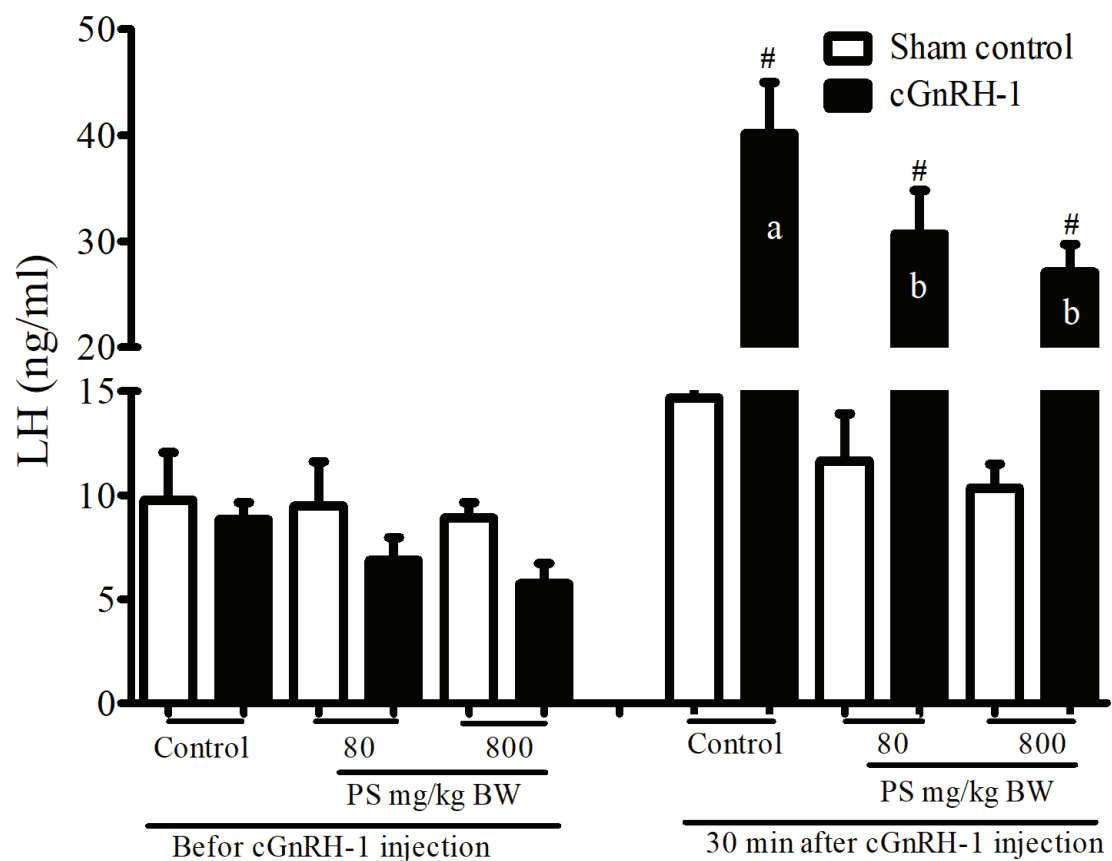


Fig. 4-2. Plasma LH levels before and 30 min after cGnRH-1 injection in control and phytosterols-treated male quails. White bars represent LH level in control (non-cGnRH -1 injection), and black bars denote LH levels in cGnRH-1 injected male Japanese quails. Different letters denote significant difference among the groups ($P < 0.05$), and Hash marks shown significant difference between sham control and cGnRH-1 challenged quails ($P < 0.001$).

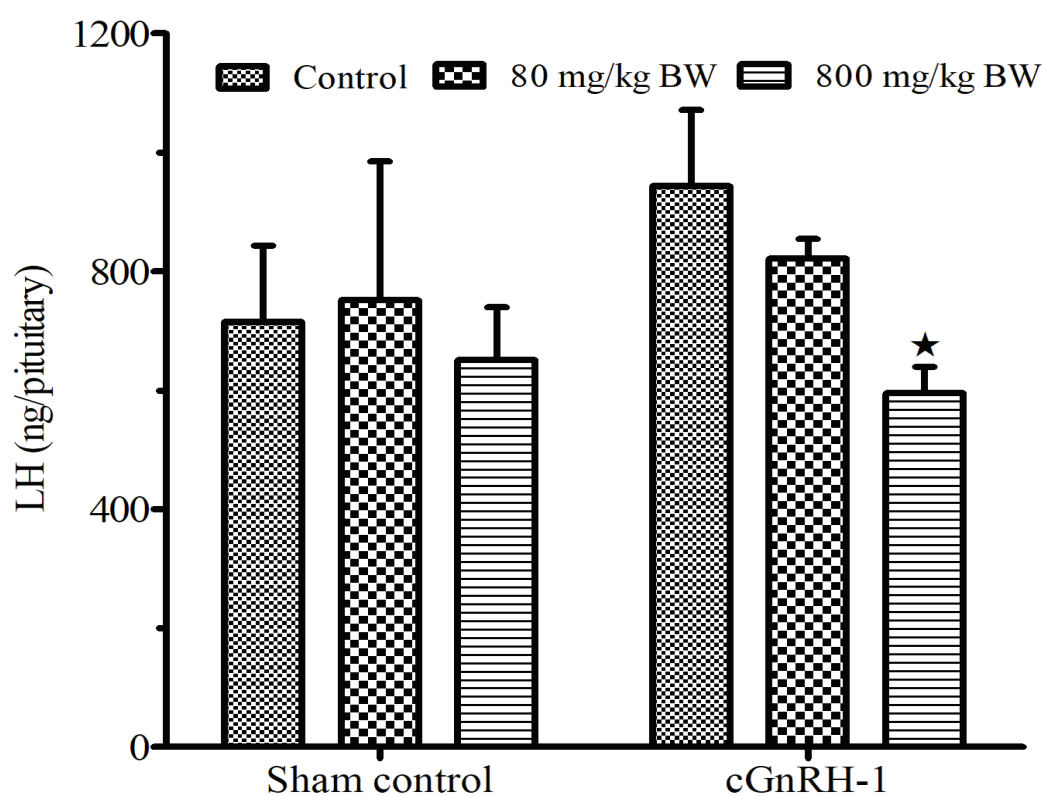


Fig. 4-3. LH levels in the extract of pituitary glands among the groups in sham control, and cGnRH-1 injected male Japanese quails. Asterisk denote significant difference from control ($P < 0.05$).

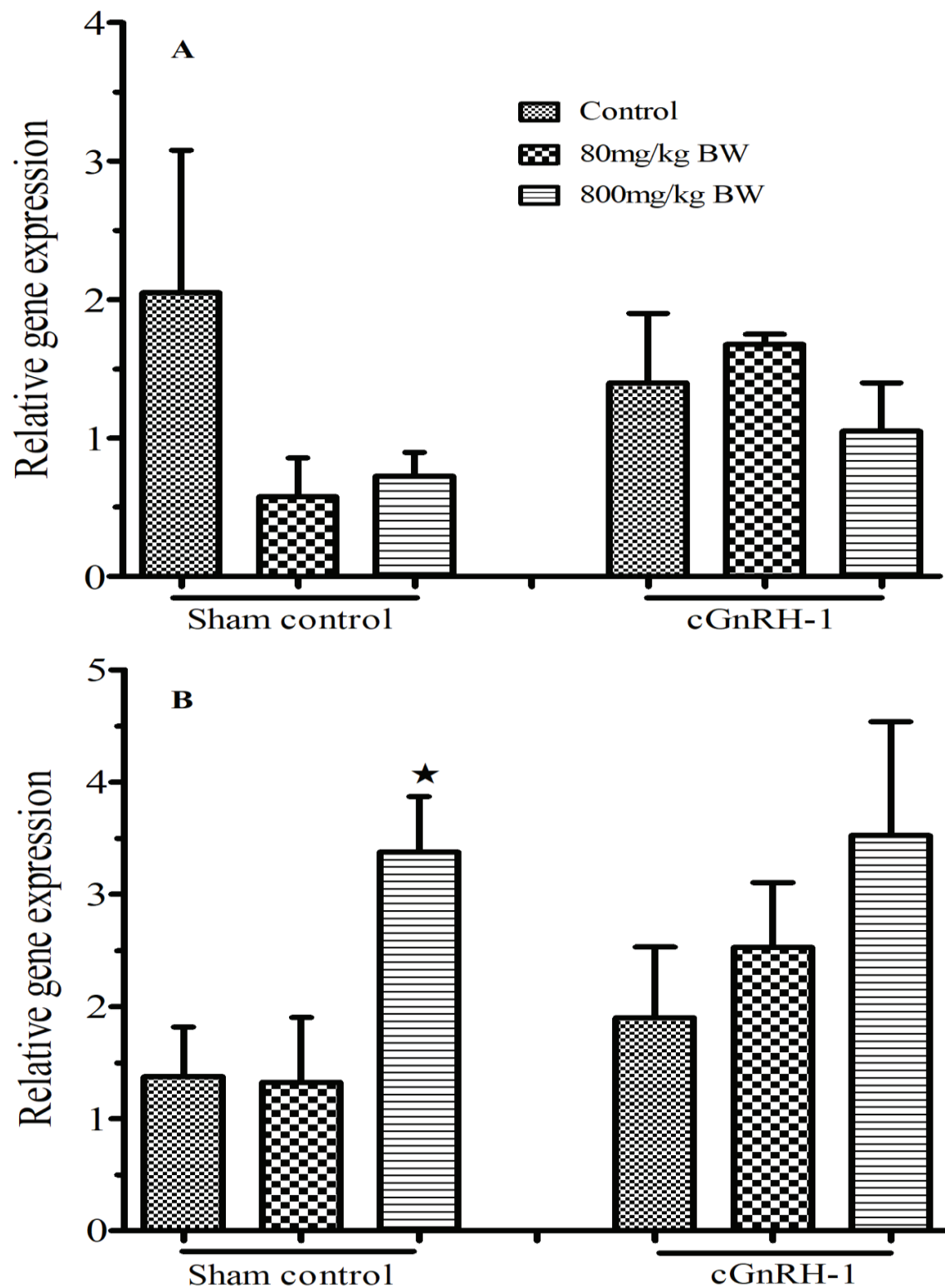


Fig. 4-4. Hypothalamic expression of GnRH-1 (A), and GnIH (B) genes among the groups in sham control and cGnRH-1 injected quails. Asterisk denote significant difference from control ($P < 0.05$).

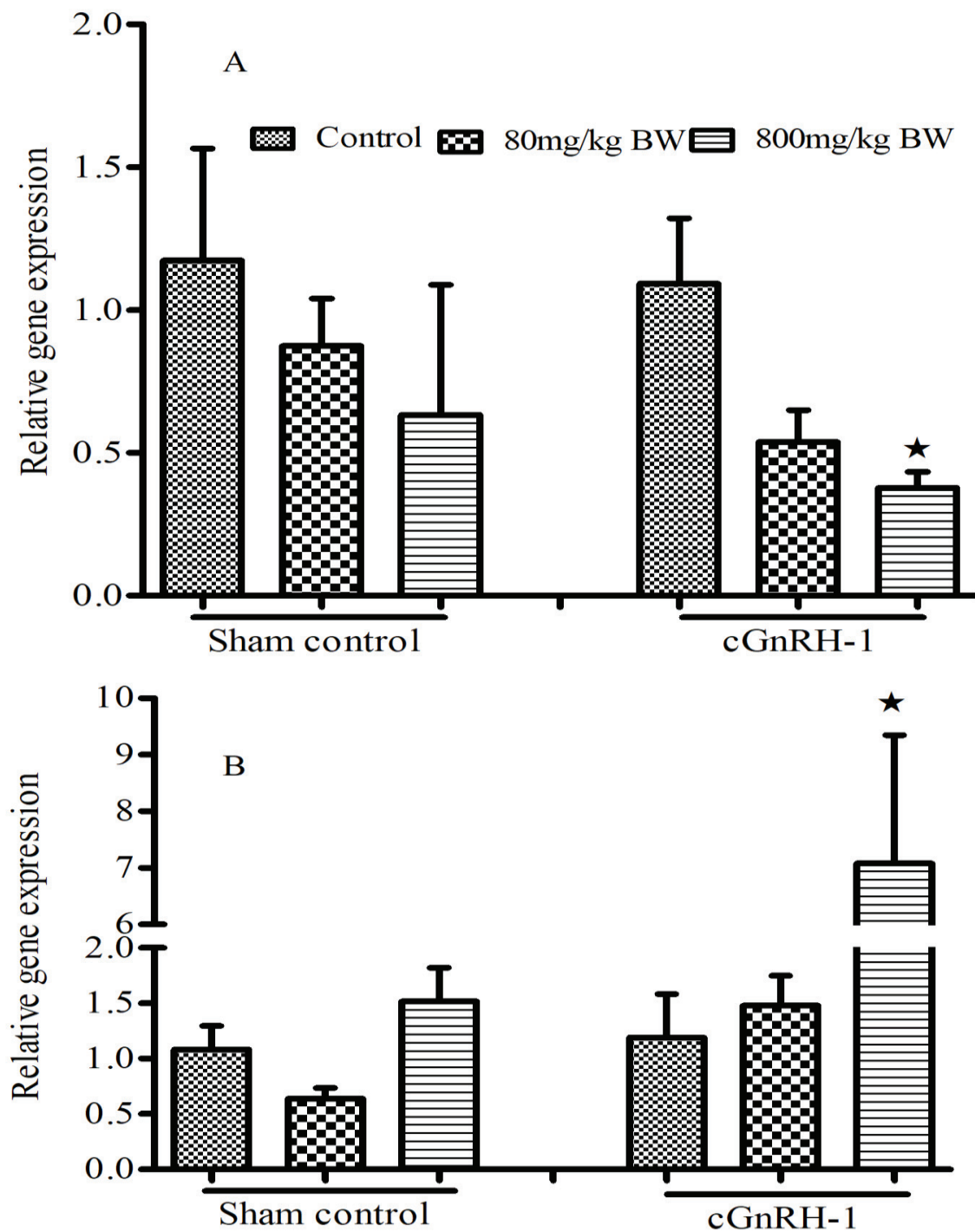


Fig. 4-5. Relative gene expressions of GnRH-1 (A), and GnIH (B) in the testes among the groups in sham control, and cGnRH-1 injected male quails. Asterisk denote significant difference from control ($P < 0.05$).

CHAPTER FIVE

Accumulation of the steroid hormones in the eggshells of Japanese quail

5.1 Background

The eggshell is a hard membrane that encloses the embryo, and mainly consists of outer (calcareous crust) and inner (mammillary) membranes (Schaafsma *et al.*, 2000; Makkar *et al.*, 2016). The eggshell plays crucial roles in avian reproduction, including (a) protecting the egg content from the surrounding physical environment, (b) controlling water and gas exchange during embryonic development in the egg, and (c) providing calcium for embryonic development once the yolk stores are depleted (Nys *et al.*, 2004). The outer layer of the eggshell is made of calcite and calcium carbonate crystals and the inner shell membrane consist of organic matters that include collagen, protein, peptides and lipids (Nys *et al.*, 2004; Makkar *et al.*, 2016).

Sex steroids are lipophilic hormones, having small molecular weight (ranged from 270-370 daltons), and are mainly produced by the adrenal cortex and gonads and transferred *via* the circulation to peripheral target tissues (Kawata, 1995). In addition, non-reproductive tissues also produce physiologically active steroids (Inoue *et al.*, 2012). In avian species, because the embryo develops outside the mother's body in the egg, mother transfer nutrients and hormones to the yolk of developing follicles (Groothuis *et al.*, 2005; Hsu *et al.*, 2016). Egg yolk contains high levels of androstenedione, testosterone and 5 α -dihydrotestosterone; while 17 β -estradiol and corticosterone are at low levels (Elf and Fivizzani, 2002). Moreover, the avian embryo is not only exposed to maternal steroids, but also to endogenously produced sex steroids which play key roles in sexual

differentiation (Tanabe *et al.*, 1979; Abdelnabi *et al.*, 2000; Ottinger *et al.*, 2001). In the chicken embryo, sex hormones were measurable as early as 3.5 d of incubation (Woods and Erton, 1978). During embryonic development, the chorioallantoic membrane (CAM) of the chicken and turtle (*Pseudemys nelsoni*) was shown to display steroidogenic function and be able to synthesize progesterone and be sensitive progesterone signaling (Albergotti *et al.*, 2009; Cruze *et al.*, 2013). Kobayashi *et al.* (2015) previously reported that embryonic sex steroids accumulate in the eggshell of the loggerhead sea turtle (*Caretta caretta*).

It is currently unknown whether the sex steroids accumulate in the avian eggshell or not, and the sources and physiologic status of the steroids are not clear for avian species. In this chapter, I attempted to quantify the eggshell steroid level during embryonic development and after hatching and find the variations between male and female.

5.2 Materials and methods

5.2.1 Sampling and eggshell processing

A total of 100 fertilized eggs were collected from our laboratory quail stock during 3 consecutive days. Before egg collection, quails were arranged in their cages in order to determine the sex-ratio, and three females and one male were placed in one cage. Stock quails were housed under a controlled environment (lights on, 0500–1900 h; temperature, $23 \pm 2^{\circ}\text{C}$; humidity, $50 \pm 10\%$; air exchanged 20 times hourly). Animals had free access to food (Cosmos Company, Aichi, Japan) and water. Stock animals were at the same age and from the same laying clutch. Eggshells were obtained from the three different stages; non-incubated eggshells (after laying), 15 d of incubation, and after hatching; and processed as follow.

5.2.1.1 After laying

Collected eggs were processed for their shells within 24 h after laying. The egg was broken into half and the yolk and albumen were gently removed from the eggshells, including inner and outer membranes. The eggshells then individually placed into 50-mL tubes and dried in an incubator at 100 °C for 24 h as previously described (Kobayashi *et al.*, 2015). Dried eggshells were pulverized using a glass stick, and then 5 mL of 80% methanol was added, vortexed for 30 min, and centrifuged for 10 min at 1700g at 4°C. The supernatant was transferred to another tube and stored for hormonal analysis.

5.2.1.2 15 days of incubation

To evaluate the variation in steroids in the eggshell during incubation and hatching, the remaining eggs were incubated at $37 \pm 5^{\circ}\text{C}$ in a humidified incubator (Showa Furanki, Tokyo, Japan). After 15 d of incubation, eggshells were obtained from the eggs in which embryos developed inside (confirmed by candling). Euthanasia of developing embryos was accomplished by freezing with ice for 30 min, a methodology approved by the Animal Care Committee of the Tokyo University of Agriculture and Technology. Collected eggshells were then processed as above. To identify the sex-related eggshells at this stage, embryos were fixed in 4% paraformaldehyde (Wako Co, Osaka, Japan) for 24 h. The morphologic (gonadal differentiation was visual) and microscopic (H&E staining) methods were used for the embryonic sex identification.

5.2.1.3 Hatching time

Before hatchling, the eggs were individually placed inside compartments made from carton box in the hatchery space. On the day of hatching, eggshells containing the CAM

were collected and cleaned residue left by the chicks by Kimwipes and processed as previous batch. Day-old chicks were tagged on their legs and kept for sexual identification through feather colors after 20 days.

5.2.2 Hormonal measurements

All procedures for hormonal measurements were performed as previously described (Kobayashi *et al.*, 2015). The concentrations of testosterone, estradiol (Taya *et al.*, 1985) and corticosterone (Kanesaka *et al.*, 1992) in the eggshell extract were measured by double-antibody RIA using ¹²⁵I-labeled radioligand. Anti-sera against testosterone (GDN250) and estradiol (GDN244) were kindly provided by Dr. G.D. Niswender (Fort Collins, CO. USA). Antiserum against corticosterone was goat anti-corticosterone produce by our laboratory (Kanesaka *et al.*, 1992).

5.2.3 Statistical analysis

For statistical analysis, GraphPad Prism5 (San Diego, CA, USA) was used. All data are expressed as means \pm SEM. The concentrations of hormones between the sexes at each time-point were statistically analyzed using the Mann-Whitney test for significance. Correlations between testosterone and estradiol, and estradiol and corticosterone were analyzed by Spearman correlation test. $P < 0.05$ was considered to be statistical significant.

5.3 Results

In this study, testosterone concentrations were at their highest levels in the eggshells of freshly laid eggs. However, concomitant with progressing incubation period and

embryonic development, testosterone levels declined. There was no significant difference in the eggshell testosterone concentrations between males and females during incubation and after hatching (Fig. 5-1).

In contrast, estradiol was undetectable in the eggshells of freshly laid eggs. However, estradiol significantly increased with incubation and embryonic development in the eggshell. Compared to male eggshells, estradiol was significantly higher ($P < 0.001$) in the female eggshells at 15 d of incubation, and slightly higher during the hatching period (Fig. 5-2).

Furthermore, low levels of corticosterone concentration were detected in the eggshells of freshly laid eggs, and increased with embryonic development. Corticosterone levels were significantly higher ($P < 0.01$) in eggshells of male juvenile compared with female eggshells (Fig. 5-3). Eggshells from the chicks brooded earlier had lower corticosterone levels than those that were hatched later in both males and females.

In addition, no significant correlation was found between testosterone and estradiol, and corticosterone and estradiol in male and female eggshells during incubation or after hatching.

5.4 Discussion

The present study is the first to report that eggshells of Japanese quails contain detectable amounts of testosterone and corticosterone after laying. The concentrations of testosterone were decreased during incubation and post hatching, and high levels of estradiol and corticosterone was detected during incubation and hatching; and the amounts were different between the female and male eggshells.

In the present study, a significant decrement (more than 5-fold) was observed from laying day up to 15 days of incubation and post hatching in eggshell testosterone concentration. No difference was observed between the male and the female eggshells during incubation or after hatching. This would be associated with decreases in the yolk and embryonic testosterone levels during late embryonic development and post hatching. Elf and Fivizzani (2002) reported high androstenedione, testosterone, and dihydrotestosterone levels in the yolk, which decreased with embryonic development, proceeded. Moreover, Ottinger *et al.* (2001) also reported low plasma testosterone levels in male and female Japanese quails' offspring during hatching. In addition, relative expression of 17 β -HSD (a key enzyme that converts androstenedione to testosterone) was significantly decreased by day 18 of incubation in the chicken CAM (Albergotti *et al.*, 2009). Our results suggest that maternal androgen levels are higher than endogenously synthesized embryonic steroids. During the incubation period, the embryos might be utilizing yolk androgen levels for their sexual differentiation and development.

I found significantly higher estradiol concentration in the eggshells obtained on 15 days of incubation and post hatching. Moreover, significant and relatively high estradiol was found in the female compared with male eggshells on the 15 days of incubation and higher after hatching, respectively. In the Japanese quail, estradiol levels increased in the embryonic period from days 10 to 16, and then decreased on the day of hatching (Abdelnabi *et al.*, 2000). Elf and Fivizzani (2002) reported that egg yolk estradiol levels were significantly increased between 14 and 20 days of chicken embryonic development. Changes in estradiol concentrations of the eggshell during embryonic development and after hatching were associated with the internal variations of this hormone in the egg.

The corticosterone level was significantly higher in the male compared with the female eggshells. Eggshells of the chicks hatched earlier also had lower corticosterone levels than those that were hatched later. In avian species, corticosterone is the major glucocorticoid released in response to stress and may allow females to bias the sex ratio of their offspring in response to environmental conditions around the time of egg production (Pike and Petrie, 2006). Moreover, in domestic chickens, male offsprings showed a great response to stressful conditions relative to females (Goerlich *et al.*, 2012). Hatchling time is also a stressful condition for juvenile chicks and the offspring need to have higher corticosterone levels for metabolism and immunity to overcome this stressor, and elevated plasma corticosterone levels have been shown to reduce testosterone and progesterone concentrations in chicken plasma and egg yolk (Petrie *et al.*, 2001; Zeman *et al.*, 2013).

Collectively, these results indicated that the steroid hormones accumulated in the eggshells of Japanese quails. Sex steroid levels in the eggshell contents were variable during embryonic development and hatchling periods. Moreover, the different levels of corticosterone and estradiol in the eggshells of male and female juveniles could be applied to distinguish the sexes of the monomorphic animals after hatching. This method will be useful and should be applicable to the wild precocial birds.

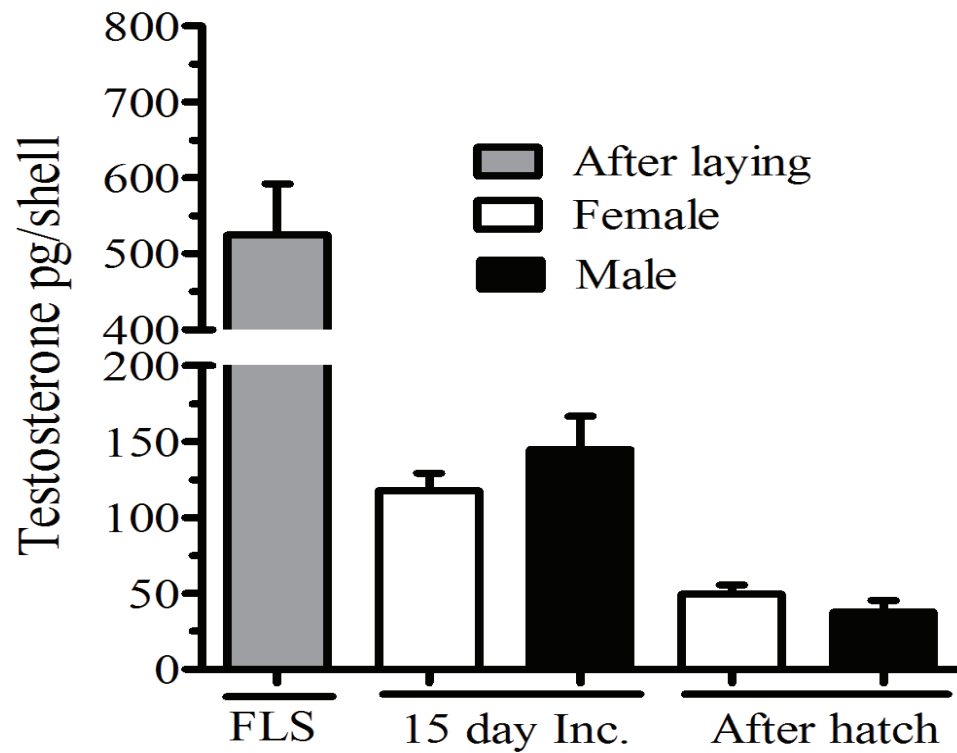


Fig. 5-1. Testosterone concentrations in the eggshell of Japanese quails. Graphs are showing the concentration of testosterone (pg/shell) in the eggshell after laying (gray bar), 15 days of incubation and post hatching. White bars represent testosterone level in the female and black bars in the male eggshells. Bars represent mean \pm SEM. FLS = Fresh laid eggshell, 15 day Inc. = 15 days of incubation.

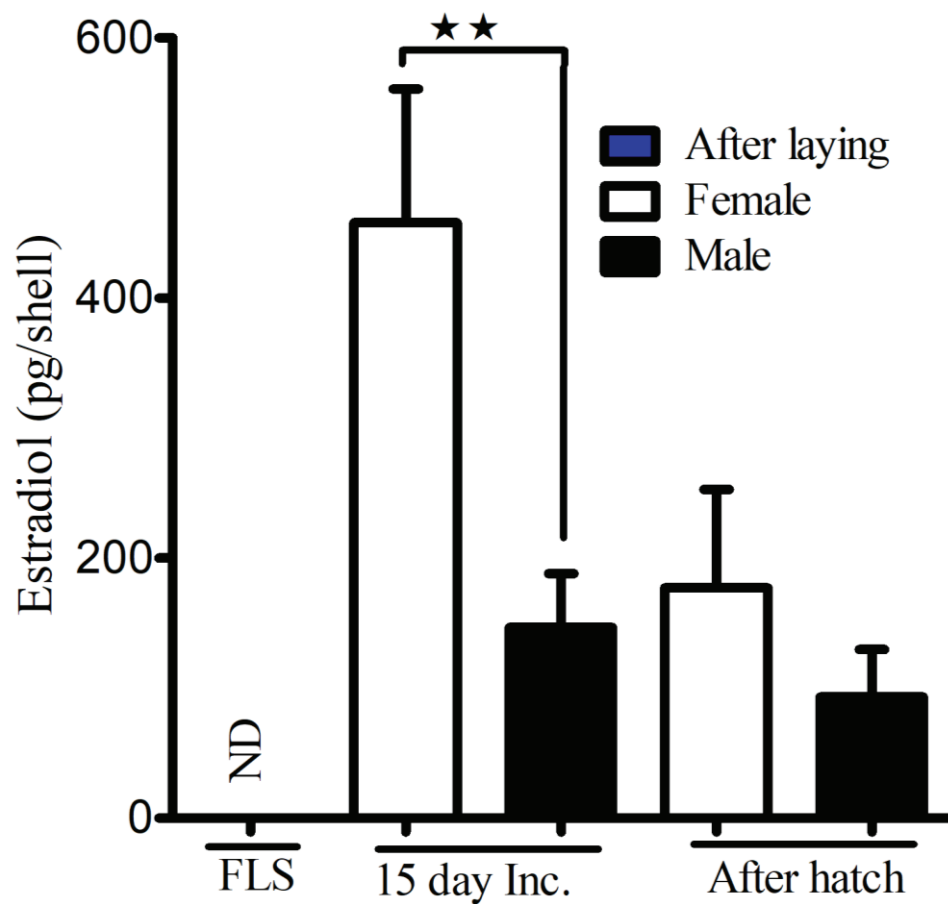


Fig. 5-2. Concentration of estradiol in the eggshells of Japanese quails. Graphs represent estradiol levels in female (white bars) and male (black bars) eggshells during the 15 days of incubation and after hatching. Bars represent mean \pm SEM, and the asterisks are denoted the significant differences ($P < 0.05$). ND = Not detected, FLS = Fresh laid eggshell, 15 day Inc. = 15 days of incubation.

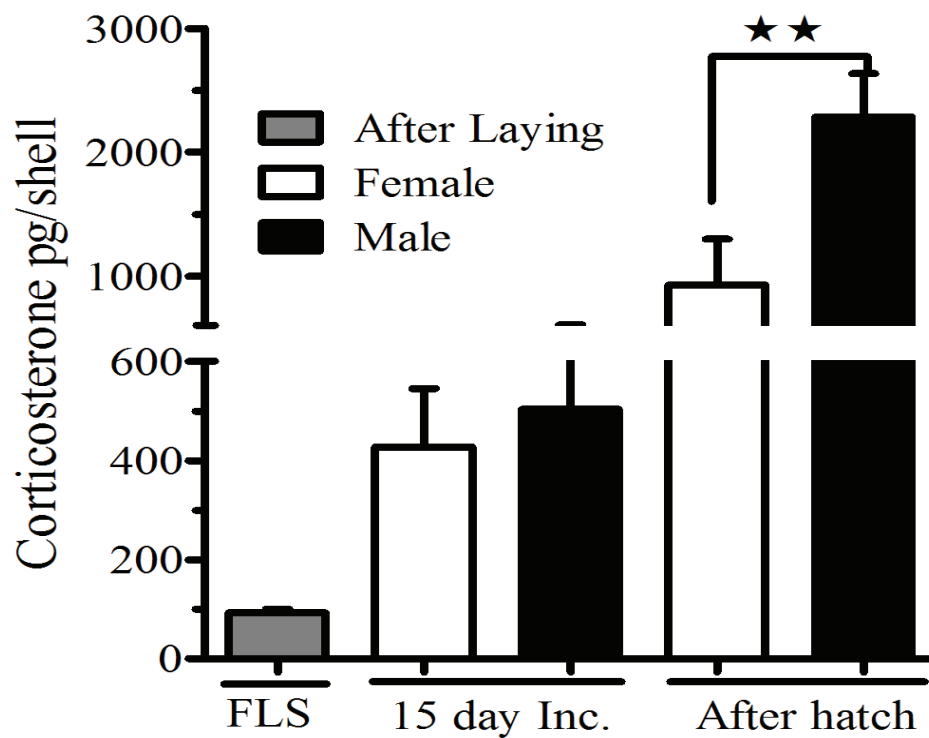


Fig. 5-3. Corticosterone levels the eggshells of Japanese quails. Bar graphs represent the eggshell corticosterone level (pg/shell) after laying (gray bar), 15 days of incubation and after hatching. White bars indicate corticosterone level in the female and black bars in the male eggshells. Bars represent mean \pm SEM, and the asterisks are denoted the significant differences ($P < 0.05$). FLS = Fresh laid eggshell, 15 day Inc. = 15 days of incubation.

GENERAL DISCUSSION

In this study, the Japanese quails are used for studying the effects of phytosterols on adrenal glands and reproductive endocrine functions during the different life stages. Phytosterols as feed additives has been shown to act as endocrine-disrupting chemicals in many laboratory animals (Awad *et al.*, 1998; Solca *et al.*, 2013; Singh and Gupta, 2016). However, the previous knowledge regarding the action mechanism(s) was basic and required further investigations.

Animals in this study were exposed to chronic and acute phytosterols treatment in the different doses did not shown signs of toxicity, none of the male and female quails were died during the different life stages. Except of growing period, no significantly difference was found in body, adrenal glands, gonads and liver weights during the entire experiments. Only the testicular weight was significantly low during the growing period in the phytosterols-treated animals. However, plasma cholesterol level was slightly decreased in quails under chronic and acute treatment of phytosterols compared to control animals.

In the current study, chronic and acute exposure to different doses of phytosterols increased corticosterone production in male and female Japanese quails during the growing period. Even quails under the long-term and high doses of phytosterols gavage did not show adrenal insufficiency. Except one study, no previous reports had been shown adrenal gland insufficiency caused by phytosterols. Mushtaq *et al.* (2007) reported that elevated plasma phytosterols in human phytosterolemic patient caused adrenal insufficiency and low corticosterone production. In the sterolin-deficient mice, phytosterols accumulations disrupt the adrenal cholesterol homeostasis and significantly

reduced adrenal gland cholesterol level (Yang *et al.*, 2004). However, phytosterols feeding induced enhancement of corticosterone production in the young male Japanese quails challenged with ACTH (Liu *et al.*, 2012). This would be associated with use of phytosterols as precursor for corticosterone production, or endogenous cholesterol was enough to compensate corticosterone production in the Japanese quails.

Acute exposure to phytosterols at different doses didn't significantly changed testosterone production in the adult male and female Japanese quails. However, testosterone concentrations were significantly decreased both in *in vivo* and *in vitro* experiments. Long-term phytosterols feeding especially at the doses of 80 mg and 800 mg/kg BW during the growing and adult period's significantly decreased testosterone concentrations both in male and female Japanese quails. Furthermore, interstitial cells were cultured with phytosterols (87.5 µg/ml) significantly decreased testosterone levels under stimulations of O-LH as compared to control group. Similar results were previously reported by Awad *et al.* (1998) in rats. Moreover, intra-peritoneal implantation of β -sitosterol significantly reduced testosterone concentrations in male goldfishes, while testosterone levels were not significantly effects in female (Sharpe *et al.*, 2006). In rats, lipoprotein-cholesterol, rather than de novo cholesterol is the major substrate for the production of steroid hormone by adrenal glands and gonads (Andersen and Dietschy, 1978). It was found that adrenals and gonads use the different sources of cholesterol (lipoprotein-cholesterol) for steroidogenesis in human and rodents. In human, rats and mice HDL cholesterol is the major source for steroid production (Yang *et al.*, 2004; Bochem *et al.*, 2013). In contrast, gonadal steroidogenesis mostly depends on continuous supply of LDL cholesterol levels. Based in previous results, low testosterone

concentrations in this study might be due to low cholesterol level in the phytosterols-treated animals.

The current results indicated that chronic and acute exposure to high doses of phytosterols did not significantly altering LH levels during the growing and adulthood periods in male and female Japanese quails. Similarly, in European polecat (*Mustela putorius*) feeding of phytosterols did not significantly changed LH level (Nieminen *et al.*, 2002). MacLatchy *et al.* (1997) also reported that water-borne β -sit exposures had no effect on the basal levels of plasma LH in goldfishes. However, phytosterols feeding to laying hens in late periods of egg production significantly reduced LH level (Yanmin and Tian, 2008). In addition, feeding of β -sitosterol significantly reduced testosterone, LH and FSH concentrations and sperm count in male albino rats (Singh and Gupta, 2016). Based on the current results the induction of sex steroid production seems to be mediated at the gonadal bases. Phytosterols feeding may directly reduce testosterone production probably by interfering to cholesterol levels or cholesterol trafficking enzymes for steroidogenesis.

Histologic examination of testes revealed that testicular maturation was affected by phytosterols treatment in male quails during the growing period. Parallel to testicular weight, seminiferous tubules in the testes of phytosterols-treated animals were smaller than in controls. Moreover, the lumen of seminiferous tubules, and mature spermatozoa were not appearing in the testes of phytosterols-treated animals. In addition, Leydig cell number was also low in the testes of adult male Japanese quails after long-term feeding of 800 mg/kg BW of phytosterols during adulthood period. In male Japanese quails, testicular maturation and cloacal gland weight have strong relationship with testosterone concentrations (Ottinger and Brinkley, 1979). Thus, reduced availability of testosterone can be an indirect reason for the weight reduction of these reproductive organs. Moreover,

phytosterols may delayed the testicular maturation and reduce testosterone productions during the growing and adult periods in male quails. On the other hand, as ligand of the LXR, phytosterols may exert apoptotic effects on the Leydig cells in the testis of male quails.

Cloacal gland size and the amounts of foam production in male quails are closely related to the gonadal activity (Biswas *et al.*, 2007; Hanafy *et al.*, 2016). The remarkable testosterone dependent functionality of the gland makes a valuable indicator for the sexual activity (Li *et al.*, 2006; Biswas *et al.*, 2007; Hanafy *et al.*, 2016). The present study revealed that phytosterols administration in higher doses has significantly decreased the cloacal gland size. However, the cloacal amounts of foam production were not significant different between the groups. The smaller size of cloacal glands in phytosterols-treated group might be the cause of low testosterone level in the circulation.

In the current study, given phytosterols for the animals had significant effects on the animals' sex libido and courtship. Male quails especially in the high dose phytosterols feeding had significantly low mounting and copulation behaviors when compared with control. In male quails, the sex-related behaviors are quite sensitive to the low androgen level, perhaps the role of testosterone is more crucial (Ball and Balthazart, 2010; Hanafy *et al.*, 2016). I assume that low sexual activities in the phytosterols groups might be the cause of low level of testosterone. Moreover, phytosterols may have central effect on the brain because phytosterols can easily pass the BBB (Saeed *et al.*, 2015).

In this study, I found that chronic feeding of phytosterols in the dose of 800 mg/kg BW slightly reduced the expression 17 β -HSD both in *in vivo* and *in vitro* studies. Previous results proposed that endocrine-disrupting activities of phytosterols are mostly related to cholesterol lowering activity and interfering with the cholesterol trafficking enzyme

responsible for steroidogenesis (MacLatchy *et al.*, 1997, Sharpe *et al.*, 2006). The current results are not coinciding to previous report showed that β -sitosterol exposure significantly reduced stAR gene expression in goldfishes (Sharpe *et al.*, 2006). These results suggested that testosterone reduction in quails might also cause by alteration of 17 β -HSD gene by feeding of high dose of phytosterols.

The results of the current study showed that hypothalamic and testicular expression of GnRH-1 decreased in phytosterols-treated animals as compared to controls. Previous reports found that phytosterols can efficiently pass the BBB, and irreversibly accumulate in brain cellular membranes (Vanmierlo *et al.*, 2012). These results are inconsistent with a previous report that found that accumulated β -sitosterol in brain membrane prevented an inflammatory reduction of GnRH in vitro (Shi *et al.*, 2015). It is possible that membrane phytosterols may alter GnRH response to feedback by low testosterone production from the testes, or hypothalamic GnRH-1 expression may be downregulated by local aromatization of testosterone to estrogen (Sun *et al.*, 2001) due to the estrogenic activity of phytosterols as previously reported (Awad and Fink, 2000).

The results of this study indicated that long-term feeding of phytosterols in the dose of 800 mg/kg BW significantly increased the expression of GnIH in the diencephalon area of hypothalamus and the testis. High expression of GnIH both in brain and testis of phytosterols-treated male quails (800 mg/kg BW) may imply the autocrine/paracrine role of GnIH in testicular function. Additionally, high expression of GnIH in phytosterols-treated animals might also reduce the expression of GnRH and LH as well.

CONCLUSION

The present study exposes a new insight on phytosterols feeding at the high doses on the adrenal and reproductive functions in male and female Japanese quails. Chronic feeding of high doses of phytosterols boosts adrenal glands' corticosterone production both in male and female quails. However, phytosterols feeding affects the reproductive functions by decreasing testosterone concentrations in both sexes, and slackens the testicular maturity during the growing period in male quails. Moreover, LH levels were not significantly changed by phytosterols feeding during development and adulthood periods. Low testosterone concentration affects animals' sex libido and sexual behaviors such as waltzing, mount and copulation. Furthermore, the testicular histology and the cloacal gland size revealed the difference among control and phytosterols-treated animals. However, phytosterols feeding did not significantly effects on semen concentration, sperm quality and fertilization ability in male quails.

Low testosterone concentrations might be the cause of low cholesterol levels or lower expression of cholesterol-converting enzymes such as 17β -HSD. Moreover, high expression of GnIH in the brain and testis may have direct effects on testosterone productions and testicular maturity. GnIH inhibits GnRH production and release from the hypothalamus and pituitary gonadotropins (LH) as consequence.

Phytosterols at the high doses for hen may change the accumulation of hormones and other biological active substance such as cholesterol in the egg yolk. The change in the egg ingredient may affect the offspring surviving and development after hatching.

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