BIOLOGICAL STUDIES OF THE REPRODUCTIVE CYCLE AND THE EFFECTS OF PHOTOPERIOD UPON THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKEN

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

Suranaree University of Technology

Academic Year 2007

การศึกษาชีววิทยาของวงจรการสืบพันธุ์และผลของช่วงแสงต่อระบบการสืบพันธุ์ในไก่พื้นเมืองไทยเพศเมีย

นางสาวสุนันทา โกศลศิริลักษณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2550

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การศึกษาการควบคุมด้วยระบบประสาทและระบบต่อมไร้ท่อและบทบาทของช่วงแสงต่อระบบสืบพันธุ์ของไก่พื้นเมืองไทยเพศเมียพบว่า ระดับของฮอร์โมนโปรแลคตินในพลาสมามีการเปลี่ยนแปลงตามวงจรการสืบพันธุ์โดยมีระดับสูงสุดในไก่ที่อยู่ในระยะนั่งฟัก แต่ไม่พบการเปลี่ยนแปลงของลูติไนซิงฮอร์โมนในพลาสมา การศึกษาโดยใช้เทคนิคอิมมูโนฮิสโตเคมิสทรีพบว่าเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์กระจายอยู่ทั่วทั้งสมองและพบมากที่สุดที่สมองส่วนไดเอนเซฟาลอน โดยพบว่าจำนวนเซลล์ประสาทที่มีวาโซแอคทีฟอินเทสทินอลเปปไทด์ที่บริเวณอินฟันดิบูลานิวเคลียร์คอมเพล็กซ์มีการเปลี่ยนแปลงตามวงจรการสืบพันธุ์และพบเป็นจำนวนมากในไก่ระยะนั่งฟักซึ่งสอดคล้องกับระดับของฮอร์โมนโปรแลคตินในพลาสมา ช่วงแสงอาจมีบทบาทต่อระบบสืบพันธุ์ของไก่พื้นเมืองไทย ผลที่ได้จากการศึกษาโดยรวมแล้วสรุปได้ว่าวาโซแอคทีฟอินเทสทินอลเปปไทด์และฮอร์โมนโปรแลคตินมีบทบาทที่สำคัญยิ่งต่อระบบสืบพันธุ์ของไก่พื้นเมืองไทยซึ่งเป็นสัตว์ที่มีถิ่นอาศัยอยู่ในแถบเส้นศูนย์สูตร

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SUNANTHA KOSONSIRILUK : BIOLOGICAL STUDIES OF THE REPRODUCTIVE CYCLE AND THE EFFECTS OF PHOTOPERIOD UPON THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKEN. THESIS ADVISOR : ASST. PROF. YUPAPORN CHAISEHA, Ph.D. 240 PP.

LUTEINIZING HORMONE/NATIVE THAI CHICKEN/PHOTOPERIOD/ PROLACTIN/REPRODUCTIVE CYCLE/VASOACTIVE INTESTINAL PEPTIDE

Neuroendocrine regulation and the roles of photoperiod upon the reproductive system of female native Thai chickens were elucidated. Plasma prolactin (PRL) levels changed throughout reproductive stages with the highest level in incubating hens (B) whereas the changes in plasma luteinizing hormone (LH) levels were not observed. Immunohistochemistry studies revealed that distributions of vasoactive intestinal peptide (VIP) immunoreactivity were found throughout the brain and predominantly in the diencephalon. The changes of VIP-immunoreactive neurons in the infundibular nuclear complex were observed across reproductive stages with the greatest density were found in B and mirrored the plasma PRL levels. Photoperiod might play a role in the reproduction. In conclusion, VIP and PRL play a pivotal role in reproduction in this equatorial species.

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ACKNOWLEDGEMENTS

I would like to express my sincere and deepest gratitude to my kind advisor, Asst. Prof. Dr. Yupaporn Chaiseha for her expert advice and guidance. She has been considerate and support throughout my time as her graduate student. I would also like to extend my appreciation to my co-advisor Prof. Dr. Mohamed El Halawani for his valuable advice and hospitality throughout the time when I was in his laboratory.

I would also like to express my appreciation to Asst. Prof. Dr. Griangsak Eumkeb, Assoc. Prof. Dr. Thaweesak Songserm, and Asst. Prof. Dr. Rungrudee Srisawat, for taking the time to serve on my thesis committee.

The production of this dissertation would not be possible without the full financial support of The Royal Golden Jubilee Ph.D. Program.

My dissertation research was facilitated by the expert technical teaching by Asst. Prof. Dr. Aree Thayananuphat and Orlan Youngren. I would also like to extend my appreciation to the Department of Pathology, Kasetsart University for providing the cryostat, Suranaree University Farm for providing the barn for the experimental animals. I am thankful to Natagarn Sartsoongnoen and Nattiya Prakobsaeng for their helps, sympathy, friendship, and sincere encouragement.

Finally, I would like to express my deep gratitude to my parents and my family for their love, support, and understanding that helped me to overcome many difficult moments. Thank you very much.

Sunantha Kosonsiriluk

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CHAPTER I

INTRODUCTION

1.1 Rational of the Study

Native Thai chicken (*Gallus domesticus*) has been raised in the countryside of Thailand for many generations. It is a small domestic animal that probably originated from one of the several jungle fowls, which still found wildly distributed throughout Southeast Asia. Nowadays, there are about 54 millions native Thai chickens in Thailand. Raising the native Thai chicken is a self-reliant activity. The main objectives of raising the native Thai chicken are for consumption, sport competition, and recreation. The native Thai chicken is easy to raise, resistant to diseases, and acclimatize to the local environments. Furthermore, it provides high quality meat with low in fat and good-taste. Thus, the native Thai chicken is not only served as a main animal protein food source but it can be sold for supplemental income for family as well.

The native Thai chicken expresses high maternal behaviors such as incubation behavior which is a heritable trait. Expression of incubation behavior affects the number of egg produced because it terminates egg laying. Normally, the native Thai hen lays eggs 3-4 times per year and 4-17 eggs per clutch. Thus, it produces about 30-40 chicks per year which is significantly lower than that of the imported hen which produces eggs all year long. The reproductive cycle of the native Thai chicken may divided into four reproductive stages; non-laying, laying, incubating, and rearing

chicks. Although, it is of little commercial importance to the poultry industry because of its poor egg production, the native Thai chicken has become the new economic domestic animal of Thailand. There are no marketing problems with the native Thai chicken because it is in high demand by consumers. Thus, the price of the native Thai chicken meat is higher than that of the imported ones.

The primary components of the integrated female reproductive system are the brain, especially the hypothalamus, the pituitary, and the ovary. This integrated system is referred to as the hypothalamic-pituitary-gonadal axis (H-P-G axis). It is very well documented that neurotransmitters, neurohormones, and hormones of the H-P-G axis play an important role in the reproductive cycle of avian species, including gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), and gonadal steroids (e.g., estradiol and progesterone). FSH and LH, anterior pituitary hormones called gonadotropins, are responsible for ovarian follicular growth, maintaining the hierachical size of the bird follicles, and trigging ovulation, respectively. Subsequently, as the follicles increase in size, their production of steroid hormones, including progesterone and estradiol increases. In birds, physiological functions of these steroid hormones are correlated with ovulation and female secondary sex characteristics. Furthermore, there are many evidences suggesting that progesterone and estradiol play an important role in modulation of gonadotropins secretion via feedback effect. It is very well established that the gonadotropins synthesis and secretion are under stimulatory control by the hypothalamic releasing factor, chicken gonadotropin releasing hormone-I (cGnRH-I). There are growing evidences indicating the involvement of hypothalamic dopamine (DA) in the regulation of cGnRH-I and the secretion of LH and FSH. It has been very

well documented that gonadotropins and cGnRH-I are essential regulators of the reproductive cycle in several avian species such as mallard, King penguin, turkey, and cockatiel.

Two stages of the native Thai chicken reproductive cycle including incubation and rearing chicks constrain the number of egg produced. The expression of such behaviors is a costly problem, resulting in substantial loss of potential egg production. There are several lines of evidence showing that hormones play an important role in the reproductive cycle of avian species, including maternal behavior. The period of egg laying in birds is associated with relatively high levels of LH, FSH, and gonadal steroids circulating in the blood. On the contrary, the onset of incubation behavior is correlated with declining levels of LH and gonadal steroids and increasing levels of prolactin (PRL).

PRL, an anterior pituitary hormone, is involved in many aspects of reproductive physiology and behavior. PRL has been implicated as a causative factor in the onset and maintenance of broodiness in birds. The onset of incubation activity is correlated with declining levels of gonadotropins and a dramatic rise in circulating PRL level. It is this rising which has been implicated as the cause of the cessation of ovulation, ovarian regression, and induction of incubation behavior, when birds shift from egg laying to broodiness. PRL is widely thought to play a role in parental behavior by mediating incubation behavior, crop milk production and secretion, feeding of the young, and nest defense. PRL has been shown to be associated with the reproductive cycle in several avian species but no studies have been conducted on the native Thai chicken.

In birds, it is very well documented that PRL is under stimulatory control by hypothalamic vasoactive intestinal peptide (VIP), the avian PRL-releasing factor (PRF). VIP, an octacosapeptide, was first isolated from porcine duodenum and subsequently found widely distributed in the central and peripheral nervous systems and considered to function as a neurotransmitter and neuroendocrine substance. Moreover, it is also well established that DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL. In addition, recent evidences indicate that dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ opioid, serotonergic, dopaminergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator.

In seasonal breeder birds, the initiation of the breeding cycle depends upon the precise prediction of environmental conditions which are for health of the mating pair and the survivability of their offspring. The control of PRL secretion in birds involves the interaction of external stimuli with endocrine mechanisms. Critical environmental stimuli include sensory information concerning photoperiod, ambient temperature, and the presence of egg and offspring. These external stimuli as well as the prevailing internal steroid milieu (estrogen and progesterone) are important in initiating and maintaining PRL secretion, although their relative importance varies with the stages of the reproductive cycle.

It is not only endocrine factors that influence the reproductive cycle of birds but also environmental factors. The jungle fowl, the ancestor of the native Thai chicken, originated in the tropical region of Southeast Asia, where its breeding season would has been timed by both photoperiodic and non-photoperiodic factors, allowing

the chick to hatch at a time of year when food was most abundant. Most of birds are highly photoperiodic and gonadal development occurs in response to increasing day length. The endocrine and environmental factors associated with the reproductive cycle of avian species are complex. The integration of the endocrine system, hypothalamic neuropeptides, pituitary hormones, ovarian steroids, environmental photoperiod, ambient temperature, and the presence of egg and chick, regulating the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken.

In Thailand, there are many research laboratories working on avian reproduction, but there are only a limit number of scientists studying the neuroendocrine regulation of reproduction in the native Thai chicken. Progesterone level has been shown to be correlated to the reproductive cycle of the native Thai chicken. The effects of lighting regimens (photoperiod) upon growth, reproductive development, laying performance, and reproductive efficiency has been reported. However, the results of these studies are contradictive and far from understood. In order to increase the production of the native Thai chicken, it is very important to understand the basic endocrinology and the environmental factor(s) influencing its reproductive activity. The focus of my dissertation research was proposed to characterize the neuroendocrine regulation of the reproductive cycle in the female native Thai chicken and the roles of photoperiod upon the neuroendocrine regulation of the reproductive system. The findings from this study will provide the base line information of the hormonal and physiological characteristics of the reproductive system in the native Thai chicken, which has never been studied. The knowledge gained will help to understand the basic neuroendocrine regulation of the reproductive

cycle and elucidate the effects of photoperiod upon the native Thai chicken reproduction. This information can be then applied commercially in poultry industry to increase reproductive efficiency and egg production of the native Thai chicken in Thailand.

1.2 Research Objectives

1.2.1 To study the roles of PRL and LH in the regulation of the reproductive cycle of the female native Thai chicken.

1.2.2 To study the distribution of VIP in the brain across the reproductive cycle of the female native Thai chicken.

1.2.3 To study the roles of photoperiod in the regulation of the reproductive system of the female native Thai chicken.

CHAPTER II

LITERATURE REVIEW

2.1 Neuroendocrine Regulation of the Avian Reproductive Cycle

It has been known that stimuli from the environment profoundly influence reproductive physiology and behavior in birds. The brain integrates sensory and endocrine information to regulate the secretion of pituitary and gonadal hormones. There are two neuroendocrine systems that play a pivotal role in the avian reproductive cycle. First, gonadotropin releasing hormone-I/follicle stimulating hormone-luteinizing hormone (GnRH-I/FSH-LH) system which involves GnRH-I and the subsequent secretion of gonadotropins (FSH and LH). Second, vasoactive intestinal peptide/prolactin (VIP/PRL) system which involves VIP and leading to the subsequent secretion of PRL. Both systems depend upon the duration of day length (photoperiod) and the transduction of photoperiodic information, resulting in either gonad recrudescence and its associated sexual activity or gonad regression and the termination of reproductive activity. The final common pathway controlling the secretion of PRL, LH, and FSH is formed by a system of peptidergic neurons whose axons terminate around portal capillaries in the external layer of the median eminence (ME). VIP and GnRH are among the best characterized hypophysiotropic neuropeptides (Chaiseha and El Halawani, 2005).

2.1.1 Gonadotropin Releasing Hormone-I/Follicle Stimulating Hormone-Luteinizing Hormone System

It is very well established that FSH and LH secretions are regulated by the central nervous system through the hypothalamus. The hypothalamus synthesizes GnRH which in turn stimulates the synthesis and secretion of the pituitary gonadotropins (Ulloa-Aguirre and Timossi, 2000; Shalev and Leung, 2003). GnRH has first been isolated from porcine hypothalamus (Peczely, 1989). Two forms of GnRH have been isolated in chicken; cGnRH-I or GnRH-I ([Gln8]-GnRH) and cGnRH-II ([His5, Trp7, Tyr8]-GnRH; King and Millar, 1982; Miyamoto et al., 1982; 1984; Millar and King, 1984; Sherwood et al., 1988). However, all the available evidence suggests that only GnRH-I has a physiological role in regulating of gonadotropins secretion (Sharp et al., 1990). Fully processed cGnRH-I messenger ribonucleic acid (mRNA) and a variant transcript with a retained intron 1 were observed in the preoptic area (preoptic-anterior hypothalamus: POA), basal hypothalamus, anterior pituitary gland, and testes of cockerel (Sun et al., 2001). Once environmental stimuli are transduced by the appropriate receptor, they influence the secretion of GnRH located in neurons of the preoptic and hypothalamic regions in birds and mammals.

FSH first primes the initial phase of follicogenesis with an increase in gene transcription that encodes growth factors, induces LH receptors on granulosa cell membranes, and promotes estradiol secretion. FSH and estradiol are required for the acquisition of LH receptors by granulosa cells, whereas the synthesis of androgen which is a precursor for estradiol is controlled by LH (Hsueh et al., 1984). After the initial phase of ovarian follicular growth, an LH surge participates in oocyte meiosis

and consequent ovulation (Levi-Setti et al., 2004). It has been reported that FSH and LH secretions and gene expressions are stimulated by long day length (Nicholls et al., 1988; Dawson et al., 2001) and required the functional integrity of the GnRH neuronal system (Katz et al., 1990; Sharp et al., 1990). It is very well established that gonadotropins secretion is influenced by GnRH. Ovarian development is found to be correlated with plasma LH level and the amount of GnRH-I content, indicating the expression of the GnRH-I gene is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). This decaneuropeptide increases LH and FSH secretion of the anterior pituitary both *in vitro* and *in vivo* (Peczely, 1989). In *in vivo* study, injection of cGnRH-I or cGnRH-II stimulated an increase in plasma LH concentration in hens (Guemene and Williams, 1999). In addition, GnRH agonists may imitate the native hormone and induce an endogenous LH surge (Shalev and Leung, 2003). In contrast, GnRH inhibits FSH-stimulated steroidogenesis in chickens as well as in mammals but enhances LH-stimulated progesterone production (Hertelendy et al., 1982). cGnRH-I did not affect circulating FSH concentrations but stimulated LH secretion when administrated to 3 weeks old cockerels (Krishnan et al., 1993). GnRH release occurs episodically from the mammalian hypothalamus, and the frequency and amplitude of GnRH release determine the pattern of gonadotropins secretion (Levine and Ramirez, 1982; Moenter et al., 1992). In Japanese quail, a pulsatile pattern of GnRH-I release was observed from the medial basal hypothalamus and POA *in vitro* (Li et al., 1994). Changes in pituitary responsiveness to GnRH are negatively correlated to changes in the circulating LH level (Balthazart et al., 1980).

It has been established for sometime that adrenergic stimulation at the hypothalamic level can release GnRH and thereby increase gonadotropins secretion

(Yu et al., 1991). GnRH-I levels decrease when birds enter the incubating stage and this decrease in GnRH-I release is thought to be implemented by the inhibitory effect of PRL, which reaches its highest level during this stage (Sharp et al., 1988). Like LH, PRL, and VIP, GnRH contents also change during the reproductive cycle. GnRH-I concentrations was significantly elevated in the POA of the hypothalamus during incubation (Millam et al., 1995). However, measurements of hypothalamic GnRH peptide in the hypothalamus during the reproductive cycle of the turkey (Millam et al., 1989; El Halawani et al., 1993; Rozenboim et al., 1993) indicate that levels do not change during incubation but the amount of GnRH in the hypothalamus decreases during photorefractoriness (Rozenboim et al., 1993) and other avian species (Dawson et al., 1985; Foster et al., 1987; Bluhm et al., 1991; Saldanha et al., 1994; Hahn and Ball, 1995). GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples were higher in laying hens than that of non-lying hens (Advis et al., 1985). GnRH perikarya and fibers are more widely distributed throughout the avian brain. Kuenzel and Blahser (1991) reported six major groups of perikarya were found including the olfactory bulb, olfactory tubercle, lobus parolfactorius, nucleus accumbens, septal preoptic hypothalamic region (three sub-nuclei), and lateral anterior thalamic nucleus. In other studies, GnRH neurons were found within the POA, anterior hypothalamus, and lateral septum (LS; Mikami et al., 1988; Millam et al., 1993). Little is known regarding the GnRH cell group(s) that project to the ME (Dawson and Goldsmith, 1997; Teruyama and Beck, 2000). Recently, it has been reported that GnRH mRNA expression was determined during the four different reproductive stages of the female turkey. GnRH mRNA was highly expressed in the organum vasculosum laminae terminalis (OVLT) and the nucleus commissurae pallii

(nCPa), and limited expression was observed in the POA, nucleus preopticus medialis (POM), and nucleus septalis lateralis (SL). Hypothalamic GnRH mRNA expression was significantly increased after subjecting the non-photostimulated female turkey to a 90 minute light period at Zeitgeber time (ZT) 14. GnRH mRNA abundance within the SL, OVLT, and nCPa areas was highest in laying hens, with decreasing abundance found in non-photostimulated and incubating hens, respectively. The lowest levels of GnRH mRNA were observed in photorefractory hens. These results indicate that hypothalamic GnRH mRNA expression may be used to precisely characterize the different reproductive stages (Kang et al., 2006).

2.1.2 Vasoactive Intestinal Peptide/Prolactin System

In response to long day length, the increase in VIP/PRL secretion is gradual, but progressive, and both their release and gene expression are up-regulated (Wong et al., 1991; El Halawani et al., 1996; Tong et al., 1997; Chaiseha et al., 1998). Activation of the GnRH/LH-FSH and VIP/PRL systems in mature photosensitive female turkey initiates the transition from reproductive quiescence to reproductive activity. When gonadotropin stimulates estrogen secretion (Wineland and Wentworth, 1975) and induces sexual receptivity (El Halawani et al., 1986), it also primes the VIP/PRL system to enhance PRL secretion as well (El Halawani et al., 1983). VIP is the hypothalamic releasing factor that has been implicated in the control of a pituitary hormone, PRL. VIP stimulates the secretion of PRL from the anterior pituitary (Macnamee et al., 1986). This hormone is modulated by stimuli from the environment and plays an important role in the control of reproduction (El Halawani et al., 1997; Chaiseha and El Halawani, 2005).

At the onset of sexual maturity, the preovulatory surge of progesterone induces the nesting behavior associated with oviposition (Wood-Gush and Gilbert, 1973; El Halawani et al., 1986). The combine action of estrogen, progesterone, and nesting activity further stimulates PRL secretion in turkey (El Halawani et al., 1983; 1986). These increasing PRL levels suppress the activity of the GnRH/FSH-LH system (Rozenboim et al., 1993; You et al., 1995), reducing ovarian steroids secretion (Porter et al., 1991; Tabibzadeh et al., 1995), terminating egg laying, inducing ovarian regression (Youngren et al., 1991), and signal the transition from sexual behavior to incubation behavior (Chaiseha and El Halawani, 2005). Elevated PRL levels and incubation behavior are maintained by tactile stimuli from the nest and eggs (El Halawani et al., 1980; 1986; Opel and Proudman, 1988). After hatching, or when eggs are replaced with poults, tactile stimuli from the young induces the emergence and maintenance of maternal responses such as brooding the young, vocalizations, nest desertion, a sharp decrease in circulating PRL, molt, and the transition to the photorefractoriness (Opel and Proudman, 1989). With the onset of photorefractoriness, circulating PRL and LH levels, pituitary PRL/LH peptide contents and their mRNA levels sharply decline, eventhough long day length continues (Wong et al., 1991; 1992; Mauro et al., 1992; El Halawani et al., 1996). A rapid decrease in PRL and LH/FSH release and their gene expression may be triggered at any time due to a lack of response to long day length or by subjecting birds to short day lighting (Nicholls et al., 1988; El Halawani et al., 1990a).

As aforementioned, it is very well documented that PRL is under stimulatory control by hypothalamic VIP, the only avian PRL-releasing factor (PRF; El Halawani et al., 1997). Avian PRL secretion and gene expression is regulated by VIP (El

Halawani et al., 1997; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005). It is also very well established that dopamine (DA) plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL (Youngren et al., 1996b; Chaiseha et al., 1997; 2003a). In addition, recent evidences indicate that dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ opioid, serotonergic, dopaminergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (El Halawani et al., 2000).

5-HT, norepinephrine (NE), and DA also involve with GnRH. It has been reported that 5-HT shows the inhibitory effect upon GnRH (Sharp et al., 1984; 1989). NE has been reported to have the stimulatory effect on GnRH secretion (Knight et al., 1984). Bilateral microinjections of 5-HT into the caudal ventromedial nucleus (VMN) of the turkey hypothalamus, but not the rostral part, notably impeded the PRL release effected by electrical stimulation in the POA. These data lead to the hypothesis that 5-HT, at least at the VMN level, may be involved in the decline in circulating PRL observed during reproductive inactivity i.e. the photorefractory state (Youngren and El Halawani, 2002). Recent study reported that electrical stimulation in the POA, which is known to stimulate LH and PRL secretion, activates GnRH and VIP immunoreactive neurons (as indicated by c-fos mRNA expression) in the POA and infundibulum nuclear complex (INF) areas, respectively (El Halawani et al., 2004). Some other evidences suggest an inhibitory role for DA upon GnRH release in mammals as well as in birds (Ramirez et al., 1984; Sharp et al., 1984). Further evidence for the involvement of DA in correlating with GnRH is derived from dense concentration of tyrosine hydroxylase (TH; the rate limiting enzyme for DA synthesis)

and GnRH-containing processes are located in the lateral and mediobasal portion of the external layer of the hen ME (Contijoch et al., 1992). Activation of dopaminergic neurons in the nucleus mamillaris lateralis (ML) is associated with the activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006). This result provides an opportunity for synaptic interaction between GnRH and DA. This is the first identification of a specific DA group that is associated with the stimulation of GnRH/LH-FSH and VIP/PRL systems.

The following literature review will be extensively reviewed in photoperiodic regulation of VIP/PRL system which is related to this dissertation research.

2.2 Prolactin: Structure, Function, and Regulation of Secretion

2.2.1 The Structure of Prolactin

PRL, a single-chain polypeptide hormone, is synthesized in and secreted from specialized cells called the lactotrophs of the anterior pituitary gland (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000). PRL is one of a family of related hormones including growth hormone (GH) and placental lactogen (PL) that are hypothesized to have arisen from a common ancestral gene about 500 millions years ago. This prominent peptide hormone was discovered more than 70 years ago by Riddle and co-workers (1931; 1932). The hormone was given its name based on the fact that an extract of bovine pituitary gland would cause growth of the crop sac and stimulate the elaboration of crop milk in pigeons or promote lactation in rabbits (Riddle et al., 1933; Bern and Nicoll, 1968).

Like other hormones, PRL is synthesized as a prohormone. Following cleavage of the signal peptide, the length of the mature hormone is between 194-199 amino

acids, depending upon species. Hormone structure is stabilized by three intramolecular disulfide bonds. Based on its genetic, structural, binding, and functional properties, PRL belongs to the PRL/GH/PL family (Horseman and Yu-Lee, 1994). Genes encoding PRL, GH, and PL evolved from a common ancestral gene by gene duplication (Niall et al., 1971). The primary structure of PRL was first elucidated in the ovine (Li et al., 1970). The complete amino acid sequence of PRLs of more than 25 species have been determined (for review, see Sinha, 1995). Their amino acid sequences which have been elucidated by protein sequencing and complementary dihydroxy nucleic acid (DNA) sequencing are given in Fig. 2.1. A comparison of the amino acid sequence of PRL from different species (Fig. 2.1) shows varying degrees of sequence homology (Fig. 2.2), reflecting to a great extent the order of the phylogenetic relationships. Some 32 residues seem to be conserved among the different species (Watahiki et al., 1989).

PRL is secreated by lactotrophs in the anterior pituitary (Velkeniers et al., 1988; Freeman et al., 2000). PRL also has been reported to be synthesized by a number of extra-pituitary tissues in mammals (Ben-Jonathan et al., 1996; Freeman et al., 2000; Soares, 2004) and in birds (Berghman et al., 1992; Ramesh et al., 2000; Chaiseha et al., 2003b), but its physiological function was poorly understood. PRL has been reported to be synthesized and secreted by a broad range of other cells in the body, most prominently various immune cells, mammary epithelium, placenta, the deciduas of the pregnant uterus, and brain (Ben-Jonathan et al., 1996). Although the major form of PRL found in the pituitary gland is 23 kDa, variants of PRL have been characterized in many mammals. PRL variants can be the results of alternative splicing of the primary transcript, proteolytic cleavage, phosphorylation, glycosylation, and other

post-translational modifications of the amino acid chain, thereby altering its biological effects (Sinha, 1995). Up to date, over 300 different physiological functions of PRL have been documented in such areas as reproduction, water and electrolyte balance, growth and development, brain and behavior, endocrinology and metabolism, and immunoregulation as well as behaviors like migration, the nurturing of the young in different vertebrate species, highlighting the importance of this pituitary hormone (Houdebine, 1983; Bole-Feysot et al., 1998; Harris et al., 2004).

PRL receptor (PRLR), a single membrane-bound protein transmembrane receptor, belongs to the member of class I cytokine receptor superfamily that includes the receptors of GH, leptin, erythropoietin, and several interleukins (Bazan, 1989; 1990; Kelly et al., 1991). PRL and GH receptors share several structural and functional features despite their low sequence homology (30%; Goffin and Kelly, 1996). PRL, as well as PL and primate GH, binds the PRLR. The first step in receptor activation is the binding of a single ligand to the receptor, resulting in receptor dimerization which subsequently activates a number of signaling cascade through which PRL exerts its biological effects (Bole-Feysot et al., 1998). Numerous PRLR isoforms have been described in different tissues in both mammals and birds (Davis and Linzer, 1989; Ali et al., 1991; Lesueur et al., 1991; Pitts et al., 2000). These isoforms are results of transcription starting at alternative initiation sites of the different PRLR promoters as well as alternative splicing of non-coding and coding exon transcripts (Hu and Dufau, 1991; Hu et al., 1998). PRLR and its mRNA were found in the mammary gland and the ovary, two of the best-characterized sites of PRL actions in mammals (Nagano and Kelly, 1994). In addition, PRLR and its mRNA were also found in numerous parts of the central nervous system (CNS). The mRNA

distribution of the PRLR has been characterized in the rat brain (Bakowska and Morrell, 1997). PRLR were also founded in a wide range of peripheral organs like the pituitary gland, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, and skin (Nagano and Kelly, 1994; Bole-Feysot et al., 1998). PRLR is widely expressed by immune cells and some types of lymphocytes synthesize and secrete PRL. These suggest that PRL may act as an autocrine or paracrine modulator of immune activity. In birds, PRLR mRNA were also found in liver, intestine, kidney, pineal gland, oviduct, infundibulum, magnum, isthmus, and testes. Turkey PRLR mRNA levels were also compared during the reproductive cycle. PRLR mRNA levels were greatest in laying hen pineal glands and in incubating hen oviducts (Pitts et al., 2000).

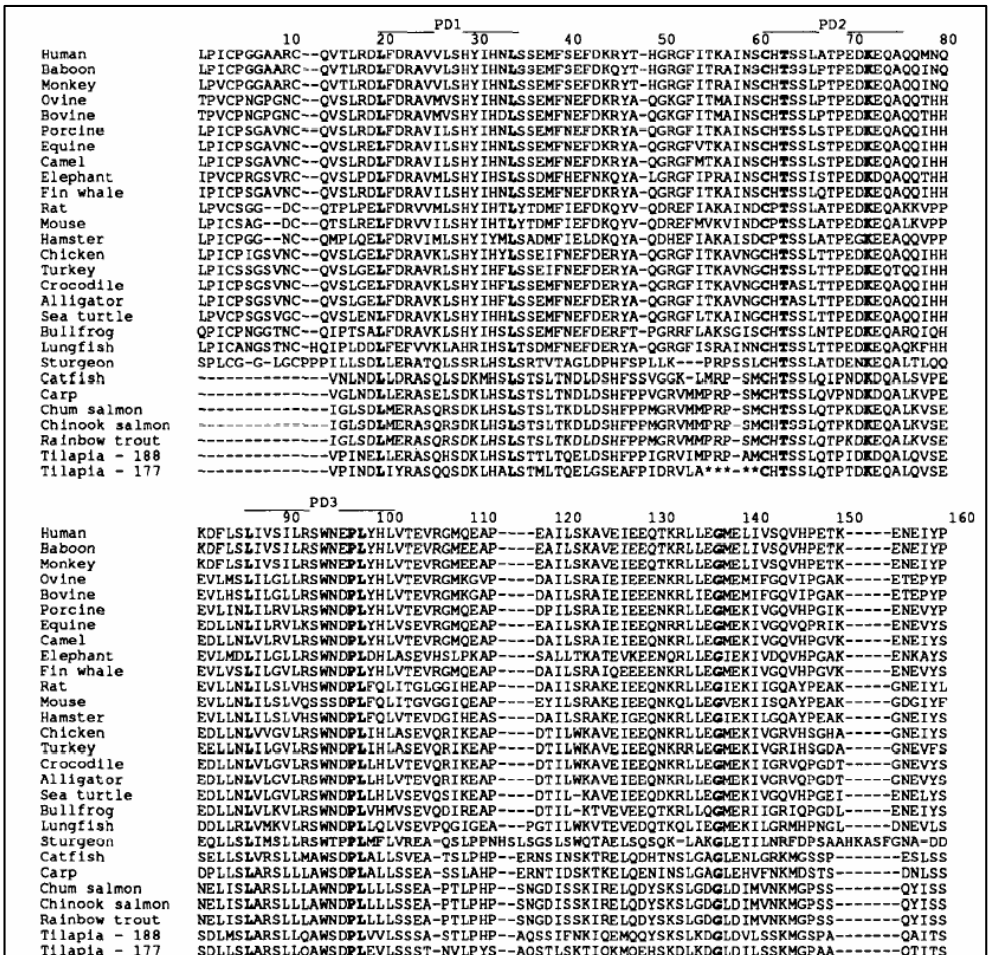

Fig. 2.1 Primary structures of the PRLs of various vertebrate species. (-) indicates positions left blank to optimize alignment of amino acid sequences. Residues common to all PRLs are shown in boldface. PD, PRL domain. PD1-PD4 indicates the four highly conserved domains of the PRLs (Sinha, 1995).

	Human	Baboon	Monkey	Ovine	Bovine	Porcine	Equine	Camel	Elephant	Fin whale	Rat	Mouse	Hamster	Chicken	Turkey	Crocodile	Alligator	Sea turtle	Bullfrog	Lungfish	Sturgeon	Catfish	Carp	Chum salmon	Chinook salmon	Rainbow trout	Tilapia-188	Tilapia-177
Human		97	97	76	76	81	82	81	67	82	64	61	62	72	70	72	73	75	65	58	36	35	36	35	35	35	34	31
Baboon			99	73	73	79	80	80	66	78	61	58	62	70	68	69	70	71	64	54	36	34	34	35	35	35	33	31
Monkey				74	73	79	78	80	66	77	61	56	60	70	67	70	70	72	64	53	37	35	34	35	35	34	34	31
Ovine					99	83	79	80	74	84	61	56	58	69	70	71	71	71	59	53	34	34	35	34	34	34	33	30
Bovine						84	80	80	73	85	62	56	59	70	70	72	71	72	60	54	35	34	35	34	34	34	33	30
Porcine							93	96	76	96	65	61	64	79	79	81	81	80	67	61	35	34	35	34	34	34	33	30
Equine								93	73	91	64	61	63	79	79	81	82	80	69	61	35	35	36	35	35	35	34	30
Camel									72	93	63	61	63	80	78	83	84	84	69	59	37	34	34	35	35	34	33	29
Elephant										76	57	54	57	67	67	66	66	69	57	55	37	36	37	36	36	36	37	31
Fin whale											64	60	61	79	79	80	82	80	66	61	36	35	36	35	35	35	34	31
Rat												85	82	59	60	60	61	60	53	52	30	31	33	31	31	31	31	30
Mouse													72	55	56	56	56	56	48	47	35	32	35	31	31	31	33	31
Hamster														58	58	62	61	60	53	47	36	29	29	29	29	30	28	28
Chicken															93	90	91	89	72	65	31	36	38	35	35	35	35	31
Turkey																89	90	85	71	64	35	35	35	35	35	35	35	30
Crocodile																	99	85	73	66	35	35	35	33	33	33	32	29
Alligator																		86	72	65	34	34	34	34	34	34	31	28
Sea turtle																			74	66	37	36	38	35	35	35	34	31
Bullfrog																				64	40	35	35	35	35	35	34	31
Lungfish																					40	35	37	37	37	37	33	31
Sturgeon																						46	45	46	47	46	43	36
Catfish																							79	68	67	68	64	53
Carp																								73	71	73	65	52
Chum salmon																									97	99	69	56
Chinook salmon																										98	68	56
Rainbow trout																											69	56
Tilapia-188																												69
Tilapia-177																												

Fig. 2.2 Sequence homology (%) among PRLs of different species (Sinha, 1995).

2.2.2 The Function of Prolactin

2.2.2.1 Prolactin Function in Mammals

Although PRL seems to be an omnipotent hormone, it is best known for its role in milk production. In addition of its essential role for the physiology of lactation in mammals, PRL also plays a significant role in reproduction, maternal care, and parental behavior in birds and mammals (Buntin, 1993; Schradin and Anzenberger, 1999). The conventional view of PRL is that its major target organ is the mammary gland, and stimulating mammary gland development and milk production pretty well define its functions. It has been demonstrated that PRL has two major roles in milk production. PRL induces lobuloalveolar growth of the mammary gland and stimulates lactogenesis or milk production (Bern and Nicoll, 1968). The initiation and maintenance of lactation following parturition is dependent on the mitogenic effects of PRL on mammary cell development, as well as its regulation of transcription and translation of casein milk proteins (Ben-Jonathan et al., 1989). It has been suggested that PRL, cortisol, and insulin act together to stimulate transcription of the genes that encode milk proteins. It appears to modulate ovulation since elevated physiological or pathological levels will result in cessation of cyclicity (Nicoll, 1974). PRL also appears to be important in several non-lactational aspects of reproduction. PRL is necessary for maintainance of corpora lutea in some species (Morishige and Rothchild, 1974). It also affects other actions related to reproduction such as mating and maternal behaviors (Dutt et al., 1994). Aside from its actions on reproductive processes, PRL plays a role in maintaining the constancy of the internal environment by regulation of the immune system, osmotic balance, and angiogenesis. PRL has been found to stimulate proliferation of oligodendrocyte precursor cells. These cells differentiate into

oligodendrocytes which responsible for myelin coating on axons in the CNS (Gregg et al., 2007).

2.2.2.2 Prolactin Function in Birds

In birds, PRL is associated with a wide range of reproductive physiology and behaviors including incubation, migration, grooming, crop milk secretion, feeding of young, nest defense, and sexual activity (Lea et al., 1981; 1986; Silver, 1984; Janik and Buntin, 1985; Buntin et al., 1991). It is very well established that PRL is associated with incubation behavior in pigeon (Riddle et al., 1935), pheasant (Breitenbach and Meyer, 1959), cowbird (Hohn, 1959), turkey (Burke and Dennison, 1980; El Halawani et al., 1988a; Youngren et al., 1991), mallard duck (Goldsmith and Williams, 1980), and chicken (Sharp et al., 1988). Changes in PRL gene expression and its plasma levels are highly correlated with the reproductive cycle in birds (Knapp et al., 1988; El Halawani et al., 1990a; Talbot et al., 1991; Wong et al., 1991; You et al., 1995; Tong et al., 1997). Plasma PRL levels are very low (5-10 ng/ml) during the reproductively quiescent stages of the turkey reproductive cycle. However, during the laying and incubating stages, circulating PRL levels increase dramatically (500-1500 ng/ml; El Halawani et al., 1984).

The onset of incubation behavior is correlated with declining plasma levels of LH and gonadal steroids and increasing plasma levels of PRL in bantam (Lea et al., 1981) and turkey hens (El Halawani and Rozenboim, 1993). LH levels begin to increase continuously and reach a peak amount at about 8 to 2 hours before ovulation (Mashaly et al., 1976). Unlike LH, plasma concentration of chicken FSH (cFSH) is low throughout the ovulatory cycle but a significant decline in cFSH occurred prior to

the pre-ovulatory LH surge and a significant increase occurred during the 3 hour prior to oviposition as LH levels decline (Krishnan et al., 1993). Thereafter, LH levels continue to decline during incubating period (Myers et al., 1989). On the contrary, during laying and incubating period, circulating PRL levels increase dramatically (El Halawani et al., 1984). It is this rising PRL level which has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior (Sharp et al., 1984; Buntin, 1986; Hall et al., 1986; El Halawani et al., 1988a). Subsequently, PRL level declines whereas LH level begin to rise when incubation behavior terminates (El Halawani et al., 1988a; Knapp et al., 1988) and as soon as molting is ended (Bluhm et al., 1983; Mauget et al., 1994). High levels of PRL may inhibit LH secretion (Zadworny and Etches, 1987). It has been suggested that PRL acts centrally to reduce LH levels by reducing GnRH levels in the hypothalamus (Rozenboim et al., 1993). Eventually, high level of PRL reduces hypothalamic release of GnRH and release of LH from the pituitary (Tabibzadeh et al., 1995). In addition, it appears that PRL may also directly inhibit ovarian steroidogenesis (Rozenboim et al., 1993). These actions of PRL would lead to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct. The abundance of LH-β subunit and PRL mRNAs shows an inverse relationship in photostimulated/laying and incubating turkey hens (Wong et al., 1992). Administration of exogenous ovine PRL suppresses the photo- and ovariectomy-induced increases in LH secretion, delays the onset of egg laying, and induces incubation behavior in laying hens (El Halawani et al., 1991). Circulating PRL declines when incubation behavior terminates (El Halawani et al., 1980; Wentworth et al., 1983). Administration of exogenous PRL leads to increase parental behaviors in birds (Buntin et al., 1981; Lea and Vowles, 1986; Macnamee et

al., 1986; Pedersen, 1989; Youngren et al., 1991). Taken together, PRL has been implicated as a causative factor for the reduced circulating gonadotropins and ovarian regression, when birds shift from egg laying to incubation behavior (El Halawani et al., 1997).

2.2.3 Neuroendocrine Regulation of Prolactin Secretion

PRL secretion from the pituitary gland is governed by the hypothalamo-adenohypophysis axis. The existence of neurosecretory neurons or neurons containing stored secretory material capable of releasing blood-borne signals is first proposed by Scharrer (1972) and set the stage for later demonstration of hypothalamic control of the pituitary gland. Studies that show the transaction of the hypophyseal stalk impaired reproductive function led to the discovery of a neurovascular pathway, the hypophyseal portal system (Harris and Donovan, 1966). This pathway is then very well documented that it facilitates the transport of hypothalamic neurohormones to the anterior pituitary and affects the release of hormones. The mammalian hypothalmic-pituitary axis includes the hypothalamus, the specialized region of the basal hypothalamus called the ME, the plexus of hypophyseal portal vessels, and the pituitary gland. The mammalian pituitary gland is comprised of the adenohypophysis; the pars distalis and pars intermedia. The neural lobe or neurohypophysis is of neural tissue origin formed by an evagination of the diencephalons. In birds, the pars intermedia does not exist as a separate entity as it does in mammals and the pars distalis, the ME and its associated capillary plexus, have distinct anterior and posterior divisions (Oksche and Farner, 1974). Although no anatomical separation exists between the cephalic and caudal lobes, histological studies have defined a distinct

distribution of hormone-secreting cells, which indicates that the adenocorticotropin, thyroid stimulating hormone, and PRL-secreting cells are confined to the cephalic lobe, whereas GH cells are found within the caudal lobe (Kobayashi and Wada, 1973; Kansaku et al., 1995; Lopez et al., 1995; Ramesh et al., 1996). Hypothalamic regulation of pituitary function is a multifaceted chain of events that ultimately involves many organ systems. External or internal stimuli are perceived by the CNS, and then transmitted via electrical or chemical signals to the hypothalamus which results in the release of hypothalamic factors that induce the secretion of pituitary hormones.

During the past three decades, several hypothalamic neurotransmitters and neuropeptides have been indicated to be involved in the regulation of PRL secretion such as VIP (El Halawani et al., 1997; Chaiseha and El Halawani, 1999; Chaiseha et al., 2004), DA (El Halawani et al., 2000; Chaiseha et al., 2003a; Al-Zailaie et al., 2006), thyrotropin-releasing hormone (TRH; Grosvenor and Mena, 1980; El Halawani et al., 1988a; Laverriere et al., 1988; Lafuente et al., 1994), 5-HT (Chaiseha and El Halawani, 2005), angiotensin II (Malarkey et al., 1987; Opel and Proudman, 1988; Myers and Steele, 1989; Steele, 1990), oxytocin/vasopressin (Hyde and Ben-Jonathan, 1988; 1989; Johnston and Negro-Vilar, 1988), peptide histidine isoleucine (PHI; Samson et al., 1983; Werner et al., 1983; Proudman and Opel, 1990; Kulick et al., 2005), and pituitary adenylate cyclase activating polypeptide (PACAP; Miyata et al., 1989; Yamauchi et al., 1995; Yoo et al., 2000). Substances of hypothalamic origin which stimulate PRL release from the anterior pituitary appear to be important in both birds and mammals. In stead of exhaustively review on each hypothalamic

neurotransmitter and neuropeptide, the regulation of PRL secretion by DA, TRH, and VIP will be focused on the following literature review.

2.2.3.1 Prolactin Regulation in Mammals

PRL secretion in mammals is regulated by both stimulatory and inhibitory control of hypothalamic factors which mainly under tonic inhibitory control (Neill, 1988; Ben-Jonathan et al., 1989; Lamberts and MacLeod, 1990). In mammals, the regulation of PRL secretion and gene expression are under the inhibitory control of tuberoinfundibular dopaminergic (TIDA) neurons in the hypothalamus (Pasqualini et al., 1988; Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001), which release DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs (Caron et al., 1978; Civelli et al., 1991). This is supported by recent evidence showing that transgenic mice with a disrupted D_2 DA receptor gene exhibit anterior lobe lactotroph hyperplasia and hyperprolactinemia (Saiardi et al., 1997). Removal of this dopaminergic inhibition results in increased PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). These and similar studies clearly attest to the physiological relevance of DA as a PRL-inhibiting factor (PIF).

DA appears to be the predominant ongoing inhibitor of PRL secretion (MacLeod and Login, 1976; Ben-Jonathan, 1985; Lamberts and MacLeod, 1990). On the contrary, TRH functions as a hypothalamic releasing factor of PRL in mammals. A wealth of functional data supports a role of TRH as a regulator of PRL secretion. TRH stimulates PRL release both *in vivo* (Grosvenor and Mena, 1980; de Greef and Visser, 1981; Lafuente et al., 1994) and *in vitro* (Maas et al., 1991). TRH also stimulates PRL gene transcription (Potter et al., 1981; Laverriere et al., 1988). PRL release by TRH

occurs during a transient depression in dopaminergic activity (Plotsky and Neill, 1982; Martinez de la Escalera et al., 1988). However, the contradictive results from some studies have led researchers to question TRH's role as the PRF.

2.2.3.2 Prolactin Regulation in Birds

The regulation of PRL secretion and gene expression are under the inhibitory control of TIDA neurons in the hypothalamus in mammals (Ben-Jonathan and Hnasko, 2001). This is not the case in birds, where removal of hypothalamic inputs results in the complete cessation of PRL secretion (Tixier-Vidal et al., 1966; Chadwick et al., 1978; Hall et al., 1986). It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus (Kargt and Meities, 1965; Bern and Nicoll, 1968) and that principal PRF is VIP (for review, see El Halawani et al., 1997). PRL in birds is under tonic stimulatory control by the hypothalamus. It is very well established that PRL is under stimulatory control by hypothalamic VIP, the only avian PRF (El Halawani et al., 1997; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999). The role of DA in the regulation of avian PRL secretion is unclear at present for comparing it to the mammalian DAergic strategy for PRL control. In apparent contrast with mammals, it has been established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptors (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003a). DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL (Youngren et al., 1996b; Chaiseha et al., 1997; 2003a). Recent evidences indicate that dynorphin, 5-HT, DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ-opioid, serotonergic,

dopaminergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (El Halawani et al., 2000; Chaiseha and El Halawani, 2005). The extensively but not exhaustively review regarding the roles of VIP and DA in PRL regulation will be elaborated in the followings.

2.2.4 Photostimulation of Prolactin Secretion

The role of light in biological activities associated with avian reproduction is very well known. Light quality can be defined by three criteria: 1) duration of light-photoperiod, 2) light intensity (brightness), and 3) spectra composition (Andrews and Zimmerman, 1990). In birds from subtropical and temperate latitudes, the gradual or abrupt increase in day length (long day, photostimulation) initiates gonad recrudescence and egg laying. Conversely, reduction in day length (short photoperiod) delays the onset of sexual maturity or terminate egg laying activity in birds (Benoit, 1964; Woodard et al., 1969). The effects of season and photostimulation upon the hypothalamic-pituitary-gonadal axis are well characterized (Dawson and Goldsmith, 1997; Cho et al., 1998; Dunn and Sharp, 1999; Peczely and Kovacs, 2000).

The control of PRL secretion in birds involves the interaction of external stimuli with endocrine mechanisms. Critical environmental stimuli include sensory information concerning photoperiod, ambient temperature, and the presence of eggs and offspring (Curlewis, 1992). In addition to its involvement in incubation behavior, PRL also appears to be associated with photoperiod (Lea et al., 1982; El Halawani et al., 1984). Circulating PRL levels are low prior photostimulation. Following photostimulation, PRL levels gradually increase and remain elevated during egg

laying. PRL levels then increase sharply with the initiation of incubation behavior (Burke and Dennison, 1980). Similar findings are reported in mallard duck (Goldsmith and Williams, 1980), chicken (Lea et al., 1981), starling (Dawson and Goldsmith, 1982), and black swan (Goldsmith, 1982). PRL declines during the transition from incubation to photorefractoriness (El Halawani et al., 1980; Wong et al., 1991). PRL and its mRNA contents in the pituitary have been shown to be positively correlated to the levels of circulating PRL. PRL and its mRNA contents are lowest in reproductively inactive birds. Following photostimulation, pituitary PRL and its mRNA contents parallel the rising circulating PRL levels, and then decline when birds become photorefractory (Saeki and Tanabe, 1955; Cherms et al., 1962; Talbot et al., 1991; Wong et al., 1991; Tong et al., 1997).

2.3 Dopamine: Structure, Function, and Regulation of PRL Secretion

2.3.1 The Structure of Dopamine

DA, a neurotransmitter/neuromodulator, is found in both central and peripheral nervous systems of many species including both vertebrates and invertebrates. It was named DA because it was a monoamine, and its synthetic precursor was 3,4-dihydroxyphenylalanine (L-DOPA; Benes, 2001). DA belongs to the catecholamine family and is a precursor of NE (noradrenaline) and then epinephrine (adrenaline) in the biosynthetic pathways. It has several important physiological functions involved in a wide variety of behaviors and reproduction.

2.3.2 The Function of Dopamine

In the brain, DA functions as a neurotransmitter, activating the five types of DA receptor: D_1, D_2, D_3, D_4, D_5, and their variants (Contreras et al., 2002). DA is produced in several areas of the brain. Dopaminergic neurons are presented in the ventral tegmental area of the midbrain, substantia nigra pars compacta, and arcuate nucleus of the hypothalamus in mammals. The anatomical distribution of the avian dopaminergic system apparently resembles that of mammals (Moons et al., 1994; Reiner et al., 1994), as DA neurons are found throughout the avian hypothalamus (Kiss and Peczely, 1987; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been shown to be immunoreactive for VIP (Mauro et al., 1989; Hof et al., 1991; Mauro et al., 1992) and VIP mRNA (Kuenzel et al., 1997; Chaiseha and El Halawani, 1999). DA is also a neurohormone released by the hypothalamus and has the main function to inhibit the release of PRL from the anterior pituitary as the principle PIF. DA can act on the sympathetic nervous system, producing effects such as increased heart rate and blood pressure. DA also plays important roles in behavior and cognition, motor activity, motivation and reward, regulation of milk secretion, sleep, mood, attention, and learning (for review, see Velasco and Luchsinger, 1998).

DA receptors (D_1-D_5), a class of metabotropic G protein-coupled receptors that are prominent in the vertebrate CNS are very well defined (Sunahara et al., 1993; Ogawa, 1995; Contreras et al., 2002). Very few anatomical data concerning the cellular localization of DA receptors are available in birds. The existence of specific DA-binding sites has been identified in the anterior and posterior hypothalamus of the hen (Macnamee and Sharp, 1989). D_1-like DA receptors have been mapped in the brain of pigeon (Richfield et al., 1987; Dietl and Palacios, 1988), European staring

(Casto and Ball, 1994), quail (Ball et al., 1995), and chick (Schnabel et al., 1997). D_2-like DA receptors have been mapped in the brain of pigeon (Richfield et al., 1987) and quail (Levens et al., 2000). Three D_1 DA receptor subtypes (D_{1A}, D_{1B}, D_{1D}) from chickens were cloned (Demchyshyn et al., 1995). Cloning of a complementary dihydroxy nucleic acid (cDNA) from turkey brain encoding D_1 and D_2 DA receptor subtypes were reported (Schnell et al., 1999a; 1999b). Recently, D_1 and D_2 DA receptors gene expression within the brain of the chick (Sun and Reiner, 2000) and turkey (Schnell et al., 1999a; 1999b) has been reported. The gross tissue distribution of D_1 and D_2 DA receptor subtypes in the turkey brain and pituitary has been determined (Schnell et al., 1999a; 1999b) and demonstrated that the expression of stimulatory D_1 DA receptor mRNA in the hypothalamus increases in hyperprolactinemic incubating hens, whereas inhibitory D_2 DA receptor mRNA increases in the pituitary of hypoprolactinemic photorefractory hens (Chaiseha et al., 2003a).

2.3.3 Dopamine as the PIF in Mammals

DA is the primary neuroendocrine regulator of the secretion of PRL from the anterior pituitary gland in mammals. DA produced by neurons in the arcuate nucleus of the hypothalamus and secreted into the hypothalamo-hypophyseal blood vessels of the ME regulates pituitary PRL secretion. Experimental evidence strongly supports the hypothesis that the predominant mammalian PIF is the catecholamine, DA, which is released from a dense network of neurons within the mediobasal hypothalamus known as the TIDA. DA binds to D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991) and attenuates PRL secretion (Ben-Jonathan et al., 1989). Removal of this

dopaminergic inhibition results in increased PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970). During proestrus or following a sucking stimulus, hypophyseal portal blood DA concentrations decrease in association with increase circulating PRL (Plotsky and Neill, 1982). Pharmacological blockade of TH led to a decrease in portal blood DA and increase circulating PRL. DA and its agonists attenuate PRL secretion both *in vivo* and *in vitro* (MacLeod and Lehmeyer, 1974; Lamberts and MacLeod, 1990). The interruption of DA and its agonists led to augmented PRL secretion. These and similar studies clearly attest to the physiological relevance of DA as the PIF. Aside from providing the role of DA as the PIF, these studies have revealed that attenuation of DAergic tone results in an augmentation of PRL release which is small compared to PRL response *in vivo* following physiological stimuli, thereby providing additional support for the role of DA as the PRF. Several studies have demonstrated that DA at low concentrations stimulates PRL secretion *in vitro* (Shin, 1978; Denef et al., 1980; Burris et al., 1991; 1992; Porter et al., 1994) and *in vivo* (Arey et al., 1993). These phenomena suggest that all pituitary lactotrophs have the potential to respond to the inhibitory and stimulatory effects of DA (Kineman et al., 1994) or that a subpopulation of lactotrophs sensitive to the stimulatory effect of DA exists (Burris et al., 1992; Burris and Freemen, 1993) and the two opposite effects of DA upon PRL secretion may be mediated by distinct G proteins (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994). Another possibility is that the various PRL-releasing and -inhibiting factors which are known to exert effects at the level of the pituitary may also interact at the hypothalamic level to control PRL secretion (Moog and Samson, 1990). The existence of mammalian PRF is supported by the fact that hypothalamic extracts do possess PRL releasing capability, and that the PRL surge is

associated with sucking and stress cannot be entirely accounted for by a concurrent decline in DA (Ben-Jonathan et al., 1989). The hypothalamic control of PRL secretion is dependent on a balance between VIP and TRH stimulation on one hand, and DA inhibition on the other hand. Those three factors as well as the peripheral hormones, estradiol, and glucocorticoids act directly on PRL cells by different receptors (Gourdji et al., 1973; Rotsztejn et al., 1980).

The association of hormones with their receptors is an essential component of their action mediating the regulation of PRL secretion. Dissociation of the inhibitory hormone DA from its receptor is an important physiological signal in PRL secretion. DA tonically suppresses PRL secretion by occupying specific receptors on lactotrophs. Lowering the concentration of DA results in rapid dissociation of DA from its receptor. Dissociation from the receptor activates multiple signaling pathways that in turn stimulate PRL secretion during the time in which DA concentration is decreased. Short-term dissociation from the receptor results in the potentiation of the stimulatory action of TRH on PRL secretion (Cronin et al., 1978).

DA concentrations in hypophyseal portal blood are maintained at physiologically active levels (Ben-Jonathan et al., 1977; Gibbs and Neill, 1978; Ben-Jonathan et al., 1980), and lactotrophs contain DA receptors (Caron et al., 1978; Cronin et al., 1978; Goldsmith et al., 1979). Moreover, PRL levels increase after treatment with DA antagonists (Smalstig et al., 1974; MacLeod and Lamberts, 1978). One signal for PRL release among other endocrine factors is the dissociation of DA from its receptors. Therefore, the removal of DA appears to play an important physiological role in the regulation of PRL secretion.

2.3.4 Dopamine Regulation of PRL Secretion in Birds

The role of DA in the regulation of avian PRL secretion is unclear at present for comparing it to the mammalian DAergic strategy for PRL control. In birds, DA has been measured and visualized in various species, including quail (Ottinger et al., 1986; Ottinger and Balthazart, 1987; Balthazart et al., 1992), zebra finches (Barclay and Harding, 1990), pigeons (Divac et al., 1994), and chickens (Moons et al., 1994). DA inhibits PRL release in chicken, pigeon, and turkey anterior pituitary cells *in vitro* (Harvey et al., 1982; Hall and Chadwick, 1983; 1984). Specific DA-binding sites identified in the anterior pituitary are found to be more abundant in laying than in incubating hens (Macnamee and Sharp, 1989). Data from *in vivo* studies also support the concept that DA is inhibitory to the neuroendocrine system which stimulates PRL secretion in laying turkey hens (El Halawani et al., 1991). However, other data clearly show that DA is not an inhibitor but a stimulator of PRL secretion in non laying turkey (Harvey et al., 1982). An advance in elucidating the neurochemical mechanisms using the turkey as a model demonstrates the dual role of DA in PRL secretion and expression, stimulating via D_1 DA receptors and inhibiting via D_2 DA receptors (Youngren et al., 1995; 1996b; Xu et al., 1996; Chaiseha et al., 1997; Youngren et al., 1998). Both D_1 and D_2 DA receptor mRNAs are abundant in the pituitary and brain (Schnell et al., 1999a), suggesting DA exhibits biphasic actions within the turkey hypothalamus and pituitary. In deed, tonic stimulation of PRL secretion and gene expression are regulated centrally via D_1 DA receptors on VIP neurons, where the expression of D_1 DA receptors is greater than that of D_2 DA receptors (Youngren et al., 1995; Al Kahtane et al., 2003; Chaiseha et al., 2003a). DA inhibits PRL secretion and gene expression by blocking the action of VIP at the level of the anterior pituitary via

D_2 DA receptors (Youngren et al., 1998). The results of the studies suggested that DA acts through D_1DA receptors in the avian hypothalamus to stimulate PRL secretion and the stimulatory effects of DA required the presence of VIP in order to stimulate PRL secretion. Intracranial infusions of DA were ineffective in releasing PRL in turkeys actively immunized against VIP, suggesting that DA affects PRL secretion by stimulating the release of VIP. To date, it can be concluded that DA may act centrally via D_1DA receptors to release VIP to the anterior pituitary to affect PRL secretion. DA may also act on pituitary D_2DA receptors to inhibit PRL secretion. It is known that the secretion of avian PRL also requires an intact serotonergic system (El Halawani et al., 1988b). These findings suggest the involvement of a complex and even redundant network in PRL control, where an integrative interaction among VIPergic, dopaminergic, and serotonergic systems exerts a stimulatory effect upon PRL secretion in birds.

There are some other evidences suggesting an inhibitory role for DA on GnRH release in mammals as well as in birds (Ramirez et al., 1984; Sharp et al., 1984). Several DA neuronl groups have been identified in the preoptic-hypothalamic areas (Kiss and Peczely, 1987; Reiner et al., 1994). Exogenous DA activates hypothalamic VIP gene expression and this increased expression is limited exclusively to the avian INF. The increased VIP mRNA in the INF is correlated with increased levels of circulating PRL and LH-β mRNAs in the anterior pituitary (Bhatt et al., 2003). DAergic neurons inhibit GnRH release through presynaptic inputs at the ME level, as has been demonstrated in the chicken (Contijoch et al., 1992; Fraley and Kuenzel, 1993). Dopaminergic neurons are not only located in a single discrete hypothalamic nucleus or region, but instead are dispersed among a variety of hypothalamic regions

(POA, anterior hypothalamus (AM), suprachiasmatic nucleus (SCN), lateral hypothalamic area (LHy), paraventricular nucleus (PVN), lateral mamillaris nucleus (ML), and dorsomedial nucleus (DM; Kiss and Peczely, 1987; Reiner et al., 1994). Given their widespread distributions, and the findings that DA axons and terminals are found intermingled with VIP neurons in the INF, GnRH neurons in the POA, and with both VIP and GnRH terminals in the external layer of the ME, it is reasonable to consider whether any regional specificity exists in those DA neurons that is neuroendocrine in nature, i.e., controlling the release and expression of VIP/PRL and GnRH/LH-FSH systems. Recently, it has been suggested that activation of DAergic cells in the ML is associated with the activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006).

2.4 Vasoactive Intestinal Peptide: Structure, Function, and Regulation of PRL Secretion

2.4.1 The Structure of Vasoactive Intestinal Peptide

VIP is a highly conserved 28 amino acid peptide originally isolated from the porcine duodenum by Said and Mutt (1970). Subsequently, it has been found to be widely distributed in the central and peripheral nervous systems (Mutt and Said, 1974; Larsson et al., 1976; Said and Rosenberg, 1976; Giachetti et al., 1977; Rosselin et al., 1982), with high concentrations found in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 1991) and is considered to function as a neurotransmitter and neuroendocrine substance. VIP belongs to a family of structurally related peptides, the glucagon-secretin family, which included secretin, glucagon, gastric inhibitory peptide (GIP), GH releasing factor, and PACAP, whose

actions are mediated via interaction with specific receptors that are coupled to adenelate cyclase and the production of cyclic adenosine monophosphate (cAMP) through a G-protein-coupled receptor (Hokfelt et al., 1980; Couvineau et al., 1990; Lutz et al., 1995).

Mammalians VIP have been cloned (Itoh et al., 1983; Nishizawa et al., 1985). VIP is coded from a single-copy gene consisting of seven exons, exon 4 codes for peptide histidine methionine (PHM), and exon 5 codes for VIP (Bodner et al., 1985; Yamagami et al., 1988; Giladi et al., 1990). Both chicken and turkey VIP cDNA have been sequenced (McFarlin et al., 1995; Talbot et al., 1995; You et al., 1995) and show a structure similar to that of the mammalian VIP gene. The open reading frame is comprised of 165 amino acids. Chicken and turkey VIP share complete amino acid homology and are 98% homologus at the nucleotide level. Chicken VIP is different from mammalian VIP in its amino acid sequence at position 11, 13, 26, 28 although the number of amino acid residue is the same (Nilsson, 1975). VIP mRNA may exist with or without PHI. Both mRNA forms were found in the chicken gut and hypothalamus. In contrast, the short form was found only in the turkey hypothalamus and comprised 4-6% of all VIP transcripts (You et al., 1995). The amino acid similarity of VIP, secretin, and glucagon is indicated in Fig. 2.3.

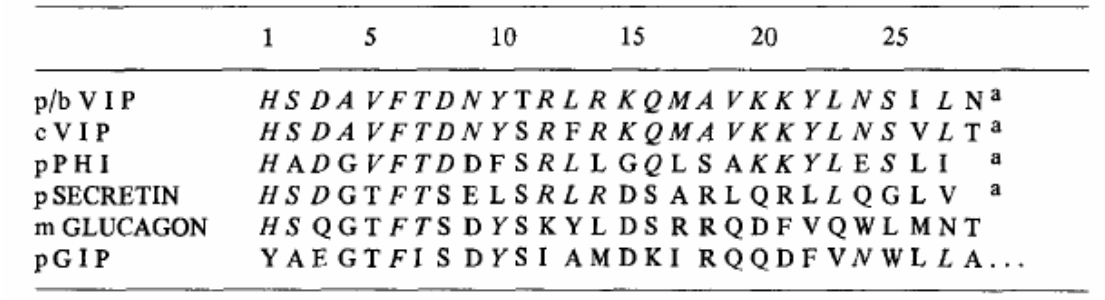

Fig. 2.3 The amino acid sequence of VIP, PHI, secretin, glucagon, and GIP.

The one-letter notation for amino residues.

a: the C-terminal amino acid is in the amide form.

p: porcine, b: bovine, c: chicken, m: mammalian (Rosselin et al., 1982).

The mammalian VIP receptors have been cloned and functionally characterized (Sreedharan et al., 1991; 1993; Ishihara et al., 1992; Lutz et al., 1993; Couvineau et al., 1994; Gagnon et al., 1994; Usdin et al., 1994). Pharmacological evidence indicates that there are two VIP receptor subtypes (VIP1 and VIP2) with different but related amino acid sequences. Each receptor is expressed in a tissue specific manner (Couvineau et al., 1994; Usdin et al., 1994; Sherward et al., 1995). This receptor is present in lung, liver, and intestine, as well as several regions of the brain (e.g. cerebral cortex and hippocampus; Besson et al., 1986; Martin et al., 1987; Csillag et al., 1993) which contain high densities of specific binding sites for VIP. Furthermore, the results from *in situ* hybridization histochemistry (ISH) study with antisense mRNA indicates that the VIP receptors are expressed in neuronal cells throughout the brain (Usdin et al., 1994; Sherward et al., 1995). It has been suggested that a single VIP receptor is expressed and functions in non-mammalians species (Kansaku et al., 2001). In birds, VIP receptors are present on the surface membranes of the anterior pituitary (Rozenboim et al., 1993; Gonzales et al., 1994a; 1994b),

hypothalamus (Gonzales et al., 1995), small intestine, and granulosa cells (Kawashima et al., 1995). Recently, avian VIP receptors were cloned and functionally characterized in chicken (Kansaku et al., 2001) and turkey (You et al., 2001). It also has been indicated that the variations in PRL secretion observed across the turkey reproductive cycle are, in part, regulated by changes in VIP receptors at the pituitary level (Chaiseha et al., 2004).

2.4.2 General Function of Vasoactive Intestinal Peptide

VIP was initially considered to be a gastrointestinal hormone (Grossman, 1974), and was believed to be present in endocrine cells of mammalian and avian species (Polak et al., 1974). The widespread distribution of VIP is correlated with its involvement in a wide variety of biological activities.#With respect to the digestive system, VIP seems to induce smooth muscle relaxation (lower esophageal sphincter, stomach, and gallbladder), stimulate secretion of water into pancreatic juice and bile, and cause inhibition of gastric acid secretion and absorption from the intestinal lumen. Significant concentrations of VIP are present in the gastrointestinal tract, heart, lung, thyroid gland, kidney, immune system, urinary bladder, and genital organs. The general physiologic effects of VIP include vasodilation (Bakken et al., 1995), broncodilation (Tam et al., 1990), exocrine secretions (Alonso et al., 1994; Nassar et al., 1995; Rodriguez-Lopez et al., 1995), smooth muscle relaxation, anti-inflammatory actions, immuno-suppression, hormonal secretion, cell proliferation, and increased of gastric motility (for reviews, see Gozes et al., 1999; Gozes and Furman, 2003).

In addition, VIP exerts a broad spectrum of immunological functions that control the homeostasis of the immune system (Gomariz et al., 2001), including the

modulation of innate and adaptive immunity, and shows a predominant anti-inflammatory action. The presence of VIPergic nerve fibers was shown in both central and peripheral lymphoid organs (Bellinger et al., 1996). These VIP-containing nerve terminals establish the anatomical link between the CNS and the immune system. VIP appears to modulate maturation of specific populations of effecter cells, T cell recognition, antibody production, and homing capabilities.

2.4.3 Neuronal Function of Vasoactive Intestinal Peptide

Although VIP was first isolated from porcine duodenum (Said and Mutt, 1970), it has been found to be widely distributed in the central and peripheral nervous systems (Larsson et al., 1976; Said and Rosenberg, 1976; Giachetti et al., 1977; Hokfelt et al., 1982; Rosselin et al., 1982), with high concentrations found in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 1991) where it may act as a neurotransmitter or neuromodulater (Said and Rosenberg, 1976). Within the CNS, VIP presents in the cerebral cortex, hypothalamus, hippocampus, corpus striatum, and vagal centers of the medulla oblongata (Gozes et al., 1999). VIP promotes neuronal survival, induces neuronal differentiation, modulates transmitter synthesis, and influences neuronal excitability (Klimaschewski, 1997). The discovery of a large population of VIP-immunoreactive neurons in the hypothalamus whose axons project to the ME (Samson et al., 1978; 1979; Polak and Bloom, 1982; Lam 1991; Dalcik and Phelps, 1993) and a high concentration of VIP in hypophyseal portal blood (Said and Porter, 1979; Shimatsu et al., 1981; Brar et al., 1985; Mutt, 1988) led to the hypothesis that VIP participates in the regulation of anterior pituitary function.

2.4.4 Vasoactive Intestinal Peptide as the PRF in Mammals

In mammals, several studies show the involvement of VIP in the regulation of PRL secretion from the pituitary (Kato et al., 1978; Rotsztejn et al., 1980; Reichlin, 1988). The presence of VIP in the hypothalamic nerve endings, the adenohypophysis (Besson et al., 1979), and the hypophyseal portal blood (Said and Porter, 1979) suggests an involvement of VIP in the regulation of pituitary secretion by a neuroendocrine pathway. VIP stimulates PRL release *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Shaar et al., 1979; Enjalbert et al., 1980; Samson et al., 1980; Matsushita et al., 1983). The administration of VIP anti-serum inhibits PRL release induced by stress, serotonin, or suckling (Shimatsu et al., 1984; Abe et al., 1985; Kaji et al., 1985a; 1985b; Ohta et al., 1985). Furthermore, VIP also appears to regulate the amount of pituitary PRL mRNA and PRL synthesis (Ben-Jonathan et al., 1989; Maas et al., 1991). The study in rat showed that hypothalamic VIP mRNA is increased during lactation (Gozes and Shani, 1986). The concentration of VIP in the hypophyseal portal blood is increased relative to in peripheral blood (Said and Porter, 1979; Shimatsu et al., 1981). VIP receptors have been identified in many tissues including the pituitary and in all of these tissues VIP receptors are coupled with adenylate cyclase, which indicates the involvement of cyclic AMP (Gourdji et al., 1979; Bjoro et al., 1987). VIP also promotes the entry of extracellular calcium ions into the PRL-secreting pituitary cells of rat and human (Bjoro et al., 1987; Prysor-Jones et al., 1987). All of these data point VIP as the PRF in mammals.

2.4.5 Vasoactive Intestinal Peptide as the PRF in Birds

In birds, it has long been established that the hypothalamic control of PRL secretion in birds involves a stimulatory mechanism rather than the inhibitory dopaminergic system found in mammals (Kragt and Meites, 1965; Bern and Nicoll, 1968; El Halawani et al., 1984; Hall et al., 1986). In birds, it is very well established that pituitary PRL secretion is tonically stimulated by VIP which is secreted from neurons located in the INF of the caudo-medial hypothalamus (El Halawani et al., 1997). VIP is implicated as the PRF and proved to be a potent releaser of PRL *in vivo* (Lea and Vowles, 1986; Macnamee et al., 1986; Opel and Proudman, 1988; El Halawani et al., 1990c; Pitts et al., 1994) and *in vitro* (Macnamee et al., 1986; Proudman and Opel, 1988; El Halawani et al., 1990b; Xu et al., 1996). VIP regulates PRL gene expression by both enhancing the transcription rate of PRL and up-regulating PRL mRNA stability (Tong et al., 1998). Variations in VIP immunoreactivity, VIP content, and VIP mRNA steady-state levels occurring within the hypothalamus are correlated with changes in the amount of circulating PRL throughout the turkey reproductive cycle (Mauro et al., 1989; You et al., 1995; Chaiseha and El Halawani, 1999). The only exception to this correlation between hypothalamic VIP and circulating PRL is found in photorefractory hens, where plasma PRL levels are extremely low in the presence of high hypothalamic VIP levels (Mauro et al., 1989). Changes in pituitary VIP receptor mRNA was also observed across the reproductive stages in turkeys. Increased VIP receptor mRNA in the pituitary was observed in turkey hens with normal (laying) or high PRL secretion (incubating), while much less VIP receptor mRNA was observed in the pituitary of hypoprolactinemic nonphotostimulated and photorefractory turkey hens (Chaiseha et

al., 2004). These results are in good agreement with studies indicating variations in VIP immunoreactivity and VIP content in the INF and ME, in VIP mRNA steady-state levels in the INF (Mauro et al., 1989; Chaiseha and El Halawani, 1999), and in VIP concentrations in turkey hypophysial portal blood (Youngren et al., 1996a). This suggests that the VIP receptors located in the INF may be involved in avian PRL secretion, and indicates that PRL secretion is principally regulated by VIP receptors at the pituitary level (Chaiseha et al., 2004).

VIP neurons are widely distributed throughout the hypothalamus (Yamada et al., 1982; Macnamee et al., 1986; Mikami and Yamada, 1984; Peczely and Kiss, 1988; Mauro et al., 1989; Chaiseha and El Halawani, 1999). Changes in the number of VIP immunoreactive (VIP-ir) cells have been demonstrated to parallel plasma concentrations of PRL in turkey (Mauro et al., 1989), chicken (Sharp et al., 1989), and dove (Cloues et al., 1990). Hypothalamic VIP immunoreactivity within the INF increases after photostimulation, reaching the highest levels in incubating hens. Similar results are found in chicken, where the number and intensity of VIP-ir neurons are greater in incubating than in laying hens (Sharp et al., 1988).

It has been demonstrated that VIP is inhibited by high concentrations of circulating PRL. Intracerebroventricular PRL injections into incubating ring dove reduce the number of infundibular VIP-like neurons, indicating the existence of a hypothalamic negative feedback loop for PRL (Saldanha and Silver, 1995). In addition, previous studies have shown that both intracranial and systemic administration of ovine PRL into laying turkey hens reduce circulating PRL levels (Youngren et al., 1991; Rozenboim et al., 1993). Systemic PRL administration also reduces hypothalamic VIP content and the number of anterior pituitary VIP binding sites

(Rozenboim et al., 1993), suggesting that PRL may act directly at the pituitary level. PRL binding sites have been observed within the avian hypothalamus (Buntin and Ruzycki, 1987; Buntin and Walsh, 1988) and PRL receptor mRNA is detected in the chicken brain (Tanaka et al., 1992) and the turkey hypothalamus (Zhou et al., 1996; Pitts et al., 2000). PRL may cross the blood-brain barrier at the choroids plexus (Buntin and Walsh, 1988) and bind to PRL receptors lining the third ventricle, thereby decreasing the number of hypothalamic VIP-containing neurons (Saldanha and Silver, 1995).

Passive immunization with anti-VIP serum decreases plasma PRL and pituitary mRNA levels and terminates incubation behavior (Talbot et al., 1991). Similarly, active immunization of female turkeys with VIP reduces circulating PRL, prevents the expression of incubation behavior (El Halawani et al., 1996; 2000). The stimulatory action of VIP occurs via specific binding sites located on turkey anterior pituitary cell membranes, which change throughout the reproductive cycle (Rozenboim and El Halawani, 1993; Chaiseha et al., 2004).

As aforementioned, it is very well documented that PRL is under stimulatory control by hypothalamic VIP, the only avian PRF. VIP meets the classical criteria for defining it as the hypophysiotrophic PRF in birds. The classical criteria for defining substances as the hypophysiotropic PRF, candidate substances should ideally fit the following criteria: (1) localization in the hypothalamic neurons which terminate within the ME; (2) release into hypophyseal portal circulation at concentrations greater than peripheral blood; (3) proven biological activity *in vivo* and *in vitro*; (4) the presence of specific receptors on anterior pituitary cells; and (5) the alteration of pituitary function, due to antagonism of the candidate substances. Several hypothalamic

neurotransmitters and neuropeptides have been studied during the past three decades for their effects upon PRL, such as TRH, angiotensin II, oxytocin, vasopressin, PACAP, and PHI. Only VIP is thought to be physiologically significant in birds.

2.4.6 The Presence of VIP-ir Neurons in the Avian Brain

The distribution of VIP-containing neurons has been conducted in the brain of several avian species such as Pekin duck (Korf and Fahrenkrug, 1984), Japanese quail (Peczely and Kiss, 1988), turkey (Mauro et al., 1989; Chaiseha and El Halawani, 1999), pigeon (Cloues et al., 1990), ring dove (Norgren and Silver, 1990), chick (Kuenzel and Blahser, 1994; Kuenzel et al. 1997), dark-eyed junco (Saldanha et al., 1994), and male zebra finch (Bottjer and Alexander, 1995). In the avian hypothalamus, VIP neurons are found in the areas of the medial preoptic nucleus (MPOA), medial hypothalamus, AM, LHy, and INF (den Boer-Visser and Dubbeldam, 2002).

Studies using a combination of electrophysiology, radioimmunoassay, immunocytochemistry (ICC), and ISH suggest that VIP in the ME is derived from neurons located within the INF (Macnamee et al., 1986; Mauro et al., 1989; Chaiseha and El Halawani, 1999; Youngren et al., 2002). VIP immunoreactivity is associated with PRL and the reproductive stage. VIP peptide and mRNA levels in the INF increase following exposure to long days and remain elevated as long as such exposure continues, declining only when the bird is subjected to short days (Chaiseha and El Halawani, 1999). ICC and ISH studies have shown that fluctuations in hypothalamic VIP immunoreactivity and expression within the INF parallel fluctuations in circulating PRL (Mauro et al., 1989; Chaiseha and El Halawani, 1999). Other studies have also shown increases in the number and size of VIP-ir neurons

within this region in the domesticated pigeon following the initiation of crop milk secretion and feeding of offspring, which are periods of elevated circulating PRL (Peczely and Kiss, 1988; Cloues et al., 1990). The number, area, and density of hypothalamic VIP-ir neurons are greater in incubating than laying chicken hens (Sharp et al., 1989). The hypothalami of incubating turkey hens contain more VIP-ir neurons than those from non-photostimulated hens. Depriving incubating birds from their nests are found to reduce circulating PRL levels and hypothalamic VIP immunoreactivity (Mauro et al., 1989). These studies demonstrate that a reproductive stage normally associates with large amounts of circulating PRL is also related to increased VIP immunoreactivity in the hypothalamus. Moreover, concentrations of VIP in portal blood plasma are significantly higher than VIP concentrations in peripheral blood plasma in all reproductive stages. VIP concentrations in portal blood plasma are lowest in non-photostimulated, reproductively quiescent turkey hens, and highest in incubating hens, with laying and photorefractory hens having intermediate levels (Youngren et al., 1996a). These differences in VIP portal blood concentrations mirror those of PRL in the general circulation, supporting the hypothesis that VIP is the avian PRF.

In general, three types of VIP fibers are described. The first consists of a large number of spindle, or bipolar neurons that connected the third ventricle to the external zone of the ME. A second set of VIP-ir fibers extends from the infundibular nucleus to the ME. The third type of VIP-ir neurons terminates upon small capillaries within the hypothalamus. VIP terminals are observed in the external portion of the ME, and the majority of VIP-containing cell bodies are found in the INF. The number of VIP-ir cells in the INF increases following a gonadal stimulatory photoperiod (Mauro et al.,

1989). These neurons project to the ME, where VIP is transported through the hypothalamic-pituitary portal vessels to the anterior pituitary (Yamada et al., 1982; Macnamee et al., 1986; Mauro et al., 1989). Moreover, the PRL increase seen after photostimulation is prevented by lesions in the INF (Youngren et al., 1989). These data indicate that the VIP neurons in the INF are an important factor in the stimulation of PRL secretion. VIP axon terminals have been found in close apposition to GnRH neurons in the lateral septal organ and POA (Teruyama and Beck, 2001), and an inverse relationship between VIP in the INF and GnRH in the POA has been reported (Deviche et al., 2000). Elevated hypothalamic VIP peptide and mRNA contents are associated with gonad regression and suppression of gonadotropin in photorefractory turkeys (Chaiseha et al., 1998; Chaiseha and El Halawani, 1999). VIP immunoneutralization up-regulates LHβ- and FSHβ subunit mRNA (Ahn et al., 2001), and delays the onset of photorefractoriness and molt in starlings (Dawson and Sharp, 1998). While the functional significance of these findings remains to be clarified, they imply that VIP exerts an inhibitory influence on the gonadotropin system. There are indications that VIP has a central inhibitory influence on GnRH/LH release (Pitts et al., 1994).

2.4.7 Photoperiodic Regulation of Vasoactive Intestinal Peptide Secretion

Quantification of hypothalamic VIP revealed an increased content following photostimulation (Mauro et al., 1992) and it has been suggested that VIP mediates the effects of photoperiod on PRL secretion in the turkey (El Halawani et al., 1996). It has been demonstrated that ME VIP content, hypothalamic cytoplasmic VIP mRNA steady-state levels and hypothalamic nascent VIP mRNA levels were all increased and

correlated with increased PRL secretion following photostimulation (Chaiseha et al., 1998). This result lends support to a hypothetical scheme for photoperiodic regulation of PRL in which VIP serves as a PRF that is intimately linked to photoperiodic mechanisms. However, it remains to be established how photoperiodic information is transduced to VIP-ir neurons located in the infundibulum region of the hypothalamus (Mauro et al., 1989). Whether photoperiodic cues directly influence VIP remains an open question. Silver and co-workers (1988) have shown that VIP is colocalized with an opsin-like pigment in the INF region of the hypothalamus. This area is thought to contain extraretinal hypothalamic photoreceptors which are important for the induction of seasonal reproductive function in birds (Oksche and Farner, 1974; Oliver and Bayle, 1976; Oliver et al., 1977). Alternatively, photoperiod may modulate VIP expression by acting upon unknown neuronal circuits that influence VIP transcription.

Immunoneutralization of VIP averts the rise in circulating PRL that follows photostimulation, prevents the induction of incubation behavior, up-regulates LHβ- and FSHβ subunit mRNAs, and extends the duration of reproductive activity (egg laying period), but does not prevent spontaneous gonad regression and molting (Sharp et al., 1989; El Halawani et al., 1995; 1996; Dawson and Sharp, 1998; Ahn et al., 2001). Despite the well established antigonadotropic effect of PRL, it appears that the high circulating PRL level of laying and non-incubating birds is not the primary cause of GnRH/gonadotropin suppression and the termination of reproduction (Juss, 1993; Dawson and Sharp, 1998).

2.5 Photoperiodic Control of the Avian Reproductive Cycle

2.5.1 Avian Reproduction Cycle: Role of Photoperiod

In many avian species, environmental information initiates reproductive development prior to the onset of optimal conditions for raising offspring while other environmental factors regulate the specific timing of reproductive behaviors and the eventual termination of reproduction. Most of birds show annual cycles of physiological and behavioral events including specific times for molt, body weight changes, migration behavior, gonadal development, and breeding behavior. The environmental cue responsible for the initiation of seasonal events is photoperiod. Generally, increasing or increased photoperiod causes seasonal and unseasonal gonadal development of most of temperate avian species (Burger, 1949; Farner, 1955; Follett, 1984) and may maintain reproductive system of some species in a continuous state of activity (Nicholls et al., 1988; Chaturvedi et al., 1993). In most of long day breeding photoperiodic species, period of reproduction is terminated abruptly by the rapid collapse of gonad during late summer when days are still long. During this phase, gonadal regression of these birds cannot be re-stimulated experimentally by any period of long day length and they are said to be absolute photorefractory (Burger, 1953; Farner and Follett, 1966; Murton and Westwood, 1974). The site of photorefractoriness is believed to be at the hypothalamic or higher level (Farner and Lewis, 1971; Storey and Nicholls, 1976) because some photorefractory birds are able to respond readily and with essentially normal gonad development to exogenous stimulatory agents such as gonadotropins (Stetson et al., 1973). It has been demonstrated that post reproductive gonadal regression is due to marked decrease in LH and FSH (Nicholls, 1974; Mattocks et al., 1976; Dawson and Goldsmith, 1982).

This photorefractoriness can be terminated by exposing the birds to short day length. Some tropical and sub tropical birds which do not experience variations in annual day length are also reported to use photoperiod as an environmental factor to time their seasonal reproduction and exhibit the phenomena of absolute photorefractoriness such as common myna, red headed bunting, red vented bulbul, and Indian rose finch, (Chaturvedi and Thapliyal, 1983; Thapliyal and Gupta, 1989; Rani and Kumar, 2000). Unlike absolute photorefractory birds, some birds remain continuously in breeding condition under constant long days but gonads regress if they are shifted to relatively short days (>15 hours). This type of refractoriness is so called "relative refractoriness" and it is seen in various species of Indian weaver bird (Thapliyal and Tewary, 1964; Chakravorty et al., 1985) and quail (Anthony, 1970; Follett, 1984).

Short days are also reported to have variable effects on the breeding cycle. In general, short photoperiod partially (Miller, 1955) or fully (Weise, 1962) block the annual cycle of gonadal development in birds. However, in certain species, incomplete or complete development of gonad may occur even under total darkness (Marshall and Serventy, 1956; Benoit, 1961). In addition, based on the short photoperiod responses of seasonal breeders and short day breeding species phenomenon of scotosensitivity and scotorefractorininess is reported (Reiter, 1972). In some species, short days had no significant effect on the annual gonadal cycle such as Japanese quail (Tewary and Thapliyal, 1962; Lofts, 1964). The rate and degree of gonadal and cloacal gland growth is directly proportional to daily photoperiod, except short day length until full reproductive condition is achieved, although under short photoperiod also full breeding condition is apparent (Chaturvedi et al., 1992).

As mentioned above, birds are highly photoperiodic and gonadal development occurs in response to increasing day length. The birds breeding outside the tropics use seasonal changes in day length to provide predictive information for the optimal time to initiate and terminate breeding (Murton and Westwood, 1977; Sharp, 1996). It has been known that stimuli from the environment profoundly influence reproductive physiology and behavior by changing levels of circulating hormones in the blood (Marshall, 1959; Lehrman, 1961; Murton and Westwood, 1977; Wingfield and Farner, 1980; Follett, 1984). Environmental light stimulates neural (photo) receptors in conjunction with an internal circadian cycle, enable the bird to respond to the most favorable time for reproduction. At the brain level, a cascade of events is involved in the integration of external environment stimuli and internal body signals. The specialized receptors for the external signals transducer the physical stimuli into the neuronal events. The signals from various receptors reach target areas in the brain and secreted neuromodulators and enzymatic factors regulating the hypothalamic releasing hormones/factors. Finally, peripheral steroid hormones act feedback in the brain to alter its responsiveness (for review, see Silver and Ball, 1989).

In conclusion, the lighting conditions to which photoperiodic birds are exposed when they begin or terminate breeding differ widely between avian species. Subtropical and temperate zone birds use a biological clock for photoperiodic time measurement that is integrated with the reproductive neuroendocrine system in different ways to generate species-specific breeding patterns.

2.5.2 Light Detection in Birds

Temperate zone birds limit their reproductive activity to a particular time of the year. This is involves responses to photoperiodic information and supplementary cues such as behavioral interactions, the availability of nest sites, nest materials, and food (Wingfield, 1983; Farner, 1986). Photoperiodic information is detected by an extraretinal encephalic photoreceptor while the supplementary information is processed by the ears and eyes (Yokoyama and Farner, 1978; Yokoyama et al., 1978; Glass and Lauber, 1981; Foster et al., 1994; Saldanha et al., 1994; Wada et al., 2000; Li et al., 2004).

The role of light in biological activities associated with avian reproduction is very well known. Light quality can be defined by three criteria: 1) pattern of light-dark exposure (photoperiod), 2) light intensity (brightness), and 3) spectra composition (Andrews and Zimmerman, 1990). In birds from subtropical and temperate latitudes, the gradual or abrupt increase in day length initiates gonad recrudescence and egg laying. Conversely, reduction in day length delays the onset of sexual maturity or terminates egg laying activity in birds (Benoit, 1964; Woodard et al., 1969).

Photoreceptors were suggested to be involved in the detection of daily or seasonal changes in photoperiod. Different types of photoreceptors responsible for transferring photic information to the H-P-G axis. Three structures that could contain the essential photoreceptors include eyes, pineal gland, and deep brain. It has been suggested that photoperiodic regulation of reproductive state is controlled by photoreceptors outside the eyes and pineal (Menaker and Keatts, 1968; Menaker et al., 1970) and the locations of extraretinal-extrapineal photoreceptors mediating the

reproductive photoperiodic responses appear to be located within the mediobasal region of the hypothalamus (MBH; Benoit and Ott, 1944; Homma et al., 1977). To date, there are few data supporting the eyes or pineal gland as viable candidates (Homma et al., 1967; Harrison and Becker, 1969; Menaker et al., 1970; Gwinner et al., 1971; Harrison, 1972; Follett et al., 1975; McMillan et al., 1975; Wilson, 1991). Therefore, an emphasis has been directed to the brain as a source of encephalic photoreceptors.

Detection of light in birds is extraretinal (Kuenzel, 1993). The existence of encephalic photoreceptors has been well documented in a variety of avian species (Oliver and Bayle, 1980). Blinded, pinealectomized, or blinded and pinealectomized birds show the same reproductive photoperiodic responses as intact controls (Sipoes and El Halawani, 1986; 1989; Wilson, 1991). It has been indicated that an extraretinal photoreceptor for reproductive function is in the basal hypothalamus (Foster et al., 1985), but the precise neural structures involved have not been conclusively identified.

All known vertebrate photoreceptors contain the protein termed opsin as part of the photopigment that transduces the photic signal. ICC studies using antibodies to proteins involved in photoreception suggest that extraretinal reception in the hypothalamus is not restricted to one locus. Several opsin photopigments have been identified and described in cells and tissues beyond the traditionally accepted retinal photoreceptors, the rods and cones, in vertebrate species (Foster and Hankins, 2002; Berson, 2003; Van Gelder, 2003). Proteins involved in photoreception are found in cerebrospinal fluid (CSF)-contacting neurons in the LS, and basal hypothalamus (Silver et al., 1988; Foster et al., 1994; Wada et al., 2000), and the ME (Saldanha et al., 2001). Furthermore, opsin-like immunoreactivity in CSF-contacting neurons in the

septal and tuberal hypothalamic regions also expresses VIP (Silver et al., 1988). In addition, opsin immunoreactive neurons have been localized in the MBH of the quail and the duck (Silver et al., 1988) where electrical stimulation induced LH secretion and gonad growth (Ohta et al., 1984; Konishi et al., 1987), and lesions block the photo-induced release of LH and testicular growth (Sharp and Follett, 1969; Davies and Follett, 1975; Ohta and Homma, 1987). Furthermore, neuronal activation, as indicated by fos-like protein expression, is shown to occur in the MBH of quail and turkey in association with a photoperiodically driven LH rise (Meddle and Follett, 1997; Millam et al., 2003). Also, the evidence is presented pointing to the MBH as an important site for the clock responsible for circadian measurement of day length (Yasuo et al., 2003).

The encephalic photoreceptors embedded in deep brain would need to response to very dim light. Data obtained that produced an action spectrum for light passing through brain tissue showed a wavelength of maximum sensitivity at 492 nm responsible for trigger the greatest reproductive response in quail, which matched the absorption spectrum for rhodopsin (Follett et al., 1985; Foster and Follett, 1985). A monoclonal antibody against the N-terminus of rhodopsin, RET-P1, identified a group of CSF-contacting neurons in the LS and in the infundibular region of the hypothalamus of dove, quail, and duck brains (Silver et al., 1988).

The photosexual responses and subsequent alterations in reproductive activities have been shown to be mediated by retinal and extra-retinal (brain) photoreceptors (Benoit and Assenmacher, 1966; Homma et al., 1972; Menaker and Underwood, 1976; Siopes and Wilson, 1980a; 1980b). In birds subjected to a gonad stimulating photoperiod, long wave radiation (630-780 nm) penetrates the tissue and

directly acts on hypothalamic extra-retinal photoreceptors to stimulate reproductive function. In contrast to the stimulatory effect of long wave radiation on reproductive activity (Benoit and Assenmacher, 1966; Menaker and Underwood, 1976), activation of retinal photoreceptors by visible radiation appears to be inhibitory to avian reproduction (Homma et al., 1972; Siopes and Wilson, 1980a; 1980b). The response to visible radiation is surmised to be mediated by the green-yellow bands of the light spectrum (545-575 nm), where the avian retina is maximally sensitive (Prescott and Wathes, 1999; Lewis and Morris, 2000).

The effects of season and photostimulation upon the hypothalamic-pituitary-gonadal axis are well characterized (Dawson and Goldsmith, 1997; Cho et al., 1998; Dunn and Sharp, 1999; Peczely and Kovacs, 2000), while the wavelength characteristic that transduces relevant photic information to neuroendocrine effector neurons are not well established and far from understood. Several studies have been indicated that the eyes may have an inhibitory effect on reproductive activities in birds. It has been demonstrated that the eyes of Japanese quail are not essential for photostimualtion and sexual development, but appear to be involved in the regressive effects of short day on the photosexual responses (Homma et al., 1972; Siopes and Wilson, 1975; 1980b). Furthermore, Yokoyama and Farner have reported on an inhibitory effect of the eye on serum LH in white-crowned sparrows (Yokoyama and Farner, 1976).

2.5.3 Photoperiodic Regulation of Reproduction in Birds

Information from photoperiodic time measurement controls seasonal changes in gonadotropins and PRL secretion. In birds, the secretion of LH and PRL are under

the stimulatory control of GnRH-I and VIP, respectively (Sharp et al., 1998). Photoinduced seasonal changes in GnRH-I and VIP release are likely to be responsible for a pattern of seasonal change in concentrations of LH and PRL found in both long and short day breeders (Sharp and Blache, 2003). Once environmental stimuli are transduced by the appropriate receptors, they influence the secretion of GnRH and VIP. Cell bodies containing GnRH-I occur in the preoptic-anterior hypothalamus, while VIP cell bodies known to control PRL secretion occur in the basal hypothalamus. Terminals containing GnRH-I or VIP are abundant in the avian ME, consistent with their functions in the regulation of gonadotrophin and PRL secretion (Saldanha et al., 2001; Teruyama and Beck, 2001).

Studies of GnRH positive neurons by radioimmunoassay (Dawson et al., 1985) and by immunohistochemistry (Foster et al., 1987) reveal striking differences in the activity of this decapeptide in reproductively active and inactive European starlings. The data demonstrated that GnRH positive neurons of the POA of the hypothalamus is 10-fold higher in photosensitive (capable of responding to long day lengths) compared to photorefractory (incapable of responding to long days) birds. Immunohistochemical evidence indicates the location of the GnRH cell bodies in the preoptic and septal regions of the brain and confirms a seasonal change in the intensity of staining. Individual neuronal perikarya in the POA increase in diameter and in the intensity of GnRH staining density in photosensitive animals. Thus, seasonal cycles of sensitivity to daylength are accompanied by changes in the synthesis of GnRH. The induction of gonadal growth associated with photosensitivity is preceded by an increase in hypothalamic GnRH contents (Goldsmith et al., 1989). Similarly, gonadal regression is preceded by a decline in GnRH (Goldsmith et al., 1989). Recently, it has been

reported GnRH mRNA was highly expressed in the OVLT and nCPa, and limited expression was observed in the POA, POM, and LS. Hypothalamic GnRH mRNA expression was significantly increased after subjecting the non-photostimulated female turkey to a 90 minute light period at ZT 14. GnRH mRNA abundance within the LS, OVLT, and nCPA areas was highest in laying hens, with decreasing abundance found in non-photostimulated and incubating hens, respectively. The lowest levels of GnRH mRNA were observed in photorefractory hens. These results indicate that hypothalamic GnRH mRNA expression may be used to precisely characterize the different reproductive stages (Kang et al., 2006).

The breeding season is preceded by an increase in circulating LH followed by a more gradual increase in circulating PRL. Birds are in full breeding condition when plasma LH level is high, and a decrease in LH secretion signals the end of the breeding season and initiates gonadal regression. PRL concentrations reach the highest values when circulating LH is beginning to decrease at the onset of the development of photorefractoriness. After plasma LH level returns to baseline values, plasma PRL level also decreases (Dawson and Sharp, 1998). The initial decrease in plasma LH level at the end of the breeding season is therefore likely to be due to a suppressive effect of high PRL concentrations.

The control of PRL secretion in birds involves the interaction of external stimuli with endocrine mechanisms. Critical environment stimuli include sensory information concerning photoperiod, ambient temperature, and the presence of eggs and poults. These external stimuli as well as the prevailing internal steroid milieu (estrogen and progesterone) are important in initiating and maintaining PRL secretion, although their relative importance varies with the stages of the reproductive cycle. In

seasonal breeder birds, the initiation of the breeding cycle depends upon the precise prediction of environmental conditions which are optimal for health of the mating pair and the survivability of its offspring (Curlewis, 1992). Photoperiod has been associated with circulating PRL in duck (Kragt and Meites, 1965), turkey (Burke and Dennison, 1980), and starling (Dawson and Goldsmith, 1982). The observation that photostimulation increased PRL to the same level in both intact and ovariectomized turkeys (El Halawani et al., 1983) suggests that ovarian hormones are not involved in the initial PRL rise, and argues in favor of direct regulation by photoperiod. PRL and VIP gene transcription and the abundance of their mRNAs have also been shown to increase in the female turkey pituitary upon exposure to a gonadal stimulating photoperiod (Wong et al., 1991; Tong et al., 1997; Chaiseha et al., 1998).

The control of reproduction is well documented for animals living at temperate, regular seasonal, and latitudes (Baker, 1938; Murton and Westwood, 1977; Bronson, 1987; Wingfield and Kenagy, 1991; Ball, 1993; Cockrem, 1995). In contrast, the timing processes of animals inhabiting environments with only slight seasonal fluctuations such as tropics are poorly understood (Gwinner and Dittami, 1985; Bronson, 1987; Dittami and Gwinner, 1990; Wingfield et al., 1992; Hau et al., 1999).

2.5.4 Seasonal Reproduction in Birds

Most temperate animals rely to a high degree on photoperiodic stimuli for controlling reproduction. A change in day length is one of the primary environmental factors regulating seasonal cycles of gonadal growth and regression. The physiological processes underlying the timing of breeding in birds have been studied extensively in temperate zone species (Murton and Westwood, 1977; Follett et al., 1985; Wingfield

et al., 1992; Ball, 1993; Cockrem, 1995; Hahn et al., 1997). Photoperiod regulates seasonal processes either directly by initiating or terminating them or indirectly by synchronizing endogenous timing mechanisms (Farner and Lewis, 1971; Follett et al., 1985; Gwinner, 1986; Wilson and Donham, 1988).

An increase in photoperiod starts the neuroendocrine cascade beginning with the secretion of releasing hormone from the hypothalamus, GnRH. GnRH then stimulates the secretion of adenohypophyseal hormones, LH and FSH, which in turn induce gonad growth and production of steroid hormones (Murton and Westwood, 1977; Ball, 1993; Wingfield and Farner, 1993). Gonadal steroids regulate important physiological changes and behaviors associated with reproduction (Balthazart, 1983; Wingfield and Ramenofsky, 1985; Wingfield et al., 1999). The reproductive period is usually terminated by a photorefractoriness process. The neuroendocrine cascade is shut down, and the gonad collapse (Farner et al., 1983; Follett et al., 1985; Nicholls et al., 1988; Wilson and Donham, 1988; Bentley, 1997).

It is still unknown whether these mechanisms are useful in more extreme environments with only slight or variable seasonality. Particularly, seasonal control mechanisms in equatorial habitats, where photoperiodic changes are minimal and poorly understood. Some near-equatorial vertebrate species do show photoperiodic responses when exposed to temperate zone-like day length changes (Gwinner and Dittami, 1985; Bronson, 1987; Hau et al., 1999). Interestingly, some studies suggest a functional relevance for such photic responses in regulating seasonal breeding in near-equatorial birds (Hau et al., 1998; Gwinner and Scheuerlein, 1999). Seasonal breeding in the near-equatorial habitat is probably made possible by possessing an exceptionally high sensitivity to the slight seasonal changes in photoperiod at this latitude (Hau et al.,

1998; Wikelski et al., 2000). A response to photoperiodic cues enables these birds to physiologically prepare for the coming breeding season ahead of time. In addition to photoperiod, equatorial birds are also very responsive to changes in food abundance and adjust gonad growth to food availability (Hau et al., 2000; Wikelski et al., 2000).

2.6 The Studies of the Native Thai Chicken Reproduction

Native Thai chicken (*Gallus domesticus*) is originated from the wild jungle fowl in Southeast Asia. It was domesticated by village people approximately 3,000 years ago. There are about 54 millions native Thai chickens out of the total 184 millions chickens in Thailand (Department of Livestock Development, 2006). Normally, the native Thai hen lays eggs 3-4 times per year and 4-17 eggs per clutch. Thus, it produces about 30-40 chicks per year which is significantly lower than that of the imported hen which produce eggs all year long. Some characteristics that inherited from the wild jungle fowl and still expressed in native Thai chicken are maternal and incubation behaviors (Beissinger et al., 1998). Native Thai hen has poor egg production and short egg laying period because the onset of incubation behavior terminates egg laying. Therefore, incubation behavior in native Thai chicken might be the cause of low egg production.

In general, animals need to adjust reproductive decisions to environmental seasonality. In contrast to species from the well-studied temperate zones, little is known for tropical birds regarding the environmental cues that stimulate reproductive activity and physiological mechanisms regulating reproduction. In Thailand, the effects of lighting regimens (photoperiod) upon growth, reproductive development, laying performance, and reproductive efficiency in native Thai chickens have been

reported (Chotesangasa et al., 1992; Chotesangasa and Gongruttananun, 1994; 1995; 1997; Choprakarn et al., 1998). However, the results of these studies are contradictive and far from understood. Up to date, in order to increase reproductive efficiency by the lighting regimens is still not successful. It has been reported that progesterone level is related to reproductive cycle of the native Thai chicken (Katawatin et al., 1997). The researchers were unable to measure plasma PRL levels due to unable to establish a chicken PRL assay. In order to increase the production of the native Thai chicken, it is very important to understand the basic endocrinology and the environmental factor(s) influencing its reproductive activity. The focus of this dissertation research was proposed to characterize the neuroendocrine regulation of the reproductive cycle in the female native Thai chicken and the roles of photoperiod upon the neuroendocrine regulation of the reproductive system. The findings from this study will provide the base line information of the hormonal and physiological characteristics of the reproductive system in the native Thai chicken, which has never been studied. The knowledge gained will help to understand the basic neuroendocrine regulation of the reproductive cycle and elucidate the effects of photoperiod upon the native Thai chicken reproduction. This information can be then applied commercially in poultry industry to increase reproductive efficiency and egg production of the native Thai chicken in Thailand.

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CHAPTER III

CIRCULATING PROLACTIN AND LUTEINIZING HORMONE LEVELS DURING THE REPRODUCTIVE CYCLE OF NATIVE THAI CHICKENS

3.1 Abstract

Unlike Gallinacous-temperate zone birds, native Thai chicken, an equatorial non-photoperiodic continuous breeder consists of four reproductive stages including non-laying (NL), laying (L), incubating (broodiness: B), and rearing of young (R). In temperate zone birds, prolactin (PRL) and luteinizing hormone (LH) levels vary during reproductive stages with the high PRL levels observed during the incubation phase are responsible for the suppression of gonadotropic hormones and ovarian steroids, follicular atresia, termination of egg laying activity, and induction of incubation behavior. The objective of this study was to establish baseline information of the neuroendocrine regulation emphasizing the plasma levels of PRL and LH profiles associated with reproductive cycles of the native Thai chickens. Chickens were divided into two experimental studies and classified into four reproductive stages: NL, L, B, and R. Daily records of egg production, nesting activity, and other behaviors were recorded during the reproductive cycle. During the experiments, blood samples were collected for determining plasma PRL and LH levels for two reproductive cycles and in each reproductive stage by Enzyme-Linked ImmunoSorbent Assay. The results

revealed that pattern of circulating PRL levels were low in NL, gradually elevated in L, reached the highest levels in B, and then declined sharply in R. The mean of plasma PRL levels (ng/ml) were significantly higher in B (351.97±37.08, $P<0.05$) than that of in L (40.40±12.60), NL (25.92±1.39), and R (23.80±2.17). In contrast, there were no changes in plasma LH levels (ng/ml) across the reproductive cycles (NL: 3.86±0.36, L: 3.44±0.09, B: 3.74±0.13, and R: 3.22±0.04). The ovary weights (g) were significantly higher in L (35.92±2.12, $P<0.05$) than that of in NL, B, and R (1.47±0.18, 3.07±0.23, and 1.92±0.19). The findings that ovarian regression observed in incubating and rearing hens in the absence of a decline in LH levels is interpreted as an adaptive mechanism(s) allowing for reinitiating egg laying in the case of nest destruction at any times and irrespective of the season. The findings further suggest that the antigonadotropic effect of PRL is limited to its effect on the ovary. In conclusion, the results of this present study provide, for the first time, the baseline information of the neuroendocrine changes during the reproductive cycles in this species and support the previous studies that PRL is associated with the reproductive cycle in avian species and play a pivotal role in the incubation behavior.

3.2 Introduction

In birds, changes in concentrations of luteinizing hormone (LH) and prolactin (PRL) during the reproductive cycles are well documented (Follett, 1984; El Halawani et al., 1988; Nicholls et al., 1988). The hypothalamic control of LH and PRL secretion in birds is mediated by a hypophyseal portal vascular system which transports regulatory neuropeptides and neurotransmitters released from the median eminence (ME) to the anterior pituitary gland (Follet, 1984). There are two neuroendocrine

systems that play a pivotal role in the avian reproductive cycle. First, gonadotropin releasing hormone-I/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) system which involves GnRH-I and the subsequent secretion of FSH and LH. Second, vasoactive intestinal peptide/prolactin (VIP/PRL) system which involves VIP and leading to the subsequent secretion of PRL. In temperate zone birds, both systems depend upon the duration of day length and the transduction of photoperiodic information, resulting in either gonad recrudescence and its associated sexual activity or gonad regression and the termination of reproductive activity. The final common pathway controlling the secretion of PRL, LH, and FSH is formed by a system of peptidergic neurons whose axons terminate around portal capillaries in the external layer of the ME. VIP and GnRH are among the best characterized hypophysiotropic neuropeptides (Chaiseha and El Halawani, 2005).

The period of egg laying in birds is associated with relatively high levels of FSH, LH, and gonadal steroids (estradiol and progesterone) circulating in the blood. High concentrations of LH have been shown to be associated with initiation of egg production (Tanabe et al., 1981; Bacon and Long, 1995; Liu et al., 2002). It has been reported that FSH and LH secretions and gene expressions are stimulated by long day length (Nicholls et al., 1988; Dawson et al., 2001) and required the functional integrity of the GnRH neuronal system (Katz et al., 1990; Sharp et al., 1990). FSH induces mainly ovarian follicular growth in birds (Chaudhuri and Maiti, 1998; Rose et al., 2000) and maintains the hierarchical size of the bird follicles. Circulating LH is directly related to gonadal activity and the control of steroidogenesis (Robinson et al., 1988). LH stimulates progesterone production by the largest follicle (F1), leading to ovulation (Pollock and Orosz, 2002). Ovarian development is found to correlate with

plasma LH and the amount of GnRH-I indicating that the expression of the GnRH-I gene is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). GnRH-I levels decrease when birds enter the incubating stage and this decrease is thought to be implemented by the inhibitory effect of PRL, which reaches its highest level during this stage (Sharp et al., 1988).

Over 300 different physiological functions of PRL have been documented, highlighting the importance of this pituitary hormone (Harriss et al., 2004). Although PRL seems to be an omnipotent hormone, it is best known for its role in milk production in mammals. PRL also plays a significant role in reproduction, maternal care, and parental behavior in birds and mammals (Buntin, 1993, Schradin and Anzenberger, 1999). In birds, PRL is associated with a wide range of reproductive physiology and behaviors, including incubation, migration, grooming, crop milk secretion, feeding of young, nest defense, and sexual activity (Lea et al., 1981; 1986; Silver, 1984; Janik and Buntin, 1985; Buntin et al., 1991). Changes in PRL gene expression and its plasma levels are highly correlated with the reproductive cycle in birds (Knapp et al., 1988; Talbot et al., 1991; Wong et al., 1991; Tong et al., 1997). Plasma PRL levels are very low (5-10 ng/ml) during the reproductively quiescent stages of the turkey reproductive cycle. However, during the laying and incubating stages, circulating PRL levels increase dramatically (500-1500 ng/ml; El Halawani et al., 1984). It is very well established that PRL is associated with incubation behavior in birds (Riddle et al., 1935; Breitenbach and Meyer, 1959; Burke and Dennison, 1980; Goldsmith and Williams, 1980; El Halawani et al., 1988; Sharp et al., 1988). PRL is widely thought to play a role in parental behavior. PRL has been shown to be associated with the reproductive cycle in several avian species (for review, see: El

Halawani et al., 1997) but no studies have been conducted on the native Thai chicken.

The onset of incubation behavior is correlated with declining plasma levels of LH and gonadal steroids and increasing plasma levels of PRL in birds (Lea et al., 1981; El Halawani and Rozenboim, 1993). LH levels begin to increase continuously and reach a peak before ovulation (Mashaly et al., 1976) and plasma concentration of FSH is low throughout the ovulatory cycle but a significant decline in FSH occurred prior to the pre-ovulatory LH surge and a significant increase occurred during the 3 hour prior to oviposition as LH levels decline (Krishnan et al., 1993). Thereafter, LH levels continue to decline during incubating period (Myers et al., 1989). On the contrary, during laying and incubating period, circulating PRL levels increase dramatically (El Halawani et al., 1984). It is this rising PRL level which has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior (Sharp et al., 1984; Buntin, 1986; Hall et al., 1986; El Halawani et al., 1988). Subsequently, PRL level declines whereas LH level begin to rise when incubation behavior terminates (El Halawani et al., 1988; Knapp et al., 1988) and as soon as molting is ended (Bluhm et al., 1983; Mauget et al., 1994). At the onset of sexual maturity, the preovulatory surge of progesterone induces the nesting behavior associated with oviposition (Wood-Gush and Gilbert, 1973; El Halawani et al., 1986). The combine action of estrogen, progesterone, and nesting activity further stimulates PRL secretion (El Halawani et al., 1983; 1986). These increasing PRL levels suppress the activity of the GnRH/FSH-LH system (Rozenboim et al., 1993; You et al., 1995), reduce ovarian steroids secretion (Porter et al., 1991; Tabibzadeh et al., 1995), terminate egg laying, induce ovarian regression (Youngren et al., 1991), and signal the transition from sexual behavior to incubation behavior. Elevated PRL levels and

incubation behavior are maintained by tactile stimuli from the nest and eggs (El Halawani et al., 1980; 1986; Opel and Proundman, 1988). After hatching, or when eggs are replaced with poults, tactile stimuli from the young induces the emergence and maintenance of maternal responses, a sharp decrease in circulating PRL (Opel and Proundman, 1989), and the transition to the photorefractory state.

It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meities, 1965; Bern and Nicoll, 1968). It is very well documented that PRL is under stimulatory control by the VIP, the only avian PRL-releasing factor (PRF; for review, see: El Halawani et al., 1997; 2000; Chaiseha and El Halawani, 2005). Variations in hypothalamic VIP immunoreactivity, VIP content, and VIP mRNA steady-state levels in the infundibular nuclear complex (INF), VIP-immunoreactive (VIP-ir) fibers in the ME, and VIP concentrations in hypophyseal portal blood are correlated with changes in the amount of circulating PRL throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha and El Halawani, 1999). Changes in pituitary VIP receptor mRNA were also observed across the reproductive stages in turkeys (Chaiseha et al., 2004). VIP and PRL release and gene expression are up-regulated in response to long day length (Wong et al., 1991; Tong et al., 1997; Chaiseha et al., 1998). The role of dopamine (DA) in the regulation of avian PRL secretion is unclear at present for comparing it to the mammalian DAergic strategy for PRL control. In contrast with mammals, it has been established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptors (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003). Recent evidences suggested that DA plays an intermediary role in PRL secretion, requiring an intact

VIPergic system in order to cause the release of PRL (Youngren et al., 1996b). In addition, recent evidences indicate that dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ opioid, serotonergic, dopaminergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (El Halawani et al., 2000).

PRL and gonadotropin secretions are controlled by closely related mechanisms. From a number of physiological or experimental reproductive conditions, an inverse relationship between PRL and gonadotropins secretion seems to emerge. The onset of incubation activity is associated with greatly enhanced circulating PRL levels and diminished LH levels in bantam hen (Sharp et al., 1979), turkey (Burke and Dennison, 1980; El Halawani et al., 1984), duck (Goldsmith and Williams, 1980; Bluhm et al., 1983), ring dove (Goldsmith et al., 1981), wild starling (Dawson and Goldsmith, 1982), spotted sandpiper (Oring et al., 1986), canary (Goldsmith et al., 1984), and white-crowed sparrows (Hiatt et al., 1987). In Thailand, there are many research laboratories working on avian reproduction, but there are only a limit number of scientists studying the neuroendocrine regulation of reproduction in the native Thai chicken. It has been reported that progesterone level is related to reproductive cycle of the native Thai chicken (Katawatin et al., 1997) but plasma PRL levels were unable to measure due to unable to establish a chicken PRL assay. Compared with other domestic animals, relatively little is known about the changes in reproductive hormones during the reproductive cycles of the native Thai chicken. Thus, this study was proposed to establish baseline information of the neuroendocrine changes across the reproductive cycles and the association with the different reproductive stages of

the native Thai chicken. The findings from this study will provide the baseline information of the hormonal profiles and physiological characteristics of the reproductive system in the native Thai chicken, which has never been studied. The knowledge gained will help to understand the basic neuroendocrine regulation of their reproductive cycle.

3.3 Materials and Methods

3.3.1 Experimental Animals

130 female and 20 male native Thai chickens, Pradoohangdam breed (fig. 3.1A-D), were used. They were reared and housed in floor pens under natural daylight (approximately 12 hours of light and 12 hours of dark; 12L:12D). Food and water were constantly available. Each hen was identified by wing band number. Chickens were randomly divided in floor pens (6-7 females:1 male/pen). The chickens were observed their behaviors in order to classify them into 4 reproductive stages as non-laying (NL; fig. 3.1A), laying (L; fig. 3.1B), incubating (broodiness: B; fig. 3.1C), and rearing chicks (R; fig. 3.1D). The criteria for the reproductive stages classification were: 1) NL: the chickens that had not reached the sexual maturity and did not lay or express incubation and maternal behaviors, 2) L: the hens that laid regularly, 3) B: the hens that exhibited persistent nesting activity, no egg production, and aggressive nest protection behavior, and 4) R: the hens that stopped nesting and laying eggs, and reared the chicks after hatching. Daily records of egg production, nesting activity, and other behaviors were recorded throughout the experiments. The animals were treated in accordance with Suranaree University of Technology Animal Care and Use Committee Guidelines.

3.3.2 Experimental Design

3.3.2.1 Experiment I

30 female and 5 male native Thai chickens at 16 weeks of age were used. The chickens were randomly divided into 5 pens (6 females:1 male/pen). Each pen was provided with nests. Chickens were observed their behaviors everyday in order to classify them into 4 reproductive stages: NL, L, B, and R. During the experiment, blood samples of 30 female native Thai chickens were collected from the brachial vein in heparinized tubes once a week from the beginning until the end of the experiment (two reproductive cycles). Plasma samples were separated by centrifugation and stored at -35°C until used for determining plasma PRL and LH levels by enzyme-linked immunosorbent assay (ELISA). Daily records of egg production, nesting activity, and other behaviors were recorded during the reproductive cycle.

3.3.2.2 Experiment II

100 female and 15 male native Thai chickens at 16 weeks of age were used. The chickens were randomly divided into 15 floor pens (6-7 females:1 male/pen). Each pen was provided with nests. Chickens were classified into 4 reproductive stages: NL, L, B, and R. Blood samples of native Thai chickens were collected in each reproductive stage (n=25): NL, L: 7 days after the first egg was laid and hens laid egg regularly, B: 10 days after hens stopped laying and were sitting on nests continuously, and R: 14 days after the first chick was hatched and hens were rearing their chicks. The blood samples were collected from the brachial vein in heparinized tubes prior to euthanize with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). After chickens were sacrificed, ovaries and oviducts were removed and

weighed. Plasma samples were separated by centrifugation and stored at -35°C until used for determining plasma PRL and LH concentrations by ELISA. Daily records of egg production, nesting activity, and other behaviors were kept during the reproductive cycle.

3.3.3 Measurement of Plasma PRL Concentrations

Plasma PRL levels of Experiment I (n=10) and II (n=10) were measured by ELISA technique (Proudman et al., 2001). Briefly, plates were coated with 100 µl of AffiniPure Goat anti-Rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) which was diluted in 0.05 M potassium phosphate buffer at the dilution 1:2,000. The plates were incubated at 4°C overnight and then blocked with blocking solution (100 µl of 0.4% casein, 0.01% thimerosal, 1 mM EDTA). 20 µl of samples, 30 µl of the assay buffer (0.1% casein, 0.01% thimerosal, 1 mM EDTA), 25 µl of anti-PRL (1:20,000, provided by Dr. John Proudman, USDA, USA), and 25 µl of β-PRL tracer (1:50,000) were added into the reaction, then incubated at 4°C overnight. The reactions were measured the absorbent at 405 nm. The plasma samples were measured in duplicate within a single assay.

3.3.4 Measurement of Plasma LH Concentrations

Plasma LH levels of Experiment I (n=10) and II (n=10) were measured by ELISA technique (Proudman et al., 2001). Briefly, plates were coated with 100 µl of AffiniPure Goat anti-Rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) which was diluted in 0.05 M potassium phosphate buffer at the dilution 1:2,000. The plates were incubated at 4°C overnight and then blocked with

blocking solution (100 µl of 0.4% casein, 0.01% thimerosal, 1 mM EDTA). 20 µl of samples, 30 µl of the assay buffer (0.1% casein, 0.01% thimerosal, 1 mM EDTA), and 50 µl of anti-LH (1:10,000, provided by Dr. John Proudman, USDA, USA) diluted in the assay buffer were added into the reactions. The plates were incubated plate overnight at 4°C. The plates were washed and then added 100 µl of LH tracer diluted in the assay buffer at the dilution 1:500 and then incubated at 4°C overnight. The reactions were measured the absorbent at 405 nm. The samples were determined in duplicate within a single assay.

3.3.5 Statistical Analysis

Differences of plasma PRL and LH levels, ovary and oviduct weights among 4 reproductive stages (treatment groups) were analyzed. Egg production, hatchability, and the duration of laying, incubating, and rearing were compared between two reproductive cycles. Results were expressed as mean ± SEM. Significant differences of mean were statistically analyzed using one way analysis of variance (ANOVA). Significance differences among treatment groups were computed utilizing Tukey's HSD test. Differences were considered as statistically significant if a P value was less than 0.05. All statistical analyses were performed using SPSS Windows Software (SPSS Windows Software, version 13.0, SPSS Inc., Chicago, IL, USA).

3.4 Results

3.4.1 Experiment I

Blood samples of the native Thai chickens were collected weekly throughout two reproductive cycles and the concentrations of plasma PRL and LH levels were

determined by ELISA. The results showed that hormonal profiles of plasma PRL and LH levels during the reproductive cycles were similar in all birds (n=10). In the first reproductive cycle, plasma PRL levels were low in NL, gradually increased in L, continued to rise and reached the highest in B, and immediately declined to the basal levels in R, whereas plasma LH levels were fluctuated throughout the two reproductive cycles. Similar patterns of plasma PRL levels were observed in the second reproductive cycle. The profiles of plasma PRL and LH levels in individual bird are illustrated in Fig. 3.2A-J.

The reproductive characteristics during the reproductive cycles of native Thai chickens were recorded. The age at the first lay, egg production, hatchability, and the duration of laying, incubating, and rearing during two reproductive cycles were shown in Table 3.1. The duration of lay, number of egg produced, and % hatchability per hen of the hens in the second reproductive cycle are higher than that of the hens in the first reproductive cycle. The significant different ($P<0.05$) of egg production and the duration of rearing between the first and the second reproductive cycle were observed (12.45±0.99 vs. 18.37±2.15 eggs and 54.83±3.53 vs. 41.93±1.88 days, respectively). However, the differences of plasma hormones between the first and second reproductive cycle of the hens were not observed.

3.4.2 Experiment II

Blood samples of the native Thai chickens in each reproductive stage were collected prior to sacrifice the chickens for determining the plasma PRL and LH levels by ELISA. The findings from this study clearly support the results of Experiment I that plasma PRL levels (Fig. 3.3A, Table 3.2, n=10, ng/ml) were low in NL

(25.92±1.39), gradually elevated in L (40.40±12.60), significantly increased in B (351.97±37.08, P<0.05), and declined sharply to the basal level in R (23.80±2.17). The changes in plasma LH levels were not observed across the reproductive stages. (Fig. 3.3B, Table 3.2, n=10, ng/ml, NL: 3.86±0.36, L: 3.44±0.09, B: 3.74±0.13, and R: 3.22±0.04).

The ovary and oviduct were removed and weighed after sacrifice all the chickens (n=25). Means ± SEM of ovary and oviduct weights, age, and body weight in each reproductive stage were summarized in Table 3.3. The highest ovary weights (g) was observed in laying hens (35.92±2.12) compared to other reproductive stages (NL: 1.47±0.18, B: 3.07±0.23, and R: 1.92±0.19) as shown in Fig. 3.4A, 3.5, and Table 3.3. The oviduct weights (g) were low in NL (2.32±0.80) and R (3.79±0.63), increased in B (6.70±0.35, P<0.05), and significantly higher in L (39.27±2.03, P<0.05) as shown in Fig. 3.4B, 3.5, and Table 3.3.

Fig. 3.1 The native Thai chickens, Pradoohangdam breed. (A) Male and female chickens. (B) Laying hen. (C) Incubating hen. (D) Rearing hen.

Fig. 3.2A Plasma PRL and LH concentrations during the reproductive cycles from Bird #21

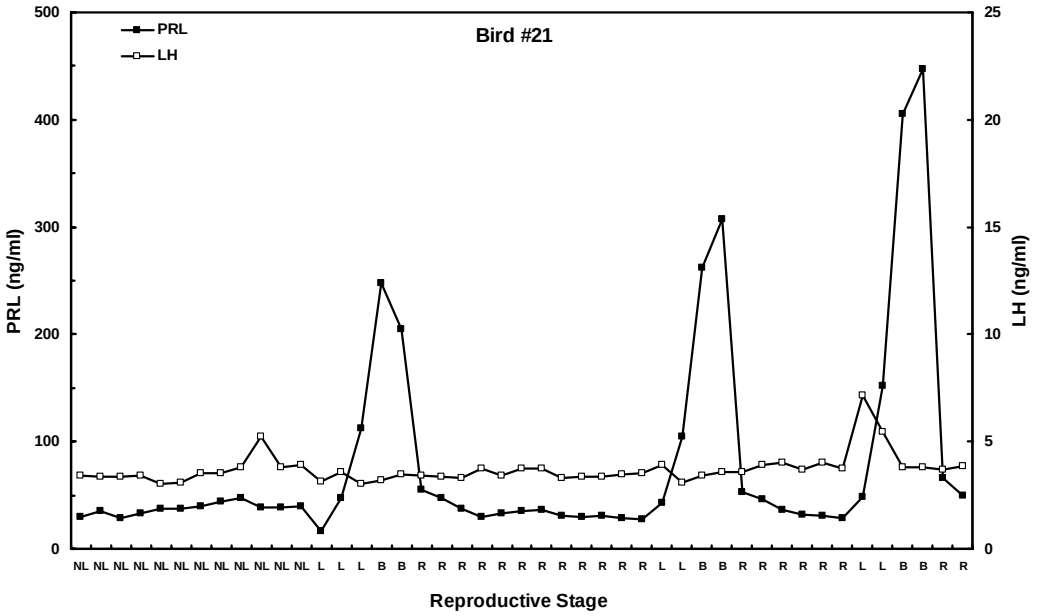

Fig. 3.2B Plasma PRL and LH concentrations during the reproductive cycles from Bird #22

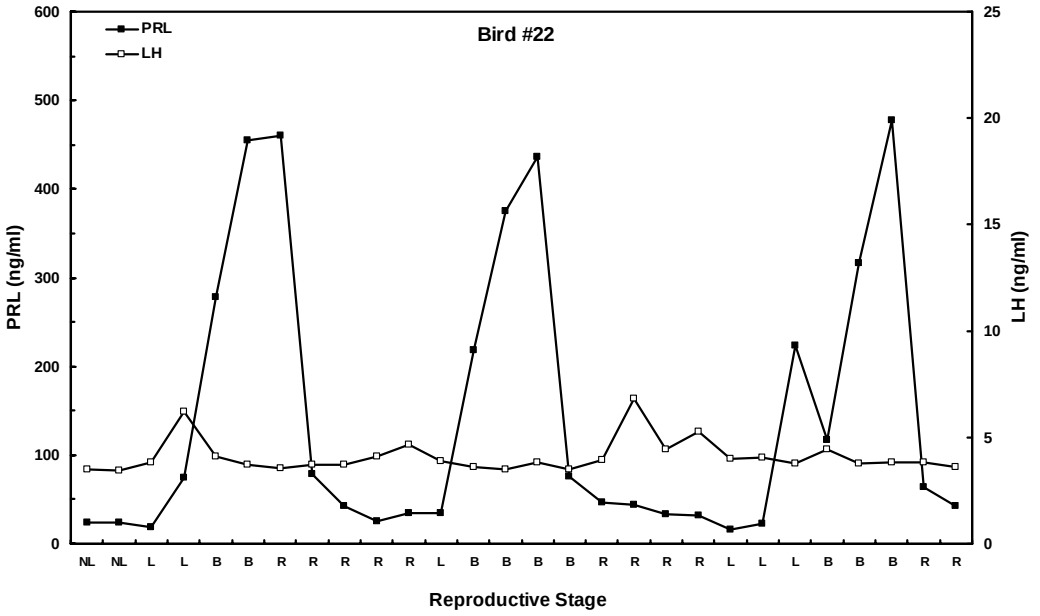

Fig. 3.2C Plasma PRL and LH concentrations during the reproductive cycles from Bird #23

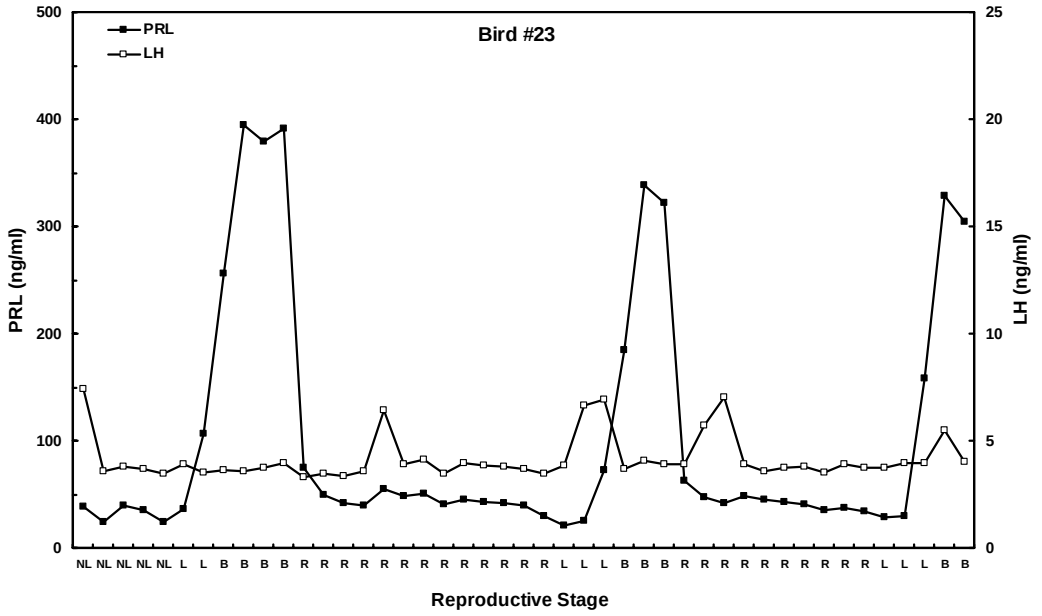

Fig. 3.2D Plasma PRL and LH concentrations during the reproductive cycles from Bird #27

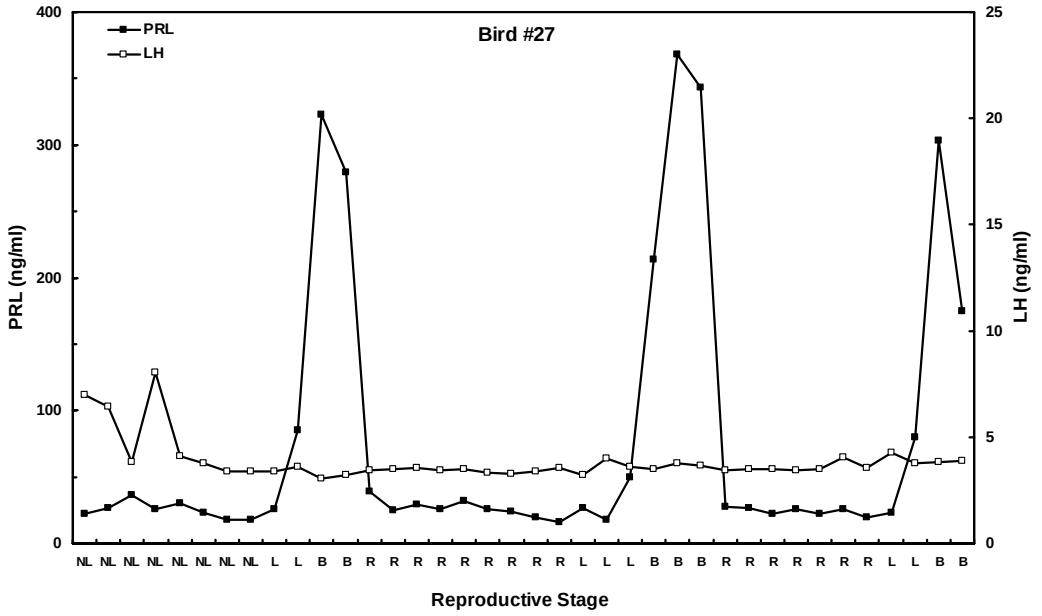

Fig. 3.2E Plasma PRL and LH concentrations during the reproductive cycles from Bird #28

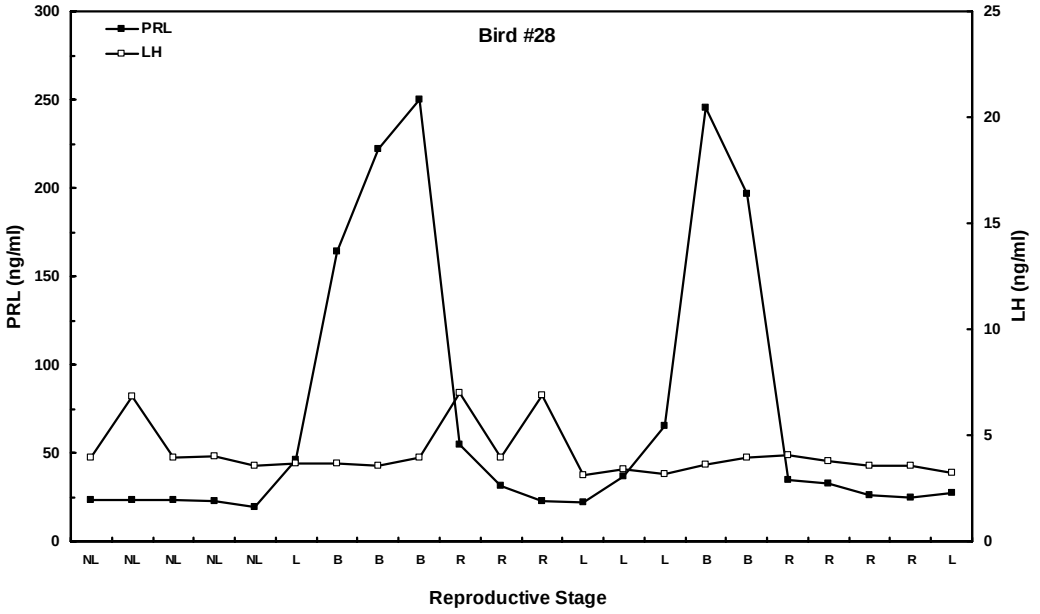

Fig. 3.2F Plasma PRL and LH concentrations during the reproductive cycles from Bird #30

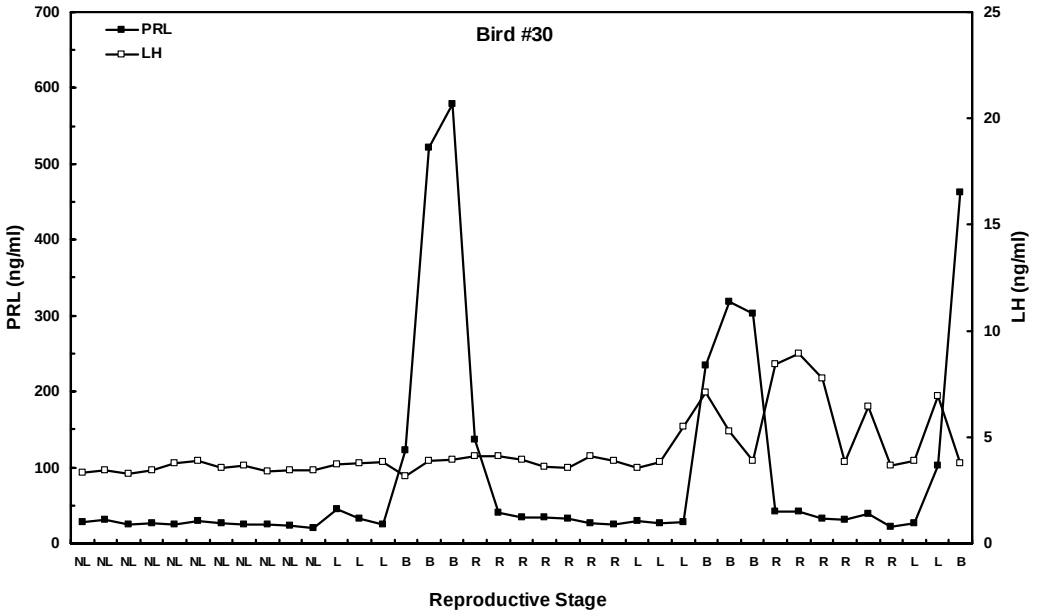

Fig. 3.2G Plasma PRL and LH concentrations during the reproductive cycles from Bird #31

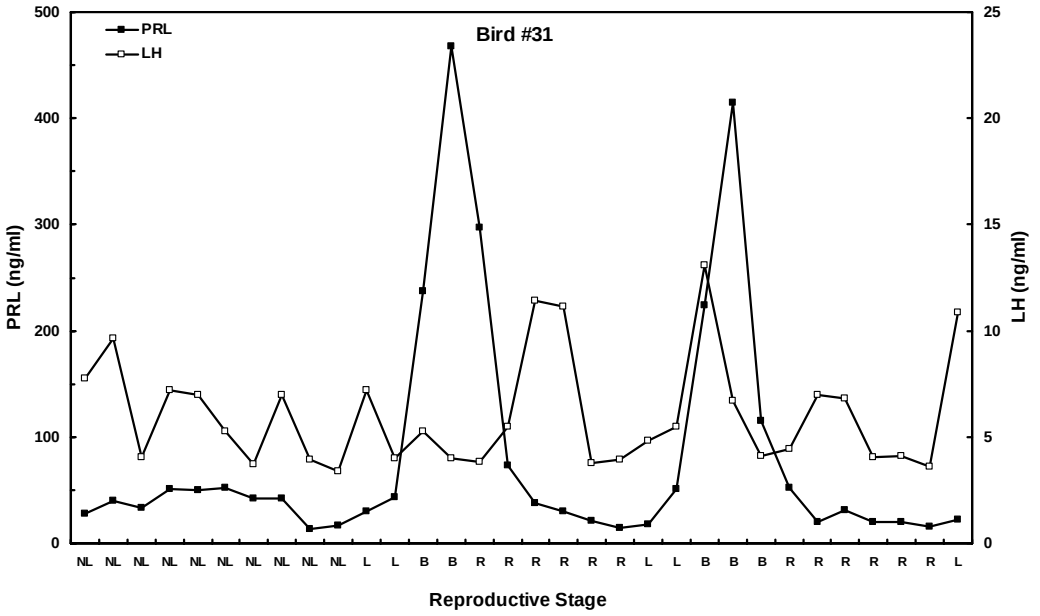

Fig. 3.2H Plasma PRL and LH concentrations during the reproductive cycles from Bird #34

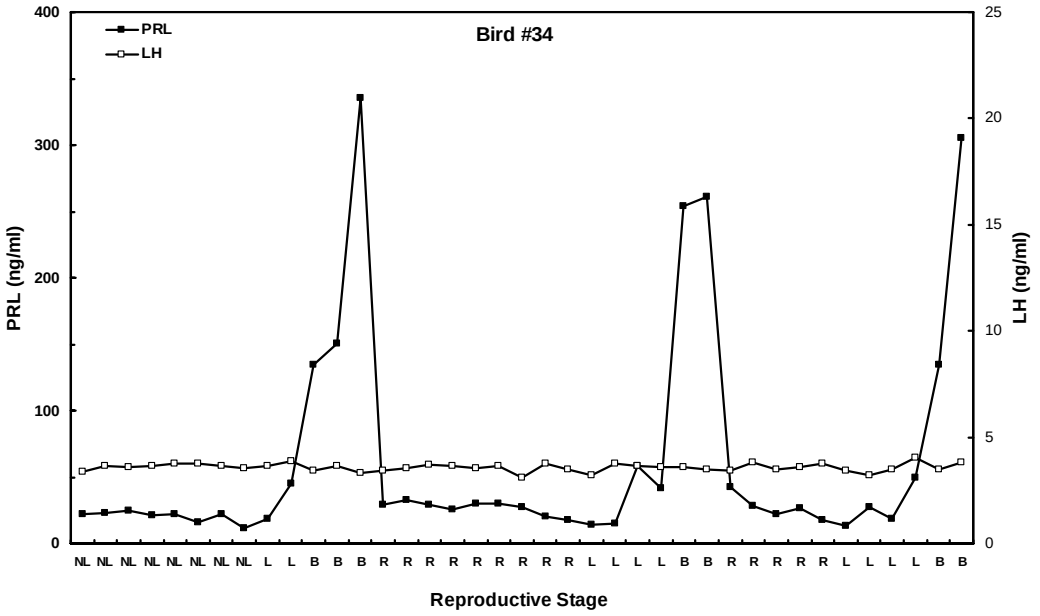

Fig. 3.2I Plasma PRL and LH concentrations during the reproductive cycles from Bird #35

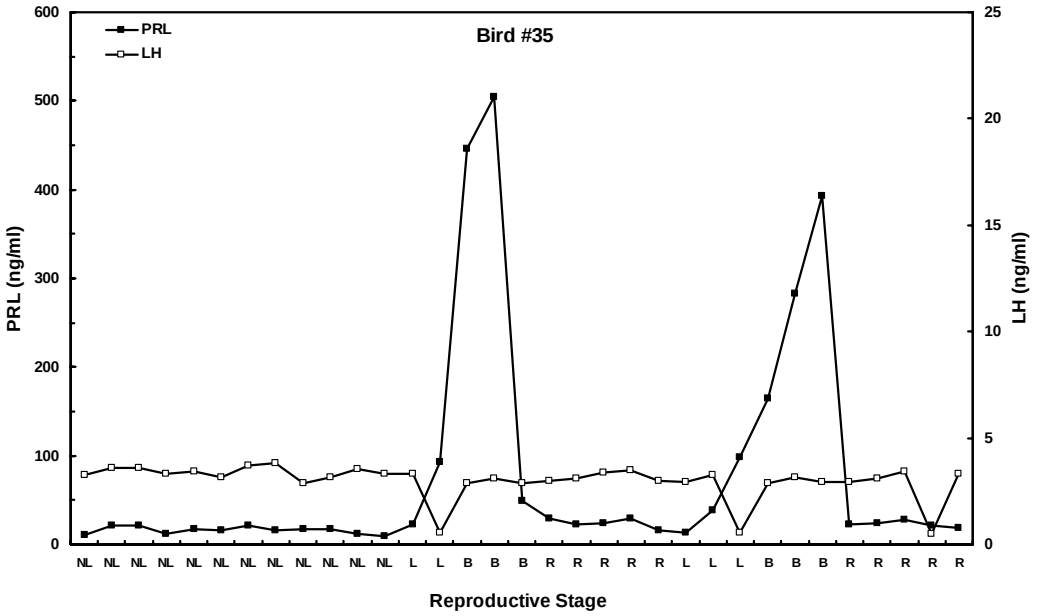

Fig. 3.2J Plasma PRL and LH concentrations during the reproductive cycles from Bird #60

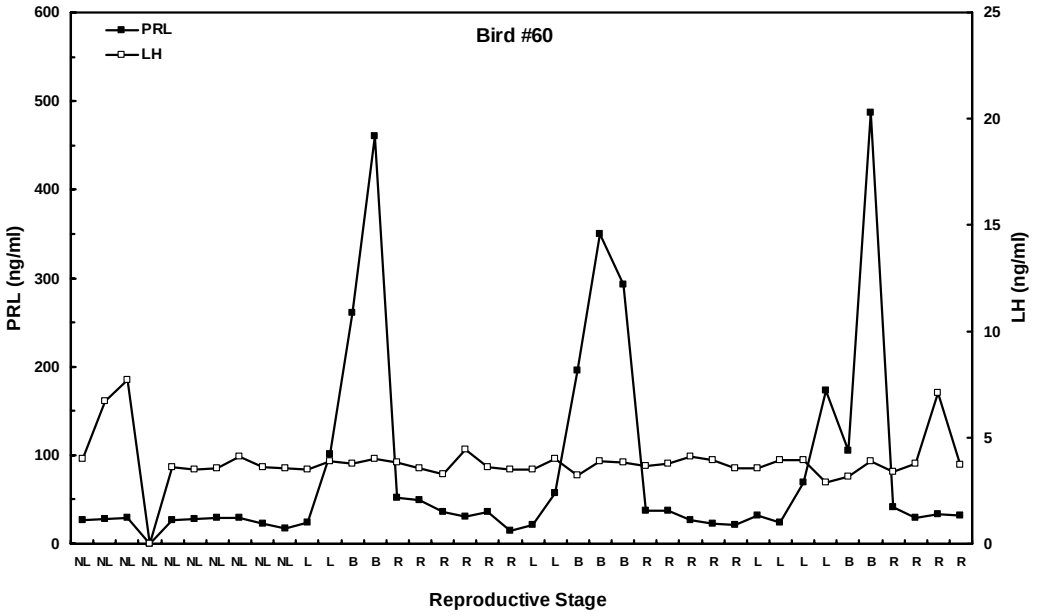

Table 3.1 Reproductive characteristics of the native Thai chickens during the reproductive cycles. Values are expressed as the Mean ± SEM. Values with different letters are significantly different (P<0.05).

Reproductive Cycle	Age at First Lay (wk)	Duration of Laying (day)	Egg Production (egg)	Duration of Incubating (day)	Hatchability (%)	Duration of Rearing (day)
Cycle 1 (n=29)	32.31 ± 0.75	18.55 ± 2.48[a]	12.45 ± 0.99[a]	20.00 ± 0.65[a]	58.10 ± 5.12[a]	54.83 ± 3.53[a]
Cycle 2 (n=27)	45.56 ± 1.13	28.59 ± 5.08[a]	18.37 ± 2.15[b]	19.00 ± 0.28[a]	65.93 ± 4.29[a]	41.93 ± 1.88[b]

Fig. 3.3A Plasma PRL levels in the native Thai chickens in each reproductive stage (n=10). Values are expressed as the Mean ± SEM. Values with different letters are significantly different (P<0.05).

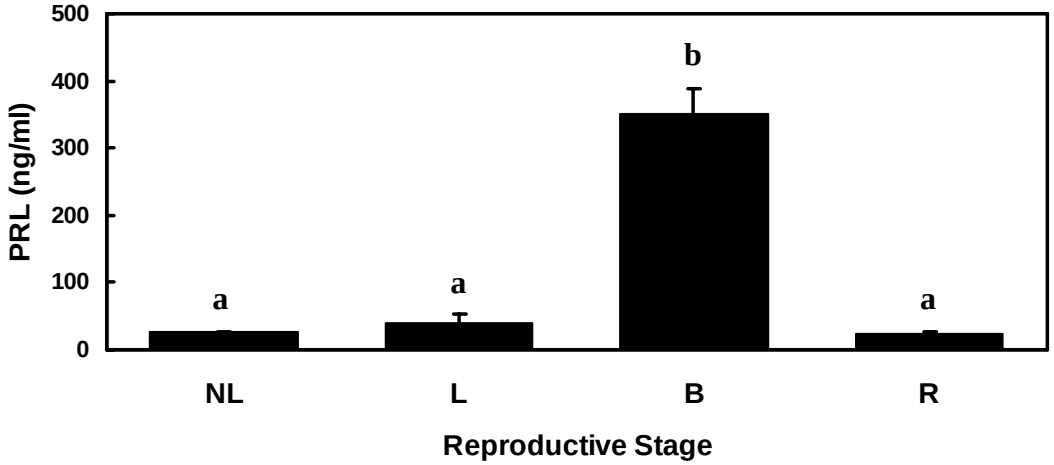

Fig. 3.3B Plasma LH levels in the native Thai chickens in each reproductive stage (n=10). Values are expressed as the Mean ± SEM. Values with different letters are significantly different (P<0.05).

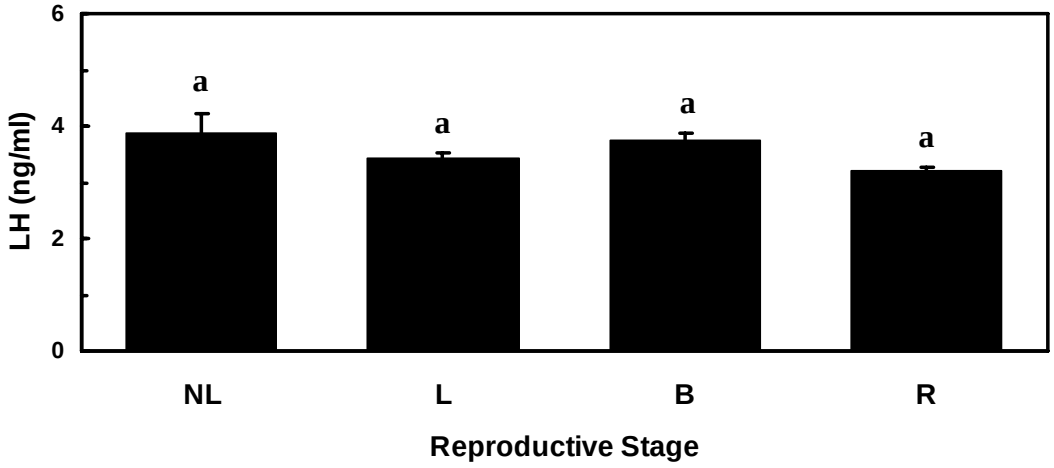

Table 3.2 Plasma PRL and LH concentrations in the native Thai chickens in each reproductive stage (n=10). Values are expressed as the Mean ± SEM. Values with different letters are significantly different (P<0.05).

Reproductive Stage	PRL Concentration (ng/ml)	LH Concentration (ng/ml)
NL	25.92 ± 1.39^{a}	3.86 ± 0.36^{a}
L	40.40 ± 12.60^{a}	3.44 ± 0.09^{a}
B	351.97 ± 37.08^{b}	3.74 ± 0.13^{a}
R	23.80 ± 2.17^{a}	3.22 ± 0.04^{a}

Fig. 3.4A The ovaries of the native Thai chickens in each reproductive stage.

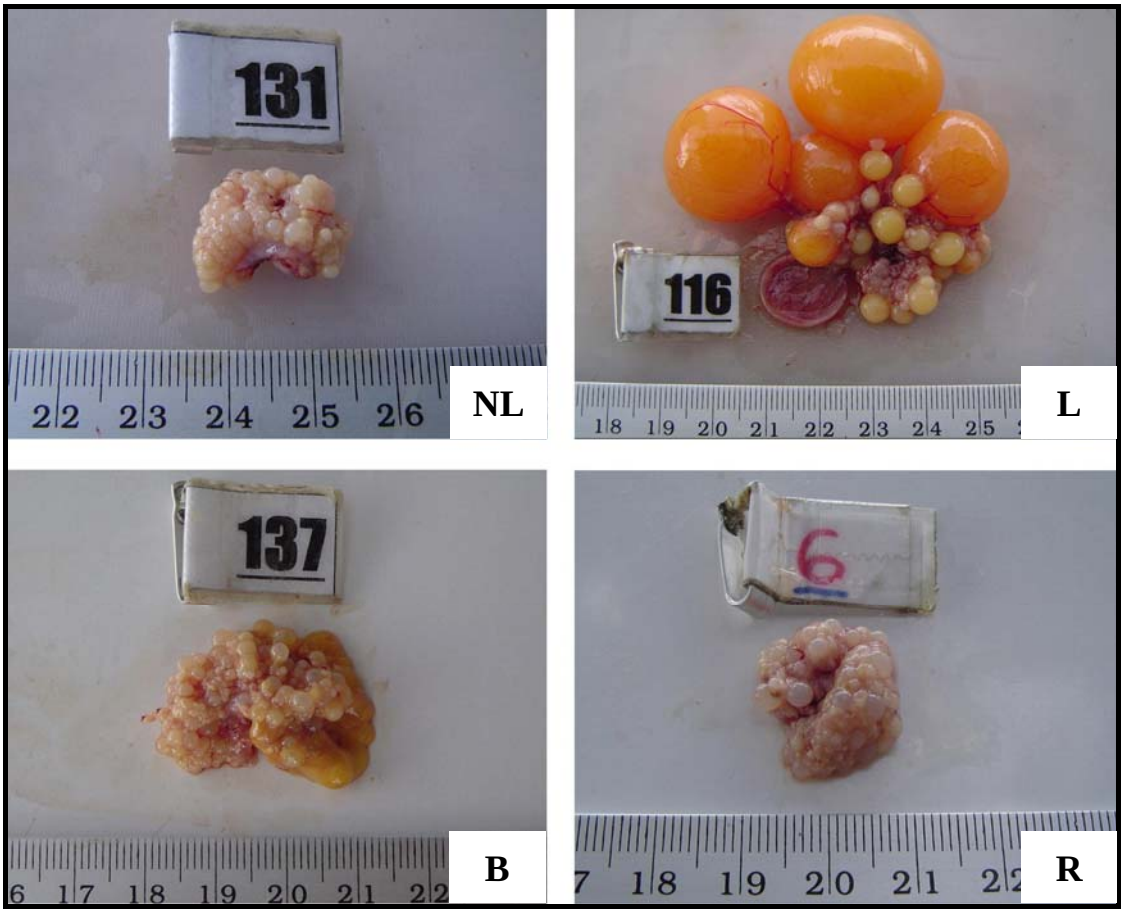

Fig. 3.4B The oviducts of the native Thai chickens in each reproductive stage.

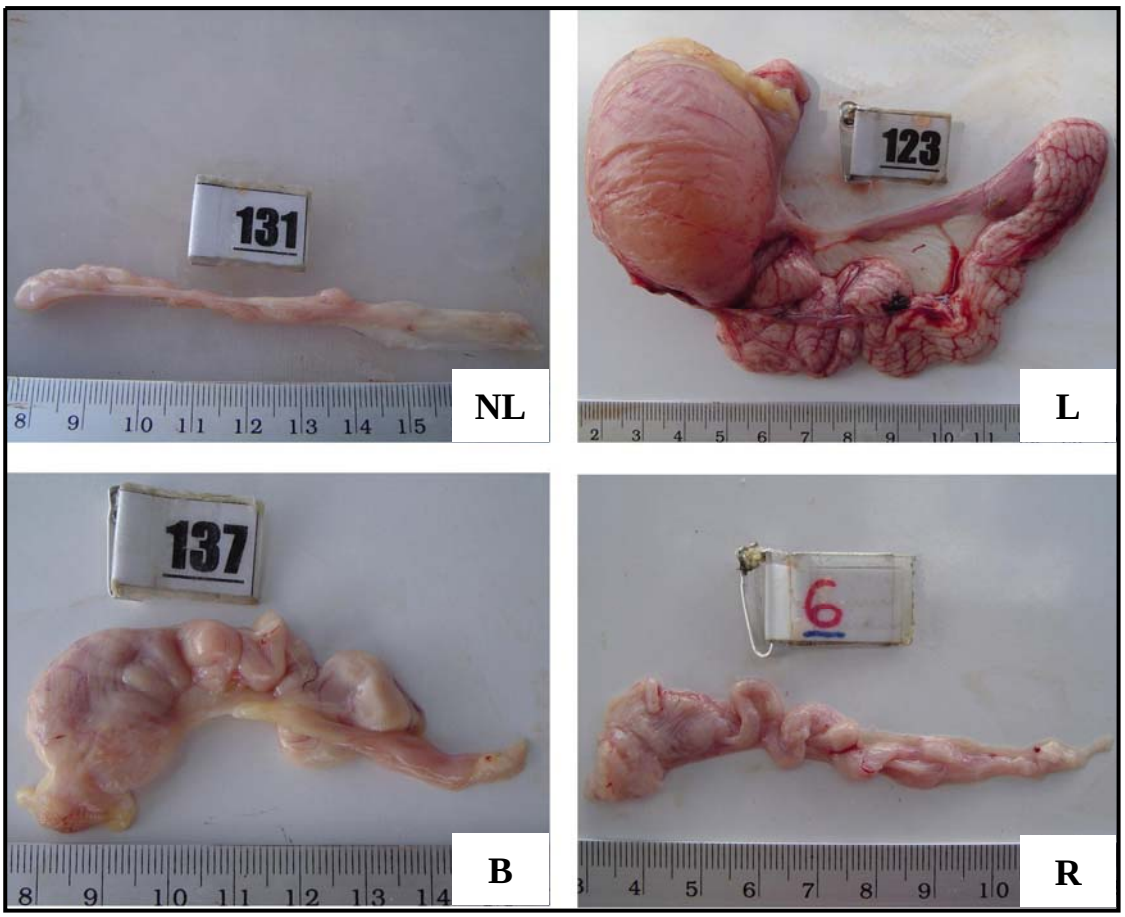

Fig. 3.5 Ovary and oviduct weights of the native Thai chickens in each reproductive stage (n=25). Values are expressed as the Mean ± SEM. Values with different letters are significantly different (P<0.05).

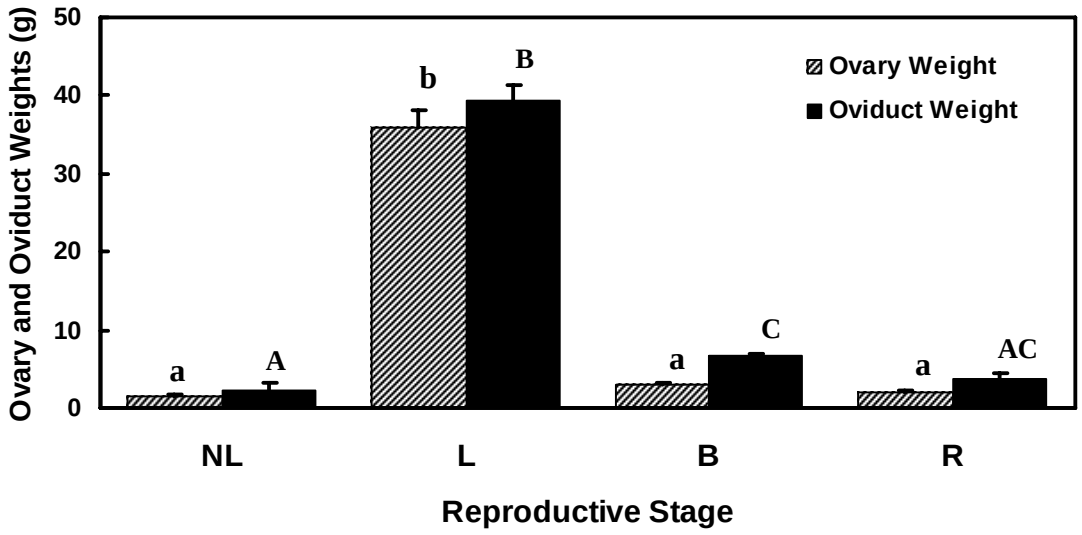

Table 3.3 Mean ± SEM of age, body weight, ovary and oviduct weights of the native Thai chickens in each reproductive stage (n=25). Values with different letters are significantly different (P<0.05).

Reproductive Stage	Age (wk)	Body Weight (kg)	Ovary Weight (g)	Oviduct Weight (g)
NL	28.67 ± 1.20	1.33 ± 0.07	1.47 ± 0.18[a]	2.32 ± 0.80[a]
L	31.50 ± 0.82	1.73 ± 0.06	35.92 ± 2.12[b]	39.27 ± 2.03[b]
B	35.07 ± 0.96	1.58 ± 0.06	3.07 ± 0.23[a]	6.70 ± 0.35[c]
R	37.88 ± 0.98	1.62 ± 0.05	1.92 ± 0.19[a]	3.79 ± 0.63[ac]

3.5 Discussion

This study reports, for the first time, the hormonal profiles of PRL and LH during the reproductive cycles, as well as the reproductive characteristics of the female native Thai chickens. During the two reproductive cycles, plasma PRL levels were low in non-laying birds, gradually increased when birds started to lay, continued to rise and reached the highest levels when birds entered incubation phase, and immediately declined to the same levels of non-laying birds in birds that rearing chicks. Plasma LH levels were fluctuated throughout reproductive cycles. The plasma PRL levels were determined in each reproductive stage. The low levels of plasma PRL were observed in NL and tended to be higher in L. However, significant different of the mean of plasma PRL levels were observed in B and plasma PRL levels were then declined in R. The changes in plasma LH levels were not observed across the reproductive stages. The ovary weights were significantly higher in L than that of in NL, B, and R. The finding that ovarian regression observed in incubating and rearing hens in the absence of a decline in LH levels is interpreted as an adaptive mechanism(s) allowing for reinitiating egg laying in the case of nest destruction at any times and irrespective of the season. The results of this present study provide the baseline information of the neuroendocrine changes during the reproductive cycle in this equatorial species and support the previous studies that PRL is associated with the reproductive cycle in avian species and play an important role in the incubation behavior. These findings further suggest that the antigonadotropic effect of PRL is limited to its effect on the ovary.

Plasma PRL levels were low in NL when the chickens had not reached their sexual maturity and the reproductive systems were not fully developed. The transition

of the reproductive stage from NL to L was associated with an increase in basal plasma PRL levels and accompanied with the start of the development of ovary and oviduct without a concomitant rise in basal plasma LH levels. The sharply increased in plasma PRL levels were observed during birds shift their reproductive stages from L to B and associated with the regression of ovaries and oviducts without the absence of a decline in plasma LH levels. In contrast to the pattern of plasma PRL, the fluctuated plasma LH levels were observed throughout the two reproductive stages. These results suggest that PRL is released in substantial amounts into the circulation according to the reproductive stage of the chicken. LH may probably arise entirely in the peripheral circulation. The duration of lay, number of egg produced, and % hatchability per hen of the hens in the second reproductive cycle is higher than that of the hens in the first reproductive cycle. The significant different of egg production and the duration of rearing between the first and the second reproductive cycle were observed. However, the differences of plasma hormones between the first and the second reproductive cycle of the hens were not observed. Taken together, the reproductive performance of the chickens in the second reproductive cycle were better than that of the first cycle.

The results of this study are in good agreement with previous studies in temperate zone birds. In temperate zone birds, PRL and LH levels vary during the four reproductive stages with the high PRL levels observed during the incubation phase are responsible for the suppression of gonadotropic hormones and ovarian steroids, follicular atresia, termination of egg laying activity, and induction of incubation behavior. PRL action on the reproductive neuroendocrine system has been shown to be mediated by its feedback effects on the hypothalamus, pituitary, and ovary (Chaiseha and El Halawani, 2005). These evidences correlated very well with the

findings of this study that plasma PRL levels were reached the highest levels in incubating hens. The hormonal profile of PRL during the reproductive cycle of the native Thai chicken was similar to the turkey (El Halawani et al., 1984) which the minor changes of plasma PRL levels were observed in the native Thai chickens, equatorial non-photoperiodic continuous breeders.

In birds, it is very well documented that PRL is associated with a wide range of reproductive physiology and behaviors including incubation, migration, grooming, crop milk secretion, feeding of young, nest defense, and sexual activity (Lea et al., 1981; Silver, 1984; Buntin et al., 1991). It has been established that PRL is associated with incubation behavior in pigeon (Riddle et al., 1935), pheasant (Breitenbach and Meyer, 1959), cowbird (Hohn, 1959), turkey (Burke and Dennison, 1980; El Halawani et al., 1988; Youngren et al., 1991), mallard duck (Goldsmith and Williams, 1980), and chicken (Sharp et al., 1988). Changes in PRL gene expression and its plasma levels are highly correlated with the reproductive cycle in birds (Knapp et al., 1988; El Halawani et al., 1990; Talbot et al., 1991; Wong et al., 1991; You et al., 1995; Tong et al., 1997). It has been indicated that PRL concentrations in the blood rises as egg laying proceed and reach the highest in incubation and these were similar to the results of this present study. In addition, it has been suggested that PRL may also directly inhibit ovarian steroidogenesis (Rozenboim et al., 1993). Thus, these actions of PRL would lead to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct. High levels of PRL are instrumental for regression of reproductive system in birds (Dawson and Sharp, 1998), and molting of feathers in birds or pelage in mammals (Lincoln, 1990; Dawson and Sharp, 1998), which also coincides with demise of reproductive system. It is very well accepted that during

incubation, high levels of PRL directly inhibit hypothalamic secretion of GnRH, which in turn reduces pituitary secretion of LH and leads to regression of the gonads (Curlewis, 1992; El Halawani and Rozenboim, 1993).

In birds, it has been very well documented that PRL secretion is tonically stimulated by VIP (for reviews, see: El Halawani et al., 1997; 2000; Chaiseha and El Halawani, 2005). Variations in hypothalamuc VIP immunoreactivity, VIP content, and VIP mRNA steady-state levels in the INF, VIP-ir fibers in the ME, and VIP concentrations in hypophyseal portal blood are correlated with changes in the amount of circulating PRL throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al. 1996a; Chaiseha and El Halawani, 1999). Pituitary VIP receptor mRNAs were changed across the reproductive stages with the highest expression found in the incubating turkey hens (Chaiseha et al., 2004). The result from this present study is in good accordance with the previous reports, changes in plasma PRL levels across the reproductive cycle were found to be paralleled with the changes in the number of VIP-ir neurons in the INF area of the native Thai chickens (Kosonsiriluk et al., 2006).

It is generally considered that LH is one of the important hormones in controlling reproduction of hens. During ovulatory cycle, plasma levels of LH and other reproductive hormones increase from a basal level to a peak level before ovulation in hen (Kappauf and van Tienhovan, 1972; Furr et al., 1973; Lague et al., 1975; Etches and Cunningham, 1976; Proudman et al., 1984), turkey (Mashaly et al., 1976; Proudman et al., 1984), Japanese quail (Doi et al., 1980), and duck (Tanabe et al., 1980). However, the changes of plasma LH levels were not observed during the reproductive cycle of the native Thai chicken. Due to relatively long intervals (one

time per week) between collected samples, it might not be possible to determine whether LH concentrations increase during ovulatory cycle of the native Thai hens. Thus, a precise conclusion concerning temporal changes in LH associated with ovulation was not possible to be established in this present study and needed to be further investigated.

The fluctuation of plasma LH levels was observed throughout the reproductive cycles of the native Thai chicken. In contrast, increased in plasma LH levels that coincided with the initiation of egg laying were reported in chicken (Furr et al., 1973; Shodono et al., 1975; Wilson and Sharp, 1975), herring gull (Scanes et al., 1974), turkey (Mashaly et al., 1976), white-crowned sparrow (Mattocks et al., 1976; Wingfield and Farner, 1978b), snow geese (Campbell et al., 1978), mallard (Donham et al., 1976; Donham, 1979; Tanabe et al., 1980), and Japanese quail (Doi et al., 1980). A fall in LH secretion at the onset of incubation has also been reported in ring dove (Cheng and Follett, 1976; Silver et al., 1980), snow geese (Campbell et al., 1978), white-crowned sparrow (Wingfield and Farner, 1978a; 1978b), bantam hen (Sharp et al., 1979), and turkey (Cogger et al., 1979). It has been established that the onset of incubation behavior is correlated with declining plasma levels of LH and gonadal steroids and increasing plasma levels of PRL in bantam (Lea et al., 1981) and turkey hens (El Halawani and Rozenboim, 1993). A suppressive effect of PRL on gonadotropin secretion is suggested by the association between high PRL levels and low LH levels in incubating birds. This is supported by the findings that the administration of PRL antiserum to incubating chicken results in an increase in circulating LH (Lea et al., 1981), and exogenous PRL suppresses LH and induces gonadal regression (Opel and Proudman, 1980; Buntin and Tesch, 1985; Sharp et al.,

1988; El Halawani et al., 1991).

No significant difference was observed in plasma LH levels of the native Thai chickens across four reproductive stages examined. This result is consistent with the LH levels previously reported in turkeys which serum LH in laying and incubating hens was not significantly different (Harvey et al., 1981; El Halawani et al., 1984; Wong et al., 1992). Previous findings indicated that the LH-releasing mechanism is not impaired under the conditions of hyperprolactimia in birds, since the high PRL levels in incubating hens do not depress LH secretion (Sharp et al., 1986; El Halawani et al., 1987). These findings, taken together with the findings of this present study that plasma LH levels were not suppressed by the high levels of plasma PRL, suggest that the interaction of PRL with LH appears to be variable and is species-specific.

In conclusion, the results of this present study provide baseline information of the neuroendocrine changes emphasizing the plasma levels of PRL and LH profiles associated with reproductive stages of the native Thai chicken and support the previous studies that PRL is associated with the reproductive cycle in avian species and play a pivotal role in the incubation behavior.

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CHAPTER IV

DISTRIBUTION OF VASOACTIVE INTESTINAL PEPTIDE IMMUNOREACTIVITY IN THE BRAIN OF THE NATIVE THAI CHICKEN

4.1 Abstract

Avian prolactin (PRL) secretion is under stimulatory control by the PRL-releasing factor (PRF) vasoactive intestinal peptide (VIP), which is secreted from neurons in the nucleus infundibuli hypothalami (IN) and the nucleus inferioris hypothalami (IH). The expression and release of the VIP/PRL system in temperate zone birds is regulated by a gonad stimulating photoperiod. The distribution of VIP immunoreactivity in several brain areas of these species has been reported. This study, utilizing indirect immunofluorescent techniques, was designed to characterize the distribution of the VIP system in the native Thai chicken brain, a non-photoperiodic, continuous breeding species. Differential VIP expression may give us insight into the mechanism(s) underlying the regulation of the reproductive cycle in this species. The results revealed that VIP-immunoreactive (VIP-ir) cells and fibers were found throughout the brain, but were predominantly located within the diencephalon where the greatest concentrations were found in the IN-IH area. VIP-ir neurons were also observed within the organum septi laterale, nucleus septalis lateralis, nucleus anterior medialis hypothalami, regio lateralis hypothalami, nucleus ventromedialis

hypothalami, and nucleus rotundus. A dense accumulation of VIP-ir fibers was found in the external layer of the eminentia mediana. The pattern of VIP distribution in this study supports the data reported previously in several avian species with some minor differences. Changes in hypothalamic VIP-ir neurons within the IN-IH area, but not other areas of the hypothalamus, were directly correlated to concentrations of circulating PRL throughout the reproductive cycle of the native Thai chicken. This extensive VIP neuronal network in the hypothalamus of the native Thai chicken suggests it is important in the regulation of reproductive behavior in equatorial birds. The prevalence of VIP in the mediobasal hypothalamus is now implicated in the control of female reproductive activities in this species. Furthermore, the results of this study provide additional evidence that VIP is also the PRF in non-photoperiodic, continuously breeding avian species.

4.2 Introduction

Vasoactive intestinal peptide (VIP) is a potent stimulator of mammalian prolactin (PRL) secretion, both *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Ruberg et al., 1978; Shaar et al., 1979; Matsushita et al., 1983). VIP occurs extensively in neurons of both the central and peripheral nervous systems (Said and Rosenberg, 1976; Hokfelt et al., 1982), with high concentrations found in the hypothalamus (Samson et al., 1979; Ceccatelli et al., 1992) and the hypophyseal portal blood (Shimatsu et al., 1981). In mammals, VIP is widely expressed throughout the body and has important regulatory effects on the circulatory, immune, reproductive, and gastrointestinal systems (Said, 1982; Gressens et al., 1993). This octacosapeptide was first isolated from the porcine small intestine (Said and Mutt, 1970) and is a

conserved peptide displaying only 4 amino acid substitutions between the swine and the chicken (Nilsson, 1975).

In birds, it has been established for some time that pituitary PRL secretion is tonically stimulated (Kragt and Meites, 1965; Bern and Nicoll, 1968) and the releasing factor responsible for this tonic stimulation is VIP, secreted from neurons located in the infundibular nuclear complex (INF) of the caudo-medial hypothalamus (El Halawani et al., 1997). Dopamine (DA) also appears to have a prominent role in PRL secretion, acting upon the VIPergic system to regulate the release of PRL (Youngren et al., 1996; Chaiseha et al., 2003). Serotonin and the opiate dynorphin also stimulate PRL release, and may act along a single pathway with DA to influence VIP secretion and ultimately circulating PRL levels (El Halawani et al., 2000).

Pituitary PRL secretion is closely correlated with the avian reproductive cycle. During the reproductively quiescent stages of the cycle, plasma PRL levels are very low (5-10 ng/ml); however, during the laying and incubating stages, circulating PRL levels increase dramatically (500-1500 ng/ml; El Halawani et al., 1984). In temperate zone birds, expression and secretion within the VIP/PRL system is regulated by gonad-stimulating photoperiod. Nascent VIP mRNA levels in the hypothalamus, cytoplasmic VIP mRNA steady-state levels in the hypothalamus and VIP content in the median eminence (eminentia mediana; ME) were all increased and correlated with increased PRL secretion following photostimulation (Chaiseha et al., 1998). These results support a hypothetical schematic involving the regulation of PRL secretion where VIP, serving as the PRL-releasing factor, is intimately linked with photoperiodic mechanisms. Immunocytochemical studies have shown that hypothalamic VIP-immunoreactive (VIP-ir) neurons within the INF and VIP-ir fibers

in the ME displayed increases corresponding to circulating PRL levels (Mauro et al., 1989). These abundant hypothalamic VIP-ir neurons extend axons into the external layer of the ME, suggesting physiological relevance for neuroendocrine regulation (Yamada et al., 1982; Macnamee et al., 1986). Other studies have also shown increases in the number and size of VIP-ir neurons within this region in the domesticated pigeon and ring dove during periods of elevated circulating PRL levels (Peczely and Kiss, 1988; Cloues et al., 1990).

VIP localization studies have been conducted in the brains of several avian species, such as the Pekin duck (Korf and Fahrenkrug, 1984), Japanese quail (Peczely and Kiss, 1988), turkey (Mauro et al., 1989; Chaiseha and El Halawani, 1999), pigeon (Cloues et al., 1990), ring dove (Norgren and Silver, 1990), chicken (Kuenzel and Blahser, 1994; Kuenzel et al., 1997), dark-eyed junco (Saldanha et al., 1994), and male zebra finch (Bottjer and Alexander, 1995). However, to date, there has been no report on the location of VIP immunoreactivity within the brain of the native Thai chicken. Thus, the objective of this study was to characterize the distribution of the VIP system in the brain of the Thai chicken, an equatorial species that breeds continuously and is unaffected by photoperiod. Differential VIP expression may lead to insight into neural mechanism(s) underlying the regulation of the reproductive cycle in a non-photoperiodic species.

4.3 Materials and Methods

4.3.1 Experimental Animals

70 female and 12 male native Thai chickens, Pradoohangdam breed, were used. They were reared and housed in floor pens under natural daylight (approximately 12

hours of light and 12 hours of dark; 12L:12D). Food and water were constantly available. Each hen was identified by wing band number. Chickens were randomly divided into floor pens (6-8 females:1 male/pen). The chickens were observed their behaviors in order to classify them into 4 reproductive stages as non-laying (NL), laying (L), incubating (broodiness: B), and rearing chicks (R). The criteria for the reproductive stages classification were: 1) NL: the chickens that had not reached the sexual maturity and did not lay or express incubation and maternal behaviors, 2) L: the hens that laid regularly, 3) B: the hens that exhibited persistent nesting activity, no egg production, and aggressive nest protection behavior, and 4) R: the hens that stopped nesting and laying eggs, and reared the chicks after hatching. Daily records of egg production, nesting activity, and other behaviors were recorded throughout the experiments. The animals were treated in accordance with Suranaree University of Technology Animal Care and Use Committee Guidelines.

4.3.2 Experimental Design

4.3.2.1 Experiment I

Blood samples of the native Thai chickens in each reproductive stage (NL, L: 7 days after the first egg was laid and hens laid egg regularly, B: 10 days after hens stopped laying and were sitting on nests continuously, and R: 14 days after the first chick was hatched and hens were rearing their chicks) were collected from the brachial vein in heparinized tubes prior to sacrifice the chickens (n=10). Plasma samples were separated by centrifugation and stored at -35°C until used for determining plasma PRL levels by enzyme-linked immunosorbent assay (ELISA). A postmortem examination of each hen was performed to confirm its reproductive status.

4.3.2.2 Experiment II

To localize the VIP distribution throughout the brain of the native Thai ckickens, the laying hens (7 days after the first egg was laid and hens lay egg regularly) were used (n=6). The brains were fixed by pressure-perfused prior to section and used for further processed by immunohistochemistry. A postmortem examination of each hen was performed to confirm its reproductive status.

4.3.2.3 Experiment III

To determine the changes of VIP-ir neurons within the INF in each reproductive stage, native Thai chickens in each reproductive stage were used (n=6, the criteria for each reproductive stage were described in Experiment I). The brains were fixed by pressure-perfused prior to section and used for further processed by immunohistochemistry. A postmortem examination of each hen was performed to confirm its reproductive status.

4.3.3 Measurement of Plasma PRL Concentrations

Plasma PRL levels were measured by ELISA technique (Proudman et al., 2001). Briefly, plates were coated with 100 µl of AffiniPure Goat anti-Rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) which was diluted in 0.05 M potassium phosphate buffer at the dilution 1:2,000. The plates were incubated at 4°C overnight and then blocked with blocking solution (100 µl of 0.4% casein, 0.01% thimerosal, 1 mM EDTA). 20 µl of samples, 30 µl of the assay buffer (0.1% casein, 0.01% thimerosal, 1 mM EDTA), 25 µl of anti-PRL (1:20,000, provided by Dr. John Proudman, USDA, USA), and 25 µl of β-PRL tracer (1:50,000) were

added into the reaction, then incubated at 4°C overnight. The reactions were measured the absorbent at 405 nm. The samples were determined plasma PRL levels in duplicate within a single assay.

4.3.4 Processing of Tissues for Immunohistochemistry

Prior to perfusion, each hen was intravenously injected with 3,000 units of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA), and euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). The head was removed and immediately pressure-perfused via the carotid arteries with phosphate buffered saline (PBS, pH 7.4) for 3-5 min, followed by a freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min according to the previously described method (Al-Zailaie et al., 2006). The brain was then dissected intact from the skull, and soaked in 20% sucrose in PBS at 4°C for 48 hrs or until saturated for cryoprotection. The brain was then frozen in powdered dry ice for 1 hr, and stored at -35°C until sectioned in the coronal plane at a thickness of 16 μm utilizing a cryostat (Leica CM1850, Leica Instruments GMbH, Nussioch, Germany). The sections were mounted onto subbed slides and stored desiccated at -20°C until further processed for immunohistochemistry.

4.3.5 Immunohistochemistry

In order to localize the VIP distribution throughout the brain and visualize the changes in VIP-ir neurons within the INF in each reproductive stage, immunohistochemistry was performed as previously described (Al-Zailaie et al., 2006). Briefly, tissue sections of different areas throughout the brains and four adjacent brain

tissue sections from each reproductive stage were thawed to room temperature prior to use. The thawed sections were then covered with 100 µl of VIP primary antibody (polyclonal anti-chicken VIP antiserum (VIP4-DYC8), generously provided by Dr. M.E. El Halawani, University of Minnesota, USA) diluted 1:1000 with PBS (pH 7.4) containing 1% bovine serum albumin and 0.3% triton-X. Tissue section slides were then incubated in a moist chamber at 4°C overnight, and then washed three times with PBS (pH 7.4) for 5 min each. After washing, 100 µl of secondary antibody diluted 1:500 Cy^{TM}3-conjugated AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was applied in dark conditions. Slides were further incubated in a moist dark chamber at room temperature for 1 hr, washed with PBS (pH 7.4) three times for 5 min each, and then mounted with DPX mountant (Sigma-Aldrich, Inc., Steinheim, Germany). Microscopic images of brain sections were visualized with a fluorescence microscope (Olympus IX71, Tokyo, Japan) at 4x, 10x, 20x, and 40x magnification using a cooled digital color camera (Olympus DP70, Tokyo, Japan). The images were then captured and stored by DP70-BSW software (Olympus, Tokyo, Japan). To aid in the documentation of neuroanatomical results illustrating VIP immunoreactivity, the nomenclature and schematic diagrams from the stereotaxic atlases of the brain of the chick (Kuenzel and Masson, 1988). The specificity of the anti-VIP serum was tested by omitting the anti-VIP serum from the primary antiserum. No immunostaining of VIP was observed in control sections.

4.3.6 Statistical Analysis

Differences in plasma PRL levels and in the number of VIP-ir neurons among

4 reproductive stages (treatment groups) were analyzed. Results were expressed as mean ± SEM. Significant differences of mean were statistically analyzed using one way analysis of variance (ANOVA). Significance differences among treatment groups were computed utilizing Tukey's HSD test. Differences were considered as statistically significant if a P value was less than 0.05. All statistical analyses were performed using SPSS Windows Software (SPSS Windows Software, version 13.0, SPSS Inc., Chicago, IL, USA).

4.4 Results

4.4.1 Experiment I

Plasma PRL levels in each reproductive stage (n=10) was measured by ELISA. The results showed that plasma PRL levels were lowest in NL (28.25±3.87 ng/ml), increased in L (42.83±8.94 ng/ml), reached the highest in B (238.38±19.77 ng/ml, $P<0.05$), and then declined sharply in R (25.67±1.25 ng/ml) as shown in Fig 4.1.

4.4.2 Experiment II

The results from the immunohistochemistry revealed that the distribution of VIP-ir cells and fibers were found throughout the brain (telencephalon, diencephalon, mesencephalon, and rhombencephalon) of the female native Thai chicken (Fig. 4.2) with the predominant accumulation found within the diencephalon.

4.4.2.1 Telencephalon

Beginning with the anterior portion of the brain, the most rostral VIP-ir fibers were found in the lobus parolfactorius (LPO). In the septal area, a cluster of

cerebrospinal fluid (CSF)-contacting VIP-ir neurons with bulb-like processes projecting into the ventriculus lateralis (VL) was observed within the organum septi laterale (LSO), a circumventricular organ (CVO) found in birds and reptiles (Kuenzel and van Tienhoven, 1982; Korf and Fahrenkrug, 1984; Hirunagi et al., 1993; Kuenzel and Blahser, 1994), at the medial portion (organum septi laterale, pars medialis: LSOm) as shown in Fig. 4.3A. A group of VIP-ir fibers were also found in and about the nucleus accumbens (Ac). In addition, there was a dense accumulation of VIP-ir fibers present in the nucleus septalis lateralis (SL) and the nucleus septalis medialis (SM), but few VIP-ir cells were seen in the SL as shown in Fig. 4.3B. Furthermore, a few uniformly dark-stained VIP-ir neurons were located in the nucleus taeniae (Tn).

4.4.2.2 Diencephalon

The greatest expression of VIP-ir neurons occurred in the diencephalons, with the highest accumulation found within the INF (Fig. 4.4A; 4.4B). Numerous VIP-ir neurons were found in the nucleus infundibuli hypothalami (IN) and the nucleus inferioris hypothalami (IH), where neuron activity has been shown to correspond to changes in circulating PRL in other avian species. Most of the VIP-ir neurons were found in the caudal portion of the IN-IH area, whereas there were only a few VIP-ir neurons found in the rostral portion. None of the VIP-ir cells in this area were CSF-contacting neurons with bulb-like processes protruding into the third ventricle, as found in the LSOm. In addition, dense accumulations of VIP-ir fibers were found in the external layer of the ME (Fig. 4.4C), where beaded fibers were distributed specifically in a palisade arrangement. There was a large group of VIP-ir neurons observed outside the hypothalamus in the nucleus rotundus (ROT) as shown in Fig.

4.5A and 4.5B. The cluster of VIP-ir cells in this area showed stained with coarse-grained granules in the cytoplasm and neurite. Scattered VIP-ir neurons were also found within the nucleus anterior medialis hypothalami (AM, Fig. 4.5C), regio lateralis hypothalami (LHy, Fig. 4.5D), and nucleus ventromedialis hypothalami (VMN). A very dense accumulation of VIP-ir fibers was observed in the LHy. Within the boundaries of the nucleus periventricularis hypothalami (PHN, Fig. 4.5E), there were beaded VIP-ir fibers, some of which extended in parallel with the third ventricle to the INF. VIP-ir fibers were observed in the nucleus paraventricularis magnocellularis (PVN) as well. In the thalamus, there were some VIP-ir fibers present in the ventral portion of the tractus cortico-habenularis et cortico-septalis (CHCS). Additional VIP-ir fibers were also found in the nucleus commissurae pallii (nCPa, Fig. 4.5F).

4.4.2.3 Mesencephalon and Rhombencephalon

There were VIP-ir neurons scattered in the substantia grisea centralis (GCt, Fig. 4.6A), nucleus intercollicularis (ICo), area ventralis (AVT, Fig. 4.6B), nucleus tegmenti pedunculo-pontinus, pars compacta (TPc, Fig. 4.6C; 4.6D), nucleus subceruleus ventralis (SCv), locus ceruleus (LoC), and nucleus interpeduncularis (IP). Moving caudally to the beginning of the pons, a dense plexus of VIP-ir fibers was observed in the area of the nucleus parabrachialis, pars ventralis (PBv), nucleus tractus solitarii (S) and some in the nucleus linearis caudalis (LC) and nucleus reticularis paragiganto-cellularis lateralis (Rpgl). Additionally, VIP-ir cells were found lining in the cortex layer of the cerebellum (Cb, Fig. 4.7A). Conversely, there was no immunostaining observed in the pituitary (Fig. 4.7B).

4.4.3 Experiment III

Immunohistochemistry revealed that VIP-ir neurons and fibers were highly expressed throughout the diencephalons, where the greatest expression of VIP-ir neurons occurred within the INF, predominantly in the IN and the IH. Changes in VIP-ir neuron populations were observed within the INF across the reproductive cycle of the native Thai chicken (Fig. 4.8; 4.9). VIP-ir neurons counts were low in NL (43.67±3.44 cells, Fig. 4.8A; 4.9), increased in L (79.17±7.56 cells, $P<0.05$, Fig 4.8B; 4.9), and markedly increased in B (117.00±10.27 cells, $P<0.05$, Fig. 4.8C; 4.9). The number of VIP-ir neurons decreased (52.42±6.86 cells, Fig. 4.8D; 4.9) when birds shifted from incubation to the rearing of chicks. These relationships was not observed within other areas of the hypothalamus.

Table 4.1 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988)

Ac	Nucleus accumbens
AM	Nucleus anterior medialis hypothalami
AVT	Area ventralis
Cb	Cerebellum
CHCS	Tractus cortico-habenularis et cortico-septalis
GCt	Substantia grisea centralis
ICo	Nucleus intercollicularis
IH	Nucleus inferioris hypothalami
IN	Nucleus infundibuli hypothalami
IP	Nucleus interpeduncularis
LC	Nucleus linearis caudalis
LHy	Regio lateralis hypothalami (Lateral hypothalamic area)
LoC	Locus ceruleus
LPO	Lobus parolfactorius
LSO	Organum septi laterale (Lateral septal organ)
LSOm	Organum septi laterale, pars medialis
ME	Eminentia mediana (Median eminence)
nCPa	Nucleus commissurae pallii
PBv	Nucleus parabrachialis, pars ventralis
PHN	Nucleus periventricularis hypothalami

Table 4.1 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988), continued.

Pit	Pituitary
PVN	Nucleus paraventricularis magnocellularis
ROT	Nucleus rotundus
Rpgl	Nucleus reticularis paragiganto-cellularis lateralis
S	Nucleus tractus solitarii
SCv	Nucleus subceruleus ventralis
SL	Nucleus septalis lateralis
SM	Nucleus septalis medialis
Tn	Nucleus taeniae
TSM	Tractus septomesencephalicus
TPc	Nucleus tegmenti pedunculo-pontinus, pars compacta (Substantia nigra)
V III	Ventriculus tertius (Third ventricle)
VL	Ventriculus lateralis
VMN	Nucleus ventromedialis hypothalami

Fig. 4.1 Changes in plasma PRL concentrations in each reproductive stage of the native Thai chickens. Values are presented as the mean ± SEM (n=10). Significant differences between means are denoted by different letters (P<0.05).

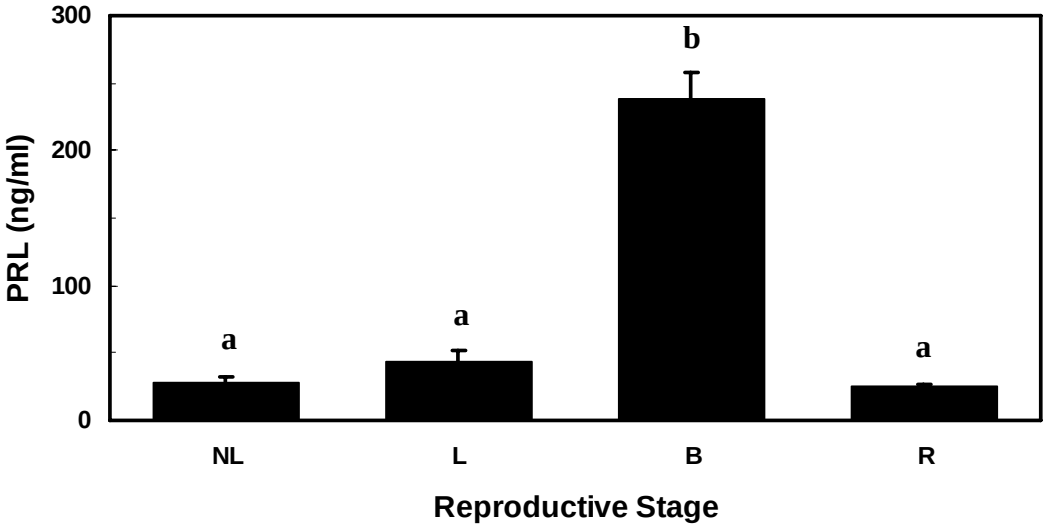


Fig. 4.2 Schematic diagrams of coronal sections from rostral to caudal (**A-F**) showing the distribution of VIP-ir cells (black dots) throughout the brain of the laying native Thai chicken. Coronal illustrations were redrawn from the chicken brain atlas (Kuenzel, 2002) with nomenclature (Kuenzel and Masson, 1988). The number in the upper right hand corner shows the anterior distance in mm from the zero coordinates given in the stereotaxic atlas of the chick brain. For abbreviations, see Table 4.1.

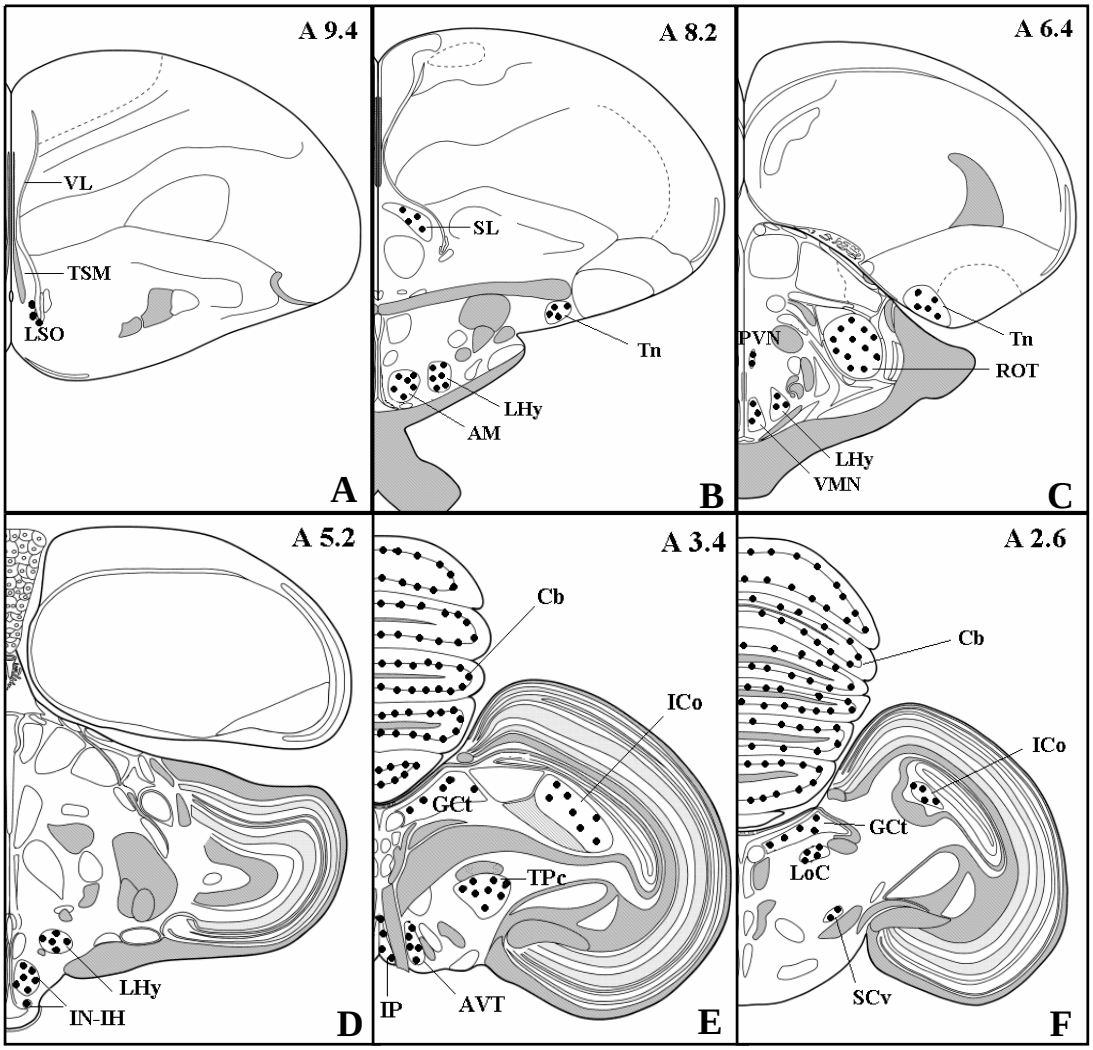


Fig. 4.3 Photomicrographs of coronal sections in the septal area of the laying native Thai chicken brain demonstratating the distribution of CSF-contacting neurons located in the LSOm (**A**) while the SL contains a dense plexus of VIP-ir fibers and a few VIP-ir cells (**B**). Original magnification X20. For abbreviations, see Table 4.1.

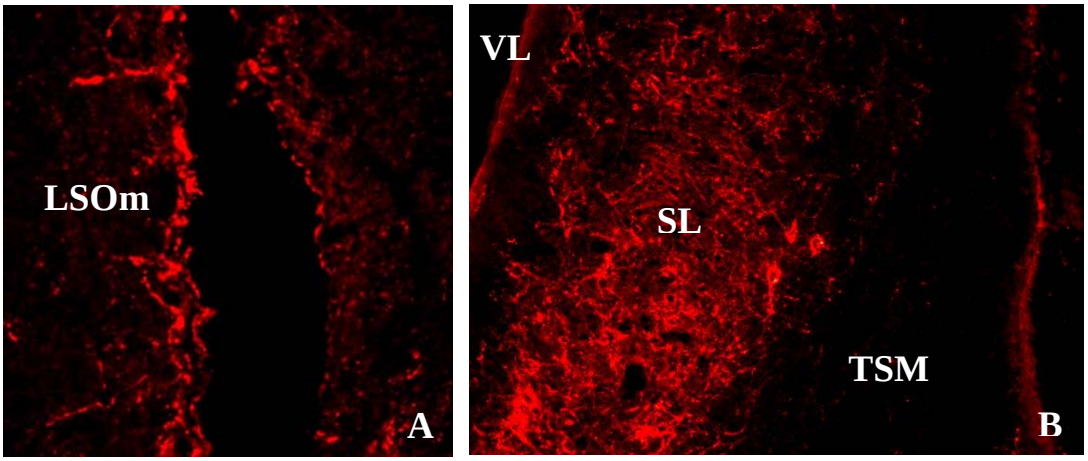


Fig. 4.4 Photomicrographs of coronal sections within the INF illustrating numerous VIP-ir cells in the IN-IH area and a dense accumulation of VIP-ir fibers in ME of the laying native Thai chicken brain (**A**; Original magnification X10). Rectangles indicate areas from which following photomicrographs were taken. Higher magnification of the VIP-ir neurons was demonstrated in the IN-IH area (**B**; Original magnification X40). Enlargement of a dense arrangement of VIP nerve terminals in the external layer of ME (**C**; Original magnification X20). For abbreviations, see Table 4.1.

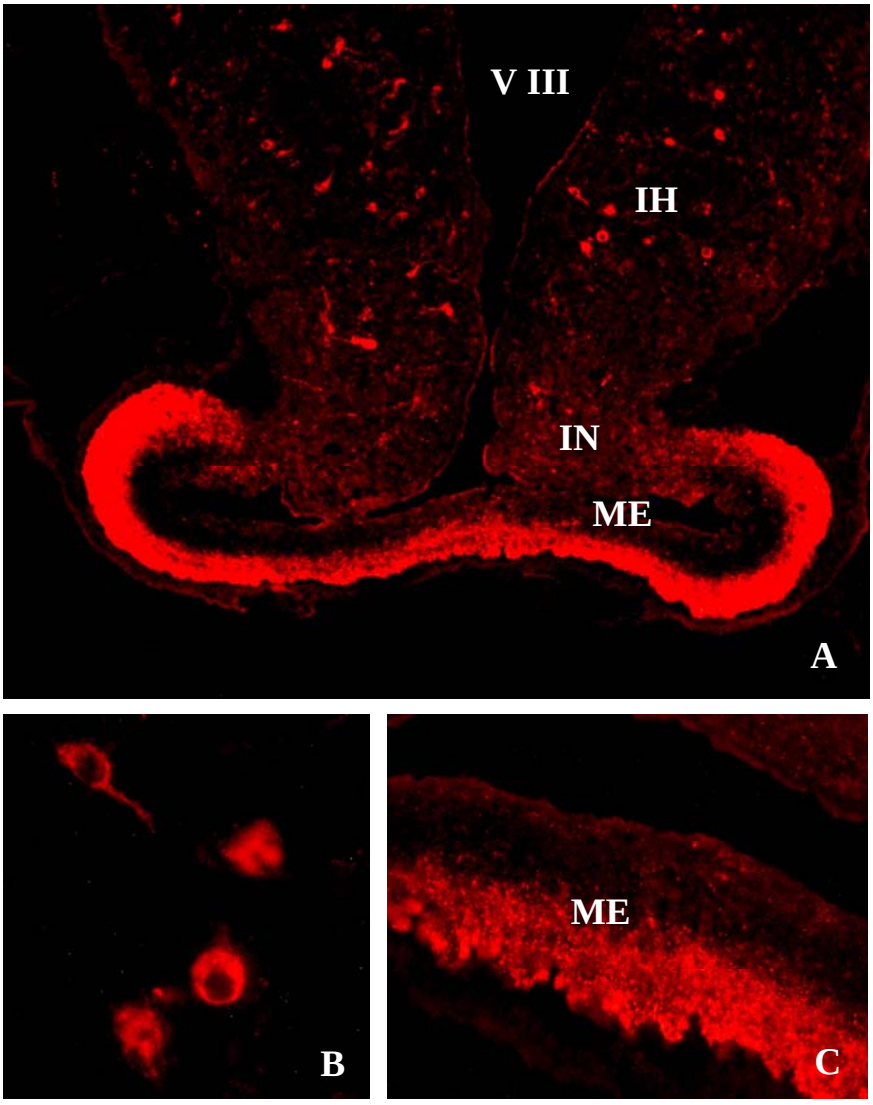


Fig. 4.5 Photomicrographs of coronal sections in the hypothalamus and surrounding areas of the laying native Thai chicken brain demonstrating the cluster of VIP-ir cells with coarse-grained granules in the cytoplasm and neurites in the ROT (**A**; Original magnification X20). Higher magnification of VIP-ir cells in the ROT (**B**; Original magnification X40). **C**, **D** Scattered VIP-ir cells located in the AM and LHy (**C**, **D**; Original magnification X20). VIP-ir fibers in the PHN and some fibers oriented parallel to the third ventricle (**E**; Original magnification X20). VIP-ir fibers found in nCPa (**F**; Original magnification X20). For abbreviations, see Table 4.1.

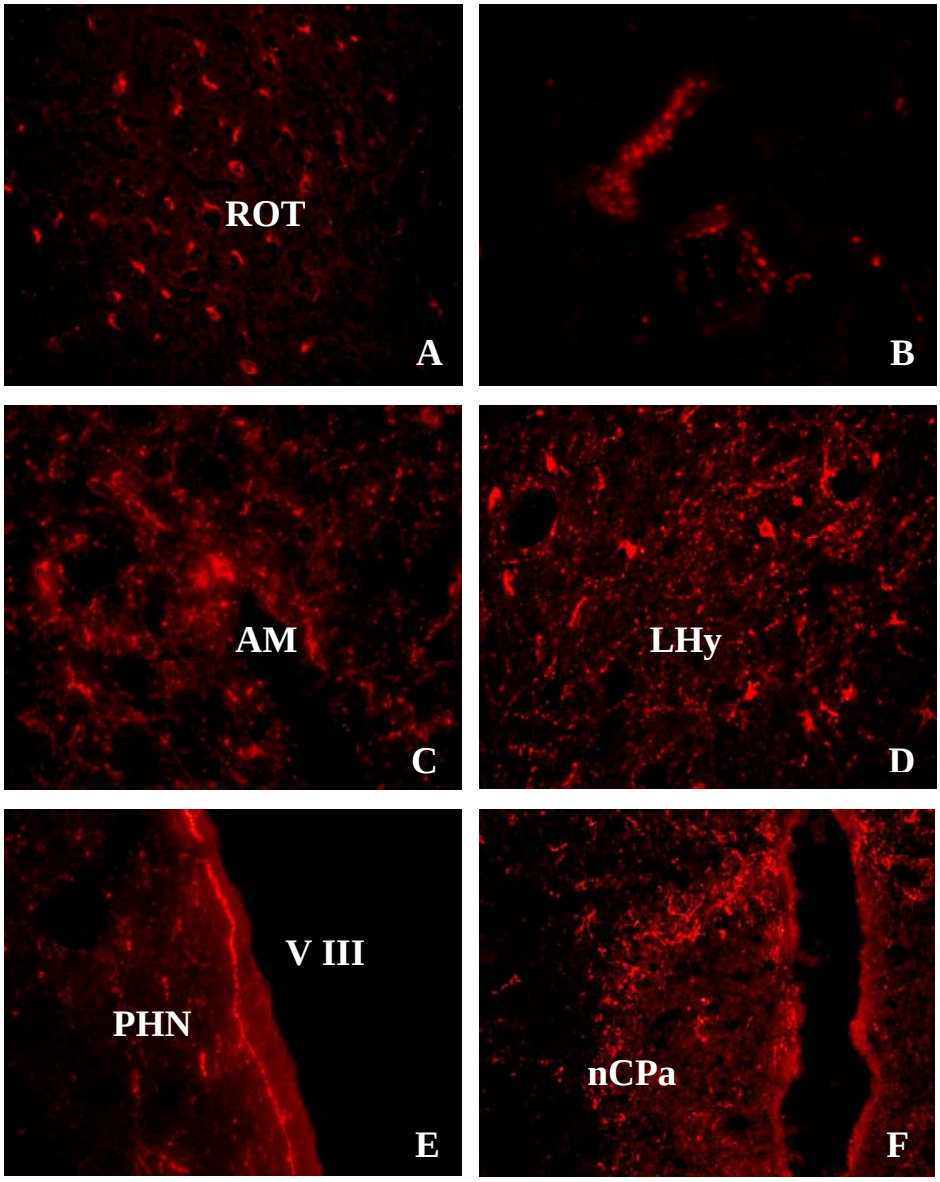


Fig. 4.6 Photomicrographs of coronal sections demonstrating the distribution of VIP-ir cells in the mesencephalon of the laying native Thai chicken brain. The specific binding of VIP antibody was observed within the GCt (**A**), AVT (**B**), and TPc (**C**; Original magnification X20). Higher magnification of VIP-ir cells from Fig. 4.6**C** (**D**; Original magnification X40). For abbreviations, see Table 4.1.

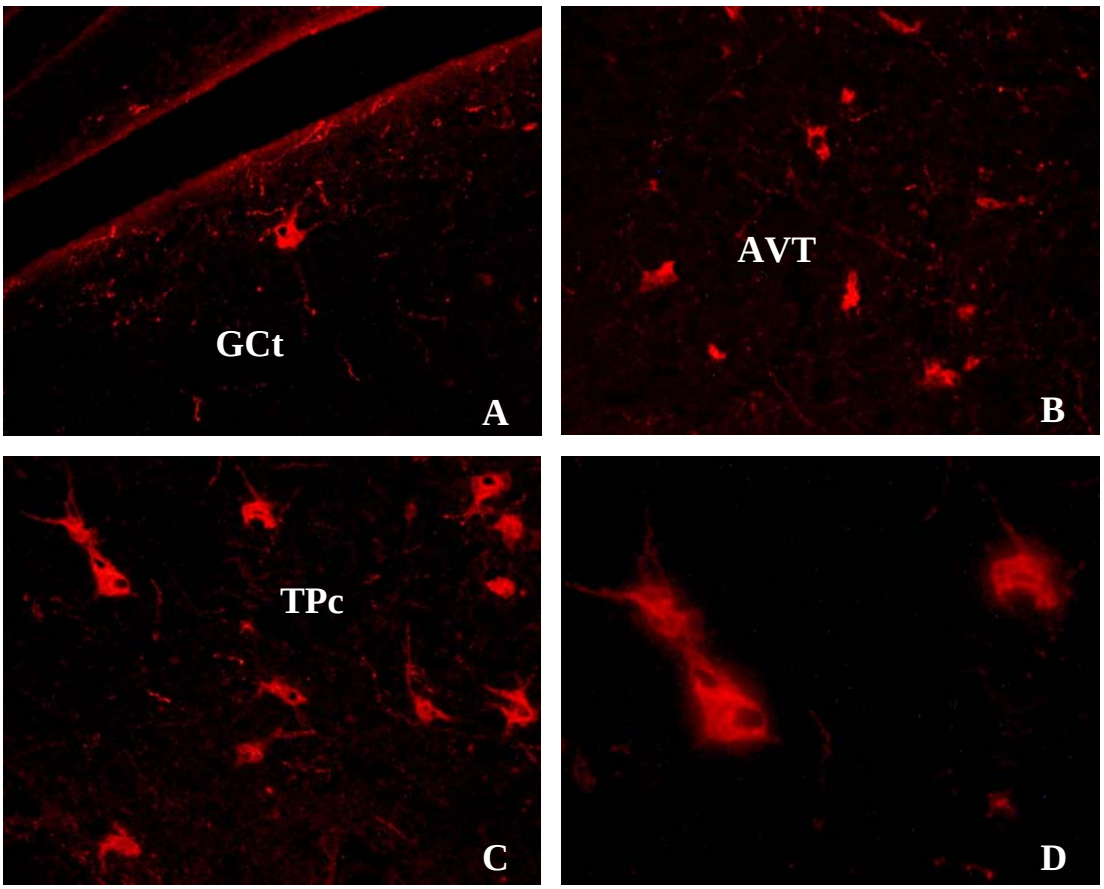


Fig. 4.7 Photomicrographs of coronal sections of the laying native Thai chicken brain demonstrating VIP-ir cells lining in the cortex layer of the Cb (**A**; Original magnification X10), whereas no immunostaining was observed in the pituitary (**B**). Original magnification X4 Insert: higher magnification of VIP-ir cells in the Cb; Original magnification X40. For abbreviations, see Table 4.1.

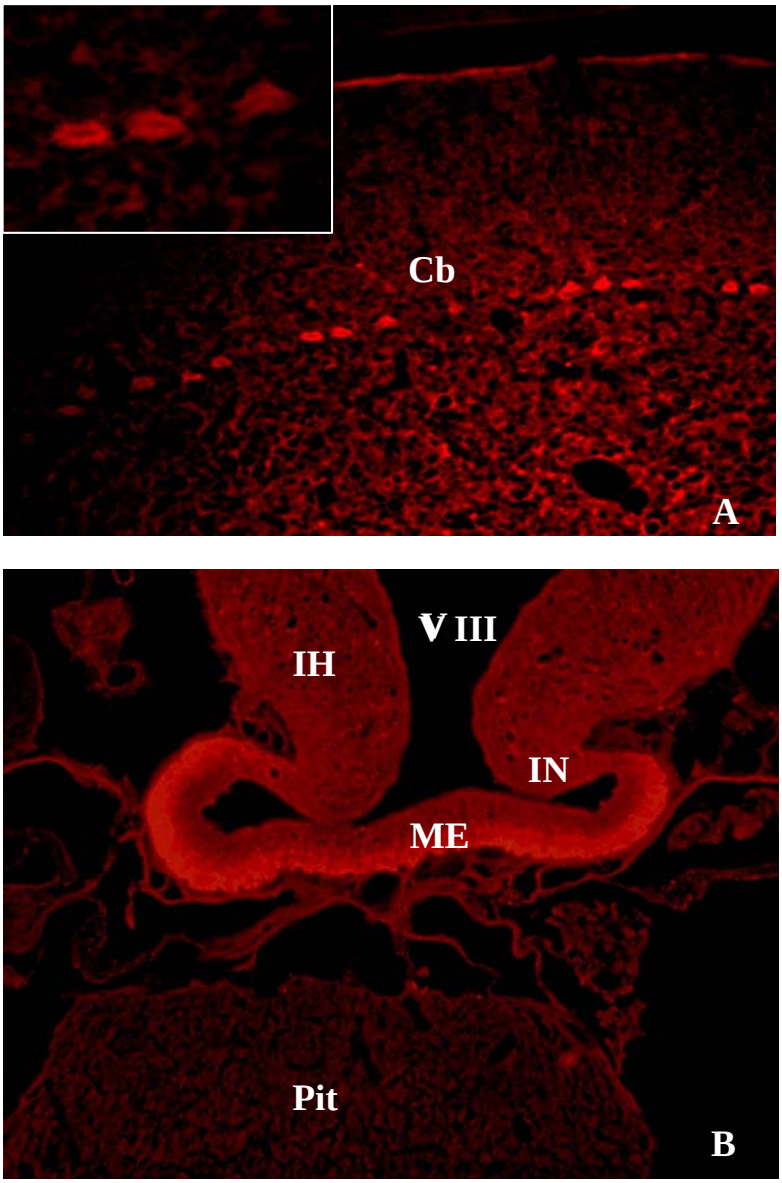


Fig. 4.8 Photomicrographs of coronal sections of the native Thai chicken hypothalamus demonstrating the distribution of VIP-ir cells and fibers within the INF at different reproductive stages (n=6). (**A**): NL, (**B**): L, (**C**): B, (**D**): R. Original magnification X10. For abbreviations, see Table 4.1.

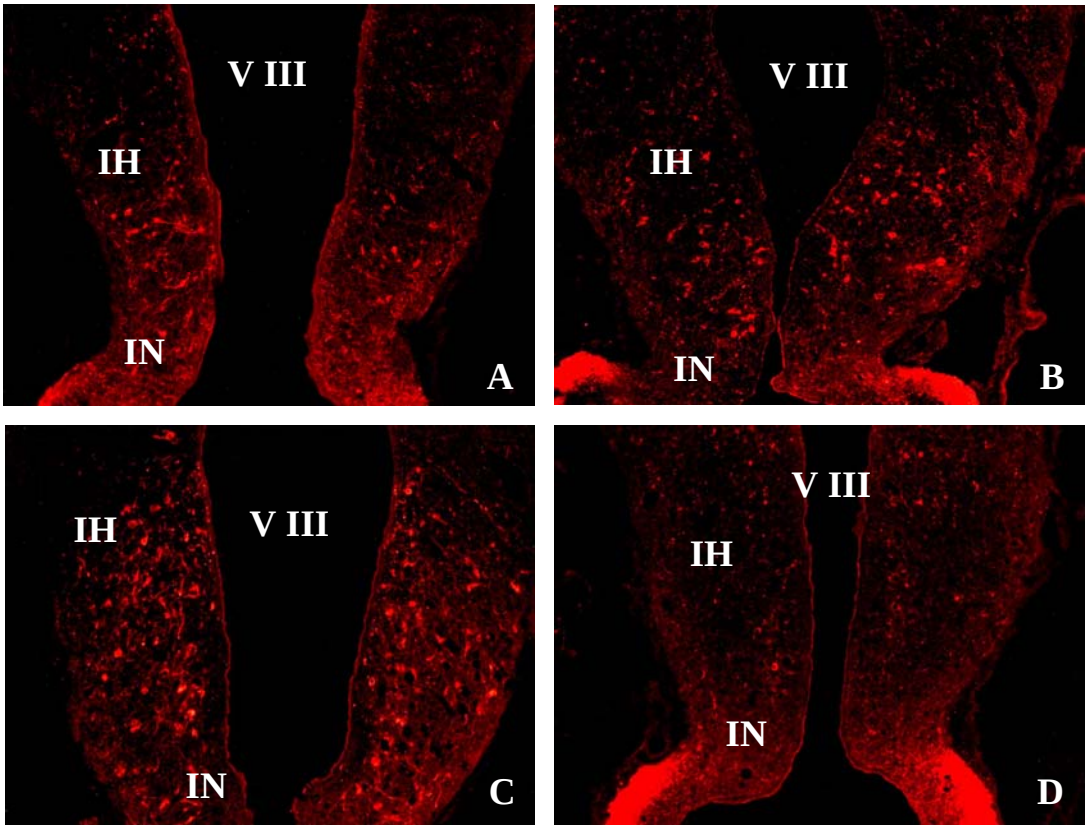
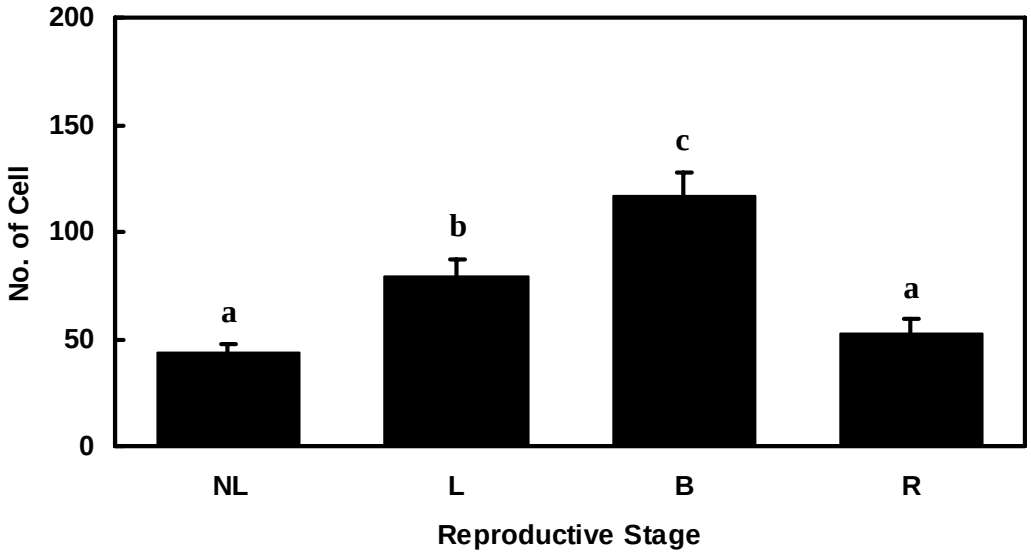


Fig. 4.9 Changes in the number of VIP-ir neurons within the INF of the native Thai chicken hypothalamus in each reproductive stage (n=6). Values with different letters are significantly different (P<0.05).



4.5 Discussion

The results of the present study revealed that VIP-ir neurons and fibers were extensively distributed throughout the brain of the native Thai chicken and predominantly expressed within the diencephalon. The greatest expression of VIP-ir neurons that occurred within the diencephalon was found within the INF. Changes in hypothalamic VIP-ir neurons within the INF, but not other areas of the hypothalamus, were observed and directly correlated to concentrations of circulating PRL throughout the reproductive cycle of the native Thai chicken. VIP-ir neurons were also found in the AM, LHy, PVN, and VMN. The findings of this study corresponded with previous studies of the distribution of VIP-containing neurons and fibers within the brains of several avian species and provides additional evidence that VIP is also the avian PRL-releasing factor (PRF) in non-photoperiodic continuous breeding species.

The immunohistochemistry survey demonstrated that VIP-ir neurons are widespread in the brain of the native Thai chicken. These results are in accordance with immunohistochemical localization of VIP in other avian species (Yamada et al., 1982; Macnamee et al., 1986; Peczely and Kiss, 1988; Mauro et al., 1989; Norgren and Silver, 1990; Esposito et al., 1993; Kuenzel and Blahser, 1994; Aste et al., 1995). In agreement with other localization studies, three major groups of VIP-ir neurons were found in this study; 1) a group of CSF-contacting neurons was observed specifically in the medial portion of LSO, 2) within the hypothalamus and surrounding areas, from medially out to the optic tectum, and 3) a group of bipolar cells in the infundibular region, especially within the IN-IH complex.

In the telencephalon, the most rostral VIP-ir fibers were found in the LPO. In the septal area, a cluster of CSF-contacting VIP-ir neurons was observed within the LSO, a CVO found in birds and reptiles (Kuenzel and van Tienhoven, 1982; Korf and Fahrenkrug, 1984; Hirunagi et al., 1993; Kuenzel and Blahser, 1994). A group of VIP-ir fibers was found in and about the Ac. There was a dense accumulation of VIP-ir fibers found in the SL and the SM with a few VIP-ir cells observed in the SL. A few uniformly dark stained VIP-ir neurons were also located in the Tn.

The LSO of the chick has been reported to have an ependymal specialization characterized by multiple layers of columnar ependymal cells at the base of the lateral ventricles. This area was proposed as an additional CVO in the avian brain (Kuenzel and van Tienhoven, 1982). CVO's are found in most vertebrates and have certain characteristics, such as specialized ependymal cells, a vascular area that has an incomplete blood-brain barrier, and CSF-contacting neurons (Vigh et al., 1977). It has been demonstrated that the LSO of the chick has an incomplete blood-brain barrier and contains VIP-like CSF-contacting neurons (Kuenzel and Blahser, 1994). Similar VIP-ir CSF-contacting neurons have been observed in the region of Ac/SL of other avian species such as the quail (Yamada et al., 1982), Pekin duck (Korf and Fahrenkrug, 1984), hen (Macnamee et al., 1986), dove (Silver et al., 1988), pegion (Hof et al., 1991), chick (Kuenzel and Blahser, 1994), and dark-eyed junco (Saldanha et al., 1994). In agreement with previous studies, a group of VIP-ir CSF-contacting neurons was also found within the LSOm of the native Thai chicken. The medial component of the LSO is the only area in the native Thai chicken brain that contains the very striking CSF-contacting neurons which immunostained with VIP, as well as having modified ependymal cells. The function of the CSF-contacting neurons in the

LSO has never been clarified. However, a subset of the CSF-contacting VIP-ir neurons has been shown to immunostain with an antibody directed against the photo pigment rhodopsin, suggesting that these cells may be stimulated by direct photoreception. A subset of VIP-ir neurons within the medial basal hypothalamus and septal region have been proposed to be encephalic photoreceptor neurons (Silver et al., 1988). In addition, the morphologic of CSF-contacting cells is similar to the sensory cells of the inner and outer segments of pinealocytes and of the developing retina in higher vertebrates (Vigh and Vigh-Teichmann, 1988). It has been suggested that the CSF-contacting neurons of the avian LSO might represent a component of the extraretinal encephalic photoreceptor involved in photoperiodic regulation (Oliver and Bayle, 1982; Foster et al., 1985). Furthermore, the occurrence of fibers of VIP-ir neurons in the lateral septum closely corresponds to the distribution of gonadotropin releasing hormone (GnRH)-containing cell bodies and fibers (Sterling and Sharp, 1982; Macnamee et al., 1986; Panzica et al., 1992). It is thus possible that VIP acts at the hypothalamic and septal levels to influence GnRH secretion in birds. This hypothesis is further reinforced by the ultrastructural demonstration that VIP nerve terminals in the lateral septum contact putative secretory GnRH neurons (Hirunagi et al., 1994), by the coexistence of VIP-ir CSF-contacting cells and GnRH-I-ir cells (Teruyama and Beck, 2001), and by the synaptic connections of chicken GnRH (cGnRH)-ir and VIP-ir cells in this region (Kiyoshi et al., 1998), suggesting the involvement of the ventricular system in cGnRH-I and VIP function.

The greatest expression of VIP-ir neurons in the diencephalon was found within the INF. Most of the VIP-ir neurons were found in the caudal portion of the IN-IH area, whereas there were only a few VIP-ir neurons found in the rostral portion.

Scattered VIP-ir neurons were observed within the AM, LHy, PVN, and VMN. In addition, dense accumulations of VIP-ir fibers were found in the external layer of the ME. A very dense accumulation of VIP-ir fibers was also observed in the LHy. None of VIP-ir cells in the diencephalon were found to be CSF-contacting neurons as found in the LSOm. There was a large group of VIP-ir neurons observed outside the hypothalamus, in the ROT. In the thalamus, there were some VIP-ir fibers located in the ventral portion of the CHCS. Additional VIP-ir fibers were also found in the nCPa.

The present findings revealed that changes in VIP-ir neurons within the INF, but not other areas of the hypothalamus were directly correlated to concentrations of circulating PRL throughout the native Thai chicken reproductive cycle. An increase in plasma PRL levels was noted during the transition from NL to L, but the greatest increase was observed in B. The plasma PRL levels declined sharply in R to the same level as NL. Changes in the number of VIP-ir neurons were observed within the INF across the reproductive stages. The findings revealed a gradual increase in the level of VIP-ir neurons within the INF during the transition from NL to L, with the greatest increase in VIP-ir neuron numbers observed in B. VIP-ir neurons decreased when birds shifted from incubation to rearing the chicks. These changes in VIP-ir neurons within the INF mirrored the levels of plasma PRL at each reproductive stage. This relationship was not observed within other areas of the hypothalamus. These results are in good agreement with previous studies (Mauro et al., 1989; Youngren et al., 1996; Chaiseha and El Halawani, 1999) indicating increased VIP immunoreactivity, increased VIP content, and VIP mRNA steady-state level in the INF, increased VIP-ir fibers in the ME, and increased VIP concentrations in hypophysial portal blood during the rise in circulating PRL in laying and incubating hens. In addition, these results

correspond with VIP binding studies (Rozenboim and El Halawani, 1993) reporting that non-photostimulated birds exhibited the least VIP high affinity binding sites, which then increased after photostimulation to maximal levels in incubating hens and then declined in photorefractory birds. Depriving incubating hens of their nests also reduced both serum PRL and anterior pituitary VIP receptors. It has been suggested that high VIP receptor mRNA levels in the anterior pituitary may account for the increased PRL gene transcription in response to VIP stimulation (Kansaku et al., 1995). Changes in pituitary VIP receptor mRNA was also observed across the reproductive stages in turkeys. Increased VIP receptor mRNA in the pituitary was observed in turkey hens with normal (laying) or high PRL secretion (incubating), while much less VIP receptor mRNA was observed in the pituitary of hypoprolactinemic nonphotostimulated and photorefractory turkey hens (Chaiseha et al., 2004). It has also been suggested that VIP receptors are located mainly in the cephalic lobe of the anterior pituitary gland, and that specific VIP binding varies according to the reproductive phase of the chicken. Therefore, more VIP receptor mRNA is expected in the incubation phase (Gonzales et al., 1994a; 1994b).

In this study, the large group of VIP-ir neurons found within the IN-IH complex corresponded to the previous studies (Yamada et al., 1982; Mikami and Yamada, 1984; Macnamee et al., 1986; Mikami, 1986; Peczely and Kiss, 1988; Silver et al., 1988; Mauro et al., 1989; Kuenzel and Blahser, 1994). It is very well established that this group of VIP neurons in the INF stimulate the release of PRL from the pituitary both *in vitro* (Proudman and Opel, 1983; Macnamee et al., 1986) and *in vivo* (Macnamee et al., 1986) and is associated with the reproductive cycle in birds (Mauro et al., 1989; Sharp et al., 1989; El Halawani and Rozenboim, 1993). Furthermore, the

differential expression of VIP within the IN-IH has been reported across the reproductive cycle of the turkey (Mauro et al., 1989; Chaiseha and El Halawani, 1999) and bantam hen (Sharp et al., 1989). In both studies, a significant correlation occurred between VIP cell number and density of fibers in the tuberal hypothalamus and the ME with shifts in circulating plasma levels of PRL during the reproductive cycle. The VIP immunoreactivity was greatest during incubation behavior in both species. Other studies have also shown increases in the number and size of VIP-ir neurons within the medio-basal hypothalamus when concentrations of plasma PRL were high, including the domesticated pigeon and ring dove during the initiation of crop milk secretion and feeding of the offspring (Peczely and Kiss, 1988; Cloues et al., 1990) and in the incubating bantam hen (Sharp et al., 1989). It has been suggested that VIP in this area might have a relevant role in the control of pituitary functions (Mikami, 1986) by projecting fibers to the external layer of the ME and affecting the release of PRL from the pituitary. Hypophysial portal blood showed differences in the concentration of VIP mirroring those of PRL in the general circulation across the turkey reproductive cycle (Youngren et al., 1996). These findings taken together suggest that the INF is the central site for PRL regulation in the turkey. The mechanism(s) underlying the changes in hypothalamic VIP mRNA during the photo-induced reproductive cycle may be due in part to alteration in the rate of VIP transcription (Chaiseha et al., 1998). In the chicken, VIP neurons within the PVN and INF are structurally adjacent to other peptidergic neurons such as somatostatin, methionine-enkephalin, and β-endorphin, suggesting complex modulating processes (Esposito et al., 1992). There are indications that the POM and PVN are involved in the control of sexual behavior (Crawford and Glick, 1975; Oliver and Bayle, 1976; Balthazart and Surlemont, 1990)

and in other physiological functions (Mills and Health, 1972; Korf et al., 1982).

Some differences of VIP-ir neuron distribution were observed compared with previous studies. The present study was not able to detect any VIP-ir cells within the optic tectum. In addition, only a few VIP-ir cells were found in other hypothalamic areas, including the AM, LHy, PVN, and VMN. This result is not in good agreement with previous reports where VIP-ir cells were found in more abundance (Kuenzel and Blahser, 1994; Teruyama and Beck, 2001). None of VIP-ir neurons found within the IN-IH complex were CSF-contacting neurons, which is consistent with the previous study in the chick (Kuenzel and Blahser, 1994). Neurons found in the Japanese quail (Yamada et al., 1982) and ring dove (Silver et al., 1988) were CSF-contacing VIP-ir neurons, and were located in both the septal region and the infundibular hypothalamic area. It could be that different orders and families of birds will show characteristic differences in the locations of VIP-like CSF-contacting neurons.

Scattered VIP-ir neurons were found in the GCt, ICo, TPc, SCv, LoC, IP, and AVT. Moving caudally to the beginning of the pons, a dense plexus of VIP-ir fibers was observed in the area of the PBv, S, and some in the LC and Rpgl. Additionally, VIP-ir cells were found lining in the cortex layer of the Cb. There was no immunostaining observed in the pituitary. When one considers the many functions attributed to VIP (Gozes and Brenneman, 1989), it is likely that VIP within these areas may be serving as a neurotransmitter/neuromodulator. The abundance of VIP-ir cells which showed staining of coarse-grained granules in the cytoplasm and neurites were found in the area of the ROT. This area was reported to be involved in the avian visual system (Con et al., 2003) and VIP-ir cells found in the ICo have been shown to elicit vocalizations in quail (Seller, 1980). Therefore, VIP-ir cells in these areas might be

related to the control of these activities. These findings illustrate the diverse roles of VIP as a neuropeptide in the brain of avian species.

In summary, the present findings, for the first time, provide the VIP localization in the brain of the native Thai chicken. VIP-ir neurons and fibers are extensively distributed throughout the brain of the native Thai chicken and are predominantly expressed within the diencephalon. Changes in hypothalamic VIP-ir neurons within the INF, but not other areas of the hypothalamus, were observed and directly correlated to concentrations of circulating PRL throughout the reproductive cycle of the native Thai chicken. The abundance of VIP neuronal networks in the native Thai chicken hypothalamus suggests its importance in the regulation of reproductive behavior in equatorial birds. Furthermore, the results of this study provide additional evidence that VIP is the PRF in avian species. In addition to the PRF function, VIP may function as a neurotransmitter and/or neuromodulator in this species, since VIP imuunoreactivity was found extensively distributed in other areas of the brain beside the hypothalamus. However, VIP is predominantly located in the posterior hypothalamus and is clearly implicated in the control of reproductive activities in the female native Thai chicken, which is a continuous breeding species that does not exhibit photoperiodic cycles.

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CHAPTER V

EFFECTS OF PHOTOPERIOD UPON THE PRODUCTIVE SYSTEM OF THE NATIVE THAI CHICKEN

5.1 Abstract

Environmental information initiates reproductive development prior to the onset of optimal conditions for raising offspring while other environmental factors regulate the specific timing of reproductive behaviors and the eventual termination of reproduction. The environmental cue responsible for the initiation of seasonal events is photoperiod. Most of birds are highly photoperiodic and gonadal development occurs in response to increasing day length. The endocrine and environmental factors associated with the reproductive cycle of avian species are complex. The integration of the endocrine system, hypothalamic neuropeptides, pituitary hormones, ovarian steroids, environmental photoperiod, ambient temperature, and the presence of egg and chick, regulating the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken. The effects of photoperiod upon reproduction of the native Thai chicken, an equatorial continuous breeder, were investigated in this present study. The study was divided into two experiments. Female native Thai chickens at 22 and 16 weeks of age were used. They were divided into 4 treatment groups with different photoperiodic treatments as control (CT), short day (SD), normal

day (ND), and long day (LD). Chickens were sacrificed after conducting the experiments for 6 and 13 weeks. The ovaries and oviducts were removed and weighed, and the presence of F1-F5 follicles, small yellow follicle (SYF), and small white follicle (SWF) were recorded. The results showed that the ovary and oviduct weights and the presence of F1-F5 follicles, SYF, and SWF in LD group are higher than that of the other groups in both experiments. In addition, the first laying hens were observed and the numbers of laying hen at the end of the experiments were highest in LD treatment group, suggesting that long day length can enhance reproductive efficiency and increase sexual maturity of the native Thai chickens. The findings of this present study suggested that photoperiod might play a role in reproduction of native Thai chicken.

5.2 Introduction

In seasonal breeder birds, the initiation of the breeding cycle depends upon the precise prediction of environmental conditions which are for health of the mating pair and the survivability of their offspring. Environmental information initiates reproductive development prior to the onset of optimal conditions for raising offspring while other environmental factors regulate the specific timing of reproductive behaviors and the eventual termination of reproduction. Most of birds show annual cycles of physiological and behavioral events including specific times for molt, body weight changes, migration behavior, gonadal development, and breeding behavior. The environmental cue responsible for the initiation of seasonal events is photoperiod. In general, increasing or increased photoperiod causes seasonal and unseasonal gonadal development of most of temperate avian species (Burger, 1949; Farner, 1955;

Follett, 1984) and may maintain reproductive system of some species in a continuous state of activity (Nicholls et al., 1988; Chaturvedi et al., 1993).

Most temperate zone animals rely to a high degree on photoperiodic stimuli for controlling reproduction. A change in day length is one of the primary environmental factors regulating seasonal cycles of gonadal growth and regression. The physiological processes underlying the timing of breeding in birds have been studied extensively in temperate zone species (Murton and Westwood, 1977; Follett et al., 1985; Wingfield et al., 1992; Ball, 1993; Cockrem, 1995; Hahn et al., 1997). Photoperiod regulates seasonal processes either directly by initiating or terminating them or indirectly by synchronizing endogenous timing mechanisms (Farner and Lewis, 1971; Follett et al., 1985; Gwinner, 1986; Wilson and Donham, 1988). Photoperiodic information is detected by an extraretinal encephalic photoreceptor while the supplementary information is processed by the ears and eyes (Yokoyama and Farner, 1978; Yokoyama et al., 1978; Glass and Lauber, 1981; Foster et al., 1994; Saldanha et al., 1994; Wada et al., 2000; Li et al., 2004). It has been known that stimuli from the environment profoundly influence reproductive physiology and behavior by changing levels of circulating hormones in the blood (Marshall, 1959; Lehrman, 1961; Murton and Westwood, 1977; Wingfield and Farner, 1980; Follett, 1984). Environmental light stimulates neural (photo) receptors in conjunction with an internal circadian rhythm, enable the bird to respond to the most favorable time for reproduction. At the brain level, a cascade of events is involved in the integration of external environment stimuli and internal body signals. The specialized receptors for the external signals transducer the physical stimuli into the neuronal events, secreting neuromodulators and enzymatic factors regulating the hypothalamic releasing hormones/factors. Finally,

peripheral steroid hormones act feedback in the brain to alter its responsiveness (for review, see: Silver and Ball, 1989).

Birds are highly photoperiodic and gonadal development occurs in response to increasing day length. The birds breeding outside the tropics use seasonal changes in day length to provide predictive information for the optimal time to initiate and terminate breeding (Murton and Westwood, 1977; Sharp, 1996). The control of reproduction is well documented for animals living at temperate, regular seasonal, latitudes (Baker, 1938; Murton and Westwood, 1977; Bronson, 1987; Wingfield and Kenagy, 1991; Ball, 1993; Cockrem, 1995). In contrast, the timing processes of animals inhabiting environments with only slight seasonal fluctuations such as tropics are poorly understood (Gwinner and Dittami, 1985; Bronson, 1987; Dittami and Gwinner, 1990; Wingfield et al., 1992; Hau et al., 1999). Some tropical and sub tropical birds which do not experience variations in annual day length are also reported to use photoperiod as an environmental factor to time their seasonal reproduction and exhibit the phenomena of absolute photorefractoriness such as common myna, red headed bunting, red vented bulbul, and Indian rose finch (Chaturvedi and Thapliyal, 1983; Thapliyal and Gupta, 1989; Rani and Kumar, 2000).

It is still unknown whether these mechanisms are useful in more extreme environments with only slight or variable seasonality. Particularly, seasonal control mechanisms in equatorial habitats, where photoperiodic changes are minimal and poorly understood. Some near-equatorial vertebrate species do show photoperiodic responses when exposed to temperate zone-like day length changes (Gwinner and Dittami, 1985; Bronson, 1987; Hau et al., 1999). Interestingly, some studies suggest a functional relevance for such photic responses in regulating seasonal breeding in near-

equatorial birds (Hau et al., 1998; Gwinner and Scheuerlein, 1999). Seasonal breeding in the near-equatorial habitat is probably made possible by possessing an exceptionally high sensitivity to the slight seasonal changes in photoperiod at this latitude (Hau et al., 1998; Wikelski et al., 2000). A response to photoperiodic cues enables these birds to physiologically prepare for the coming breeding season ahead of time. In addition to photoperiod, equatorial birds are also very responsive to changes in food abundance and adjust gonad growth to food availability (Hau et al., 2000; Wikelski et al., 2000).

The effects of season and photostimulation upon the hypothalamic-pituitary-gonadal axis are well characterized (Dawson and Goldsmith, 1997; Cho et al., 1998; Dunn and Sharp, 1999; Peczely and Kovacs, 2000). Information from photoperiodic time measurement controls seasonal changes in gonadotropins and prolactin (PRL) secretion. In birds, the secretion of luteinizing hormone (LH) and PRL are under the stimulatory control of gonadotropin releasing hormone I (GnRH-I) and vasoactive intestinal peptide (VIP), respectively (Sharp et al., 1998). Photoinduced seasonal changes in GnRH-I and VIP release are likely to be responsible for a pattern of seasonal change in concentrations of LH and PRL found in both long and short day breeders (Sharp and Blache, 2003). Once environmental stimuli are transduced by the appropriate receptors, they influence the secretion of GnRH and VIP. Cell bodies containing GnRH-I occur in the preoptic-anterior hypothalamus, while VIP cell bodies known to control PRL secretion occur in the basal hypothalamus. Terminals containing GnRH-I or VIP are abundant in the avian median eminence (ME), consistent with their functions in the regulation of gonadotropins and PRL secretion (Saldanha et al., 2001; Teruyama and Beck, 2001). Recently, it has been reported hypothalamic GnRH mRNA expression was significantly increased after subjecting

the non-photostimulated female turkey to a 90 minute light period at Zeitgeber time (ZT) 14. The lowest levels of GnRH mRNA were observed in photorefractory hens. These results indicate that hypothalamic GnRH mRNA expression may be used to precisely characterize the different reproductive stages (Kang et al., 2006).

In general, animals need to adjust reproductive decisions to environmental seasonality. Numerous studies in the 1950's and 1960's were conducted to investigate the effect of day length manipulation during the starting, growing, and holding periods on the reproductive performance of breeding hens. From all these studies, it was agreed that the day length had to be restricted to 6-9 hours of light per day prior to application of a gonadal-stimulatory light (14-16 hours/day). Little is known in tropical birds regarding the environmental cues that stimulate reproductive activity and physiological mechanisms regulating reproduction. In deed, the lighting conditions to which photoperiodic birds are exposed when they begin or terminate breeding differ widely between avian species. Subtropical and temperate zone birds use a biological clock for photoperiodic time measurement that is integrated with the reproductive neuroendocrine system in different ways to generate species-specific breeding patterns.

The jungle fowl, the ancestor of the native Thai chicken, originated in the tropical region of Southeast Asia, where its breeding season would has been timed by both photoperiodic and non-photoperiodic factors, allowing the chick to hatch at a time of year when food was most abundant. Most of birds are highly photoperiodic and gonadal development occurs in response to increasing day length. The endocrine and environmental factors associated with the reproductive cycle of avian species are complex. The integration of the endocrine system, hypothalamic neuropeptides,

pituitary hormones, ovarian steroids, environmental photoperiod, ambient temperature, and the presence of egg and chick, regulating the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken. Photoperiod studies on equatorial birds such as native Thai chicken are very limited. The effects of lighting regimens (photoperiod) upon growth, reproductive development, laying performance, and reproductive efficiency in native Thai chickens have been reported (Chotesangasa et al., 1992; Chotesangasa and Gongruttananun, 1994; 1995; 1997; Choprakarn et al., 1998). However, the results of these studies are contradictive and far from understood. Up to date, in order to increase reproductive efficiency by the lighting regimens is still not successful. The objective of this study was to investigate the effects of photoperiod upon the reproductive system of the native Thai chicken, an equatorial non-photoperiodic continuous breeder. The findings from this study will provide the information that help to understand the environmental factor(s) influencing its reproductive activity. The information can be then applied to increase reproductive efficiency and egg production of native Thai chicken in Thailand.

5.3 Materials and Methods

5.3.1 Experimental Animals

120 female and 8 male native Thai chickens, Pradoohangdam breed, were used. They were reared and housed in floor pens under different photoperiodic treatments. Food and water were constantly available. Each hen was identified by wing band number. Chickens were randomly divided into floor pens (15 females:1 male/pen). Each pen was provided with nests. The chickens were observed their behaviors in order to classify them into 4 reproductive stages as non-laying (NL), laying (L),

incubating (broodiness: B), and rearing chicks (R). The criteria for the reproductive stages classification were: 1) NL: the chickens that had not reached the sexual maturity and did not lay or express incubation and maternal behaviors, 2) L: the hens that laid regularly, 3) B: the hens that exhibited persistent nesting activity, no egg production, and aggressive nest protection behavior, and 4) R: the hens that stopped nesting and laying eggs, and reared the chicks after hatching. Daily records of egg production, nesting activity, and other behaviors were recorded throughout the experiments. The animals were treated in accordance with Suranaree University of Technology Animal Care and Use Committee Guidelines.

5.3.2 Experimental Design

5.3.2.1 Experiment I

To determine the roles of photoperiod upon the reproductive system emphasizing the laying performance of the native Thai chickens, 60 female native Thai chickens at 22 weeks of age and 4 mature roosters were used. The chickens were randomly divided into 4 treatment groups (15 females:1 male/pen) as the followings:

1) Control (CT): chickens were housed in floor pen under the natural light (approximately 12 hours of light and 12 hours of dark; 12L:12D, Fig. 5.1).

2) Short day (SD): chickens were housed in floor pen under the lighting regimen of 8L:16D.

3) Normal day (ND): chickens were housed in floor pen under the lighting regimen of 12L:12D.

4) Long day (LD): chickens were housed in floor pen under the lighting regimen of 16L:8D.

The chickens were reared in separate rooms under four different photoperiodic treatments for 6 weeks. Each pen was provided with nests. Chickens were observed their behaviors everyday in order to classify them into 4 reproductive stages: NL, L, B, and R. During the experiment, body weights of chickens were recorded weekly. At the end of the experiment, chickens were euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). After chickens were sacrificed, ovaries and oviducts were removed and weighed. The presence of F1-F5 follicles, small yellow follicle (SYF), and small white follicle (SWF) were recorded. The criteria that used to classify the follicles were revised from Etches (1993). The ovary of laying hen contains a hierarchy of yellow yolky follicles with the diameter longer than 1 cm, identified as F1, F2, F3, F4, and F5 and several smaller follicles from which the large yolky follicles are recruited. The small follicles are classified according to their diameters as SYF (5-10 mm) and SWF (1-4 mm).

5.3.2.2 Experiment II

To determine the roles of photoperiod upon the sexual maturity of the native Thai chickens, 60 female native Thai chickens at 16 weeks of age and 4 mature roosters were used. The chickens were randomly divided into 4 treatment groups (15 females:1 male/pen) as described in Experiment I (CT, SD, ND, and LD).

The chickens were reared in separate rooms under four different photoperiodic treatments for 13 weeks. Each pen was provided with nests. Chickens were observed their behaviors everyday in order to classify them into 4 reproductive stages: NL, L, B, and R. During the experiment, body weights of chickens were recorded weekly. At the end of the experiment, chickens were sacrificed as described above. Ovaries and

oviducts were removed and weighed. The presence of F1-F5 follicles, SYF, and SWF were recorded as aforementioned.

5.3.3 Statistical Analysis

Differences in ovary and oviduct weights among 4 photoperiodic treatments (treatment groups) were analyzed. Results were expressed as mean ± SEM. Significant differences of mean were statistically analyzed using one way analysis of variance (ANOVA). Significance differences among treatment groups were computed utilizing Tukey's HSD test. Differences were considered as statistically significant if a P value was less than 0.05. All statistical analyses were performed using SPSS Windows Software (SPSS Windows Software, version 13.0, SPSS Inc., Chicago, IL, USA).

5.4 Results

5.4.1 Experiment I

The ovaries and oviducts were removed and weighed after sacrifice all chickens at the end of the experiment. The ovary of the laying native Thai chicken was shown (Fig. 5.2). Means and SEM of ovary and oviduct weights in each treatment group (n=15) were shown in Fig. 5.3 and Table 5.1 The results showed that there was no significant different of the ovary and oviduct weights (g) among treatment groups (ovary weight; CT: 6.51±2.96, SD: 4.56±2.78, ND: 11.80±4.28, and LD: 12.67±3.95 and oviduct weight; CT: 13.62±4.29, SD: 7.20±2.91, ND: 15.63±5.14, and LD: 18.39±5.27). The number of laying hens at the end of the experiment, and the presence of F1-F5 follicles, SYF, and SWF were shown in Table 5.2. The results showed that the number of laying hen and the presence of F1-F5 follicles, SYF, and SWF were

found the highest in LD, intermediate in ND and CT, and the least in SD. The first laying hen was presented in the second week of in the chickens subjected to LD treatment group. At the end of the experiment, the number of laying hens was higher in LD (5 hens) than that of in ND, SD, and CT (2, 1, and 1 hens) as shown in Fig. 5.4. The body weight of all chickens was recorded weekly and showed that the body weight of SD group tended to be higher than other groups (Fig. 5.5).

5.4.2 Experiment II

The ovaries and oviducts were removed and weighed after sacrifice all chickens at the end of the experiment. Means and SEM of ovary and oviduct weights in each treatment group (n=15) were shown in Fig. 5.6 and Table 5.3. The results showed that there was no significant different of the ovary and oviduct weights (g) among treatment groups (ovary weight; CT: 22.44±4.81, SD: 21.84±7.20, ND: 12.09±4.28, and LD: 23.10±6.73 and oviduct weight; CT: 25.30±5.08, SD: 25.38±4.91, ND: 15.13±4.70, and LD: 26.41±5.68). The number of laying hens at the end of the experiment and the presence of F1-F5 follicles, SYF, and SWF were shown in Table 5.4. The results showed that the number of laying hens and the presence of F1-F5 follicles, SYF, and SWF tended to be higher in LD and CT than that of in SD and ND. Daily record of egg production showed that after subjecting the chickens to the experiment for 8 weeks, the first laying hens were presented in CT and LD treatment groups. At the end of the experiment, the number of laying hens was higher in CT (7 hens) than that of in LD, SD, and ND (6, 5, and 3 hens, respectively) as shown in Fig. 5.7. The data showed that the body weight of SD group tended to be higher than other groups (Fig. 5.8).

Fig. 5.1 The photoperiod of Nakhon Ratchasima province across the year 2006 (Hydrographic Department, 2006).

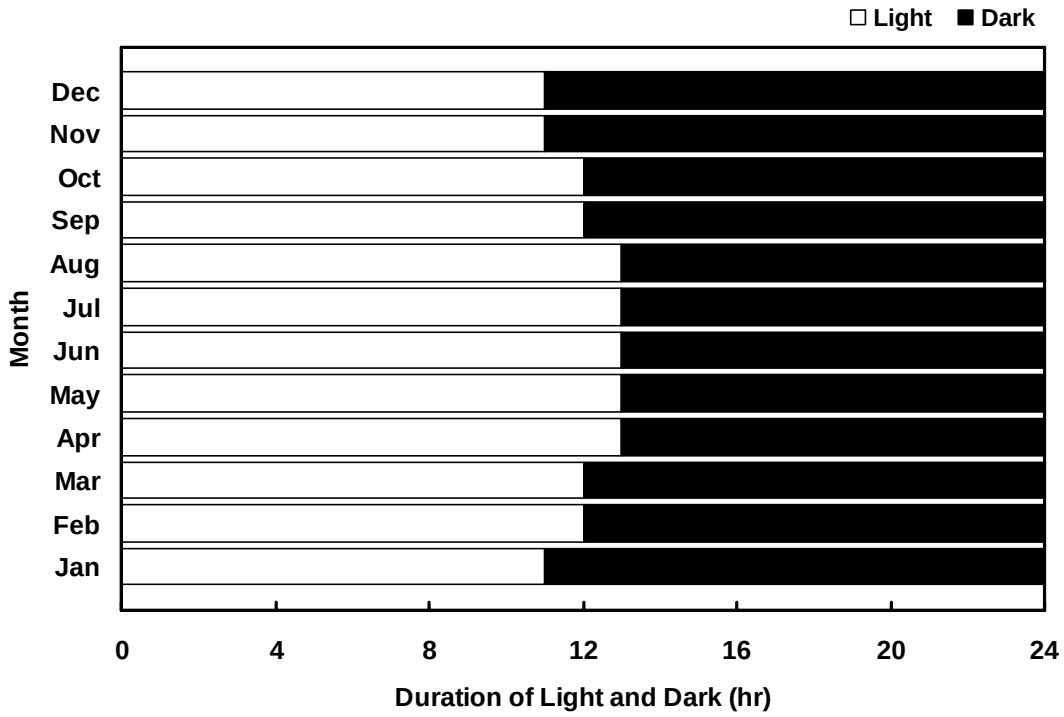


Fig. 5.2 The ovary of the laying native Thai hen shows the presence of F1-F5 follicles, small yellow follicles (SYF), small white follicles (SWF), and post-ovulatory follicles (POF).

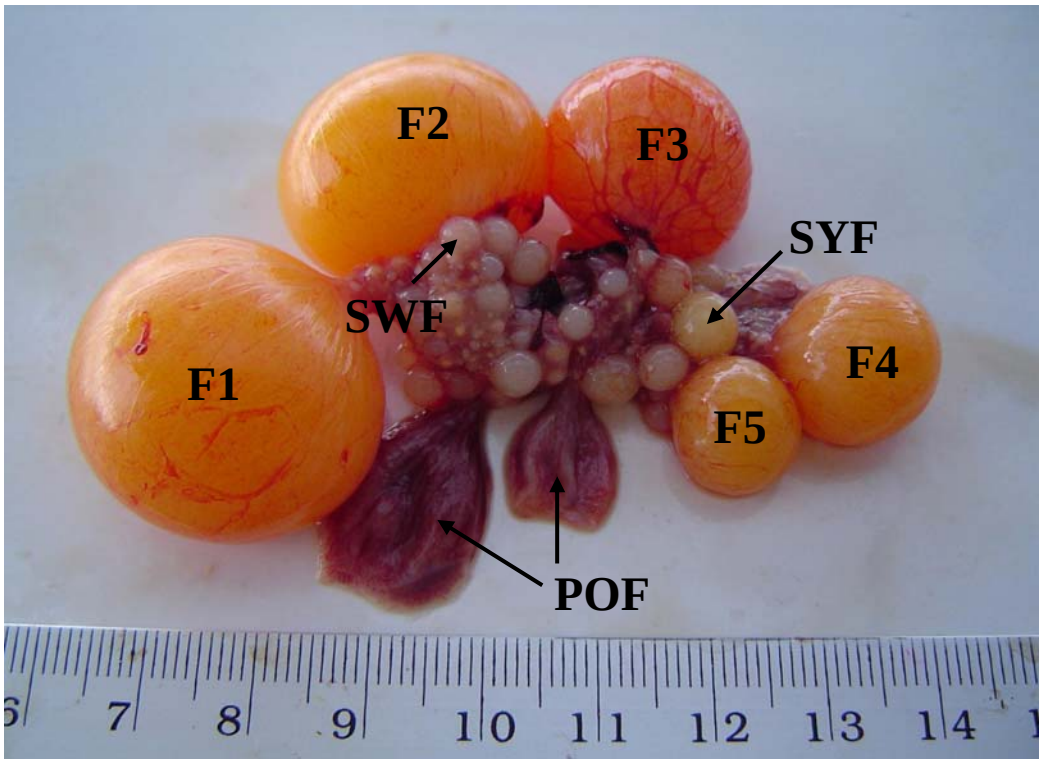


Fig. 5.3 Ovary and oviduct weights of the native Thai chickens in each treatment group of Experiment I (n=15). Values (Mean ± SEM) with different letters are significantly different (P<0.05).

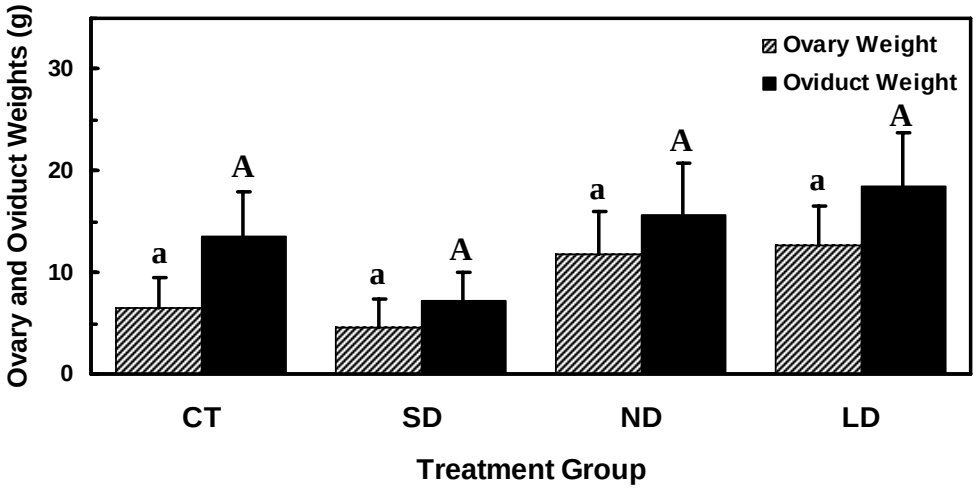


Table 5.1 Ovary and oviduct weights of the native Thai chickens in each treatment group of Experiment I (n=15). Values (Mean ± SEM) with different letters are significantly different (P<0.05).

Treatment Group	Ovary Weight (g)	Oviduct Weight (g)
CT	6.51 ± 2.96^{a}	13.62 ± 4.29^{A}
SD	4.56 ± 2.78^{a}	7.20 ± 2.91^{A}
ND	11.80 ± 4.28^{a}	15.63 ± 5.14^{A}
LD	12.67 ± 3.95^{a}	18.39 ± 5.27^{A}

Table 5.2 Percentages of the number of laying hen, the presence of F1-F5 follicles, and the presence of SYF and SWF of the native Thai chickens in each treatment group of Experiment I.

Treatment Group	No. of Laying Hen (%)	The Presence of F1-F5 Follicles (%)	The Presence of SYF and SWF (%)
CT	6.67	40.00	40.00
SD	7.14	7.14	28.57
ND	13.33	40.00	40.00
LD	38.46	46.15	53.85

Fig 5.4 The number of cumulative laying hens in each treatment group of Experiment I.

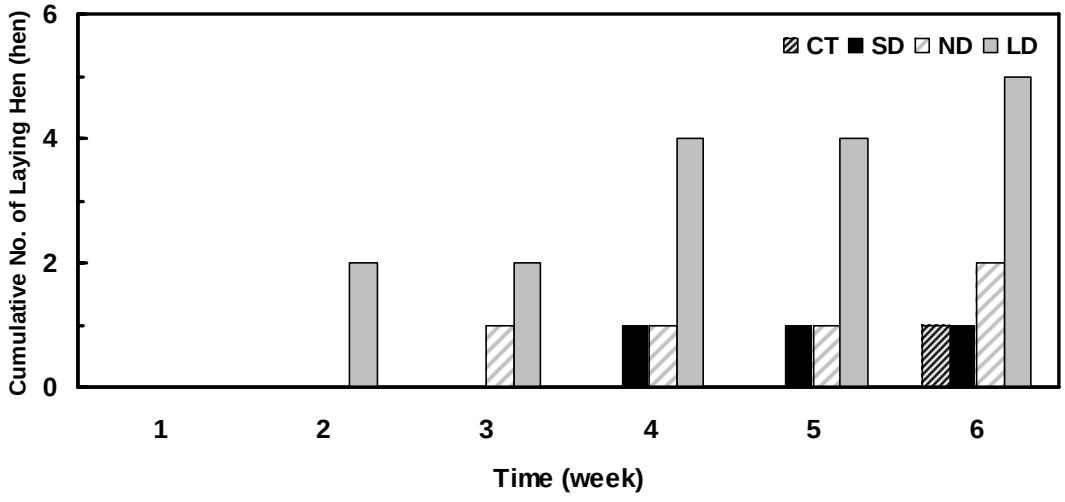


Fig. 5.5 Body weights (Mean ± SEM) of the native Thai chickens in each treatment group of Experiment I.

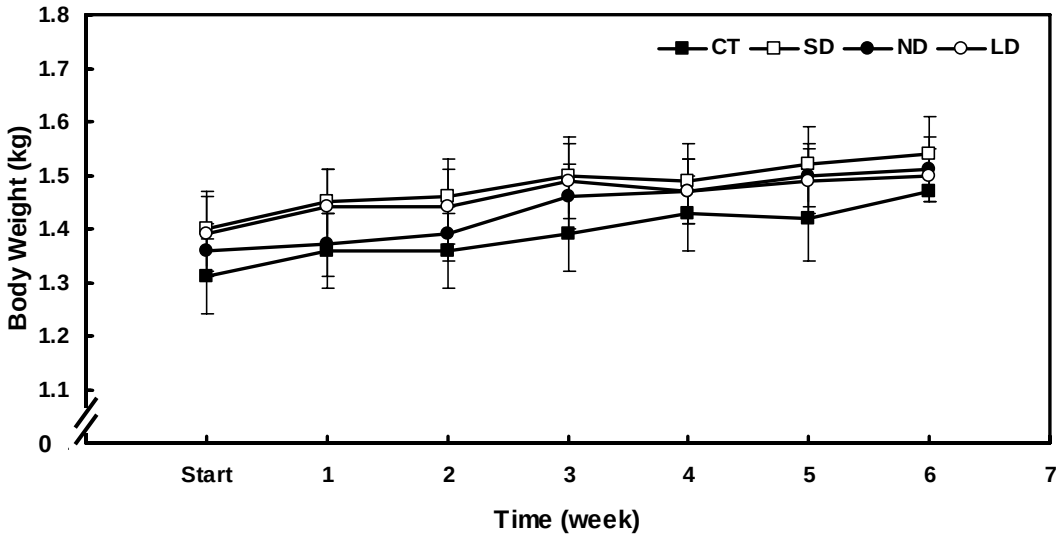


Fig. 5.6 Ovary and oviduct weights of the native Thai chickens in each treatment group of Experiment II (n=15). Values (Mean ± SEM) with different letters are significantly different (P<0.05).

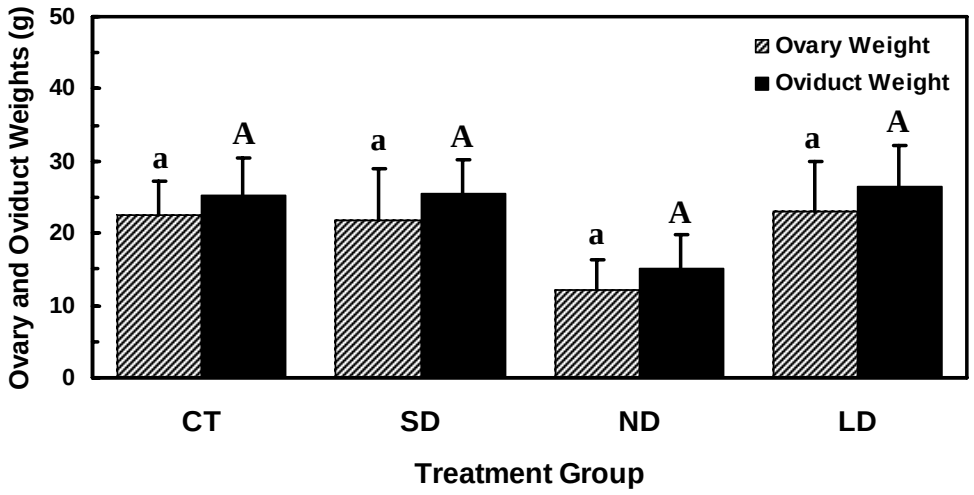


Table 5.3 Ovary and oviduct weights of the native Thai chickens in each treatment group of Experiment II (n=15). Values (Mean ± SEM) with different letters are significantly different (P<0.05).

Treatment Group	Ovary Weight (g)	Oviduct Weight (g)
CT	22.44 ± 4.81^{a}	25.30 ± 5.08^{A}
SD	21.84 ± 7.20^{a}	25.38 ± 4.91^{A}
ND	12.09 ± 4.28^{a}	15.13 ± 4.70^{A}
LD	23.10 ± 6.73^{a}	26.41 ± 5.68^{A}

Table 5.4 Percentages of the number of laying hen, the presence of F1-F5 follicles, and the presence of SYF and SWF of the native Thai chickens in each treatment group of Experiment II.

Treatment Group	No. of Laying Hen (%)	The Presence of F1-F5 Follicles (%)	The Presence of SYF and SWF (%)
CT	58.85	61.54	69.23
SD	41.67	50.00	58.33
ND	21.43	35.71	35.71
LD	50.00	66.67	66.67

Fig. 5.7 The number of cumulative laying hens in each treatment group of Experiment II.

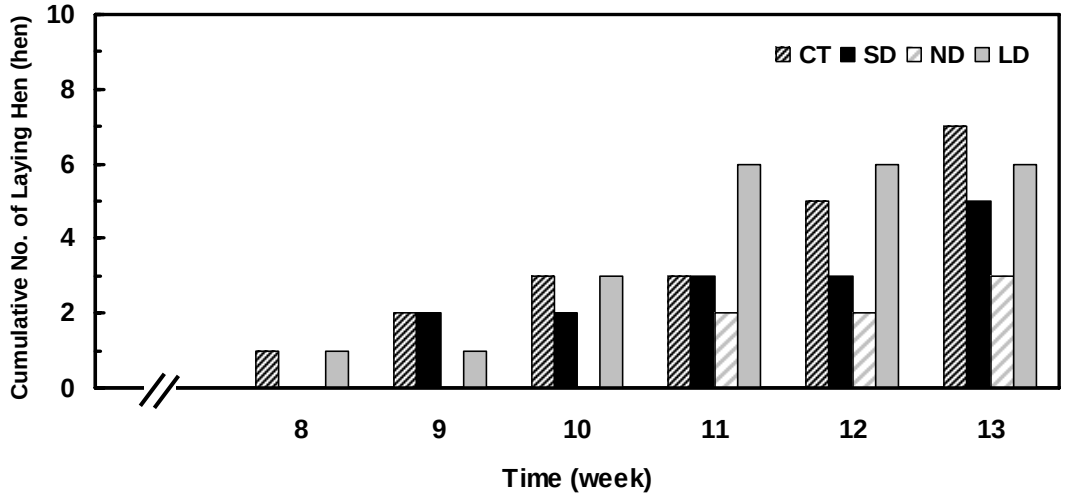
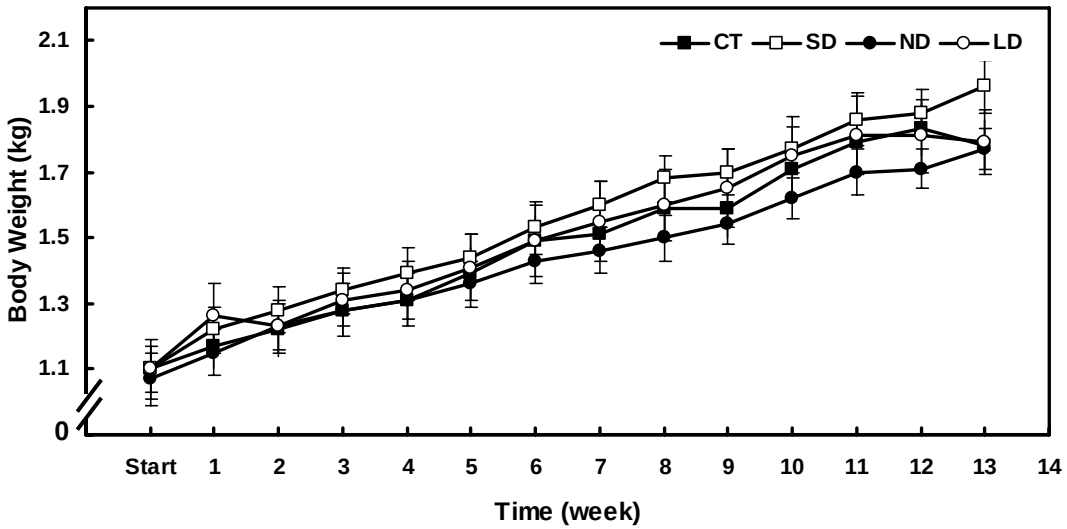


Fig. 5.8 Body weights (Mean ± SEM) of the native Thai chickens in each treatment group of Experiment II.



5.5 Discussion

The effects of photoperiod upon the laying performance and the sexual maturity of the native Thai chickens were determined by subjecting chickens into four lighting regimens as CT, SD, ND, and LD treatment groups. The results showed that ovary and oviduct weights in LD treatment group were higher than that of the other groups, suggesting that long day length can enhance reproductive efficiency of the native Thai chickens. The findings of this present study suggested that photoperiod might play a role in reproduction of native Thai chicken.

The presence of F1-F5 follicles, SYF, and SWF implicated the development of the reproductive system (Etches, 1993). The results showed that the presence of F1-F5 follicles, SYF, and SWF were found the highest in LD group, indicating that the gonads of the chickens in LD treatment group tended to develop earlier than that of the other groups. In agreement with the gonad recrudescence, the first laying hens were observed in LD treatment group in both experiments and the number of laying hens at the end of the experiments was found the highest in LD group. Taken together, the results were in good agreement with previous studies (Chaturvedi and Thapliyal, 1983; Thapliyal and Gupta, 1989; Rani and Kumar, 2000) and suggested the roles of photoperiod upon the reproduction of native Thai chicken. Long day length may, in parts, play a role on the reproduction of the native Thai chicken by enhance the sexual maturity and the reproductive system of the native Thai chickens. However, at this present time, it is not clearly to state that the photoperiod regulates the reproduction of native Thai chicken since the development of the gonad was still observed in the SD group.

In general, birds are highly photoperiodic and gonadal development occurs in response to increasing day length. The birds breeding outside the tropics use seasonal changes in day length to provide predictive information for the optimal time to initiate and terminate breeding (Murton and Westwood, 1977; Sharp, 1996). Various species of Indian weaver bird (Thapliyal and Tewary, 1964; Chakravorty et al., 1985) and quail (Anthony, 1970; Follett, 1984) remain continuously in breeding condition under constant long days but gonads regress if they are shifted to relatively short days which are different from the equatorial birds, the native Thai chicken. The gonads of the native Thai chickens still can develop even after subjecting them to short day length and the laying hens were observed in both experiments. In most of temperate avian species, increasing or increased photoperiod causes seasonal and unseasonal gonadal development (Burger, 1949; Farner, 1955; Follett, 1984) and may maintain reproductive system of some species in a continuous state of activity (Nicholls et al., 1988; Chaturvedi et al., 1993). Some tropical and sub tropical avian species which do not experience variations in annual day length are also reported to use photoperiod as an environmental factor to time their seasonal reproduction such as common myna, red headed bunting, red vented bulbul, and Indian rose finch (Chaturvedi and Thapliyal, 1983; Thapliyal and Gupta, 1989; Rani and Kumar, 2000). Photoperiod might not be the main environmental component that is responsible for timing the initiation or termination of gonad recrudescence of native Thai chickens, long day length can enhance the reproductive efficiency of native Thai chickens.

Weekly record of body weight showed that the body weight of SD group showed a trend to be higher than that of the other groups during the experiments. This data suggested that photoperiod may have effects on the growth rate of native Thai

chickens. Daily observation showed that SD chickens had less activity than that of the other groups, especially at the dark period, resulting in less energy used and high body weight. These data suggested that photoperiod might play a role in growth rate by affecting behaviors of the chickens.

Interestingly, the reproductive performance of chickens in the control group of the first study that were raised under natural light was lower than that of the other groups. This experiment was conducted in summer, resulting in the significantly higher ($P<0.05$) of ambient temperature compare to the treatment groups that were raised under the close system (CT: 31.64±0.46°C, treatment groups: 29.19±0.34°C). In addition, the body weight of the CT group was also lower than that of the treatment groups. The decline in the rate of egg production at high environmental temperature is well recognized but the physiological basis is far from clear. Reduced food consumption may account for some of the impairment in reproduction, however, the effect of high environmental temperatures on the rate of egg laying appear largely unrelated to food intake (Donoghue et al., 1990; Servatius et al., 2001). The regulatory mechanism(s) for the reduced reproductive efficiency in the heat-stressed hens appear to be modulated in part at the level of the hypothalamus and the anterior pituitary. Changes in reproductive hormone release and gene expression represent the final sequence in the neuroendocrine pathway that leads to the diminished reproductive performance associated with stress. The impaired reproductive performance in heat-stressed poultry may, in part, be related to increased PRL secretion (El Halawani et al., 1984; Donoghue et al., 1989). It is important to point out that the effects of high temperature on PRL levels are independent of the stage of the reproductive cycle, since high temperature stress increases PRL secretion in ovariectomized birds (Gahali

et al., 2001). Taken together, ambient temperature also plays the important role on the reproductive system and the growth rate of the native Thai chickens.

It has been known that stimuli from the environment profoundly influence reproductive physiology and behavior. Critical environmental stimuli include sensory information concerning photoperiod, ambient temperature, rain fall, and the presence of egg and the offspring. These external stimuli as well as the prevailing internal steroid milieu are important in initiating and maintaining the reproductive cycle of avian species. Therefore, if year-around production of eggs for hatching to be achieved, it is obvious that lighting programs and environmental temperature and their interaction must be considered and further investigations of the effects of photoperiod upon the reproductive system of native Thai chickens are needed. In addition, the effects of other environmental conditions such as ambient temperature, rain fall, the presence of male, nest, egg, and the offspring should be further investigated. The information can be then applied to increase reproductive efficiency and egg production of the native Thai chicken in Thailand.

In conclusion, the lighting conditions to which photoperiodic birds are exposed when they begin or terminate breeding differ widely between avian species. Unlike temperate zone birds, the reproductive system of the native Thai chicken, an equatorial bird, is not absolutely controlled by photoperiod. However, the reproductive performance can be enhanced by increasing long day length. The result from this study is clearly demonstrated that photoperiod influences the reproductive efficiency of the native Thai chickens.

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