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NEUROENDOCRINE REGULATION OF REARING BEHAVIOR IN THE NATIVE THAI HEN

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

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NEUROENDOCRINE REGULATION OF REARING BEHAVIOR

IN THE NATIVE THAI HEN

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ไก่พื้นเมืองไทยมีพฤติกรรมความเป็นแม่ที่ถูกถ่ายทอดจากต้นตระกูลไก่ป่าในเขตศูนย์สูตร พฤติกรรมความเป็นแม่ประกอบด้วย การนั่งฟักไข่ การกกลูกไก่ การหาอาหาร การเลี้ยงดู และปกป้องลูกไก่ การแสดงออกของพฤติกรรมความเป็นแม่ถูกควบคุมโดยระบบประสาทและระบบต่อมไร้ท่อ พฤติกรรมความเป็นแม่ส่งผลให้แม่ไก่พื้นเมืองด้อยประสิทธิภาพในการผลิตไข่ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษากลไกการควบคุมพฤติกรรมความเป็นแม่โดยระบบประสาทและระบบต่อมไร้ท่อของไก่พื้นเมืองไทยโดยเปรียบเทียบการเปลี่ยนแปลงจำนวนเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์และเซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนในแม่ไก่เลี้ยงลูกและแม่ไก่ที่ถูกพรากลูกหลังการฟักเพื่อขัดขวางการแสดงออกของพฤติกรรมความเป็นแม่ โดยใช้เทคนิคอิมมูโนฮิสโตเคมิสทรี รวมถึงตรวจสอบระดับฮอร์โมนโปรแลคตินในพลาสมาด้วยเทคนิคเอนไซม์ลิงค์อิมมูโนซอร์เบนท์แอสเสย์ ผลการศึกษาพบว่าเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์บริเวณนิวเคลียสอินเฟอริโอริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไมในวันที่ลูกไก่ฟักมีจำนวนมากและคงระดับต่อเนื่องเป็นระยะเวลาหนึ่งสัปดาห์ก่อนจะลดจำนวนลงอย่างรวดเร็ว การเปรียบเทียบจำนวนเซลล์ที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ระหว่างไก่เลี้ยงลูกและไก่ไม่เลี้ยงลูกพบว่าจำนวนเซลล์ประสาทในไก่เลี้ยงลูกมีมากกว่าไก่ไม่เลี้ยงลูกตลอดระยะเวลาสามสัปดาห์ของการทดลอง จำนวนเซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนบริเวณนิวเคลียสคอมมิสซูรีพาลลิไอในไก่ไม่เลี้ยงลูกมีจำนวนมากกว่าไก่เลี้ยงลูกอย่างมีนัยสำคัญสิบเจ็ดวันหลังการฟัก ระดับฮอร์โมนโปรแลคตินในพลาสมาของไก่เลี้ยงลูกลดลงอย่างต่อเนื่องในช่วงสัปดาห์แรกหลังการฟักไข่และคงอยู่ในระดับต่ำตลอดช่วงระยะเวลาการเลี้ยงลูก การเปรียบเทียบระดับฮอร์โมนโปรแลคตินในพลาสมาของไก่เลี้ยงลูกและไก่ไม่เลี้ยงลูกพบว่า ฮอร์โมนโปรแลคตินในไก่เลี้ยงลูกมีระดับสูงกว่าไก่ไม่เลี้ยงลูกอย่างชัดเจนตลอดระยะเวลาห้าสัปดาห์หลังการฟักไข่ การขัดขวางพฤติกรรมความเป็นแม่โดยการพรากลูกจากแม่ไก่ ส่งผลให้น้ำหนักของรังไข่และท่อนำไข่ จำนวนฟอลลิเคิลในรังไข่เพิ่มขึ้น และพบจำนวนไก่ที่กลับมาไข่รอบใหม่ ผลการศึกษาบ่งชี้ว่าการปรากฏของลูกไก่และปฏิสัมพันธ์ระหว่างแม่ไก่และลูกมีส่วนในการรักษาระดับฮอร์โมนโปรแลคตินให้คงอยู่ในระดับสูงหลังการฟักของลูกไก่และอาจส่งเสริม

หรือรักษาการดำรงอยู่ของพฤติกรรมการเลี้ยงลูกในไก่พื้นเมืองไทย การพรากลูกไก่ออกจากแม่ไก่ทำให้ระดับฮอร์โมนโปรแลคตินลดลง ซึ่งเป็นไปในทิศทางเดียวกันกับการลดลงของจำนวนเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์บริเวณนิวเคลียสอินเฟอริโอริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไม และพร้อมด้วยการเพิ่มขึ้นของจำนวนเซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนบริเวณนิวเคลียสคอมมิสซูรีพาลลิไอ แสดงให้เห็นถึงความเชื่อมโยงกันระหว่างระบบประสาทและระบบต่อมไร้ท่อซึ่งเป็นปัจจัยสำคัญที่ควบคุมระบบสืบพันธุ์และพฤติกรรมการเลี้ยงลูกของไก่พื้นเมืองไทย

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษา_____

ORN-ANONG CHAIYACHET : NEUROENDOCRINE REGULATION OF REARING BEHAVIOR IN THE NATIVE THAI HEN.
THESIS ADVISOR : ASSOC. PROF. YUPAPORN CHAISEHA, Ph.D.
304 PP.

GONADOTROPIN RELEASING HORMONE/NATIVE THAI CHICKEN/ PROLACTIN/REARING BEHAVIOR/VASOACTIVE INTESTINAL PEPTIDE

Native Thai chickens highly express maternal behaviors including incubation behavior and broodiness or rearing behavior. The expression of such behavior is a costly problem, resulting in substantial loss of potential egg production. Neuroendocrine regulation of rearing behavior in the native Thai hen was investigated. Changes in the numbers of vasoactive intestinal peptide-immunoreactive (VIP-ir), and gonadotropin releasing hormone-I-immunoreactive (GnRH-I-ir) neurons were compared in the brain of rearing hens (R) and non-rearing hens (NR) using immunohistochemistry. Plasma prolactin (PRL) levels were determined by enzyme-linked immunosorbent assay. Changes in the numbers of VIP-ir neurons in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) were high after the day of the chicks were hatched until 7 days of rearing and sharply decreased from day 10 to day 21. When the chicks were removed from the hens, VIP-ir neurons counted were markedly decreased on day 4 and continued to be lower than those of the R hens through day 21. The number of GnRH-I-ir neurons in the nucleus commissurae pallii (nCPa) was significantly increased by day 17 after removal of chicks from the hens compared to those of the R hens. Plasma PRL concentrations

remained at high levels on the day the chicks were hatched, then rapidly decreased after the first week of hatching and remained at low levels throughout the eight weeks of the rearing period. During the five weeks of the rearing period, the levels of plasma PRL were markedly decreased in hens that had their chicks removed and reached the lowest levels by the third week of separation from chicks. In the R hens, plasma PRL levels were higher than those of the NR hens during the five weeks of the observation period. Disruption of rearing behavior by removing chicks from the hens induced ovarian and oviduct recrudescence and the initiation of a new laying cycle. The present study indicated an association between VIP neurons in the IH-IN, GnRH-I neurons in the nCPa, plasma PRL levels, and rearing behavior. The relationship between hens and chicks while hens took care of their young might maintain circulating PRL levels, which correlated with the gradual decline in the number of VIP-ir neurons in the IH-IN which, in turn, facilitates/stimulates and/or maintains rearing behavior in the native Thai chickens. Taken together, the present findings indicate that disruption of rearing behavior by removing the chicks from the hens markedly decreases plasma PRL levels, a parallel decline in the number of VIP-ir neurons in the IH-IN, and an accompanying increase in the number of GnRH-I-ir neurons in the nCPa, suggesting that the VIPergic system in the IH-IN and the GnRH system in the nCPa may be involved in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of rearing behavior in the native Thai chickens.

School of Biology

Academic Year 2012

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CHAPTER I

INTRODUCTION

1.1 Rational of the Study

The native Thai chicken (*Gallus domesticus*) belongs to genus Gallus of the family Phasianidae. It is a small domestic animal that probably originated from the red jungle fowl found wildly distributed throughout Southeast Asia and is domesticated approximately 3,000 years ago. They have been raised in the countryside of Thailand for many generations and the main objectives for raising native Thai chickens are for consumption, sport competition, and recreation. It is not only a main protein food source, but it also can be sold for supplemental income for families. Its meat is firm in texture and contains high proteins as well as low fat and cholesterol contents, resulting in high demand by consumers who prefer low fat and antibiotic-free white meat. This provides a good opportunity for producing them in industrial scale. Recently, the native Thai chicken has become a new economic domestic animal of Thailand with presently growing demand and relatively high price. The market price of the native Thai chickens is two to three times higher than those of imported broilers. To date, there are about 76 millions native Thai chickens in Thailand, which are raised by 2.7 millions farmers. This exported goods gains income about 2.2 million baht per year.

The native Thai chickens can be raised under poor environmental conditions in the backyard with local feeds, minimum care and management. Furthermore, recent

Thai government policies encourage the development and the use of natural resources in supporting of His Majesty the King's concept for self-sufficiency in agriculture. The farmers focus on "mixed farming" that is a strategy for helping rural farmers to increase self-sufficiency. The native Thai chicken is one of the significant resources that needs to be developed. However, the native Thai chickens have low productivity. The reproductive performance of native chicken is much lower than that of cross breeds and hybrids, especially egg laying performance. One of the main causes of low reproductive performance in the native Thai chickens is the incidence of maternal behaviors such as incubating, brooding or rearing behaviors. The establishment of maternal behaviors affects egg production, because it terminates egg laying. These cause a problem for producing native Thai chickens commercially in the poultry industry in Thailand. Presently, market demands for the native Thai chickens cannot be met by supply, mainly because of their low egg laying performance. They tend to lay eggs in clutches rather than evenly distributed over the year, leading to irregular production of the chicks.

The native Thai chicken was domesticated without genetic selection and always expresses high maternal behaviors, heritable traits from their ancestors. Maternal behaviors are hormonal dependent, initiated with the onset of incubation behavior, and continue through the period when the young are taking care of by the mother (broody/rearing behavior). These behaviors constrain the number of eggs produced. The maternal behaviors are defined as the behaviors of the mothers that contribute to the survival of their offspring. Most mothers display the maternal behaviors after parturition and serve the immediate provision of care and defense for their young. In birds, the maternal behaviors are composed of incubating eggs and

brooding or rearing of the young. One or both parents must incubate their eggs until hatching and then provide post-hatching care.

There are several lines of evidence indicating that neurotransmitters, neurohormones, and hormones play an important role in the maternal behaviors of avian species. The parental hormone, prolactin (PRL), an anterior pituitary hormone, has been shown to be associated with the reproductive cycle in several avian species such as turkeys, quails, bantams, ring doves, pigeons, mallard ducks, and native Thai chickens. PRL has been implicated as a causative factor in the onset and maintenance of maternal behaviors. Rearing behavior is maintained by high levels of PRL and upon hatching. This pituitary hormone is widely thought to play a role in maternal behaviors by mediating incubation behavior, crop milk production and secretion, feeding of the young, and nest defense. In temperate zone birds, it is very well documented that PRL is under stimulatory control by hypothalamic vasoactive intestinal peptide (VIP), the avian PRL-releasing factor. Some evidence indicates that the dopaminergic (DAergic) system plays an intermediary role in PRL secretion. In many avian species, the onset and maintenance of maternal behaviors is correlated with low levels of gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), and luteinizing hormone (LH) and high levels of circulating PRL.

Recently, the presence of hypothalamic VIP-immunoreactive (-ir), tyrosine hydroxylase (TH)-ir (as a marker for DA), and GnRH-I-ir neurons at different reproductive stages and throughout the incubation period have been reported in the native Thai chickens. Changes in the number of VIP-ir, TH-ir, and GnRH-I-ir neurons in the native Thai chickens are observed across the reproductive stages and during incubation and nest deprivation period, which is correlated directly with variations in

plasma PRL levels. VIP-ir neurons and fibers are extensively distributed throughout the brain of the native Thai chickens and are predominantly expressed in the diencephalon, where VIP-ir neurons are highly concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in numbers of VIP-ir neurons within the IH-IN are directly correlated with changing of plasma PRL levels throughout the reproductive cycle, suggesting that VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity of the native Thai chickens. Further studies revealed that an association exists between DA neurons in the nucleus intramedialis (nI) and the regulation of the reproductive system in the native Thai chickens, suggesting that the differential expression of DA neurons in the nI might play a role in the control of VIP secretion and subsequent PRL release in the native Thai chickens. Moreover, it has been further demonstrated that changes in the number of VIP-ir neurons in the IH-IN are associated with DAergic neurons within the nI and nucleus mamillaris areas, resulting in PRL release to induce and maintain incubation behavior in the native Thai hens. It is further suggested that nesting activity stimulates PRL secretion by the activation of the DAergic system, which in turn stimulates the VIPergic system. These elevated PRL levels increase nesting activity and maintain incubation behavior.

In several avian species, PRL secretion has been shown to be stimulated by the presence of chicks. This hormone is indicated to be correlated with parental care, and is known to decline steadily immediately after hatching in precocial bird species or drop at the end of the rearing period in altricial bird species. In the native Thai hens, circulating PRL levels decrease sharply during rearing stage, but changes in plasma LH levels are not observed. This decline in plasma PRL levels corresponds with a

decrease in the numbers of VIP-ir neurons in hens rearing chicks. GnRH-I, the primary hypophysiotrophic factor involved in avian reproductive regulation, has been investigated in the native Thai chickens, revealing that the number of GnRH-I-ir neurons decline to the lowest level during rearing stage, and plasma LH levels are fluctuated and essentially the same levels throughout the reproductive cycle. The distributions of the DA-ir neurons in the hypothalamic areas of the native Thai chickens have been elucidated. The differential expression of DA-ir neurons in the nI are observed across the reproductive cycle and the number of DA-ir neurons decrease slightly during the transition from incubating to rearing stage, whereas plasma PRL levels decline dramatically to the same level of non-egg laying in hens that rearing chicks.

As aforementioned, the expression of maternal behaviors including brooding and rearing behaviors is a costly problem, resulting in substantial loss of potential egg production. Some evidence suggests that plasma PRL levels also play a role in terminating egg laying and regulating clutch size in species that lay clutches of more than two eggs. Cessation of egg laying is associated with increased PRL concentrations in the turkey and domestic fowl. Obviously, the reproductive efficiency of the native Thai chickens is low in comparison to those of the imported breeds. Thus, in order to increase the production of native Thai chickens in Thailand, it is very important to understand the neuroendocrine regulation of the maternal behaviors. To date, it has been well established that incubation behavior in this species is regulated by the VIP/PRL, GnRH/FSH-LH, and DAergic systems. However, little is known about the relationship between these neuroendocrine systems and rearing behavior in this species. Therefore, this dissertation was carried out to

investigate the neuroendocrine regulation of rearing behavior in the native Thai chickens. The results gained from this study will provide an insight into the neuroendocrine mechanism(s) underlying the regulation of rearing behavior in the native Thai chickens. The findings gained from this dissertation will provide, for the first time, the information of neuroendocrine regulation of rearing behavior in the native Thai chickens, which could help to improve the productivity of the native Thai chickens in Thailand.

1.2 Research Objectives

1.2.1 To study the changes in plasma PRL levels in the regulation of rearing behavior in the female native Thai chickens.

1.2.2 To study the differential expression of VIP that is associated with the neuroendocrine regulation of rearing behavior in the female native Thai chickens.

1.2.3 To study the differential expression of cGnRH-I that is associated with the neuroendocrine regulation of rearing behavior in the female native Thai chickens.

CHAPTER II

LITERATURE REVIEW

2.1 Native Thai Chicken

The native Thai chicken or Thai indigenous chicken (*Gallus domesticus*), belongs to genus Gallus of the family Phasianidae, originated from the wild jungle fowl. It has been suggested that red jungle fowl (*Gallus gallus*) might be the ancestor of all domestic chickens which is found wildly distributed throughout Southeast Asia (Austic and Nesheim, 1990; Fumuhito et al., 1994; Hillel et al., 2003; Sawai et al., 2010). Approximately, it was domesticated by village people 3,000 years ago. Some characteristics of native Thai chickens inherited from the ancestry and still expressed are maternal behaviors such as incubation and rearing behaviors (Beissinger et al., 1998). Historically, native Thai chickens have been in the countryside of Thailand, and the main objectives of raising native Thai chickens are for consumption, sport competition, and recreation. Indeed, it is not only a main protein food source, but it can be also sold for additional income for families. Generally, the native Thai chickens are easy to raise, resistant to diseases, and acclimatized to the local environments. However, the native Thai chicken has a slower growth rate than that of the imported commercial broiler when raised under the same conditions, but it can be raised with lower production costs by raising it as free range using organic local feed. It has been reported that high performance breeds lose their advantages over native Thai chickens in terms of weight gain when treated with local feeds (Leotarakul and

Pimkamlia, 1999). Moreover, the native Thai chicken is well adapted to the poor condition of small farm or simple rural environment. Its resistance to diseases and tolerance to heat stress are considerably higher than those of high performance or hybrid breeds, resulting in high potential for raising native Thai chickens in rural areas (Kajaroen et al., 1989). The native Thai chickens can adapt to high temperature, and imported broilers are less tolerant to the high temperature than those of Thai indigenous chickens crossbred and Thai indigenous chickens (Aengwanich, 2008).

In Thailand, to date, there are about 76 millions native Thai chickens or 24 % of total chicken production, which are broilers 55 %, layers 15 %, commercial broiler breeders 5 %, and commercial layer breeders 1 % (Department of Livestock Development, 2011). Native Thai chickens provide higher meat quality with less fat and low cholesterol than those of imported commercial broilers, resulting in high demand by consumers (Wattanachant et al., 2004; Jaturasitha et al., 2008; Teltathum and Mekchay, 2009). The textural characteristics of the indigenous chicken meat are similar to spent hen meat but are much different from imported broiler meat (Wattanachant et al., 2004; Chuaynukool et al., 2007). The indigenous chicken muscles contain higher protein and collagen contents but lower fat content than those of broiler muscles (Wattanachant et al., 2004; Wattanachant, 2008). In addition, the shear values of indigenous chicken muscles either raw or cooked are higher than those of broiler muscles (Wattanachant et al., 2004; 2005; Jaturasitha et al., 2008). The comparison between two indigenous chicken strains, black-boned and native Thai chickens with two imported, Bresse, and Rhode Island Red (Rhodes, a layer breed) found that the imported breeds are heavier at slaughter and have higher contents of fat and cholesterol than those of indigenous strains (Jaturasitha et al., 2008). Thus, there

are many factors such as genotype, rearing system, feed, age, muscle pH, chemical composition, microstructure of muscle, postmortem aging, and processing methods can influence on the quality of indigenous chicken meat (Chotesangasa and Gongruttananun, 1999a; Jaturasitha et al., 2002; Wattanachant et al., 2005; Wattanachant, 2008). The firm and low fat meat, free of drug residues such as antibiotics, makes consumers prefer these meat types (Choprakarn et al., 2000). This advantage of native Thai chicken meat leads to a higher price, about two or three times higher than those of the imported commercial broilers in Thailand, Hong Kong, China, and Japan (Chotesangasa and Gongruttananun, 1999a; Jaturasitha et al., 2008). It has been further suggested that the native Thai chickens, especially Pradu Hang Daum breed, is suitable to be developed as a meat type chicken due to its lower genetic distance to broiler strains (Dorji et al., 2011).

The production of native Thai chickens is suited to the small farm raising system, but improving the supply of chicks for fattening needs to be developed (Haitook et al., 2003). The reproductive performance of native Thai chickens is much lower than those of cross breeds and hybrids, especially egg laying performance which is critical to secure a sufficient number of chicks for fattening (Chotesangasa et al., 1994b). In commercial systems, hatchability is not the problem for producing the chicks, if the number of eggs per hen is not limited. Normally, the native Thai hen lays eggs 3-4 times per year, 4-17 eggs per clutch rather than laying eggs continuously all year long. The hen-day egg production of the native Thai hen is lower than that of the commercial laying hen at all times, with the peak production for native Thai hens and commercial laying hens being 38.0 % and 75.5 %, respectively (Chotesangasa et al., 1994b). The total number of eggs per hen of native Thai hen is

between 30-92 eggs per year, which is significantly lower than that of 243 eggs/hen/year of the imported commercial hen (Chotesangasa et al., 1994b). With a hatching rate of 80-85 %, a typical hen produces 25-40 chicks annually (Klinhom et al., 2005). The low potential in egg production of the native Thai chickens causes the problem in order to be produced commercially in poultry industry in Thailand. The main cause of low egg production and short egg laying period in the native Thai chickens is the expression of the maternal behaviors (incubation and rearing behaviors). These behaviors are high during egg laying, nesting, and brooding, which are not desired for commercial scale production (Choprakarn and Wongpichet, 2007). Generally, the native Thai chicken takes about 2 weeks for laying, 3 weeks for hatching, and 6-10 weeks for taking care of the chicks. Thus, the hen spends 10-15 weeks for each reproductive cycle (Katawatin et al., 1997; Choprakarn et al., 1998). In addition, growth rate of the native Thai chicken is significantly slower than that of the imported chicken, taking about 4-5 months to reach marketable size with a 80-85 % carcass (Choprakarn and Wongpichet, 2007). Therefore, improving the efficiency of native Thai chicken production would benefit the poultry industry in Thailand.

2.2 Neuroendocrine Regulation of the Avian Reproductive Cycle

The regulation of the avian reproductive system involves the interaction of external stimuli with neuroendocrine mechanisms. The avian reproductive system is regulated by the integration of the hypothalamus, the pituitary, and the gonads. This system is referred to as the hypothalamo-pituitary-gonadal (HPG) axis. It is very well documented that neurotransmitters, neurohormones, neuromodulators, and hormones of the HPG axis play an important role in the reproductive cycle of avian species. This

HPG axis involves two major neuroendocrine systems controlling avian reproduction. These systems include the gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH), and vasoactive intestinal peptide/prolactin (VIP/PRL) systems and both systems are influenced by dopaminergic (DAergic) neurotransmission (Bhatt et al., 2003; Chaiseha et al., 2003; Chaiseha and El Halawani, 2005). In addition, in temperate zone birds, both neuroendocrine systems depend on the 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 regulating these GnRH/FSH-LH and VIP/PRL systems is formed by a system of peptidergic neurons whose axons terminate around portal capillaries in the external layer of the median eminence (eminentia mediana, ME; Chaiseha and El Halawani, 2005). GnRH stimulates pituitary gonadotrophs to secrete gonadotropins, FSH and LH, which in turn are responsible for ovarian follicular growth and ovulation at the period of egg laying. In contrast, at the period of egg incubation, VIP stimulates pituitary lactotrophs to synthesize PRL, stimulates PRL secretion, and then regression of the gonads. In addition, GnRH and VIP can directly affect the gonads via the appropriate gonadal receptors as well (Asem and Novero, 1993; Johnson, 2000; Sun et al., 2001).

It has been studied and well documented that FSH, LH, and PRL are associated with the reproductive cycle in several avian species (turkeys: Mashaly et al., 1976; El Halawani et al., 1984b; 2001; Wong et al., 1992b; mallards: Bluhm et al., 1983a; canvasback ducks: Bluhm et al., 1983b; cockatiels: Myers et al., 1989; emperor penguins: Lormee et al., 1999; king penguins: Mauget et al., 1994; tropical seabirds:

Lormee et al., 2000; geese: Boos et al., 2007; Huang et al., 2008; native Thai chickens: Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). During reproductively quiescent stages (non-egg laying and rearing stages) of the native Thai chickens and turkeys, plasma PRL levels are very low (El Halawani et al., 1984b; 1997; Karatzas et al., 1997; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). At the onset of incubation, circulating progesterone and LH levels begin to rise continuously and reach a peak amount at about 8 to 2 hours before ovulation (Mashaly et al., 1976). Plasma levels of FSH are low throughout the ovulatory cycle, but a significant decrease in FSH concentrations occurs right before the preovulatory LH surge and a significant increase occurs during 3 hours prior to oviposition as plasma LH levels decrease (Krishnan et al., 1993). Subsequently, circulating LH levels continue to drop during the incubating period (Myers et al., 1989). In contrast, during the periods of laying and incubating, circulating PRL levels increase dramatically (El Halawani et al., 1984b; Kosonsiriluk et al., 2008).

It is well documented that PRL is a causative factor for the reduced circulating gonadotropin levels and ovarian regression, when birds shift from egg laying to incubation behavior in bantam hens, canaries, domestic chickens, cowbirds, ducks, mallard ducks, native Thai chickens, pheasants, pigeons, ring doves, spotted sandpipers, turkeys, white-crowned sparrows, and wild starlings (Sharp et al., 1977; Burke and Dennison, 1980; Goldsmith and Hall, 1980; Goldsmith et al., 1981; 1984; Dawson and Goldsmith, 1982; Bluhm et al., 1983a; El Halawani et al., 1984b; 1997; Oring et al., 1986; Hiatt et al., 1987; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). It has been suggested that PRL acts centrally to reduce LH concentrations by reducing GnRH concentrations in the hypothalamus (Rozenboim et al., 1993b), and

the abundance of LH-β subunit and PRL mRNAs expression shows an inverse relationship in photostimulated/laying and incubating turkey hens (Wong et al., 1992b). Ovine PRL administration suppresses the photo- and ovariectomy-induced increases in LH secretion and delays the onset of egg laying and induces incubation behavior in laying hens (El Halawani et al., 1991). Changes in LH and PRL concentrations during the avian reproductive cycle are well established (Follett, 1984; El Halawani et al., 1988b). Plasma PRL and LH levels are low in reproductively quiescent birds, while the levels are increased in reproductively active laying hens. During the incubating stage, circulating PRL levels are sharply elevated (El Halawani et al., 1984b; Sharp et al., 1989; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008), while plasma LH levels are gradually suppressed (Lea et al., 1981; El Halawani and Rozenboim, 1993). Abundant evidence indicates that an increased in PRL secretion is the causative factor for the reduced circulating gonadotropins and has been observed in several avian species. For example, in galliform birds, the onset of incubation behavior is associated with declining levels of gonadotropins and ovarian steroids (Sharp et al., 1979; Burke and Dennison, 1980; Bedrak et al., 1981; Lea et al., 1981) and a dramatic rise in circulating PRL levels (Goldsmith, 1985; 1991; Lea, 1987; El Halawani et al., 1988b; Sharp et al., 1988), and it is this rising PRL levels which has been implicated as the cause of cessation of ovulation, ovarian regression, induction and maintenance of incubation behavior. Subsequently, circulating PRL levels decline, whereas circulating LH levels begin to rise when incubation behavior terminates (El Halawani et al., 1988b; Knapp et al., 1988) and as soon as molting is ended (Bluhm et al., 1983a; 1983b; Mauget et al., 1994). It has been further suggested that high concentrations of PRL inhibit LH secretion (Zadworny and Etches, 1987). Studies *in*

vitro demonstrate the antigonadotropic action of PRL by reducing gonadotropic actions of LH and FSH (Taira and Beck, 2006). Further studies have reported that the breeding season terminates after circulating of PRL levels increase above a critical threshold to depress GnRH neuronal and LH activities (Sharp and Blache, 2003). Therefore, seasonal reproductive activity is inhibited by increasing circulating PRL levels, which in turn suppresses LH secretion, inhibits follicular development, then finally terminates egg laying (Huang et al., 2008). Furthermore, administration of mammalian PRL into laying turkeys causes ovarian regression (Opel and Proudman, 1980; Hargis et al., 1987), and inhibits the exogenous gonadotropin-stimulated secretion of ovarian steroids (Camper and Burke, 1977). Moreover, it has been indicated that immunization against PRL slows down ovarian follicular development in large white follicles into small yellow follicles and reduces hen egg laying performance (Li et al., 2011).

2.2.1 Gonadotropin Releasing Hormone/Follicle Stimulating Hormone-Luteinizing Hormone System

It is very well established that pituitary gonadotropins (FSH and LH) secretion is controlled by the central nervous system (CNS) 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). In both birds and mammals, when environmental stimuli are transduced by specific receptors, they influence the synthesis and secretion of hypothalamic GnRH. GnRH release occurs episodically from the mammalian hypothalamus. The frequency and amplitude of GnRH release determine the pattern of gonadotropins secretion

(Levine and Ramirez, 1982; Moenter et al., 1992). Like in mammals, GnRH is synthesized by hypothalamic neurosecretory cells, released from the ME into the hypophysial portal vessels, and then transported to the pituitary gland, where it stimulates the secretion of gonadotropins in birds. GnRH increases LH and FSH secretions of the anterior pituitary cells both *in vitro* and *in vivo* (Millar et al., 1986; Peczely, 1989). *In vivo* studies reveal that injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH levels in hens (Guemene and Williams, 1999; Proudman et al., 2006). Incubation of turkey anterior pituitary cells with GnRH results in an increase in LH-β-subunit mRNA expression and stimulates LH secretion (You et al., 1995a). A pulsatile pattern of GnRH release is observed from the medial basal hypothalamus and the preoptic area (POA) *in vitro* (Li et al., 1994). In contrast, GnRH inhibits FSH-stimulated steroidogenesis in chickens but enhances LH-stimulated progesterone production (Hertelendy et al., 1982). GnRH agonists may imitate the native hormone and induce an endogenous LH surge (Shalev and Leung, 2003). GnRH-I does not affect circulating FSH levels, but stimulates LH secretion when administrated to 3 weeks old cockerels (Krishnan et al., 1993). Changes in pituitary responsiveness to GnRH are negatively correlated with changes in circulating LH levels (Balthazart et al., 1980). In addition, it has been suggested that adrenergic stimulation at the hypothalamic level can release GnRH and subsequently increase gonadotropins secretion (Yu et al., 1991).

In birds, the egg laying period is associated with relatively high levels of circulating FSH, LH, and gonadal steroids levels and is regulated by hypothalamic GnRH (El Halawani et al., 1988b). GnRH-I is the primary hypophysiotropic factor stimulating the release of LH, since active immunization against GnRH-I causes a

decline in plasma LH levels and complete regression of the reproductive system (Sharp et al., 1990). However, seasonal changes in the GnRH-II-immunoreactive (-ir) neurons are noted, indicating an involvement of GnRH-II in the control of avian reproduction (Teruyama and Beck, 2000; Stevenson and MacDougall-Shackleton, 2005). As mentioned above, it has been reported that GnRH neuronal activity is regulated by photoperiod (Sharp and Blache, 2003). Photostimulatory inputs to GnRH neurons have the potential to increase GnRH mRNA transcription and its secretion (Dunn and Sharp, 1999) as well as increase the pituitary cells sensitivity to GnRH (Davies and Follett, 1975). The amount of hypothalamic GnRH contents increases during long day stimulation and decreases during photorefractoriness in many avian species (Dawson et al., 1985; Foster et al., 1987; Bluhm et al., 1991; Rozenboim et al., 1993a; Saldanha et al., 1994; Hahn and Ball, 1995; Dunn et al., 1996; Kang et al., 2006). In addition, gonadal steroids, hypothalamic VIP, DA, and gonadotropin inhibitory hormone (GnIH) are thought to be involved in the regulation of GnRH secretion (Ramirez et al., 1984; Sharp et al., 1984; Deviche et al., 2000; Tsutsui et al., 2000). Moreover, active VIP immunoneutralization increases pituitary contents of LH-β and FSH-β mRNAs and is accompanied by a decline in PRL mRNA expression (Ahn et al., 2001). Thus, GnRH plays a pivotal role in the control of avian reproduction, and changes in hypothalamic GnRH contents are observed during the avian reproductive cycle. GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples are higher in laying hens than those of non-laying hens (Advis et al., 1985). In turkeys, temperate zone birds, it has been reported that GnRH-I mRNA is abundance within the nucleus commissurae pallii (nCPa), organum vasculosum lamina terminalis (OVLT), and nucleus septalis lateralis (SL), and is greater in laying

hens than those of non-photostimulated and incubating hens, while lesser GnRH-I mRNA expression is observed in photorefractory hens (Kang et al., 2006).

2.2.2 Vasoactive Intestinal Peptide/Prolactin System

The regulation of PRL secretion in avian species involves the interaction of external stimuli with neuroendocrine mechanisms. Critical environmental stimuli include sensory information concerning photoperiod, ambient temperature, and the presence of eggs and offspring. These external stimuli and 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 (Curlewis, 1992). It is very well established that the regulation of avian PRL secretion and its gene expression are influenced by hypothalamic VIP, the avian PRL-releasing factor (PRF; El Halawani et al., 1997; 2001; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005; Kosonsiriluk et al., 2008). It has been established for a long time that PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968) and that the principal PRF is VIP (El Halawani et al., 1997; 2001), which is secreted from neurons located in the infundibular nuclear complex (INF) of the caudo-medial hypothalamus (Sharp et al., 1989; El Halawani et al., 1997; 2001; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005). It has been very well established that VIP is associated with the reproductive cycle in birds (Chaiseha and El Halawani, 1999). To date, VIP is very well accepted as the avian PRF because it meets the classical criteria for defining it as the hypophysiotrophic PRF in birds (El Halawani et al., 1997; 2001).

Variations in VIP immunoreactivity, VIP mRNA steady-state levels occurring within the hypothalamus, and VIP content in the ME as well as of VIP levels in hypophysial portal blood are correlated with changes in the circulating PRL levels throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999). 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). In contrast with mammals, it has been established that DAergic system influences are involved in both stimulating and inhibiting avian PRL secretion, depending on multiple subtypes of DA receptors (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003). 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). Dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate PRL secretion along a pathway expressing κ opioid, 5-HTergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (El Halawani et al., 2001; 2004). In birds, VIP acts directly on the anterior pituitary gland to stimulate PRL secretion during the reproductive cycle (Lea and Vowles, 1986; Macnamee et al., 1986; Proudman and Opel, 1988; El Halawani et al., 1990c; 1997; Kosonsiriluk et al., 2008). Immunocytochemical studies have shown that hypothalamic VIP-ir neurons in the INF and VIP-ir fibers in the ME correspond to the enhanced circulating PRL levels in turkeys and native Thai chickens (Mauro et al., 1989; Kosonsiriluk et al., 2008). Other studies have also shown increases in the number and cell size of VIP-ir neurons within this region in the domesticated pigeons and ring doves during the periods of

elevated circulating PRL levels (Peczely and Kiss, 1988; Cloues et al., 1990). Changes in pituitary VIP receptor mRNA expression are also observed across the reproductive stage in the turkeys. Increased VIP receptor mRNA expression in the pituitary gland is observed in the turkey hens with normal (laying) or high PRL secretion (incubating), while much less VIP receptor mRNA expression is observed in the pituitary gland of hypoprolactinemic non-photostimulated and photorefractory turkey hens (Chaiseha et al., 2004). This suggests that the VIP receptors located in the INF are involved in PRL secretion and indicates that PRL secretion is principally regulated by VIP receptors at the pituitary level (Chaiseha et al., 2004). In response to long day photoperiod, VIP/PRL secretion is increased gradually and progressively and both their release and gene expression are up-regulated in turkey hens (Wong et al., 1991; El Halawani et al., 1996; Tong et al., 1997; Chaiseha et al., 1998). Activation of the GnRH/FSH-LH system in photosensitive female turkeys initiates the reproductive activity. When gonadotropins stimulate estrogen secretion and induce sexual receptivity, they also prime the VIP/PRL system to enhance PRL secretion (Wineland and Wentworth, 1975; El Halawani et al., 1983; 1986).

2.3 Neuroendocrine Regulation of the Reproductive Cycle in the Native Thai Chicken

In contrast to the temperate zone seasonal breeding species, the native Thai chicken is a continuously breeding species found in the equatorial zone that produces eggs all year, independent of photoperiodic cues (Konsonsiriluk, 2007; Sartsoongnoen, 2007; Kosonsiriluk et al., 2008). The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying (NL), egg laying

(L), incubating eggs (INC), and rearing chicks (R; Figure 2.1; Kosonsiriluk, 2007). The native Thai hens highly express maternal behaviors including incubation behavior and broodiness (Prakobsaeng et al., 2011). It has been reported that progesterone and PRL plasma levels are related to the reproductive cycle of the native Thai chickens (Katawatin et al., 1997; Sangkaew, 1999; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). Circulating levels of progesterone and estradiol are higher in hens that have hen-day egg production recorded more than 80 % than those of hens with their egg production recorded below 25 % and the hen which laid no egg or non-layer (Chotesangasa et al., 1994a). However, plasma LH levels do not change across the reproductive stage (Kosonsiriluk et al., 2007). The secretion pattern of circulating PRL levels of the native Thai chickens have been determined across the reproductive cycle, revealing that plasma PRL levels are found to be low in NL, gradually elevated in L, and reached the highest levels in INC, and then quickly dropped to a low level again in R hens, equaling the level of NL birds (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008).

The presence of hypothalamic VIP-ir, tyrosine hydroxylase (TH)-ir (as a marker for DA), and GnRH-I-ir neurons at different reproductive stage and throughout the incubation period have been reported in the native Thai chickens. Changes in the number of hypothalamic VIP-ir, TH-ir, and GnRH-I-ir neurons in the native Thai chickens are observed across the reproductive cycle and during incubation and nest deprivation period, which is correlated directly with variations in plasma PRL levels (Sartsoongnoen et al., 2006; 2008; 2012; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011). VIP-ir neurons and fibers are extensively distributed throughout the brain of the native Thai chickens and are predominantly expressed in

the diencephalon, where VIP-ir neurons are concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in numbers of VIP-ir neurons within the IH-IN are directly correlated with changing plasma PRL levels throughout the reproductive cycle, suggesting that VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity of the native Thai chickens (Kosonsiriluk, 2007; Kosonsiriluk et al., 2008). Further studies reveal an association between DA neurons in the nucleus intramedialis (nI) and the regulation of the reproductive system in the native Thai chickens, suggesting that the differential expression of DA neurons in the nI might play a role in the control of VIP secretion and subsequent PRL release in the native Thai chicken, a tropical non-seasonally breeding avian species (Sartsoongnoen et al., 2008). Moreover, it has been further demonstrated that changes in the numbers of VIP-ir neurons in the IH-IN are associated with DAergic neurons within the nI and nucleus mamillaris (ML) areas, resulting in PRL release to induce and maintain incubation behavior in the native Thai hens. It is further suggested that nesting activity stimulates PRL secretion through activation of the DAergic system, which in turn stimulates VIPergic system. The elevated PRL levels increase nesting activity and maintain incubation behavior (Prakobsaeng et al., 2011).

To date, findings demonstrate the distributions and changes in the number of GnRH-I-ir neurons are observed across the reproductive cycle and during incubation and nest deprivation period of the native Thai chickens. The GnRH-I-ir neurons are distributed in a discrete region lying close to the third ventricle (V III) from the level of POA through the nucleus anterior medialis hypothalami (AM) with the greatest number found within the nCPa. Changes in the number of GnRH-I-ir neurons within

the nCPa are correlated with the reproductive cycle of the native Thai chickens. The number of GnRH-I-ir neurons in the nCPa is highest in laying hens when compared with those in the other reproductive stages. Moreover, nest deprivation causes an increase in the number of GnRH-I-ir neurons in the nCPa of nest-deprived hens when compared with incubating hens, suggesting that GnRH-I expression is correlated with the reproductive stage of native Thai chickens and may be, in part, regulated by it. These findings confirm a pivotal role of GnRH-I in controlling avian reproduction of this non-seasonal breeding, equatorial species (Prakobsaeng et al., 2009; Sartsoongnoen et al., 2012).

Immunohistochemical studies of the native Thai chickens suggest that the expression of VIP neurons in the IH-IN following hatching of the young may, in part, account for the difference in reproductive neuroendocrine response of this bird (Kosonsiriluk et al., 2008). It has been further demonstrated that the increase of GnRH-ir neurons caused by nest deprivation is related to the DAergic system (Sartsoongnoen et al., 2012). DA-ir neurons in the nI increase significantly during the incubating stage (Sartsoongnoen et al., 2008), and nest deprivation results in decreased DA-ir neurons in this area, paralleling decreased VIP-ir neurons in the IH-IN (Prakobsaeng et al., 2011). These data well support an association of the neuronal interactions between GnRH-Iergic, VIPergic, and DAergic systems in the regulation of reproductive activity in the native Thai chickens.

The effects of photoperiod on growth, carcass quality, reproductive development, laying performance, and reproductive efficiency have been documented (Chotesangasa and Santipong, 1994; Chotesangasa and Gongruttananun, 1996a; 1996b; 1999a; 1999b; Kosonsiriluk, 2007; Sartsoongnoen, 2007). Egg production is

higher in native Thai chickens raised under short day photoperiod (8L : 16D) and long day photoperiod (15L : 9D) lighting regimen during growing and laying periods, respectively (Chotesangasa and Santipong, 1994). Moreover, hens raised under a long day photoperiod (16L : 8D) show higher ovary and oviduct weights and numbers of ovarian hierarchical follicles than those of the other groups (Kosonsiriluk, 2007).

Therefore, in order to increase the production of native Thai chickens in Thailand, growth and reproductive performances need to be improved. As mentioned above, it is very important to understand the basic neuroendocrinology influencing its reproductive activities. To date, there are limited data regarding the neuroendocrine regulation of rearing behavior in this non-temperate zone gallinaceous bird. Recently, it has been well established that incubation behavior in this species is regulated by the VIP/PRL and the DAergic systems (Sartsoongnoen et al., 2006; 2008; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011). However, little is known about the relationship between these neuroendocrine systems and rearing behavior in the native Thai hens.

Figure 2.1 The reproductive cycle of the native Thai chickens; non-egg laying (NL), egg laying (L), incubating eggs (INC), and rearing chicks (R; Kosonsiriluk, 2007).

2.4 Prolactin: Structure, Function, and Regulation of Secretion

2.4.1 The Structure of Prolactin

PRL, a polypeptide hormone, was discovered by Riddle and co-workers (1931; 1932). Its name was based on findings that an extract of bovine pituitary gland caused the growth of crop sac and stimulated the elaboration of crop milk in pigeons or promoted lactation in rabbits (Riddle et al., 1933; Bern and Nicoll, 1968). It is synthesized in and secreted from the specialized cells, the lactotrophs, of the anterior pituitary gland (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000).

Molecular weight (MW) of the major form of PRL found in the pituitary gland is about 23 kDa, and it is encoded by a gene consisting of 5 exons and 4 introns (Cooke et al., 1981; Truong et al., 1984). Variants of PRL have been characterized in many mammals. Its variants can be the result of alternative splicing of the primary transcript, proteolytic cleavage, phosphorylation, glycosylation, and other posttranslational modifications, thereby altering its physiological functions (Sinha, 1995). PRL is synthesized as a preprohormone consisting of 227 amino acids in most mammals (Miller and Eberhardt, 1983). The mature hormone contains 194 to 199 amino acids, depending on species. Hormone structure is stabilized by three intramolecular disulfide bonds. The primary structure of PRL was first reported in the ovine (Li et al., 1970). The complete amino acid sequences of PRLs of more than 25 species have been identified (for review, see Sinha, 1995). A comparison of the amino acid sequence from different species shows varying degrees of sequence homology, reflecting to a great extent the order of the phylogenetic relationships. Some 32 amino acids seem to be conserved among different species (Watahiki et al., 1989). The homology of sequences of PRLs among different species and their primary structures are shown in Figures 2.2 and 2.3, respectively.

PRL belongs to the families of related hormones including growth hormone (GH) and placental lactogen (PL). Its amino acid sequence is similar to those of GH and PL sharing genomic, structure, and biological features (Boulay and Paul, 1992; Horseman and Yu-Lee, 1994). Genes encoding PRL, GH, and PL are evolved from a common ancestral gene by gene duplication (Niall et al., 1971) about 500 million years ago. Among the avian species, it has been suggested that the mechanisms, which regulate its gene expression may be wildly conserved in this species (Kansaku

et al., 2005; Hiyama et al., 2009b). However, it has been demonstrated that PRL is also synthesized by a number of extra-pituitary cells/tissues in both mammals (Ben-Jonathan et al., 1996; Freeman et al., 2000; Soares, 2004) and birds (Berghmam et al., 1992; Ramesh et al., 2000; Chaiseha et al., 2012), but its physiological function(s) in these extra-pituitary tissues is poorly understood and needed to be further elucidated.

PRL is synthesized and secreted by a broad range of other cells including most prominently various immune cells, mammary epithelium, placenta, the deciduas of the pregnant uterus, and brain (Ben-Jonathan et al., 1996). In addition, PRL synthesis is also found in the lacrimal gland, adrenal gland, corpus luteum, prostate gland, testis, and pancreas (Ben-Jonathan et al., 1996; Freeman et al., 2000). To date, over 300 different physiological functions of PRL have been documented (Houdebine, 1983; Bole-Feysot et al., 1998; Harris et al., 2004) 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. Furthermore, it has been suggested that functions and biological activities of PRL are, at least in part, regulated by additionally post-translational modifications such as phosphorylation in the various physiological stages (Hiyama et al., 2009a).

PRL receptor (PRLR), a single membrane-bound protein transmembrane receptor, is a member of the Class I cytokine receptor superfamily that includes the receptors for GH, leptin, erythropoietin, and interleukins (Bazan, 1989; 1990; Kelly et al., 1991). PRL, PL, and primate GH bind the PRLR. PRL and GH receptors share some structural and functional features despite their low sequence homology (30 %;

Goffin and Kelly, 1996). The PRLR is activated by the binding of a single ligand to the receptor to dimerize two identical receptor subunits, leading to activation of Jak2-kinase associated with the cytoplasmic domain, which subsequently activates a number of signalling cascades through which PRL exerts its physiological effects (for review, see Bole-Feysot et al., 1998; Freeman et al., 2000). Jak2 phosphorylates tyrosine residues on different target proteins, the best identified is named signal transducers and activators of transcription (Stats). The Jak2-Stat cascade is the major signalling pathway of the PRLR, but other signal transducing pathways are also involved in signal transduction by this receptor as well. Activation of mitogen-activated protein kinases pathway has been reported in different cellular systems under PRL stimulation (Bole-Feysot et al., 1998). In addition, activation of the nucleotide exchange protein Vav has been reported (Clevenger et al., 1995).

Numerous PRLR isoforms have been identified in different tissues in both mammals and birds (Davis and Linzer, 1989; Ali et al., 1991; Lesueur et al., 1991; Pitts et al., 2000). Alternative splicing of the PRLR gene results in the multiple isoforms which differ in the length and composition of their cytoplasmic tails and are referred to as the short (291aa; Boutin et al., 1988) and long (591aa; Shirota et al., 1990) PRLR isoforms (Harris et al., 2004). These isoforms are results of transcription starting at alternative initiation sites of the different promoters and alternative splicing of non-coding and coding exon transcripts (Hu and Dufau, 1991; Hu et al., 1998). PRLR and its mRNA are found in the mammary gland and the ovary, the best characterized sites of PRL biological actions in mammals (Nagano and Kelly, 1994). cDNAs encoding the PRLR gene have been cloned in chickens (Tanaka et al., 1992), doves, pigeons (Chen and Horseman, 1944), and turkeys (Zhou et al., 1996; Pitts et

al., 2000). Tissue distributions of PRLR mRNA have been reported in rats (Nagano and Kelly, 1994; Bakowska and Morrell, 1997), turkeys (Zhou et al., 1996; Pitts et al., 2000), and chickens (Ohkubo et al., 1998).

In mammals, the PRLR is found in the CNS and a wide range of peripheral organs including the pituitary gland, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, prostate gland, epithelial cells, bone, and skin (Nagano and Kelly, 1994; Nevalainen et al., 1997; Bole-Feysot et al., 1998; Clement-Lacroix et al., 1999). In rats, PRLR mRNA expression is found in the CNS, choroid plexus, bed nucleus of the stria terminalis (BSTM) , amygdala, central gray of the midbrain, thalamus, hypothalamus, cerebral cortex, and olfactory bulb. The PRLR is also extensively expressed by immune cells and some types of lymphocytes synthesized and secreted PRL, suggesting that PRL may act as an autocrine or paracrine modulator of immune activity (Freemark et al., 1995; 1996).

In birds, PRLR is found in the crop sac, brood patch, thyroid gland, liver, kidney, leg, skin, large and small intestine, adipose tissue, adrenal gland, thymus, lymphoid tissues, spleen, heart, brain, pineal gland, ovary, testis, seminal duct, and oviduct (Tanaka et al., 1992; Chen and Horseman, 1994; Zhou et al., 1996; Ohkubo et al., 1998; Pitts et al., 2000; Kang et al., 2007; Wang et al., 2009; Xing et al., 2011). Indeed, it has been reported that PRLR mRNA levels are the greatest in the pineal gland of laying and the oviduct of incubating turkey hens (Pitts et al., 2000).

	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																												

Figure 2.2 The percentage of homology sequence of PRLs among different species (Sinha, 1995).

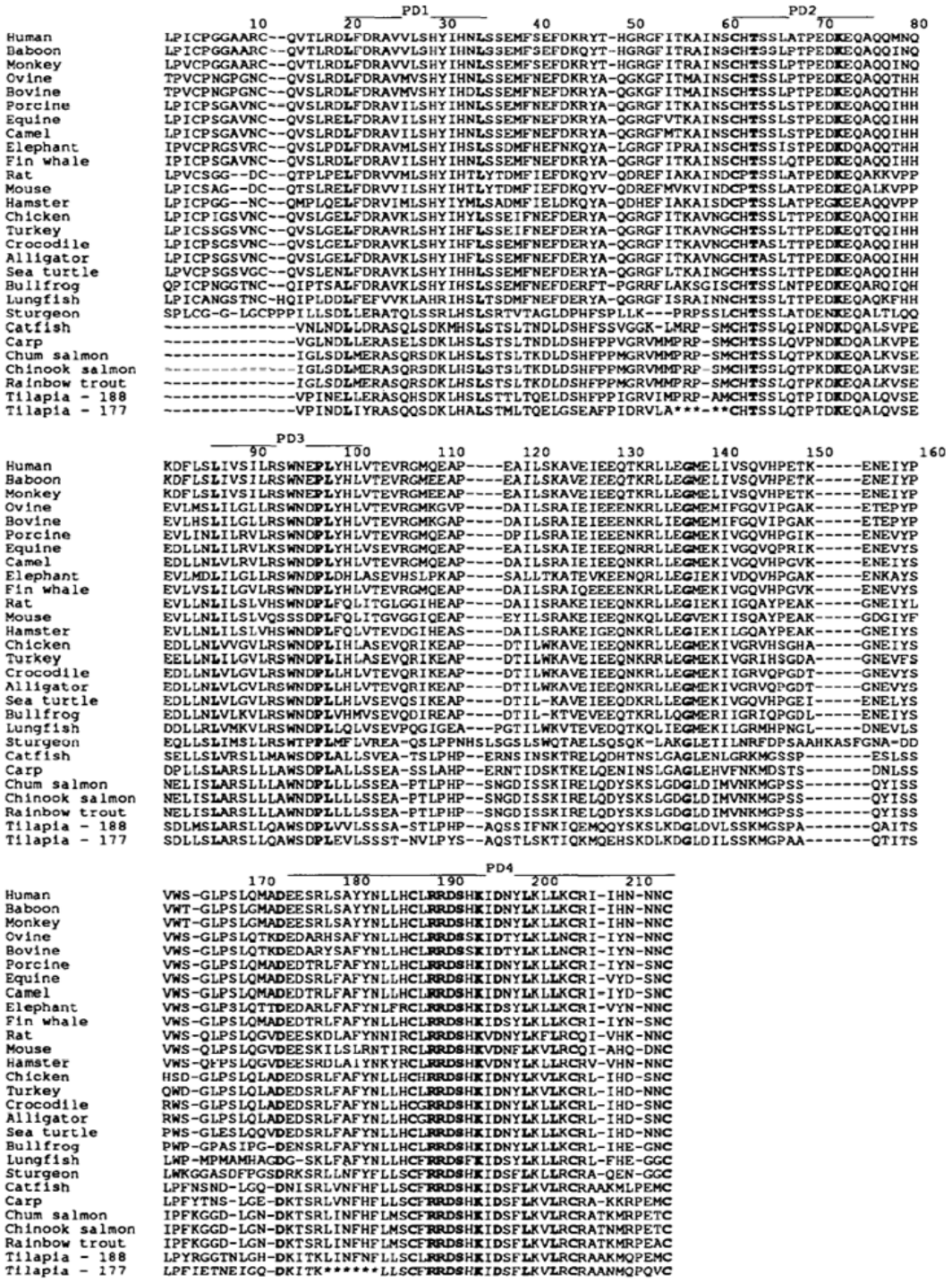

Figure 2.3 Primary structures of PRLs of different species. (-) indicates positions left blank to optimize alignment of amino acid sequences. (*) indicates absence of residues from a genetic variant of tilapia PRL. PD is PRL domain. PDI-PD4 indicates the four highly conserved domains of the PRLs (Sinha, 1995).

2.4.2 The Function of Prolactin in Birds

It has been well documented that PRL is associated with the reproductive cycle in birds (turkeys: Mashaly et al., 1976; El Halawani et al., 1984a; 1997; Wong et al., 1992b; mallards: Bluhm et al., 1983a; Boos et al., 2007; canvasback ducks: Bluhm et al., 1983b; cockatiels: Myers et al., 1989; king penguins: Mauget et al., 1994; emperor penguins: Lormee et al., 1999; tropical seabirds: Lormee et al., 2000; geese: Huang et al., 2008; native Thai chickens: Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). During reproductively quiescent stages (non-egg laying and rearing stages) of the native Thai chickens (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008) and turkeys (El Halawani et al., 1984b; 1997), plasma PRL levels are very low. During the periods of laying and incubating, circulating PRL levels increase dramatically (El Halawani et al., 1984b; Kosonsiriluk et al., 2008). It is this rising PRL level that causes the cessation of ovulation, ovarian regression, and induction and maintenance of incubation behavior. Changes in PRL gene expression 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., 1995b; Tong et al., 1997). The onset of incubation behavior is correlated with decreasing plasma LH levels and gonadal steroids and increasing plasma PRL levels (Cogger et al., 1979; Burke and Dennison, 1980; Lea et al., 1981; Rozenboim et al., 1993a). 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 in bantam hens, canaries, chickens, cowbirds, ducks, mallard ducks, native Thai chickens, pheasants, pigeons, ring doves, spotted sandpipers, turkeys, white-crowned sparrows, and wild starlings (Riddle et al., 1935; Breitenbach and Meyer, 1959; Hohn, 1959;

Sharp et al., 1977; 1988; Burke and Dennison, 1980; Goldsmith and Hall, 1980; Goldsmith and Williams, 1980; Goldsmith et al., 1981; 1984; Dawson and Goldsmith, 1982; Bluhm et al., 1983a; El Halawani et al., 1984a; 1988a; 1997; Oring et al., 1986; Hiatt et al., 1987; Youngren et al., 1991; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). PRL concentrations increase at the onset of incubation behavior and are maintained at high levels during incubation period (Saeki and Tanabe, 1955; Proudman and Opel, 1988) and decline when incubation behavior is terminated (El Halawani et al., 1980; Wentworth et al., 1983). Moreover, PRL is involved in many aspects of reproductive physiology and behaviors. It is thought to play a pivotal role in parental behaviors by mediating increases in incubation, crop milk secretion, feeding of young, and nest defense (Silver, 1984; Janik and Buntin, 1985; Lea et al., 1986; Buntin et al., 1991). Active immunization against recombinant-derived PRL reduces the incidence, delays the development, or prevents the occurrence of incubation behavior (March et al., 1994), whereas administration of exogenous PRL leads to increase parental behaviors (Lea and Vowles, 1986; Macnamee et al., 1986; Pedersen, 1989; Buntin et al., 1991; Youngren et al., 1991).

Some evidence suggests that PRL plays a role in terminating egg laying, therefore, it regulates clutch size in species that lay more than two eggs per clutch. Cessation of egg laying is associated with an increase plasma PRL levels (Etches et al., 1979; Burke and Dennison, 1980; Lea et al., 1981; Bluhm et al., 1983a; Hall and Goldsmith, 1983; Silverin and Goldsmith, 1983). Several studies have been reported that an increase in plasma PRL levels during incubating period may suppress LH secretion (Zadworny and Etches, 1987; Porter et al., 1991; El Halawani et al., 1993; Sharp et al., 1998). Administration of exogenous PRL suppresses concentrations of

gonadotropins in the turkeys (El Halawani et al., 1991) and domestic fowls (Sharp et al., 1988). It is suggested that PRL acts centrally to reduce LH concentrations by reducing hypothalamic GnRH levels (Rozenboim et al., 1993b). In incubating birds, suppression of gonadotropins release involves in a mechanism independent of increased PRL secretion (Sharp et al., 1988; 1989; Lea and Sharp, 1989; Lea et al., 1996). In addition, PRL may directly inhibit ovarian steroidogenesis as well (Rozenboim et al., 1993b), leading to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct (Porter et al., 1991). Furthermore, seasonal reproductive activities during long day photoperiod-stimulated PRL secretion occurred with increased PRL gene expression in the pituitary gland, which suppress LH secretion, inhibit follicular development, terminate laying and induced molting (Huang et al., 2008).

2.4.3 The Regulation of Prolactin Secretion

In mammals, PRL secretion is regulated by both stimulatory and inhibitory hypothalamic factors but it is mainly under tonic inhibitory control (MacLeod and Login, 1976; Neill, 1988; Ben-Jonathan et al., 1989; Lamberts and MacLeod, 1990). It is well documented that the predominant mammalian PRL-inhibiting factor (PIF) is DA, which is released from a dense network of neurons within the mediobasal hypothalamus (MBH) named as the tuberoinfundibular dopaminergic (TIDA) neurons and serves as the PIF of PRL secretion (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). DA released from the TIDA neurons acts directly on D_2 DA receptors located on the pituitary lactotrophs (Caron et al., 1978; Civelli et al., 1991). Removal of this DAergic inhibition can increase PRL secretion and hyperprolactinemia (Nicoll

and Swearingen, 1970; Nicoll, 1977). DA and its agonists inhibit the release and gene expression of PRL and proliferation of the lactotrophs (Birge et al., 1970; Shaar and Clemens, 1974; Pawlikowski et al., 1978; Maurer, 1981), indicating that the regulation of PRL secretion and its gene expression are under inhibitory control of the TIDA neurons (Pasqualini et al., 1988; Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). In *in vivo* studies, a brief fall of DA levels occurring immediately after suckling (Chiocchio et al., 1979; Selmanoff and Wise, 1981; Demarest et al., 1983) is necessary for PRL secretion (Grosvenor et al., 1980). Similarly, *in vitro* studies indicate that pituitary PRL secretion is stimulated after short term exposure of DA (Fagin and Neil, 1981; Denef et al., 1984). These studies confirm the physiological role of DA as the PIF. However, it has been reported that a much lower concentration of DA than those required for inhibition of PRL secretion can stimulate 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 evidences suggest that all pituitary lactotrophs have the potential to respond to the inhibitory and stimulatory effects of DA (Kineman et al., 1994), and the two opposite effects of DA on PRL secretion may be mediated by distinct guanine nucleotide binding proteins (G proteins) depending on its specific receptor subtypes (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994).

In mammals, VIP has been shown to be involved in the regulation of PRL secretion from the pituitary gland for many decades (Kato et al., 1978; Rotsztejn et al., 1980; Reichlin, 1988). VIP has been suggested to regulate pituitary secretion by a neuroendocrine pathway since the presence of VIP in the hypothalamic nerve endings, the anterior pituitary gland (Besson et al., 1979), and the hypophysial portal system

(Said and Porter, 1979). It is also reported that VIP can stimulate PRL release both *in vitro* (Shaar et al., 1979; Enjalbert et al., 1980; Samson et al., 1980; Matsushita et al., 1983) and *in vivo* (Kato et al., 1978; Frawley and Neill, 1981). Administration of VIP antiserum inhibits PRL release induced by stress, 5-HT, or suckling (Shimatzu et al., 1984; Abe et al., 1985; Kaji et al., 1985a; 1985b; Ohta et al., 1985). In addition, the contents of pituitary PRL mRNA and protein appear to be regulated by VIP (Ben-Jonathan et al., 1989; Maas et al., 1991). VIP mRNA expression is increased during the lactation period in rats (Gozes and Shani, 1986). An increase in VIP concentrations in the hypophysial portal blood is relative to the peripheral blood (Said and Porter, 1979; Shimatsu et al., 1981). VIP also promotes the entry of extracellular calcium ions into the PRL-secreting pituitary cells (Bjoro et al., 1987; Prysor-Jones et al., 1987). As aformentioned, VIP is proposed as a mammalian PRF.

Thyrotropin-releasing hormone (TRH) acts as a hypothalamic PRF in mammals as well. TRH stimulates PRL secretion both *in vitro* (Maas et al., 1991) and *in vivo* (Grosvenor and Mena, 1980; de Greef and Visser, 1981; Laverriere et al., 1988; Lafuente et al., 1994) and also PRL gene transcription (Potter et al., 1981; Laverriere et al., 1988). The secretion of PRL by TRH occurs during a transient depression in DAergic activity (Plotsky and Neill, 1982; Martinez de la Escalera et al., 1988). However, there are many contradictive results that have let the researchers to question its role as the PRF.

To date, various PRFs and PIFs have been observed both in birds and mammals such as 5-HT (Chaiseha and El Halawani, 2005; Chaiseha et al., 2010), angiotensin II (Malarkey et al., 1987; Opel and Proudman, 1988a; 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, 1988; 1990; Chaiseha and El Halawani, 1999; Kulick et al., 2005), and pituitary adenylate cyclase activating polypeptide (PACAP; Miyata et al., 1989; Yamauchi et al., 1995; You et al., 2000).

The regulation of PRL in birds also involves the interaction of external stimuli with neuroendocrine mechanisms. Critical environmental stimuli include sensory information concerning photoperiod, ambient temperature, and the presence of eggs and offspring. These external stimuli and steroid hormones such as estrogen and progesterone are important in initiating and maintaining PRL secretion, although their relative importance varies with the stages of the reproductive cycle (Curlewis, 1992). In incubating hens, tactile stimuli from the nests and eggs maintain the elevated circulating PRL levels and up-regulate VIP expression (Janik and Buntin, 1985; Lea et al., 1986; Silver et al., 1988; Buntin et al., 1991; Massaro et al., 2007).

As mentioned above, the regulation of PRL secretion and its gene expression are under the inhibitory control of the TIDA neurons in mammals (Ben-Jonathan and Hnasko, 2001). However, this is not the case in birds, where removal of these 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 long been well established that the secretion of PRL in birds involves in a tonic stimulatory control by the hypothalamus rather than the inhibitory DAergic system that found in mammals (Kragt and Meites, 1965; Bern and Nicoll, 1968; El Halawani et al., 1984a; Hall et al., 1986). The regulation of avian PRL secretion and its gene expression is influenced by hypothalamic VIP, the avian PRF (El Halawani et al., 1997; 2001; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005). In the past six decades, several studies

confirm the pivotal role of VIP as the only avian PRF. Immunoneutralization of VIP prevents an increase in circulating PRL levels that follows photostimulation, prevents the induction of incubation behavior, up-regulates content of LH-β- and FSH-β-subunit mRNAs, and extends the duration of egg laying period, but does not prevent spontaneous gonadal regression and molting (Sharp et al., 1989; El Halawani et al., 1995; 1996; Dawson and Sharp, 1998; Ahn et al., 2001). Variations in VIP immunoreactivity, VIP peptide contents in the INF and ME, and VIP mRNA steady-state levels in the INF are correlated with changes in circulating PRL levels throughout the avian reproductive cycle (Mauro et al., 1989; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; Kosonsiriluk et al., 2008).

In contrast with mammals, DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending on multiple DA receptor subtypes (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). In turkeys, stimulatory D_1 DA receptor mRNA expression has been found to increase in the hypothalamus of hyperprolactinemic incubating hens and in the pituitary gland of laying hens. However, inhibitory D_2 DA receptor mRNA expression increases in the pituitary gland of hypoprolactinemic photorefractory hens (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003). The stimulatory effect of DA on PRL secretion is regulated via the D_1 DA receptors residing in the INF, where the VIP neurons are located. In contrast, DA inhibits PRL release and synthesis by blocking the action of VIP at the pituitary level via the D_2 DA receptors (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). Indeed, changes in the DAergic activity during the turkey reproductive cycle parallel changes in plasma PRL levels, number of VIP-ir neurons, VIP peptide content, and its mRNA

expression within the INF (El Halawani et al., 1980; 1984b; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003). 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). It is very well established 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, there are evidence indicates that dynorphin, 5-HT, DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (for review, see El Halawani et al., 2001).

2.5 Vasoactive Intestinal Peptide: Structure, Function, and Regulation of Secretion

2.5.1 The Structure of Vasoactive Intestinal Peptide

VIP, an octacosapeptide, was first isolated from porcine duodenum (Said and Mutt, 1970; Mutt and Said, 1974). Subsequently, 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; Rosselin et al., 1982), with high concentrations found in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 1991) and its function is considered as a neurotransmitter and neuroendocrine substance (Larsson et al., 1976; Marley and Emson, 1982). The discovery of a large population of VIP-ir 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 the hypophysial portal blood (Said and Porter, 1979; Shimatsu et al., 1981; Brar et al., 1985; Mutt, 1988) led to the hypothesis that VIP involves in the regulation of anterior pituitary functions.

VIP is a neuropeptide belonged to the VIP/glucagon/secretin superfamily including secretin, glucagon, gastric inhibitory peptide (GIP), GH releasing factor, PHI, and PACAP. VIP exerts its biological effects by binding to its specific receptors that are coupled to the G-proteins, whose actions mediated via the adenylate cyclase and the production of cyclic adenosine monophosphate (cAMP; Hokfelt et al., 1980; Couvineau et al., 1990a; Lutz et al., 1995). The peptides of this family are probably the results of exon duplication coupled to gene duplication. VIP gene contains 7 exons and each exon encodes a different functional domain in the final mRNA and protein. Two adjacent exons in the genome encoding VIP and the related peptide histidine methionine (PHM) or PHI are exon 5 and exon 4, respectively (Bodner et al., 1985; Linder et al., 1987; Yamagami et al., 1988; Giladi et al., 1990). To date, mammalian VIP cDNAs (Itoh et al., 1983; Nishizawa et al., 1985), chicken and turkey VIP cDNAs (McFarlin et al., 1995; Talbot et al., 1995; You et al., 1995b) have been cloned and characterized. The open reading frame of mammalian VIP is comprised of 165 amino acids. Chicken VIP is different from mammalian VIP in its amino acid sequences at position 11, 13, 26, and 28, but the number of amino acid residue is the same (Nilsson, 1975). In addition, the chicken and turkey VIP share complete amino acid homology and are 98 % homologus at the neucleotide level. VIP mRNA may exist with or without PHI. Both mRNA forms are found in the chicken digestive tract and hypothalamus. The short form is found only in the turkey hypothalamus and comprises 4 to 6 % of all VIP transcripts (You et al., 1995b). The amino acid

sequences of VIP and the member in VIP/glucagon/secretin family are shown in Figure 2.4.

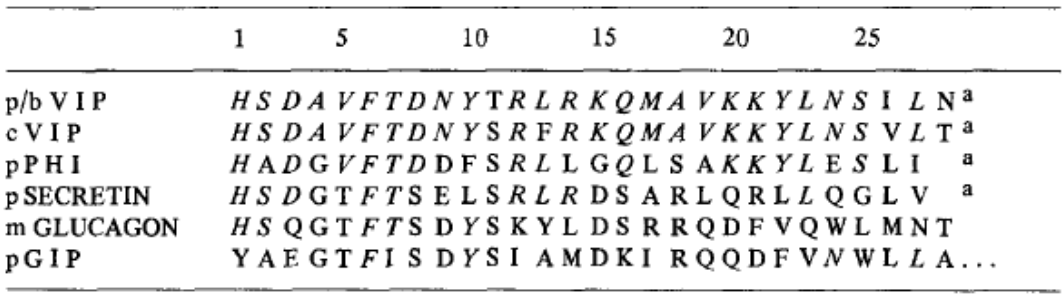

Figure 2.4 The amino acid sequences of VIP, PHI, secretin, glucagon, and GIP. p: porcine, b: bovine, c: chicken, m: mammalian, a: the C-terminal amino acid is in the amide form (Rosselin et al., 1982).

VIP receptors are purified from porcine liver with the MW is 53 kDa (Couvineau et al., 1990b). Subsequently, it has been cloned and characterized in mammals (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). Pharmacologically, two subtypes of VIP receptors; VPAC1 and VPAC2 are expressed in a tissue specific manner (Couvineau et al., 1994; Usdin et al., 1994; Sherward et al., 1995) and bind both VIP and PACAP, which these receptors are high affinity receptors for VIP. VIP receptors belong to the members of a seven transmembrane spanning domain G protein-coupled family. The biological action of VIP is mediated via its specific receptors that are coupled to adenylate cyclase and the production of intracellular cAMP (Gourdji et al., 1979; Bjoro et al., 1987; Couvineau et al., 1990a; Lutz et al., 1995). VIP receptors are presented in several organs including the lung, liver, small

intestine, and many regions of the brain such as cerebral cortex and hippocampus (Besson et al., 1986; Martin et al., 1987; Csillag et al., 1993; Usdin et al., 1994; Sherward et al., 1995; Zawilska et al., 2004). In addition, a single VIP receptor is expressed and functioned in non-mammalian species as well (Kansaku et al., 2001). Avian VIP receptors have been cloned and characterized in the chickens (Kansaku et al., 2001) and turkeys (You et al., 2001). It has been reported that the circulating PRL variations that observed across the turkey reproductive cycle are, in part, regulated by changes in VIP receptors at the pituitary level (Chaiseha et al., 2004). In birds, VIP receptors are presented on the surface membranes of the anterior pituitary cells (Rozenboim et al., 1993b; Gonzales et al., 1994a; 1994b), hypothalamus (Gonzales et al., 1995), small intestine, and granulosa cells (Kawashima et al., 1995). The sequence homology and amino acid similarities of VIP receptors in vertebrates are shown in Figure 2.5. Comparison of partial amino acid sequences of VIP receptors from different vertebrates are shown in Figure 2.6.

	Human	Porcine	Rat	Chicken	Frog	Goldfish
Human		89.5	84.7	67.4	65.5	65.0
Porcine	86.9		84.1	68.7	66.3	65.1
Rat	84.1	82.2		67.3	65.0	63.7
Chicken	65.6	65.8	66.4		71.1	67.6
Frog	66.2	67.0	65.8	77.0		67.1
Goldfish	60.3	59.6	59.7	65.2	67.1	

Figure 2.5 Sequence homology and amino acid similarlities (%) of VIP receptors. Values above and under the diagonal line represent homology of the cDNA sequence and amino acid, respectively (Kansaku et al., 2001).

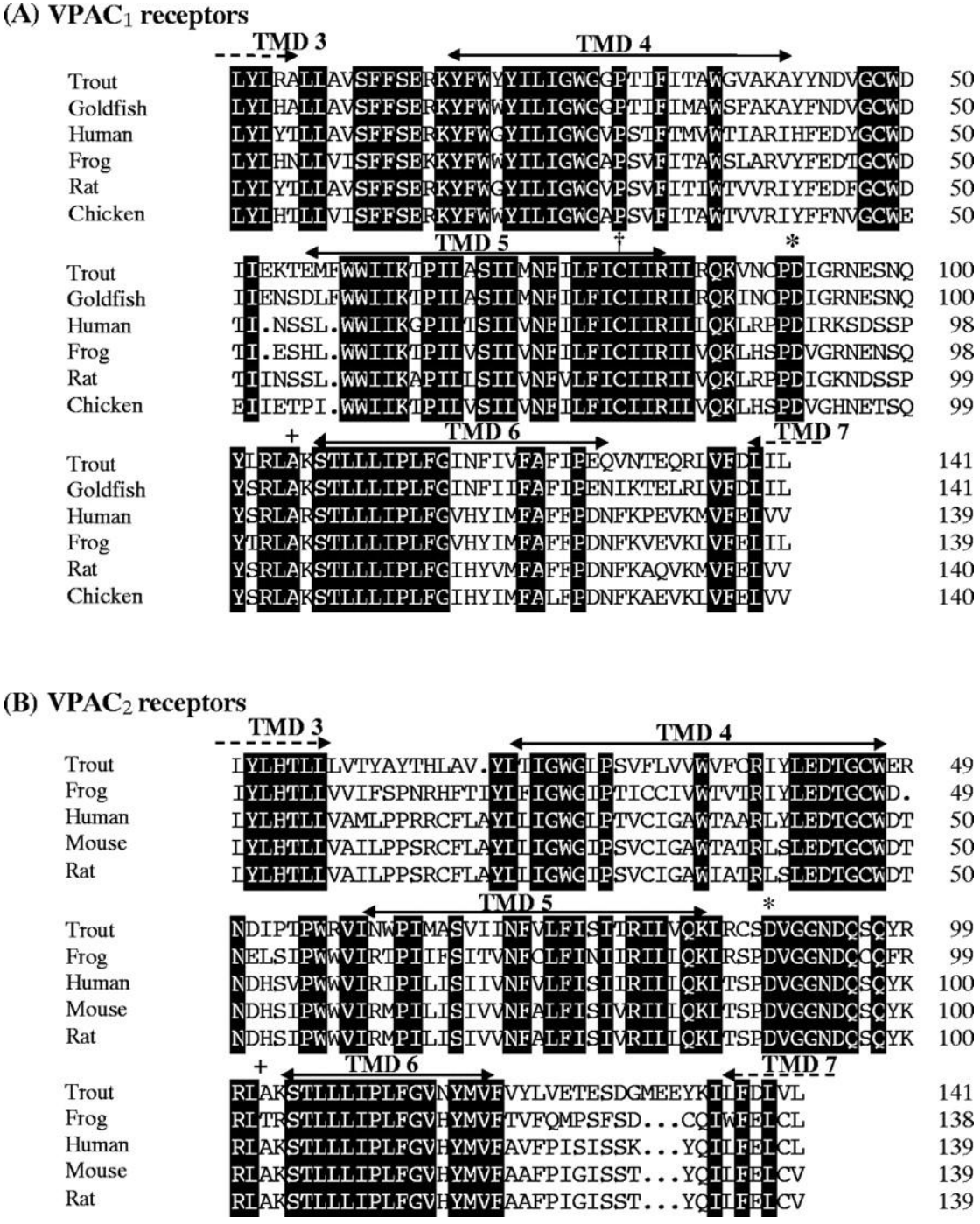

Figure 2.6 Comparison of partial amino acid sequences of $VPAC_1$ (A) and $VPAC_2$ (B) receptors from different vertebrates. Conserved amino acids (100 % identity) among taxa are highlighted. The putative transmembrane domains (TMDs) are denoted by horizonatal arrows (Montpetit et al., 2003).

2.5.2 The Localization of Vasoactive Intestinal Peptide in the Avian Brain

Well documentedly, the distributions of VIP-containing neurons have been conducted in the brain of many avian species including Pekin ducks (Korf and Fahrenkrug, 1984), Japanese quails (Peczely and Kiss, 1988), turkeys (Mauro et al., 1989; Chaiseha and El Halawani, 1999), pigeons (Cloues et al., 1990), ring doves (Norgren and Silver, 1990), chicks (Kuenzel and Blahser, 1994; Kuenzel et al., 1997), dark-eyed juncos (Saldanha et al., 1994), zebra finches (Bottjer and Alexander, 1995), and native Thai chickens (Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011). In birds, VIP neurons are extensively distributed throughout the hypothalamus (Yamada et al., 1982; Mikami and Yamada, 1984; Macnamee et al., 1986; Peczely and Kiss, 1988; Mauro et al., 1989; Hof et al., 1991; Chaiseha and El Halawani, 1999; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011), especially in the areas of the medial preoptic area (MPOA), medial hypothalamic region, AM, hypothalamus pars lateralis, and INF (den Boer-Visser and Dubbeldam, 2002). Generally, three types of VIP-ir neurons and fibers are described. The first set consists of a large number of spindle or bipolar neurons that connected the V III to the external layer of the ME. The second set of VIP-ir fibers extends from the infundibular nucleus to the ME. The third set of VIP-ir neurons terminates upon small capillaries within the hypothalamus. It has been suggested 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 terminals are observed in the external portion of the ME and the majority of VIP-containing cell bodies are located in the INF. The number of the VIP-ir neurons in the INF increases following a gonadal stimulatory photoperiod. The hypothalami of incubating turkey hens contain more VIP-ir neurons than those of

non-photostimulated hens. Circulating PRL levels and hypothalamic VIP immunoreactivity are found to reduce in nest-deprived incubating birds (Mauro et al., 1989; Prakobsaeng et al., 2011). Fluctuations in hypothalamic VIP immunoreactivity and its gene expression within the INF paralleled fluctuations in circulating PRL levels during the avian reproductive cycle (Chaiseha and El Halawani, 1999). The number, distributed area, and density of hypothalamic VIP-ir neurons are greater in incubating hens than those of laying hens (Sharp et al., 1989; Kosonsiriluk et al., 2008). In addition, in domesticated pigeons, increases in the number and cell size of VIP-ir neurons within this region following the periods of elevated circulating PRL levels have been reported (Peczely and Kiss, 1988; Cloues et al., 1990). Thus, these VIP neurons project to the ME, where VIP is transported through the hypothalamic-pituitary portal vessels to the anterior pituitary gland (Yamada et al., 1982; Macnamee et al., 1986; Mauro et al., 1989). In addition, lesions in the INF can prevent the PRL increase after photostimulation (Youngren et al., 1989).

All these above data indicate that VIP neurons in the INF are an important factor in the stimulation of PRL secretion. It has been further established that VIP axon terminals have been found in close apposition to GnRH neurons in the lateral septal organ (LSO) 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). A subset of VIP-ir neurons within the MBH and septal region of the dove brain has been proposed to be encephalic photoreceptors (Silver et al., 1988; Norgren and Silver, 1990). Recently, it has been implicated that turkey melanopsin (tOPN4x) in the hypothalamic premammillary nucleus (PMM) DA-melatonin (DA-MEL) neurons

acts as an important component of the photoreceptive system regulating reproductive activity in temperate zone birds (Kang et al., 2010).

2.5.3 The Function of Vasoactive Intestinal Peptide in Birds

In birds, it has long been established that the hypothalamic control of PRL secretion involves a stimulatory mechanism rather than the inhibitory DAergic system found in mammals (Kragt and Meites, 1965; Bern and Nicoll, 1968; El Halawani et al., 1984a; Hall et al., 1986). Many evidences confirm and support VIP as the most important avian PRF (Macnamee et al., 1986; Opel and Proudman, 1988b; Mauro et al., 1989; El Halawani et al., 1990b; 1990c; 1997; Talbot et al., 1991). It is well established that avian 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; 2001). VIP meets the classical criteria for defining it as the hypophysiotrophic PRF in birds. These criteria are; 1) the presence of VIP-ir neurons in the hypothalamus, 2) the secretion of VIP into hypophysial portal blood, 3) the modulation of VIP secretion into hypophysial portal blood, 4) the presence of VIP receptors on anterior pituitary cells, 5) the ability of VIP to regulate anterior pituitary lactotrophs, and 6) the alteration of pituitary function, due to antagonism of VIP (for review, see El Halawani et al., 1997). Further evidences have been provided by the findings that immunoneutralization of endogenous VIP reduces circulating PRL levels and its mRNA expression and totally inhibits the PRL release affected by electrical stimulation of the MPOA (El Halawani et al., 1990b; Youngren et al., 1994) and blocks the hormonal and behavioral characteristics of incubating hens (El Halawani et al., 1995). Several hypothalamic neurotransmitters and neuropeptides have been

studied during the past six decades for their effects on PRL such as TRH, angiotensin II, oxytocin, vasopressin, PACAP, and PHI. Only VIP is thought to be physiologically significant PRF in birds.

VIP is a potent releaser of PRL *in vivo* (Lea and Vowles, 1986; Macnamee et al., 1986; Opel and Proudman, 1988b; 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). Thus, VIP plays a pivotal role in the regulation of PRL in birds. VIP regulates PRL gene expression by enhancing the transcription rate of PRL and up-regulating PRL mRNA stability (Tong et al., 1998). Variations in hypothalamic VIP immunoreactivity, VIP contents, VIP mRNA steady-state levels, VIP mRNA expression in the INF, VIP receptor mRNA in the pituitary cells, and VIP concentrations in hypophysial portal blood are correlated with the changes in circulating PRL levels in many avian species such as turkeys (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha et al., 1998; 2004; Chaiseha and El Halawani, 1999), chickens (Sharp et al., 1989a), doves (Cloues et al., 1990), and native Thai chickens (Kosonsiriluk et al., 2008). Passive immunization with VIP anti-serum decreases plasma PRL and pituitary mRNA levels and terminates incubation behavior (Talbot et al., 1991). Similarly, active immunization with VIP also reduces circulating PRL levels and prevents the expression of incubation behavior in the turkeys (El Halawani et al., 1996; 2001). It has been suggested that the stimulatory action of VIP occurs via specific receptors located on anterior pituitary plasma membranes, which change throughout the reproductive cycle of the turkeys (Rozenboim and El Halawani, 1993; Chaiseha et al., 2004). As mentioned above, it is confirmed the role of VIP as the avian PRF.

Increased hypothalamic VIP mRNA and its peptide contents are associated with gonadal regression and suppression of gonadotropins in photorefractory turkeys (Chaiseha et al., 1998; Chaiseha and El Halawani, 1999). Immunoneutralization with VIP up-regulates LH-β- and FSH-β-subunit mRNAs expression (Ahn et al., 2001) and delays the onset of photorefractoriness and molt in starling (Dawson and Sharp, 1998). These findings imply that VIP also exerts an inhibitory effect on the GnRH/FSH-LH system. In deed, it has been indicated that VIP has a central inhibitory effect on GnRH/FSH-LH release in birds (Pitts et al., 1994).

2.5.4 The Regulation of Vasoactive Intestinal Peptide Secretion

Due to this research dissertation is conducted in the native Thai chicken, this Section will discuss only the regulation of VIP secretion in birds. In fact, the regulation of VIP in mammals is very well documented worldwide. It has been also suggested that VIP mediates the effects of photoperiod on PRL secretion in the turkeys (El Halawani et al., 1996) and quantification of hypothalamic VIP reveals an increased VIP content following photostimulation (Mauro et al., 1992). It has been further demonstrated that VIP peptide contents in the ME, hypothalamic cytoplasmic VIP mRNA steady-state levels, and hypothalamic nascent VIP mRNA levels are all increased and correlated with increased PRL secretion following photostimulation (Chaiseha et al., 1998). These results lend support to a hypothetical scheme for photoperiodic regulation of PRL in which VIP serves as the PRF that is intimately linked to photoperiodic mechanisms. Furthermore, the results also imply that VIP transcription is coupled to the photoperiodic state of the birds. However, it remains to be clarified how photoperiodic information is transduced to VIP-ir neurons and

whether photoperiodic cues directly influence VIP remains an open question. Silver et al. (1988) has shown that VIP is colocalized with an opsin-like pigment in the INF. This area is thought to contain extra-retinal 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. Recently, tOPN4x is found in the DA-MEL co-localized neurons in the PMM and is implicated as an important component of the photoreceptive system regulating reproductive activity in temperate zone birds (Kang et al., 2010).

VIP is also inhibited by high concentrations of circulating PRL. Intracerebroventricular (ICV) PRL injections into incubating ring doves reduce the number of infundibular VIP-like neurons, which indicates the existence of a hypothalamic negative feedback loop for PRL (Saldanha and Silver, 1995). Intracranial and systemic administrations of ovine PRL into laying turkey hens reduce circulating PRL concentrations (Youngren et al., 1991; Rozenboim et al., 1993b). In addition, systemic PRL administration reduces hypothalamic VIP contents and the number of anterior pituitary VIP binding sites (Rozenboim et al., 1993a), indicating that PRL may act directly at the pituitary level. PRL binding sites have been found within the avian hypothalamus (Buntin and Ruzycki, 1987; Buntin and Walsh, 1988) and PRLR mRNA is also detected in the brain of chickens (Tanaka et al., 1992) and the hypothalamus of turkeys (Zhou et al., 1996; Pitts et al., 2000). Furthermore, PRL may cross the blood-brain barrier at the choroids plexus (Buntin and Walsh, 1988) and binds to the PRLRs lining the V III, thus decreasing the number of hypothalamic

VIP-containing neurons (Saldanha and Silver, 1995). VIP immunoneutralization averts the rise in circulating PRL levels that follows photostimulation, prevents the induction of incubation behavior, up-regulates LH-β- and FSH-β subunit mRNAs content, and extends the duration egg laying period, but does not prevent spontaneous gonadal regression and molting (Sharp et al., 1989; El Halawani et al., 1995; 1996; Dawson and Sharp, 1998; Ahn et al., 2001). However, despite the well established antigonadotropic effects of PRL, it appears that the high circulating PRL levels of laying and non-incubating birds is not the primary cause of the suppression of GnRH/FSH-LH system and the termination of reproduction (Juss, 1993; Dawson and Sharp, 1998).

2.6 Gonadotropin Releasing Hormone: Structure, Function, and Regulation of Secretion

2.6.1 The Structure of Gonadotropin Releasing Hormone

GnRH, referred to as type one mammalian GnRH (mGnRH), was first isolated and sequenced from porcine hypothalamus (Peczely, 1989; Rivier, 2001). A second molecular form of GnRH has been later identified in several mammals and other non-mammalian vertebrate species (for review, see Bakker and Baum, 2000). This second form has been identified as chicken GnRH-II (cGnRH-II). GnRH is a hypothalamic neuronal secretory decapeptide that important for the control of reproduction in vertebrates. To date, it has been reported that GnRH consists of a family at least 25 isoforms, there are 15 and 11 isoforms of GnRH from representative vertebrate and invertebrate species, respectively (Gorbman and Sower, 2003; Kavanaugh et al., 2008; Zhang et al., 2008). Two to three isoforms have been identified in

representative species of all classes of vertebrates (Powell et al., 1994; Kah et al., 2007). All these forms consist of 10 amino acids with conserved amino acids in position 1, 4, 9, and 10 (Figure 2.7; Kavanaugh et al., 2008), and share at least 50 % sequence identity (for review, see Limonta et al., 2003). The most recognized and common structural variation among the different forms of GnRH resides in amino acids between position 5 and 8 in the sequence. There are three types of GnRH and two types of GnRH receptor have been found in the avian brain (Sun et al., 2001; Shimizu and Bedecarrats, 2006). Two distinct forms of GnRH have been isolated in chicken; cGnRH-I or GnRH-I ([Gln8]-GnRH) and cGnRH-II ([His5, Trp7, Ty8]-GnRH; King and Millar, 1982; Miyamoto et al., 1982; 1984; Millar and King, 1984; Sherwood et al., 1988). The gene encoding cGnRH-I has been cloned and characterized (Dunn et al., 1993). To date, GnRH-III which is first demonstrated in lamprey is also found in the brain of songbirds (Bentley et al., 2004). Of the three forms, GnRH-I is the form that is directly involved in controlling reproduction in birds (Sharp et al., 1990).

Type	1	2	3	4	5	6	7	8	9	10
GnRH-I (Mammal)	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH2
GnRH-I (Guinea Pig)	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	GlyNH2
GnRH-I (Chicken - I)	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	GlyNH2
GnRH-I (Rana)	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	GlyNH2
GnRH-I (Seabream)	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	GlyNH2
GnRH-III (Salmon)	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	GlyNH2
GnRH-I (Whitefish)	pGlu	His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	GlyNH2
GnRH-I (Medaka)	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	GlyNH2
GnRH-I (Catfish)	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	GlyNH2
GnRH-I (Herring)	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	GlyNH2
GnRH-I (Dogfish)	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	GlyNH2
GnRH-II (Chicken -II)	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	GlyNH2
Lamprey - II	pGlu	His	Trp	Ser	His	Gly	Trp	Phe	Pro	GlyNH2
GnRH-IV (Lamprey - III)	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	GlyNH2
GnRH-IV (Lamprey - I)	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	GlyNH2

Figure 2.7 Primary structures of GnRHs in vertebrates. Yellow highlight, Amino acids changed, compared with mammalian GnRH; gray highlight, GnRHs occurring in the sea lamprey; orange highlight, single amino acid difference between dogfish, chicken-II, and lamprey GnRH (Kavanaugh et al., 2008).

GnRH regulates gonadotropins (FSH and LH) secretion via binding to the specific receptors on the cell surface of pituitary gonadotrophs. GnRH receptors have been cloned from several mammalian species (for review, see Ramakrishnappa et al., 2005). GnRH receptor belongs to a member of the large superfamily of seven transmembrane domain receptors that bind to the G proteins. Upon the binding, GnRH activates the G_q/G_{11} subfamily of the G proteins, resulting an increase in phospholipase C activity and generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium ions from intracellular stores, whereas DAG activates protein kinase C, leading to the synthesis and release of gonadotropins (Stojilkovic and Catt, 1995). In mammals, type II GnRH receptor is shown to be

highly selective for GnRH-II and is widely expressed in reproductive tissues and the CNS (Millar et al., 2001; Neill et al., 2001). High expression of GnRH-II receptors in the gonadotrophs suggests its role in the regulation of gonadotropin secretion. The cloned of a novel GnRH receptor from chicken pituitary differs from the mGnRH receptor in its primary structure, ligand selectivity, and in the agonistic behavior of certain mGnRH receptor antagonists (Tanaka et al., 1980; Harris et al., 2004). Particularly, some antagonists of mGnRH receptors act as agonists of the chicken GnRH receptor, stimulating LH release from chicken pituitary cells (Kuo et al., 2002).

2.6.2 The Localization of Gonadotropin Releasing Hormone in the Brain

It is very well established that each GnRH form has its unique distributions within the brain, indicating a difference in developmental origin and/or adult functions (Sherwood et al., 1993; White et al., 1995; Lescheid et al., 1997; Dubois et al., 2002). In mammals, GnRH-I neurons are distributed in a loose rostral-caudal continuum in the ventral forebrain. The principle projection of these neurons is toward the ME, where their terminals have been found in the hypophysial portal plexus. However, GnRH-II-expressing neurons locate in the midbrain and the major terminal field for these neurons is the medial habenula. In contrast to the forebrain GnRH-I fibers, few GnRH-II fibers are observed in the ME (for review, see Bakker and Baum, 2000).

Like in mammals, GnRH neurons and fibers are more widely distributed throughout the avian brain. Many previous studies have examined the distributions of cGnRH-I throughout the avian brain including chickens (Jozsa and Mess, 1982; Sterling and Sharp, 1982; Mikami et al., 1988; Kuenzel and Blahser, 1991), ducks

(McNeill et al., 1976; Bons et al., 1978), white-crowned sparrows (Blahser et al., 1986; 1989), Japanese quails (Foster et al., 1988; Mikami et al., 1988; Perera and Follett, 1992; van Gils et al., 1993; Teruyama and Beck, 2000), European starlings (Dawson et al., 1985; Foster et al., 1987; Goldsmith et al., 1989), garden warblers (Bluhm et al., 1991), great tits and ring doves (Silver et al., 1992), turkeys (Millam et al., 1993), dark-eyed juncos (Saldanha et al., 1994), house sparrows (Hahn and Ball, 1995), cockerels (Sun et al., 2001), canaries (Bentley et al., 2004), and native Thai chickens (Sartsoongnoen et al., 2012). There are three major groups of GnRH-I-ir neurons; 1) a telencephalic group medial to the lateral ventricles, 2) a basotelencephalic group located ventral to the tractus septomesencephalicua (TSM) and extending laterally and dorsocaudally, and 3) a distinctive group of neurons located along the midline extending from the POA to septal regions (Foster et al., 1987; Millam et al., 1993; 1998; Teruyama and Beck, 2000). Six major groups of cGnRH-I perikarya are found including the olfactory bulb, olfactory tubercle, lobus parolfactorius, nucleus accumbens (Ac), septal preoptic hypothalamic region (three sub-nuclei), and lateral anterior thalamic nucleus (Kuenzel and Blahser, 1991). Fully processed cGnRH-I mRNA and a variant transcript with a retained intron 1 are observed in the POA, basal hypothalamus, anterior pituitary gland, and testis of the cockerels (Sun et al., 2001). The main group of cGnRH-I cell bodies is located in the POA with fibers extending along the V III and then entering the area of GnRH secretion, the ME (Foster et al., 1987; Meddle and Follett, 1997). Specific GnRH-I-ir neurons are found in several hypothlalamic areas including the POA, nucleus preopticus medialis (POM), AM, paraventricular nucleus (PVN), and nCPa. Additional scattered neurons are also found in the SL and around the OVLT. Several

studies have reported the distributions of the cGnRH-I mRNA and its protein in the brain of several species (Millam et al., 1989; Dunn and Sharp, 1999; Sun et al., 2001; Dawson et al., 2002; Kang et al., 2006). cGnRH-I mRNA expressions are the greatest in the nCPa and around the OVLT. cGnRH-I mRNAs abundance within the nCPa, OVLT, and SL are greater in laying hens than those of in non-photostimulated and incubating turkey hens (Kang et al., 2006).

The presence of cGnRH-II in the ME has been identified, but its involvement in the control of reproduction is remained controversial. A large group of cGnRH-II-ir neurons in the oculomotor region and basal lateral hypothalamus are observed in the turkeys and Japanese quails (Millam et al., 1993; 1995; 1998; Teruyama and Beck, 2000). cGnRH-II fibers are found in the ME of Japanese quails and chickens (van Gils et al., 1993; Teruyama and Beck, 2000; Clerens et al., 2003). cGnRH-II-ir fibers are found prominently in the POA, lateral septum (LS), both medial and lateral to the TSM at the level of the POA and in limbic structures, olfactory areas, and forebrain (Millam et al., 1993).

Several evidences in the Japanese quails, chickens (Mikami et al., 1988; Sharp et al., 1990), ostriches (Powell et al., 1987), and turkeys (Millam et al., 1989) reveal that cGnRH-I and cGnRH-II are distributed differently in the brain and other tissues. GnRH-II-immunoreactivity has not been observed in the ME of Japanese quails, chickens, and turkeys (van Gils et al., 1993; Millam et al., 1998; D'Hondt et al., 2001; Clerens et al., 2003), suggesting that cGnRH-II does not directly promote pituitary gonadotropins secretion (Millam et al., 1993). The release of cGnRH-II from the ME has been reported (Millam et al., 1998), but this release is less amount than that of cGnRH-I. In contrast, study in white-crowned sparrows does not find GnRH-II in the

ME (Meddle et al., 2006). In addition, GnRH-III neurons are distributed in the hypothalamus and forebrain of songbirds, especially in the telencephalon including the hippocampus and song control areas (Bentley et al., 2004).

2.6.3 The Function of Gonadotropin Releasing Hormone in Birds

Like in mammals, GnRH plays a pivotal role in the control of avian reproduction. At the peak level of reproductive activity, birds have more GnRH-ir neurons and fibers than those of sexually inactive or photorefractory ones (Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998; Sharp et al., 1990; Sartsoongnoen et al., 2012). GnRH contents also change during the avian reproductive cycle. GnRH-I levels decrease when birds enter the incubating period and this decrease is thought to be regulated by the inhibitory effect of PRL (Sharp et al., 1988). GnRH-I concentrations is significantly elevated in the POA during incubation period (Millam et al., 1995). GnRH peptide contents in the hypothalamus during the reproductive cycle of the turkeys (Millam et al., 1989; El Halawani et al., 1993; Rozenboim et al., 1993a) and chickens (Dunn et al., 1996) do not change. However, the amount of hypothalamic GnRH mRNA in incubating hens is lower than those of in laying hens (Dunn et al., 1996). GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples are higher in laying hens than those of in non-laying hens (Advis et al., 1985). In the turkeys, GnRH-I mRNA abundance within the nCPa, OVLT, and SL is greater in laying hens than those of in non-photostimulated and in incubating ones, while GnRH mRNA expression is the least in photorefractory hens, suggesting that hypothalamic GnRH mRNA expression may be used to precisely characterize the different reproductive stages in birds (Kang et al., 2006). Moreover, an increase in LH

secretion is resulted from removal of incubating hens from their nests and associated with an increase in the amount of hypothalamic GnRH mRNA (Dunn et al., 1996).

Like in mammals, GnRH is synthesized by neurosecretory cells in the hypothalamus, released from the ME into the hypophysial portal vessels, and then transported to the pituitary gland, where it stimulates the secretions of FSH and LH. GnRH increases FSH and LH secretion of the adenohypophysis both *in vitro* and *in vivo* (Peczely, 1989). Injection of GnRH increases plasma LH levels *in vivo* (Wingfield et al., 1979; McNaughton et al., 1995). Incubation of turkey anterior pituitary cells with GnRH results in an increase in LH-β-subunit gene expression and stimulates LH secretion (You et al., 1995a). A pulsatile pattern of GnRH release is observed from the MBH and POA *in vitro* (Li et al., 1994). It has been indicated that the expression of GnRH gene is important to maintain pituitary-ovarian function in the chickens (Dunn et al., 1996). In contrast, GnRH inhibits FSH-stimulated steroidogenesis in chickens, but enhances LH-stimulated progesterone production (Hertelendy et al., 1982). GnRH does not affect circulating FSH levels, but stimulates LH secretion in 3 weeks old cockerels (Krishnan et al., 1993). There are growing evidences indicating GnRH influence avian gonadotropins secretion, but its ability is different amoung the three forms of GnRH. cGnRH-I and cGnRH-II can differential stimulate the release of FSH and LH from chicken pituitary *in vitro* (Millar et al., 1986). An injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH concentrations (Guemene and Williams, 1999).

To date, it is suggested that cGnRH-I has a physiological role in regulating of gonadotropins secretion and cGnRH-II may not be involved in releasing avian pituitary gonadotropins (Sharp et al., 1990), confirming by passive immunization with

anti-cGnRH-I, but not anti-cGnRH-II causes a decrease in the plasma LH levels and complete regression of the reproductive system. cGnRH-II-immunoreactivity in the ME of Japanese quails, chickens, and turkeys has not been observed (van Gils et al., 1993; Millam et al., 1998; D'Hondt et al., 2001; Clerens et al., 2003), supporting that cGnRH-II does not directly stimulate pituitary gonadotropins secretion (Millam et al., 1993). However, seasonal changes in the cGnRH-II-ir neurons are noted, suggesting an involvement of cGnRH-II in the control of reproduction (Teruyama and Beck, 2000). The role of cGnRH-II in the stimulation of female courtship behavior has been reported since ICV infusion of cGnRH-II enhances courtship behavior in female white-crowned sparrows, but cGnRH-I does not show the same effect (Maney et al., 1997). The various distributions of cGnRH-II and GnRH-III in the avian brain suggest their functional significances. cGnRH-II may act as a neurotransmitter (Jones, 1987), and GnRH-III may act as a potential mediator in transducing song-related stimuli to areas that control gonadotropins secretion (Bentley et al., 2004).

2.6.4 The Regulation of Gonadotropin Releasing Hormone Secretion

In birds, it has been reported that photoperiod is the main regulator of the GnRH neuronal activities (Sharp and Blache, 2003). Photostimulatory inputs to GnRH neurons have the potential to increase GnRH mRNA transcription and its secretion (Dunn and Sharp, 1999) as well as pituitary sensitivity to GnRH (Davies and Follett, 1975). Indeed, there are growing evidences to confirm that photoperiod is associated with the GnRHergic system. The stimulatory effect of long day photoperiod appears to be associated with an increased GnRH contents or increased GnRH immunoreactivity in the hypothalamus and ME in birds (Dawson et al., 1985;

Foster et al., 1987; 1988; Goldsmith et al., 1989; Perera and Follett, 1992; Saldanha et al., 1994; Hahn and Ball, 1995). The amount of hypothalamic GnRH increases during long day photostimulation and decreases during photorefractoriness in many avian species such as European starlings (Dawson et al., 1985; Foster et al., 1987; Dunn et al., 1996), garden warblers (Bluhm et al., 1991), house sparrows (Hahn and Ball, 1995), dark-eyed juncos (Saldanha et al., 1994), and turkeys (Rozenboim et al., 1993a; Kang et al., 2006). This decrease in hypothalamic GnRH contents occurs at a time when the amount of hypothalamic VIP is increased (Mauro et al., 1992; Rozenboim et al., 1993a; Saldanha et al., 1994). Providing of light pulse is shown to induce GnRH mRNA expression in the nCPa of the reproductively quiescent turkeys maintained under a short day lighting regimen (Kang et al., 2006). Consistency with this finding, the nCPa responds to the photoperiod and a diet supplemented with sulfamethazine, a compound that augments the effect of long day photostimulation, with a significant increase in number of GnRH neurons compared with birds fed control diets and exposed to a short day photoperiod (Kuenzel and Golden, 2006). Taken together, these above findings support the role of photoperiod in correlated with GnRH to regulate the reproductive system in birds. During photorefractoriness, gonadal regression associated with a decrease in FSH and LH secretions is related to a decrease in hypothalamic GnRH-I contents (Dawson et al., 2001; 2002; Hua, 2001). Another study reveals that the reduction in hypothalamic GnRH contents in photorefractory birds is associated with a reduction in GnRH precursor and proGnRH-guanine triphosphatase activating protein, suggesting the development of photorefractoriness is promoted by the inhibition of GnRH synthesis rather than requiring inhibition of GnRH release from the ME (Parry et al., 1997).

There are other environmental cues such as access to food or local tropical climate play a pivotal through supplemental role in regulation of the activity of GnRH neurons (Bruggeman et al., 1998; Ciccone et al., 2006; Deviche et al., 2006; Moore et al., 2006). The HPG axis is known to be activated by the combined environmental factors. GnRH stimulates pituitary release of gonadotropins, which in turn increases gonadal production of steroid hormones. Increased levels of gonadal steroids exert a negative feedback on the GnRH/FSH-LH system. Gonadectomy increases the synthesis of hypothalamic GnRH and the release of LH (Knight et al., 1983). In addition, it has been established that testosterone decreases the number of cGnRH-I-ir neurons in the POA and cGnRH-I-ir fibers in the ME (Knight et al., 1983). In juvenile cockerels, cGnRH-I mRNA expression and its peptide contents in the POA and cGnRH-I receptors in the pituitary cells are suppressed by estrogen (Dunn and Sharp, 1999; Sun et al., 2001).

Hypothalamic VIP is also thought to inhibit GnRH gene expression (Deviche et al., 2000). It is very well documented that VIP receptor mRNA expression and its peptide co-exist with cGnRH-I neurons and fibers in the LS and caudal most septal area (Teruyama and Beck, 2001; Chaiseha et al., 2004). Furthermore, synaptic connections between VIP axons and GnRH cell bodies or dendrites in the lateral septal-POA are observed (Kiyoshi et al., 1998). In comparison to GnRH, the delayed maximal photoperiodic activation of VIP neurons enables the VIPergic system to inhibit GnRH after the annual peak of the latter is reached (Sharp and Blache, 2003). There is an inverse relationship between VIP and GnRH peptide contents in the MBH at the beginning of the photorefractoriness period (Deviche et al., 2000) and it lasts as the period progresses (Rozenboim et al., 1993a). VIP immunoneutralization

significantly increases pituitary contents of LH-β and FSH-β mRNAs and is accompanied by a decline in PRL mRNA expression (Ahn et al., 2001). It is suggested that PRL acts concomitantly with VIP to inhibit LH through the reduction of GnRH at the hypothalamic level (Rozenboim et al., 1993b).

It is well known that adrenergic stimulation at the hypothalamic level can release GnRH and increase gonadotropins secretion (Yu et al., 1991). There are evidences suggest an inhibitory role of DA on GnRH release in both mammals and birds (Ramirez et al., 1984; Sharp et al., 1984). GnRH axons are terminated in the external layer of the ME, which is closed proximity to the terminals of TIDA neurons (Ajika, 1979; Merchenthaler et al., 1984; Ugrumov et al., 1989), but little is known about these GnRH neuronal group(s) that project to the ME (Dawson and Goldsmith, 1997; Teruyama and Beck, 2000). In addition, 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 (Contijoch et al., 1992; Fraley and Kuenzel, 1993). Moreover, the interactions between the VIP and GnRH systems are innervated by VIP-ir fibers and GnRH-I-ir neurons in the LSO in the Ac, the caudalmost septal area, and the ME (Teruyama and Beck, 2001). Activation of DAergic neurons in the ML is associated with the activation of GnRH-I and VIP neurons and the subsequent release of LH and PRL (Al-Zailaie et al., 2006). Therefore, it is reasonable to consider whether any regional specificity exists in those DA neurons that are neuroendocrine in nature, for example, controlling the secretion and expression of VIP/PRL and GnRH/FSH-LH systems.

Another factor affecting GnRH secretion is a GnIH (Tsutsui et al., 2000). It has been indicated that GnIH is a regulator of gonadotropins release both *in vitro* and

in vivo. A direct effect of GnIH on pituitary release of LH in the Japanese quails is indicated in *in vitro* study which demonstrates that GnIH inhibits LH and FSH syntheses and their release (Ciccone et al., 2004). GnIH also inhibits circulating LH levels *in vivo*. Intraperitoneal administration of GnIH into the Japanese quails via osmotic pump results in significantly reduced plasma LH levels (Ubuka et al., 2006). GnIH injected concurrently with GnRH inhibits the LH surge above the baseline in song sparrows (Osugi et al., 2004). GnIH injections also decrease plasma LH levels in breeding free living Gamble's white-crowned sparrows (Osugi et al., 2004). ICV infusion of GnIH into the V III induces a sharp decrease of plasma LH levels in photostimulated female white-crowned sparrows (Bentley et al., 2006a; 2006b). Moreover, it has been demonstrated that GnIH also inhibits gonadotropins production and their release (Ciccone et al., 2004; Tsutsui et al., 2005; 2006; Ubuka et al., 2006). The expression of GnIH receptor mRNA is found in the diencephalon and the ME closed to cGnRH-I fibers terminal in the quails (Bentley et al., 2006b) and in the pituitary gland (Yin et al., 2005). In addition, an increase in MEL levels during a short day photoperiod induces an increase in GnIH gene expression (Bentley et al., 2003; Ciccone et al., 2004), suggesting that MEL seems to act directly since GnIH neurons are equipped with MEL receptors (Ubuka et al., 2005).

2.7 Maternal Behaviors in Birds

Most mothers display maternal behaviors after parturition and serve the immediate provision of care and defense for their offspring (Brunton and Russell, 2008). In fact, the life maintenance of the species is depended on the presence of precise maternal care in the period that the child is dependent on the mother (Swain et

al., 2007). In mammals, the patterns of maternal care consist of internal incubation of eggs during gestation, delivery of the young at parturition, and maternal care until weaning (Rosenblatt, 2003). The mechanisms underlying the control of maternal behaviors may be derived from the processes involving in gestation, parturition, or the regulation of lactation in mammals including changes in circulating levels of progesterone, estrogen, oxytocin, and PRL (Numan, 1994; Ziegler, 2000; Rosenblatt, 2002). These hormonal activities increase in the POA, the area that important for the expression of maternal behaviors (Featherstone et al., 2000). Furthermore, the experience that new mothers gain as they interact with their newborn young has long-term consequences, and stimuli from the young promote maternal responsiveness and facilitate the maternal behaviors (Fleming and Sarker, 1990).

Maternal care in birds is included incubation and brooding or rearing behaviors. The term incubation refers to the maternal care of unhatched eggs and brooding is the maternal care of chicks after hatching (El Halawani et al., 1988a). Incubation behavior in birds is qualified by sitting continually on their eggs until they hatch, while brooding or rearing behavior is directed to the care of newly hatched chicks (Richard-Yris et al., 1983; El Halawani et al., 1988a; Ruscio and Adkins-Regan, 2004; Sharp, 2009). Generally, the hens develope maternal behaviors gradually in four stages; brooding, titbitting, clucking, and normal broody behavior (Ramsay, 1953). The incidence of maternal behaviors concurs with a pause in laying and a significant long-term fall in the plasma levels of gonadal steroids (Richard-Yris et al., 1983; 1988). Brooding behavior or rearing behavior consists of sheltering chicks under the wing, leading the chicks to food or away from danger, and in some species, calling to the young (Cain et al., 1978). Birds that exhibit brooding behavior

allow chicks to access and remain underneath their wings, whereas birds that do not show brooding behavior actively avoided the chicks (Ruscio and Adkins-Regan, 2004).

2.7.1 Incubation Behavior

It has been well established that the physiology and behavior associated with incubation behavior are the complex ones. Some of the physiological changes include elevated circulating PRL levels, reduced circulating gonadotropins levels, reduced circulating ovarian steroids levels, ovarian regression, cessation of laying, and altered neurotransmitter activity in the brain. The behavioral patterns that associated with incubation behavior include nesting activity, nest protection activity, and anorexia (for review, see El Halawani et al., 1988a).

The onset of incubation behavior is related to nesting frequency and egg laying. Nesting frequency increases in association with the development of an increase in PRL concentrations at night until the first day of incubation, when the hens stop laying, nesting activity progressively extends to occupy the nest most of the day and has transformed to full incubation behavior (Lea et al., 1981). The hens sit on their clutches and persistently turn their eggs, rearranging the eggs to guarantee that they are well covered. This behavior is associated with the cessation of egg laying, clucking, and loss of feathers from the breast to form a brood patch. Normally, the incubation behavior and the cessation of egg laying begin after the hens accumulated a full clutch of the eggs. The bantam hens have accumulated about 10-20 eggs per clutch. However, the turkey hens may incubate their eggs although not stop egg laying (Lea and Sharp, 1982). In some birds, the same number of eggs is laid whether

or not eggs are removed from the nests, while the birds are still laying (Moss and Watson, 1982).

Most avian species that incubate their eggs develop a defeathered, edematous, and hyperemic area of skin, which includes most of the caudal ventral thoracic and portion of the cranial ventral abdominal regions, named brood patch. This brood patch develops prior to initiation of the incubation behavior and functions to facilitate heat transfer from the hen to the eggs and the transmission of tactile stimuli to the hen (for review, see El Halawani et al., 1988a). In the turkeys, tactile stimuli at the brood patch appear to be mediated the suppression of PRL concentrations than by auditory or visual stimuli (Opel and Proudman, 1985). It has been reported that anesthesia applied to the brood patch of incubating ducks suppresses the PRL concentrations (Hall and Goldsmith, 1983). Further evidence suggests that the brood patch formation begins about 5 days prior to the onset of incubation behavior (Lea et al., 1981). Administration of estrogen accompanied with PRL results in the development of the brood patch in the canaries and white crowned sparrows (Bailey, 1952; Steele and Hinde, 1963). Birds eat and drink very little and lose their weights during the incubation period. This weight loss has been reported in the turkeys (Zadworny et al., 1985), bantam chickens (Savory, 1979), geese (Akesson and Raveling, 1981), ducks (Gatti, 1983), and native Thai chickens (Kosonsiriluk, 2007). Normally, incubation behavior is terminated when the chicks are hatched, but may persist for a prolonged period if the nest contains unhatched eggs. Many wild birds species that incubate infertile eggs persist for about 50 % longer than that of normally require hatching them (Skutch, 1962). During extended incubation period, the bantam hens demonstrate more ingestive behavior such as feeding and drinking than searching

behaviors such as foraging or random walking. These behaviors are reversed when the duration of incubation increase (Sharp, 1997).

2.7.2 Neuroendocrine Regulation of Incubation Behavior

The onset of incubation behavior is correlated with declining concentrations of LH and gonadal steroids and increasing concentrations of PRL (Lea et al., 1981; El Halawani et al., 1988a; El Halawani and Rozenboim, 1993). It is this rising PRL level which has been implicated as the cause of cessation of ovulation, ovarian regression, and induction and maintenance of incubation behavior. Subsequently, PRL level declines, whereas LH level begins to increase when incubation behavior terminates (El Halawani et al., 1988a; Knapp et al., 1988), and as soon as molting is stopped (Bluhm et al., 1983a; 1983b; Mauget et al., 1994). LH concentration begins to increase at the onset of hatching the young (Sharp et al., 1979; Goldsmith and Williams, 1980; Hall, 1987; Zadworny et al., 1988; Kuwayama et al., 1992) or when presence of the chicks (Richard-Yris et al., 1987a; 1987b; 1995; Sharp et al., 1988; Leboucher et al., 1990; 1993). It has been well established that the increased in PRL concentrations maintains incubation behavior (Sharp et al., 1988). In the turkeys, incubation behavior is facilitated by the combined physiological action of estradiol, progesterone, and PRL (El Halawani et al., 1986). Stimulus of nesting maintains high circulating PRL levels in incubating hens. Removal of incubation turkeys and native Thai hens from their nests results in a dramatic decline in plasma PRL levels (El Halawani et al., 1980; Proudman and Opel, 1981; Prakobsaeng et al., 2009; 2011; Prakobsaeng, 2010). It has been further reported that ovarian hormones suppress LH secretion during incubation behavior and high level of plasma PRL supports this

suppression (Lea et al., 1996). The expression rate of incubation behavior and the plasma levels of PRL and LH are depended on rearing conditions in the turkey hens (Bedecarrats et al., 1997). In addition, the peripheral nervous inputs act on the onset of incubation behavior (Book et al., 1991). The areas of the brain involved in the expression of incubation behavior are the nucleus tuberis, nucleus preopticus medialis, nucleus ovoidalis, and paleostriatum primitivum (Georgiou et al., 1995).

2.7.3 Rearing Behavior

It is well documented that maternal experience, neurotransmitters, neurohormones, hormones, and stimuli from the young interact in a complex fashion to promote maternal responsiveness in mammals and birds. The expression of rearing behavior in avian species results from the presence of chicks. The influence of the presence of chicks induces the emergence of specific maternal behaviors and produces maternal vocalizations such as clucking and food calling. The maternal hens display physical contact with the chicks by brooding the chicks for longer durations after hatching, while clucking and food calling are regularly behavior emitted in hens rearing of the young (Richard-Yris et al., 1995; 1998; Ruscio and Adkins-Regan, 2004). In many galliform birds, newly hatched chicks are precocial and can walk, feed, see, and hear upon hatching, but they cannot effectively thermoregulate during 2 weeks after they are hatched (Mills et al., 1997). Therefore, brooding by the maternal hens can help them to survive. Brooding behavior consists of the hens allowing the chicks to nestle underneath its slightly raised wings, while assuming a distinct crouching porture (Hess et al., 1976). Stimuli from the young chicks are clearly involved in the appearance and maintenance of this maternal care behavior (Richard-

Yris and Leboucher, 1987; Opel and Proudman, 1989). Several galliform birds including the chickens, turkeys, and Japanese quails can be induced brooding behavior by introducing the newly hatched chicks to them, which the hens present immediate maternal responses (Richard-Yris et al., 1983; Richard-Yris and Leboucher, 1986; 1987; Opel and Proudman, 1988a; Leboucher et al., 1990; 1991; 1993; Ruscio and Adkins-Regan, 2004). It has been suggested that physical contact between hen and chicks, alone or in combination with visual and/or auditory stimuli originating from the chick, induces brooding behavior (Maier, 1963; Richard-Yris and Leboucher, 1987; Richard-Yris et al., 1998). In a broody hen with chicks, a bond is formed and the chicks learn to respond to the maternal food calling, distress call, and to the hens purring sound (Wauters and Richard-Yris, 2002; 2003; Edgar et al., 2011). Repeated exposure of the chicks to the hen, accompanied by food, guidance and protection, strengthen the maternal-offspring bonds (Wauters and Richard-Yris, 2001). During embryonic development, exposure to maternal calls may be impotant for the development of post-hatch species-specific maternal call recognition (Gottleib, 1976; Jain et al., 2004). In precocial species, birds are self-sufficient after hatching, but parents serve an important protective function while also teaching the chicks about food avoidance and food preference (Nicol and Pope, 1996; Nicol, 2004). During the incubation period, the comsumption of feed and water of mother hens decreases compared with laying hens, and then the intake of feed and water increases rapidly after the chicks are hatched (Zadworny et al., 1985). It has been suggested that changes in the concentrations of PRL may be related to the large changes in intermediary and water metabolism that occurred during broodiness (Zadworny et al., 1985). Moreover, the relationships between the mothers and the precocial young has

been elucidated during their first days of life, the characteristics of mothers influence the emotional and social behavioral development of their young (Bertin and Richard-Yris, 2005; Richard-Yris et al., 2005). Precocial chicks can be filial imprinting on their parents in the first few days of life (Rodgers, 1995; Mills et al., 1997).

In addition to its well established role as an incubation promoting hormone, PRL has also been implicated in the control of behavior patterns associated with care of the young after hatching (Buntin, 1986; Vleck, 1998). PRL secretion is stimulated by exposure of the mother hens tactile and visual stimuli from the chicks, while PRL facilitates/stimulates the expression of maternal behaviors such as incubating, brooding, or feeding (Angelier and Chastel, 2009). An increase in PRL secretion is involved in the transition from sexual to parental activity (Sharp et al., 1998). Thus, the levels of PRL are most elevated during the rearing period (Buntin, 1996), and this is reflected in changes in circulating PRL levels in birds with different maternal care behaviors. Depending on the species, either sharp drops or slow decreases of PRL levels after hatching have been reported (Goldsmith and Williams, 1980; Dittami, 1981; Oring et al., 1986; Hall, 1987; Oring et al., 1988, Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). In many galliform birds, PRL facilitates the induction of rearing behavior. Injection of ovine PRL induces the display of the maternal covering stance normally adopted during brooding of chicks (Opel and Proudman, 1980). PRL concentrations usually decline rapidly after the chicks are hatched in species with precocial young, and the presence of the chicks can modify this rate of decline (Dittami, 1981; Opel and Proudman, 1989). In incubating hens, the substitution of chicks for eggs, or the appearance of chicks at hatching, is associated with an increase in plasma LH levels and a marked decrease in plasma PRL from the high levels

presented during incubation period (Zadworny et al., 1988; Leboucher et al., 1991; Richard-Yris et al., 1998). Indeed, exposure to chicks can induce maternal behavior in the incubating, non-incubating, and ovariectomized hens, which exhibit marked differences in circulating levels of gonadal steroids and patterns of PRL secretion (Richard-Yris et al., 1987a; Leboucher et al., 1991; Lea et al., 1996). Unlike the domestic chickens, which rear the precocial young, the altricial species are reared jointly by both parents. The columbiform birds such as pigeons and doves feed the newly hatched chicks by regurgitating crop milk, which is produced by epithelial mucosa cells that proliferated in response to PRL and ultimately slough from the crop sac wall (Buntin, 1996; Wang and Buntin, 1999). In addition to stimulating crop milk production, PRL concentrations is elevated during the early post-hatching phase may also promote the display of parental behaviors that are essential for transferring the crop milk to the young squabs (Buntin et al., 1991). PRL concentrations begin to decrease after the chicks achieve thermal independence and do not require constant brooding (Goldsmith, 1991).

2.7.4 Neuroendocrine Regulation of Rearing Behavior

In avian species, PRL is involved in many aspects of reproductive physiology and behaviors. It is thought to play a pivotal role in parental behaviors by mediating increases in incubation, crop milk production/secretion, feeding of the young, and nest defense (Silver, 1984; Janik and Buntin, 1985; Lea et al., 1986; Buntin et al., 1991). It is very well established that PRL play a significant role in maternal behaviors. The roles of PRL in the induction and maintenance of maternal behaviors have been extensively investigated. In birds, the concentrations of PRL are low before and

during egg laying, increase before incubation, are maintained at high levels during incubation and then decrease sharply to basal levels immediately after the young are hatched (bantams: Sharp et al., 1979; mallard ducks: Goldsmith and Williams, 1980; Japanese bantams: Zadworny et al., 1985; 1988; native Thai chickens: Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). In Gifujidori hens, circulating PRL levels reach the minimum value about 1 week after hatching (Kuwayama et al., 1992).

There are abundant evidences exist to link maternal behaviors in several species of birds with an increase in PRL secretion. High circulating PRL levels are known to be associated with rearing behavior in the chickens (Sharp et al., 1979; 1988; Bedrak et al., 1981; Lea et al., 1981; Hoshino and Wakita, 1989), turkeys (Burke and Dennison, 1980; Proudman and Opel, 1981), mallard ducks (Goldsmith and Williams, 1980), and Auatralian black swans (Goldsmith, 1982). PRL is generally accepted as a crucial factor to the onset and maintenance of broodiness in avian species. LH secretion is depressed and PRL secretion is at its highest levels in incubating birds (Goldsmith, 1985). It has been indicated that the patterns of PRL secretion are regulated by the expression of parental behaviors (Dawson and Goldsmith, 1982). PRL also acts, in part, at the gonadal level to reduce estradiol production and causes the regression of large ovarian follicles in the chickens and turkeys (Opel and Proudman, 1980; Zadworny et al., 1989). The studies in which broody chickens (Sharp et al., 1979), ducks (Goldsmith and Williams, 1980), and Auatralian black swans (Goldsmith, 1982) have been allowed to hatch and rear the young have shown that PRL concentrations decline when the incubation period is terminated. Further evidence in the duck suggests that this decline may occur on or before the day of hatching (Goldsmith and Williams, 1980). Similar studies in the

turkeys have been reported that the drop in PRL levels at the end of incubation period could be related to the pipping and hatching eggs, and the consequent shift to maternal behaviors (Wentworth et al., 1983; Opel and Proudman, 1989). Moreover, brooding behavior is also characterised by low levels of LH and gonadal steroids. LH secretion may also be inhibited by increased plasma PRL levels, suggesting that high levels of circulating PRL is involved in the initiation or maintenance of brooding behavior and the possibility of an antigonaldal role in birds (Bedrak et al., 1981; Sharp et al., 1988; Zadworny et al., 1988; 1989).

The establishment of maternal behaviors is accompanied by a significant long term fall in plasma LH levels. The previous studies have been demonstrated the relationship between circulating PRL levels and brooding behavior in the parents of precocial young. The gradual decrease of plasma PRL levels is related to the decline in brooding behavior with the age of chicks (Dittami, 1981; Opel and Proudman, 1989). In shorebirds and red-necked phalaropes, facultatively polyandrous and only males care for the eggs and young, plasma PRL levels in broody males decline gradually with increasing age of the broods (Gratto-Trevor et al., 1990). In several species, circulating PRL levels decline dramatically at the time of hatch (Goldsmith and Williams, 1980; Dittami, 1981; Goldsmith, 1982; Hall and Goldsmith, 1983; Wentworth et al., 1983). In the pigeons and ring doves, PRL stimulates the growth and development of specialized epithelial cells lining the crop sac, leading to formation of crop milk, which is fed to the newly hatched. A rise in PRL concentration is associated with the onset or maintenance of egg incubation and care of young in a number of free-living passerine species (Goldsmith, 1991; Buntin, 1996). The expression of brooding behavior changes in endocrinological parameters

and production performances in the turkey hens (Guemene and Williams, 1992). During the hens exhibit broodiness, plasma LH concentrations decrease progressively while plasma PRL concentrations increase. High concentrations of PRL are maintained for a long period throughout the brooding behavior and cause the decrease in ovulation rate and egg production. In addition, *in vitro* study demonstrates that PRL synthesis and release are high in the pituitary gland of broody hens. These changes in hormonal synthesis, release, and concentrations are related to brooding behavior (Hoshino and Wakita, 1989).

In galliform birds, PRL is involved in maternal behaviors and PRL release is presented throughout the rearing period. The presence of chicks induces the emergence of specific maternal behaviors in many avian species (Maier, 1963; Richard-Yris et al., 1983; Richard-Yris and Leboucher, 1987; Leboucher et al., 1990; 1993; Wang and Buntin, 1999). Replacement of the eggs by chicks induces maternal behaviors in incubating, non-incubating, and ovariectomized hens (Richard-Yris et al., 1987a; 1995; 1998; Leboucher et al., 1990; 1993; Lea et al., 1996). At the same time, PRL concentrations decline, whereas concentrations of ovarian steroid hormones remain at low levels (Richard-Yris et al., 1987a; Sharp et al., 1988, Leboucher et al., 1990). In addition, physical contact with newly hatched chicks during brooding bouts slows down the decrease of PRL secretion and inhibits LH and estradiol release in maternal hens (Leboucher et al., 1993). PRL secretion in broody hens is facilitated by the presence of chicks and those high levels of plasma PRL concentrations maintain rearing behavior (Sharp et al., 1988). Moreover, on the day when chicks are introduced, brooding hens immediately show maternal responses along with plasma estradiol levels slightly decrease. It is possible that coexistence of

newly hatched chicks may suppress LH secretion of the hen in the natural breeding cycle (Kuwayama et al., 1992). When chicks grow older and become fledged, brooding behavior progressively declines, leading to a fall in PRL concentrations (Richard-Yris et al., 1987a; 1989; Sharp et al., 1988). Therefore, plasma PRL levels decrease after the eggs hatched, the levels remain high for several days then decline gradually as the young are reared, and then reach the basal levels by the time the young are fledged. The expression of maternal behaviors results from the presence of chicks and the introduction of chicks induces a fall in plasma PRL levels and a moderate increase in concentrations of LH and steroid hormones in the incubating domestic chickens (Richard-Yris et al., 1987a; 1995; Opel and Proudman, 1989; Leboucher et al., 1990; 1993; Sharp et al., 1988; Lea et al., 1996) and turkeys (Opel and Proudman, 1988a). These features suggest that maternal care and particularly physical contact with the young chicks may play a key role in producing these differences (Richard-Yris et al., 1995).

It is also very well established that the presence of chicks inhibits the adenohypophysial-ovarian axis in incubating (Richard-Yris et al., 1987a; Sharp et al., 1988) and non-brooding hens (Richard-Yris et al., 1983; 1987b). On the other hand, physical contact with the chicks induces brooding behavior, an immediate drop in PRL concentrations and a gradual rise in LH concentrations (Richard-Yris et al., 1998). After the chicks are hatched, the levels of LH begin to increase gradually while plasma PRL levels decline (Sharp et al., 1979; Zadworny et al., 1988). It is stated that PRL is not secreted at an increased rate during the hens are caring for their young. The bantam hens stop showing broody behavior between 4 to 10 weeks after the chicks are hatched and correspond to the time when the concentrations of LH increase

to the levels found in laying hens (Sharp et al., 1979). In contrast, plasma PRL levels remain at high levels after hatching and then decline when body mass and structure size of the young are closed to those of the hen, the maternal care behavior decline linearly with brooding behavior as well (Boos et al., 2007). In some species, stimuli from the young or from the parent-young interactions may promote or sustain the elevated in PRL levels. It has been suggested that a definite threshold in circulating PRL levels is necessary to promote and/or maintain post-hatching maternal behavior in precocial birds. On the other hand, rearing chick-hens and subsequent hens that are removed their entire chicks exhibit an abruptly increase in plasma LH levels concurrently with the decrease of plasma PRL levels (Leboucher et al., 1990). Similarly with brooding Gifujidori hens, plasma PRL levels decrease rapidly on the day of hatching, and reach minimum values about 1 week after hatching while concentrations of LH and estradiol gradually increase after hatching and reach the peak values immediately after the removal of chicks (Kuwayama et al., 1992).

Immunocytochemical studies have shown that the expression of progesterone receptor (PR) immunoreactivity in the tuberal hypothalamic area (TR) declines in brooding birds as VIP immunoreactivity increases (Askew et al., 1997; Clark et al., 1999), suggesting that progesterone may act via the PR in the TR to inhibit VIP secretion and subsequently to delay PRL secretion until incubation behavior has become firmly established (Lea et al., 2001). It has been further suggested that the PR in the POA mediates the expression of incubation behavior while PR in the TR is involved in the control of neuroendocrine function(s) (Askew et al., 1997). During the transition from egg laying to the parenting phase, PR immunoreactivity decreases in the TR and significant increases in the DAergic activity and the numbers of

mesotocin-ir neurons (MT-ir; the avian analog of mammalian oxytocin) occurred in specific neural regions including the PVN, nucleus dorsomedialis anterior thalami, and nucleus supraopticus, pars ventralis (SOv) of incubating hens compared to the laying hens (Lea et al., 2001; Thayananuphat et al., 2011). When the hens shift from incubating eggs to brooding of the young, the majority of c-fos mRNA expression by MT-ir neurons is observed within the PVN and SOv while the majority of c-fos mRNA expression in the DAergic neurons is observed in the ventral part of the POM (Thayananuphat et al., 2011). The brain areas that have been implicated in the mediation of parental behaviors in birds are the POA, ventromedial nucleus of the hypothalamus, and PVN (Slawski and Buntin, 1995; Schoech et al., 1998; Lea et al., 2001). Effects of the POA lesions disrupt PRL-induced parental feeding behavior in ring doves (Slawski and Buntin, 1995). Patterns of the expression of an immediate early gene protein products, fos-like immunoreactivity (fos-ir), in the brains of brooding ring doves and Japanese quails during given tactile to their young show more fos-ir in the POA, lateral hypothalamus, LS, MPOA, and the BSTM than those of parents not allowed to contact with their young (Ruscio and Adkins-Regan, 2004; Buntin et al., 2006). Following ICV injections of D_2 DA or oxytocin receptor antagonists into hens brooding poults, over 80 % of those hens fail to brood their poults and they also have less c-fos mRNA in the dorsal part of POM and the medial part of the BSTM areas, indicating that the DAergic, through its D2 DA receptor and MTergic systems may play a role in regulating brooding behaviors in birds (Thayananuphat et al., 2011).

Up to date, there are limited available that describe the interrelationship and the functional aspect of the changes in the VIPergic and the GnRH systems with those

in PRL levels during rearing behavior in the native Thai chickens. Therefore, this dissertation was designed to investigate an association between the VIPergic and the GnRH-I systems as well as the important role of PRL in the regulation of rearing behavior in the native Thai chickens. The findings gained from this dissertation will provide, for the first time, information on neuroendocrine regulation of rearing behavior in the native Thai chickens, which could help to improve the productivity of the native Thai chicken in Thailand.

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CHAPTER III

EFFECTS OF REARING BEHAVIOR ON THE NEUROENDOCRINE REGULATION OF THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKENS: ROLE OF PROLACTIN

3.1 Abstract

Prolactin (PRL) plays a significant role in reproduction, maternal care, and parental behavior in many vertebrate species. The role of PRL in the induction and maintenance of maternal behaviors has been extensively investigated. In birds, an increase in circulating PRL levels has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior. However, there are limited data regarding the neuroendocrine regulation of rearing behavior in the native Thai chicken, a continuously breeding species found in the equatorial zones, which highly expresses maternal behaviors including incubation behavior and broodiness. The objectives of this study were to elucidate the circulating PRL levels in hens rearing chicks during the rearing period as well as to compare the changes in plasma PRL levels of rearing (R) hens with those of non-rearing (NR) hens. Native Thai hens were divided into 2 groups, R hens which were allowed to rear their chicks and NR hens which were not allowed to rear their chicks by removing the chicks from them after hatching. Blood samples were collected in R and NR hens for

determining plasma PRL levels by enzyme-linked immunosorbent assay. The ovaries and oviducts were collected, weighed, and the presences of follicles were recorded after the hens were sacrificed. The results revealed that plasma PRL concentrations remained at high levels on the day the chicks hatched then rapidly decreased after the first week of hatching and remained at low levels throughout the eight weeks of the rearing period. During the first five weeks of the rearing period, plasma PRL levels of R hens were compared with those of NR hens. The levels of plasma PRL were markedly decreased in hens that had their chicks removed and reached the lowest levels by the third week of separation from chicks. In the R hens, plasma PRL levels were high when compared to those of the NR hens. Disruption of rearing behavior by removing their chicks increased the ovary and oviduct weights, the presence of ovarian follicles, and the number of egg laying hens. In conclusion, this study indicates that plasma PRL levels are associated with rearing behavior in native Thai chickens. External cues such as physical contact with chicks and the presence of chicks are involved in the maintenance of plasma PRL levels and rearing behavior in this equatorial non-photoperiodic continuous breeder bird.

3.2 Introduction

Prolactin (PRL), a polypeptide hormone, is synthesized and secreted from the lactotrophs, specialized cells of the anterior pituitary gland (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000). To date, over 300 different physiological functions of PRL have been proposed, highlighting the importance of this pituitary hormone (Harris et al., 2004). In mammals, PRL plays a significant role in reproduction, maternal care, and parental behaviors. It has an essential role for

lactation, since it is involved in the development of mammary gland (Bern and Nicoll, 1968), milk synthesis, and maintenance of milk secretion. In birds, PRL is involved in many aspects of reproductive physiology and behaviors. It plays a pivotal role in parental behaviors by mediating the increases in incubation, crop milk production/secretion, feeding of young, and nest defense (Silver, 1984; Janik and Buntin, 1985; Lea et al., 1986; Buntin et al., 1991; Prakobsaeng et al., 2011). In addition, 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 in chickens, turkeys, pigeons, pheasants, mallard ducks, cow birds, and native Thai chickens (Breitenbach and Meyer, 1959; El Halawani et al., 1997; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011). It has been well documented that gonadotropins and PRL are associated with the reproductive cycle in several avian species (turkeys: Mashaly et al., 1976; El Halawani et al., 1984; 2001; Wong et al., 1992; mallards: Bluhm et al., 1983a; canvasback ducks: Bluhm et al., 1983b; cockatiels: Myers et al., 1989; emperor penguins: Lormee et al., 1999; king penguins: Mauget et al., 1994; tropical seabirds: Lormee et al., 2000; geese: Boos et al., 2007; Huang et al., 2008; native Thai chickens: Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). Changes in circulating PRL levels during the avian reproductive cycle are well documented (El Halawani et al., 1988; 2001; Kosonsiriluk et al., 2008). Plasma PRL levels are low in reproductively quiescent birds, with levels increasing in reproductively active laying hens. Circulating PRL levels are elevated throughout incubation (El Halawani et al., 1984; Sharp et al., 1989; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008).

Abundant evidence implicating that an increase in PRL secretion is the causative factor for the reduced circulating gonadotropins exists in several avian species. For example, in galliform birds, the onset of incubation behavior is associated with declining levels of gonadotropins and ovarian steroids (Sharp et al., 1979; Burke and Dennison, 1980; Bedrak et al., 1981; Lea et al., 1981) and a dramatic rise in circulating PRL levels (Goldsmith, 1985; 1991; Lea, 1987; El Halawani et al., 1988; Sharp et al., 1988; Porter et al., 1991) and it is this rising PRL level which has been implicated as the cause of cessation of ovulation, ovarian regression, induction and maintenance of incubation behavior. Subsequently, PRL levels decline, whereas luteinizing hormone (LH) levels 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., 1983a; 1983b; Mauget et al., 1994). It has been suggested that high levels of PRL inhibit LH secretion (Zadworny and Etches, 1987). Furthermore, administration of mammalian PRL into laying turkeys causes ovarian regression (Opel and Proudman, 1980; Hargis et al., 1987), and inhibits exogenous gonadotropin-stimulated secretion of ovarian steroids (Camper and Burke, 1977).

The control of PRL secretion in avian species 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. These external stimuli and 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 (Curlewis, 1992). It is very well established that the regulation of avian PRL secretion and PRL gene expression is influenced by hypothalamic vasoactive

intestinal peptide (VIP), the avian PRL-releasing factor (PRF; El Halawani et al., 1997; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005; Kosonsiriluk et al., 2008). In birds, PRL secretion is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968) and that principal PRF is VIP (El Halawani et al., 1997; 2001). Variations in VIP immunoreactivity, VIP contents, and VIP mRNA steady-state levels occurring within the hypothalamus as well as of VIP levels in hypophysial portal blood are correlated with changes in circulating PRL levels throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999). 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). In contrast with mammals, it has been established that dopaminergic (DAergic) influences are involved in both stimulating and inhibiting avian PRL secretion depending on multiple subtypes of DA receptors (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003). It is very well established 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). Dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, 5-HTergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (El Halawani et al., 2001; 2004).

It has been known for a long time that hormonal control of maternal behaviors in various species includes PRL. The role of PRL in the induction and maintenance of maternal behaviors has been extensively investigated. In mammals, PRL begins to

increase toward the end of gestation, when it is crucial for inducing milk production. In combination with progesterone and estrogen, PRL reduces the latency of onset of maternal behaviors (Bridges and Ronsheim, 1990). In birds, PRL concentrations are low before and during egg laying, increase before incubation, are maintained at high levels during incubation and then decrease rapidly to basal levels immediately after the young are hatched (bantams: Sharp et al., 1979; mallard ducks: Goldsmith and Williams, 1980; Japanese bantams: Zadworny et al., 1988; turkeys: El Halawani et al., 2001; native Thai chickens: Kosonsiriluk et al., 2008). There is abundant evidence indicating a correlation of maternal behaviors in several avian species with an increase in PRL secretion. For example, high PRL levels are known to be associated with incubation behavior in the chickens (Sharp et al., 1979; 1988; Bedrak et al., 1981; Lea et al., 1981; Hoshino and Wakita, 1989), turkeys (Burke and Dennison, 1980; Proudman and Opel, 1981; Porter et al., 1991; El Halawani et al., 2001), mallard ducks (Goldsmith and Williams, 1980) and Australian black swans (Goldsmith, 1982a). PRL is generally accepted as a crucial factor to the onset and maintenance of incubation behavior in avian species. LH secretion is depressed and PRL secretion is at its highest levels in incubating birds (Goldsmith, 1985). It has been indicated that patterns of PRL secretion are controlled by the expression of parental behaviors (Dawson and Goldsmith, 1982), and PRL causes regression of large ovarian follicles in the chickens and turkeys (Opel and Proudman, 1980). The studies in which broody chickens (Sharp et al., 1979), ducks (Goldsmith and Williams, 1980), and swans (Goldsmith, 1982a) have been allowed to hatch and rear the young have shown that PRL levels decline at the end of the incubation period. Further evidence in ducks suggests that this decline may occur on or before the day of

hatching (Goldsmith and Williams, 1980). Similar studies have been reported in the turkeys that the decline in PRL levels at the end of incubation could be related to the pipping and hatching of eggs and the consequent shift to maternal brooding behaviors (Wentworth et al., 1983).

In several avian species, PRL levels decline dramatically at the time of hatching (Goldsmith and Williams, 1980; Dittami, 1981; Goldsmith, 1982b; Hall and Goldsmith, 1983; Wentworth et al., 1983). In pigeons and doves, PRL stimulates the growth and development of specialized epithelial cells lining the crop sac, leading to formation of crop milk, which is fed to the newly hatched birds. The rise in PRL levels is associated with the onset or maintenance of egg incubation and care for the young in a number of free-living passerine species (Goldsmith, 1991; Buntin, 1996). In addition, the expression of incubation behavior changes endocrinological parameters and production performances in the turkey hens (Guemene and Williams, 1992). When the hens exhibit incubation behavior, plasma LH levels decrease progressively while plasma PRL levels increase (Porter et al., 1991). High circulating levels of PRL are maintained for a long period throughout incubation behavior and cause the decrease in ovulation rate and egg production. Moreover, *in vitro* studies demonstrate that PRL synthesis and release are high in the pituitary gland of incubating hens and these changes are related to incubating behavior (Hoshino and Wakita, 1989).

In contrast of the temperate zone seasonal breeding species, the native Thai chicken is a continuously breeding species found in the equatorial zone that produces eggs all year, which is independent of photoperiodic cues (Kosonsiriluk, 2007; Sartsoongnoen, 2007; Kosonsiriluk et al., 2008). The native Thai hens highly express

maternal behaviors including incubation behavior and broodiness (Prakobsaeng et al., 2011). There are limited data regarding the neuroendocrine regulation of rearing behavior in this gallinaceous bird from the non-temperate zone. To date, it has been well established that incubation behavior in this species is regulated by the VIP/PRL, gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH), and DAergic systems (Sartsoongnoen et al., 2006; 2008; 2012; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011). Plasma PRL and LH levels across the reproductive cycle of the native Thai chickens have been reported. Changes in numbers of VIP-ir neurons within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas are directly correlated with changing plasma PRL levels throughout the reproductive cycle, suggesting that VIP expression in the IH-IN of the native Thai chickens plays a regulatory role in year-round reproductive activity (Kosonsiriluk, 2007; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). Moreover, it has been demonstrated that changes in the number of VIP-ir neurons in the IH-IN are associated with DAergic neurons within the nucleus intramedialis (nI) and nucleus mamillaris (ML) areas, resulting in PRL release to induce and maintain incubation behavior in the native Thai chickens. It is further suggested that nesting activity stimulates PRL secretion by the activation of the DAergic system, which, in turn stimulates the VIPergic system. The subsequently elevated PRL levels are thought to increase nesting activity and maintain incubation behavior (Prakobsaeng et al., 2011).

Little is known about the relationship between the VIP/PRL and GnRH/FSH-LH systems and the rearing behavior, in contrast to incubation behavior. Therefore, the present study was designed to further investigate the role of PRL in the regulation

of rearing behavior in the native Thai chickens. Changes in plasma PRL levels as well as ovary and oviduct weights were compared between rearing and non-rearing native Thai hens. The findings gained from this study will provide, for the first time, information on neuroendocrine regulation of rearing behavior in the native Thai chickens, which could help to improve the productivity of the native Thai chicken.

3.3 Materials and Methods

3.3.1 Experimental Animals

Female and male native Thai chickens (*Gallus domesticus*), Pradoohangdam breed, 22-24 weeks old, were used. They were reared and housed (9-11 females : 1 male) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of darkness; 12L : 12D). Each hen was identified by wing band number. Feed and water were given *ad libitum*. After the chicks were hatched, the hens were then randomly divided into two treatment groups; hens rearing chicks (R) and hens that had been removed from their chicks (NR). Hens in the R group were allowed to rear their chicks naturally. Hens in the NR group were disrupted from rearing behavior and not allowed to rear their chicks by removing them from the chicks to another pen. Egg production, nesting activity, and other behaviors were recorded daily throughout the experiments. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee.

3.3.2 Experimental Design

3.3.2.1 Experiment I

Fifty four female and 6 male native Thai chickens, 22-24 weeks old, were used. The chickens were randomly divided into 6 floor pens (9 hens : 1 rooster) and their behaviors observed daily. After hatching, the hens were allowed to rear their chicks. Blood samples were collected from the brachial vein of each hen prior to euthanization with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France) at different time periods (day of hatch, weeks 1, 2, 3, 4, 5, 6, 7, and 8 after the chicks were hatched). At the end of the experiment, the ovaries and oviducts were collected and weighed after the hens were sacrificed and the presence of F1-F5 follicles, small yellow follicle (SYF), and small white follicle (SWF) were recorded. The criteria used to classify the follicles were revised from Etches (1993). The ovary of laying hens that contained a hierarchy of yellow yolky follicles with the diameter longer than 1 cm were identified as F1, F2, F3, F4, and F5 and several smaller follicles from which the large yolky follicles are recruited. The small follicles were classified according to their diameters as SYF (5-9 mm) and SWF (1-4 mm). Blood samples were fractionated by centrifugation, and the plasma samples were stored at -20 °C until used to determine plasma PRL levels by enzyme-linked immunosorbent assay (ELISA). Egg production, nesting activity, and other behaviors were recorded daily throughout the experiment.

3.3.2.2 Experiment II

Fifty five female and 5 male native Thai chickens, 24 weeks old, were used. The chickens were randomly divided into 5 floor pens (11 hens : 1 rooster) and their

behaviors observed daily. After hatching, the hens were divided into two groups; R and NR. Blood samples were collected from the brachial vein of each hen prior to euthanize with pentobarbital sodium (Nembutal) at different time periods (day of hatch, weeks 1, 2, 3, 4, and 5 after hatching) of R or NR hens. At the end of the experiment, the ovaries and oviducts were collected and weighed after the hens were sacrificed and the presence of F1-F5 follicles, SYF, and SWF were recorded. The criteria used to classify the follicles were revised from Etches (1993) as aforementioned.

3.3.3 PRL Hormone Assay

Plasma PRL levels were determined using an ELISA according to a previously described method (Kosonsiriluk et al., 2008). 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 (pH 7.4) at the dilution of 1:2,000 and incubated overnight at 4 °C. Surfaces were blocked by addition of blocking solution (100 µl per well of 0.4 % casein in 0.15 M PBS (pH 7.2) containing 1.0 mM EDTA and 0.02 % thimerosal). After incubation, plates were washed three times in 0.03 M PBS containing 0.05 % Tween 20. The assay buffer was 0.15 M PBS (pH 7.2) containing 0.1 % casein, 1.0 mM EDTA, and 0.02 % thimerosal. Fifty microliters of samples (10 µl plasma diluted in 40 µl of assay buffer) or standards containing chicken PRL (Dr. A.F. Parlow, National Hormone and Peptide Program, USA) were added and 25 µl each of biotinylated PRL (1:50,000 dilution), and rabbit anti-chicken PRL (Dr. John Proudman, USDA, USA) at 1:20,000 dilution were then added into the reaction, and

incubated overnight at 4 °C. After incubation, plates were washed and 0.1 ml of streptavidin horseradish peroxidase (1:5,000 dilution) were added. After 2 h at room temperature, plates were washed and 0.1 ml ABTS reagent (0.04 % 2,2'-azino-bis-3-ethylbenzthizoline-6-sulfonic acid and 0.015 % H_2O_2 in 0.1 M citrate phosphate buffer, pH 4) were added. After 1 h incubation at room temperature, the color reaction was measured at 405 nm in a Tecan Sunrise ELISA reader (Tecan Group Ltd., Mannedorf, Switzerland). The assay of plasma PRL levels in native Thai chickens was validated. Pooled plasma samples of native Thai chickens produced a dose-response curve that paralleled a chicken PRL standard curve. Plasma samples were determined in duplicate within a single assay. The intraassay coefficient of variation was 5.0 %, and the sensitivity was 3.9 ng/ml.

3.3.4 Statistical Analysis

Significant differences in plasma PRL levels and ovary and oviduct weights (means ± SEM) according to each treatment group were compared utilizing one-way analysis of variance (ANOVA). Significant differences between treatment groups were computed utilizing Tukey's HSD Test. $P<0.05$ was considered as statistically significant. All statistical tests were analyzed employing the SPSS for Windows Software (version 13.0, SPSS Inc., Chicago, IL, USA).

3.4 Results

3.4.1 Experiment I

The profiles of plasma PRL levels during the eight-week rearing period are shown in Figure 3.1 and Table 3.1. Plasma PRL concentrations remained at high levels on the day the chicks were hatched ($P<0.05$; 115.36 ± 35.56 ng/ml), then rapidly decreased after the first week of hatching (42.23 ± 6.51 ng/ml) and remained at low levels throughout the eight-week rearing period.

The reproductive characteristics of hens rearing chicks at different time periods were also recorded. The presence of F1-F5 follicles, SYF, and SWF (Figure 3.2) were observed. During the rearing period, the presences of F1-F5 follicles, SYF, and SWF at different time are shown in Table 3.2. The results showed that F1-F5 follicles were not observed in hens that were rearing their chicks for 1 to 5 weeks. A few of the hens rearing chicks for 5 weeks exhibited the presence of SYF. However, the presence of SWF was found in all of hens rearing chicks. The hens showed the presence of F1-F5 follicles between weeks 6 to 8 of the rearing period. A number of hens started to lay, the new laying cycle, in week 8 of the rearing period.

The ovaries of hens rearing chicks are shown in Figure 3.3. The ovary weights of hens rearing chicks are shown in Figure 3.4 and Table 3.3. The ovary weights were significantly decreased since the chicks were hatched and remained low until week 4 of the rearing period. The ovary weights of hens rearing chicks were significantly increased and reached the highest weight at week 8 of the rearing period ($P<0.05$; 24.33 ± 9.72 g).

The oviducts of hens rearing chicks are shown in Figure 3.5. Similarly, the oviduct weights of hens rearing chicks are shown in Figure 3.6 and Table 3.4. The

oviduct weights of hens rearing their chicks were significantly increased at week 8 of the rearing period ($P<0.05$; 24.69 ± 9.20 g).

3.4.2 Experiment II

The plasma PRL concentrations of R and NR hens at different time periods are shown in Figure 3.7 and Table 3.5. During the first five weeks of the rearing period, PRL levels of R hens were compared with those of NR hens. The levels of plasma PRL (ng/ml) significantly decreased at week 1 after the chicks were removed from the hens ($P<0.05$; R1 vs NR1; 34.20 ± 1.78 vs 27.36 ± 1.69) and week 3 ($P<0.05$; R3 vs NR3; 41.08 ± 8.61 vs 18.54 ± 1.54). However, plasma PRL levels were not different between R and NR hens at weeks 2, 4, and 5. Taken together, in the R hens, plasma PRL levels were high when compared to those of the NR hens. Disruption of rearing behavior by removing their chicks was accompanied by a decline in plasma PRL levels.

The reproductive characteristics of R and NR hens at different time periods were also recorded. The presence of F1-F5 follicles, SYF, and SWF (Figure 3.2) were observed in both groups. In the R hens, the presences of F1-F5 follicles, SYF, and SWF at different weeks of rearing period are shown in Table 3.6. The results showed that F1-F5 follicles were not observed in R hens for 1 to 5 weeks. A few R hens that were rearing their chicks for 5 weeks exhibited the presence of SYF. However, the presence of SWF was found in all R hens. In contrast, the numbers of NR hens that exhibited the presence of F1-F5 follicles, SYF, and SWF are shown in Table 3.7. More than 80 % of NR hens showed the presence of F1-F5 follicles within 2 weeks of removing the chicks. Most of the NR hens exhibited the presence of SYF and all of

them exhibited the presence of SWF. A number of NR hens started to lay, the new laying cycle, in week 3 after removal the chicks. Moreover, at weeks 3 to 5 of separation the chicks from hens, 100 % of NR hens returned to lay (Table 3.7).

The ovaries of R and NR hens are shown in Figure 3.8. The ovary weights of R and NR hens are shown in Figure 3.9 and Table 3.8. When the hens were rearing their young, the ovary weights showed no increase throughout five weeks of the rearing period. In contrast, in the NR group, the ovary weights displayed a significant increase at weeks 2, 3, and 4 ($P<0.05$), and reached the highest weight at the third week after removing the chicks ($P<0.05$; 47.32 ± 6.77 g).

The oviducts of R and NR hens are shown in Figure 3.10. The oviduct weights of R and NR hens are shown in Figure 3.11 and Table 3.9. Similarly, the oviduct weights of R hens showed no increase during the first five weeks of the rearing period. Whereas, in hens that had their chicks removed after hatching, the oviduct weights were significantly increased at week 2 after removing the chicks ($P<0.05$) and reached the highest weight at week 4 ($P<0.05$; 55.66 ± 3.54 g). When compared between both groups, both ovary and oviduct weights of the NR hens were significantly increased by week 2 after removing the chicks and were higher than those of R hens throughout week 1 to week 5 of the observation period.

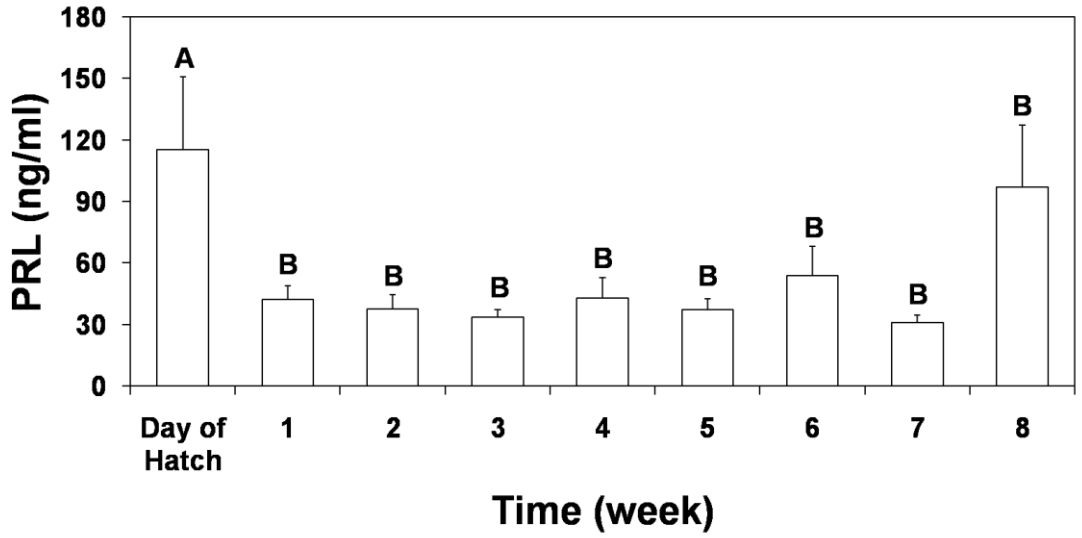

Figure 3.1 Changes in plasma PRL concentrations during eight weeks of the rearing period (n=6) of native Thai hens. Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$).

Table 3.1 Mean ± SEM of the plasma PRL levels (ng/ml) of native Thai hens rearing chicks at different times during the rearing period (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$).

Rearing Time	**PRL (ng/ml)**
Day of Hatch	115.36 ± 35.56^{A}
Week 1	42.23 ± 6.51^{B}
Week 2	37.44 ± 7.09^{B}
Week 3	33.52 ± 3.66^{B}
Week 4	42.74 ± 10.01^{B}
Week 5	37.36 ± 5.21^{B}
Week 6	53.73 ± 14.29^{B}
Week 7	30.87 ± 3.70^{B}
Week 8	96.92 ± 30.24^{B}

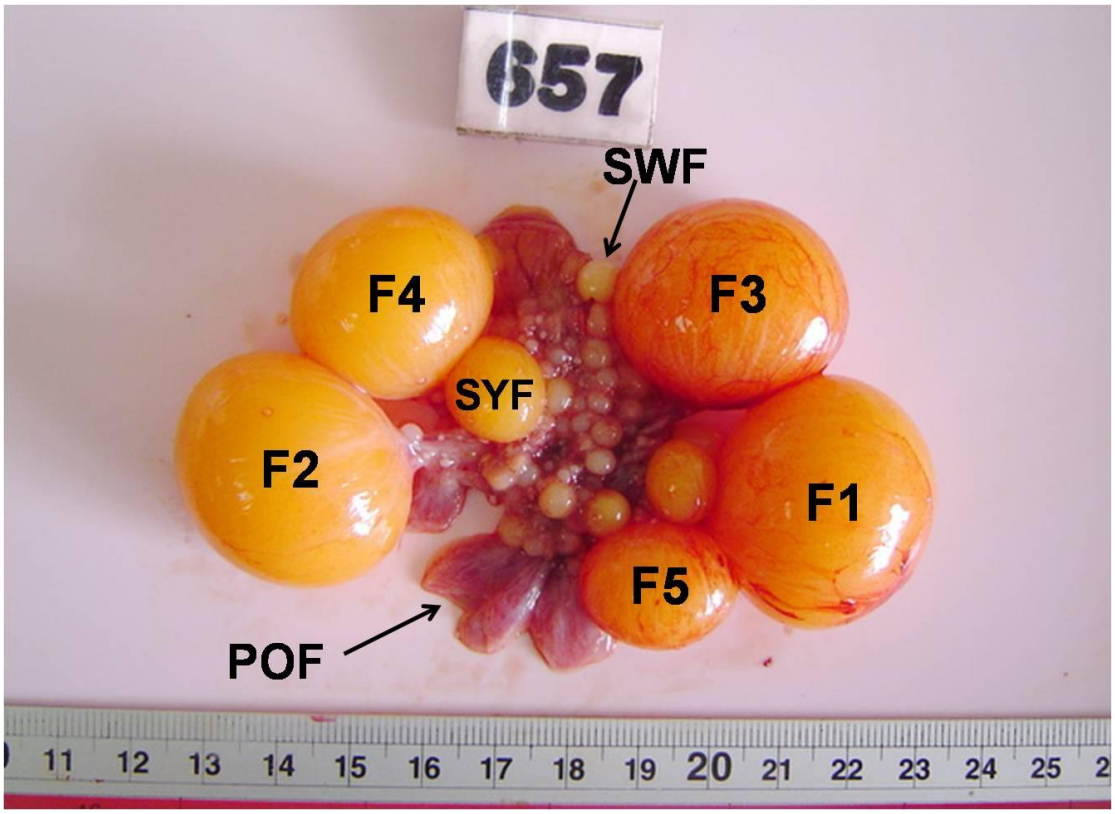

Figure 3.2 Photograph of the ovary of the native Thai hen during the egg laying period showing the F1-F5 follicles, small yellow follicles (SYF), small white follicles (SWF), and post-ovulatory follicles (POF).

Table 3.2 The number of native Thai hens that had the F1-F5 follicles, small yellow follicles (SYF), and small white follicles (SWF) at different times during the rearing period and the number of hens came back to lay in each period (n=6).

Follicles	**Rearing Time (Week)**								
	Day of Hatch	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
F1	0	0	0	0	0	0	3	3	3
F2	0	0	0	0	0	0	0	1	3
F3	0	0	0	0	0	0	0	1	3
F4	0	0	0	0	0	0	0	1	3
F5	0	0	0	0	0	0	0	1	3
SYF	0	0	0	0	0	3	5	3	5
SWF	6	6	6	6	6	6	6	6	6
Laying hens	0	0	0	0	0	0	0	0	1

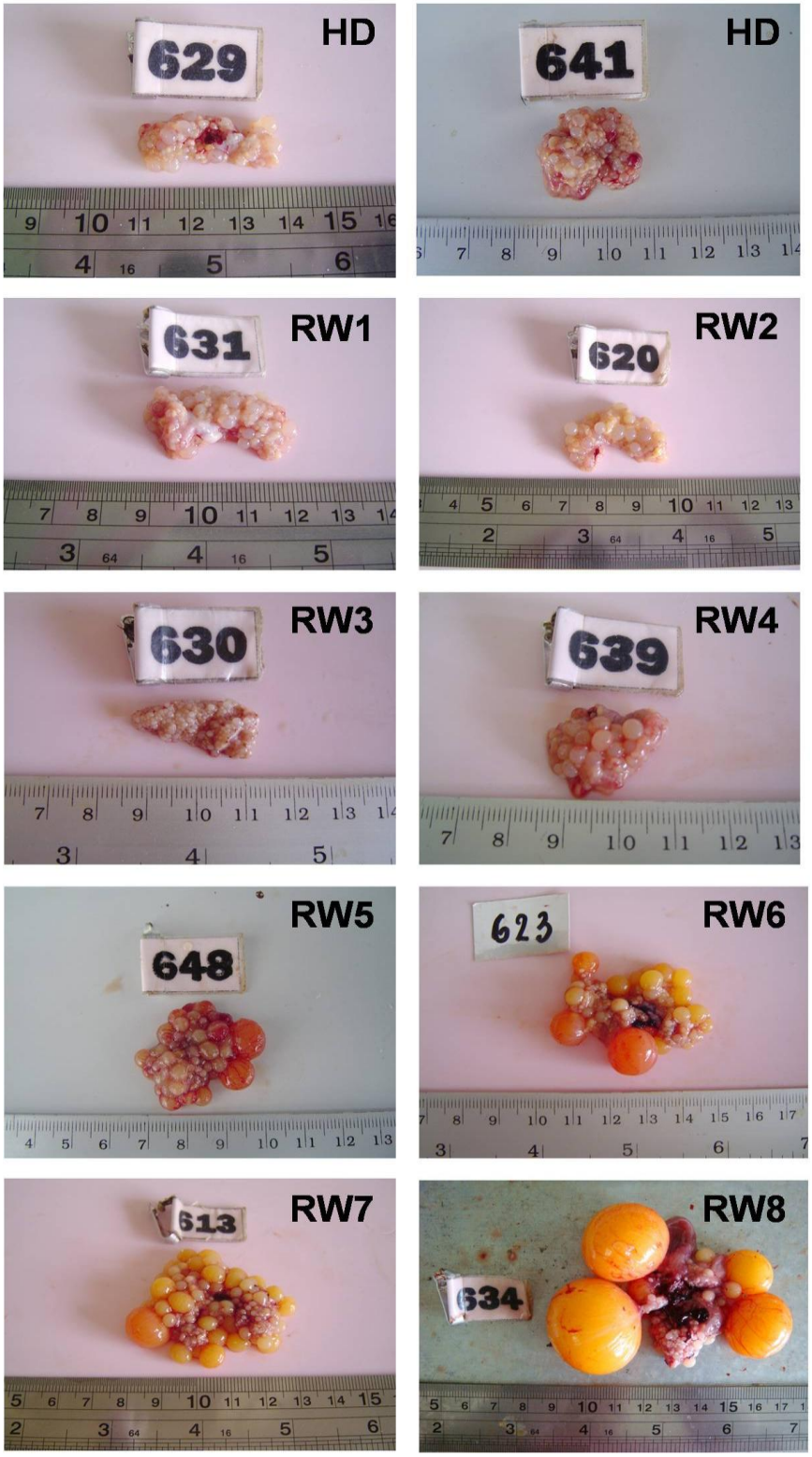

Figure 3.3 Photographs of the ovary of native Thai hens that rearing chicks at day of hatch (HD) and different times during the rearing period.

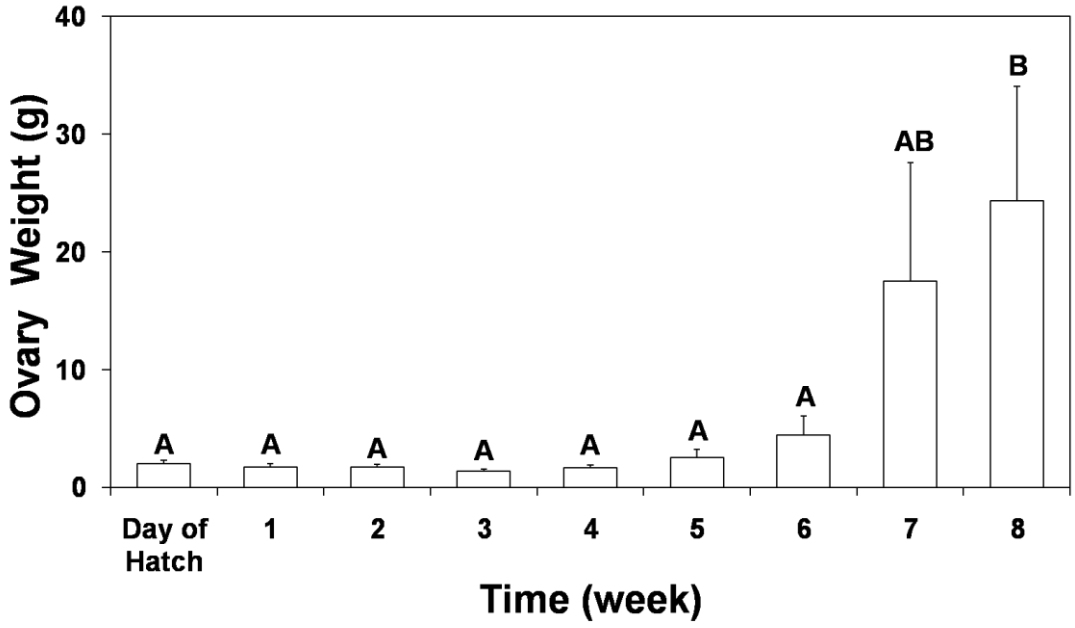

Figure 3.4 Changes in the ovary weight of native Thai hens that rearing chicks. Values are presented as means ± SEM (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$).

Table 3.3 Mean ± SEM of the ovary weight (g) of native Thai hens that rearing chicks at different times during the rearing period (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$).

Rearing Time	**Ovary Weight (g)**
Day of Hatch	2.01 ± 0.27^{A}
Week 1	1.73 ± 0.26^{A}
Week 2	1.76 ± 0.18^{A}
Week 3	1.39 ± 0.16^{A}
Week 4	1.68 ± 0.18^{A}
Week 5	2.52 ± 0.67^{A}
Week 6	4.44 ± 1.62^{A}
Week 7	17.54 ± 10.06^{AB}
Week 8	24.33 ± 9.72^{B}

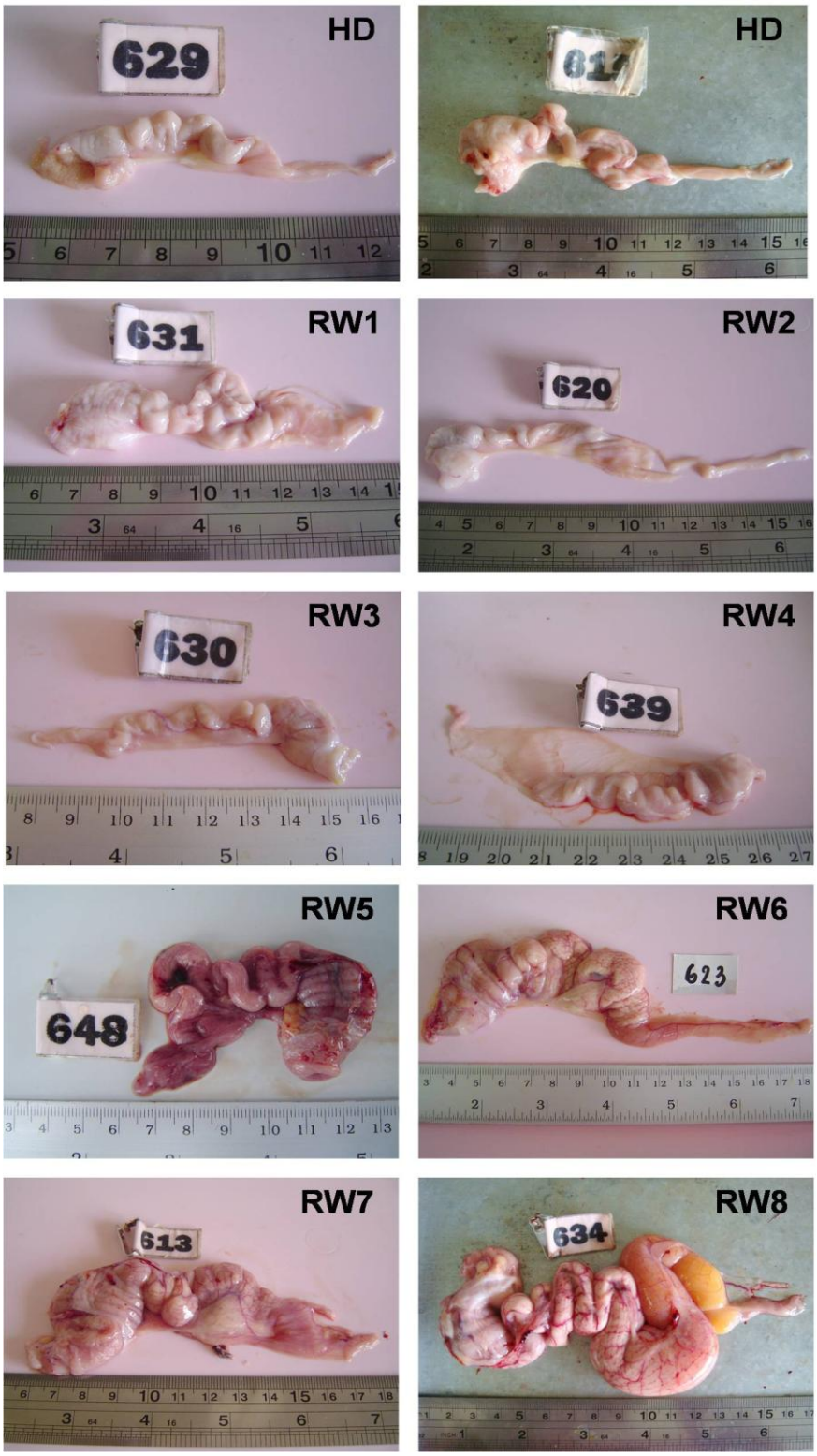

Figure 3.5 Photographs of the oviducts of native Thai hens that rearing chicks at day of hatch (HD) and different times during the rearing period.

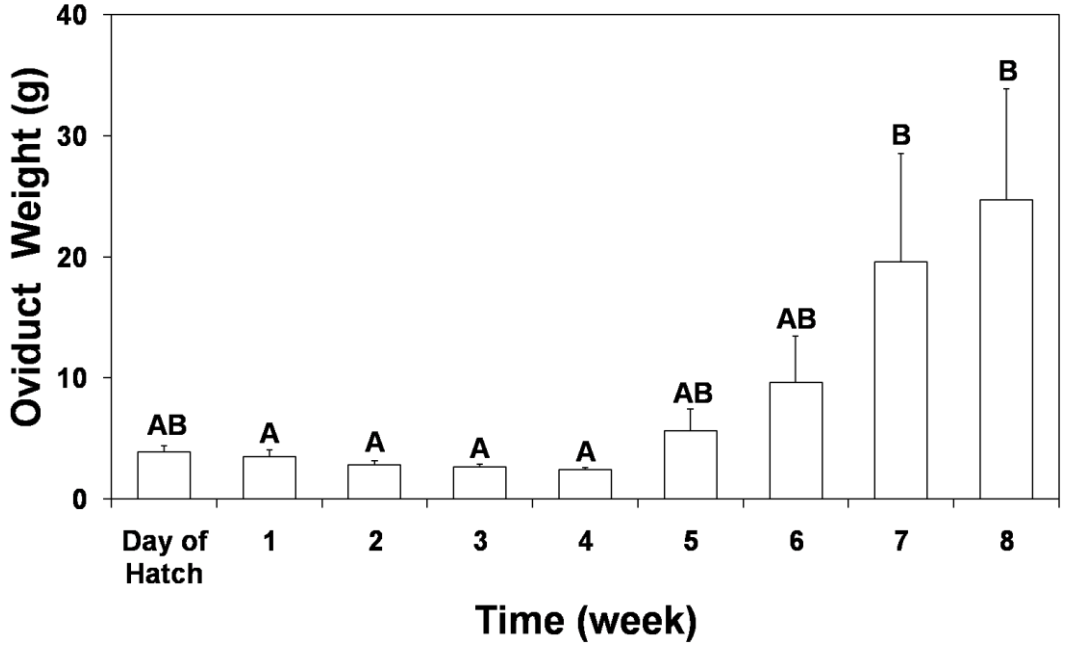

Figure 3.6 Changes in the oviduct weight of native Thai hens that rearing chicks. Values are presented as means ± SEM (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$).

Table 3.4 Mean ± SEM of the oviduct weight (g) of native Thai hens that rearing chicks at different times during the rearing period (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$).

Rearing Time	**Oviduct Weight (g)**
Day of Hatch	3.89 ± 0.49^{AB}
Week 1	3.51 ± 0.55^{A}
Week 2	2.81 ± 0.34^{A}
Week 3	2.64 ± 0.26^{A}
Week 4	2.41 ± 0.18^{A}
Week 5	5.62 ± 1.81^{AB}
Week 6	9.62 ± 3.83^{AB}
Week 7	19.58 ± 8.97^{B}
Week 8	24.69 ± 9.20^{B}

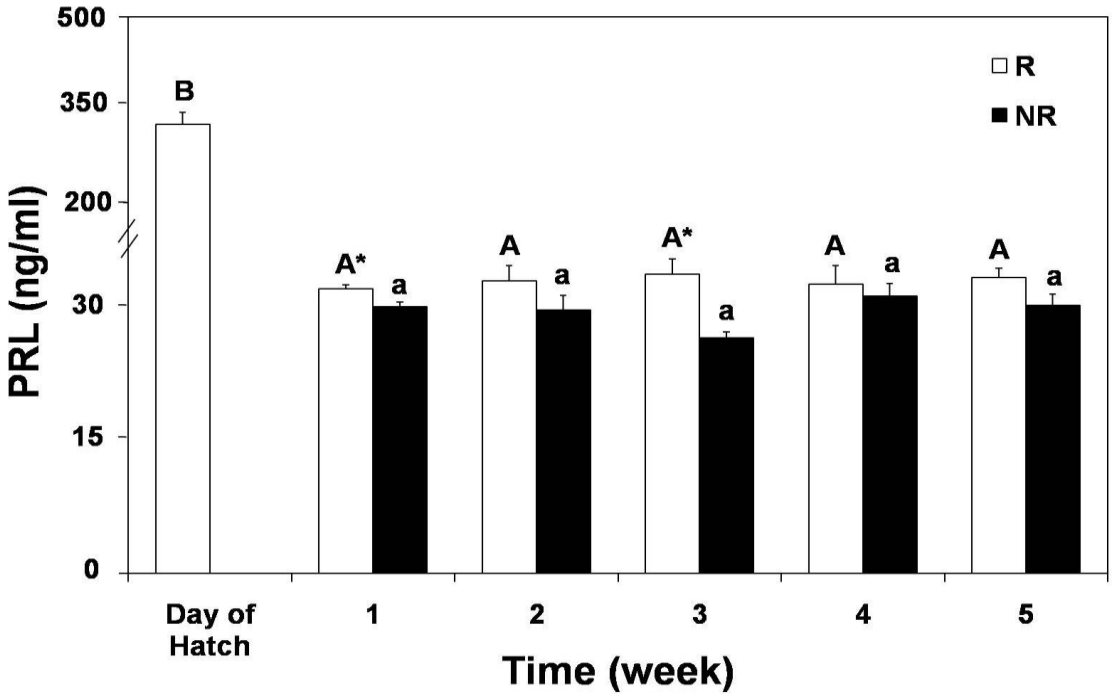

Figure 3.7 Changes in plasma PRL concentrations of rearing (R; n=6) and non-rearing (NR; n=6) native Thai hens. Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 3.5 Mean ± SEM of the plasma PRL concentrations (ng/ml) of rearing (R) and non-rearing (NR) native Thai hens at different times of rearing (n=6) or non-rearing their chicks (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Group	**Time (Week)**					
	Day of Hatch	**1**	**2**	**3**	**4**	**5**
R	262.85 ± 43.51^{B}	$34.20 \pm 1.78^{A*}$	37.80 ± 7.83^{A}	$41.08 \pm 8.61^{A*}$	36.30 ± 9.64^{A}	39.18 ± 4.97^{A}
NR	N/A	27.36 ± 1.69^{a}	26.37 ± 5.21^{a}	18.54 ± 1.54^{a}	31.28 ± 5.33^{a}	27.91 ± 4.26^{a}

Table 3.6 The number of native Thai hens that had the F1-F5 follicles, small yellow follicles (SYF), and small white follicles (SWF) at different times of rearing (n=5).

Follicles	**Rearing Time (Week)**					
	Day of Hatch	**1**	**2**	**3**	**4**	**5**
F1-F5	0	0	0	0	0	0
SYF	0	0	0	0	0	1
SWF	5	5	5	5	5	5

Table 3.7 The number of native Thai hens that had the F1-F5 follicles, small yellow follicles (SYF), and small white follicles (SWF) at different times of non-rearing and the number of hen came back to lay in each period (n=5).

Follicles	**Rearing Time (Week)**					
	Day of Hatch	**1**	**2**	**3**	**4**	**5**
F1	0	1	4	5	5	5
F2	0	1	3	5	5	5
F3	0	1	2	5	5	5
F4	0	1	1	5	5	5
F5	0	1	1	5	5	5
SYF	0	1	4	5	5	5
SWF	5	5	5	5	5	5
Laying hens	0	0	0	5	5	5

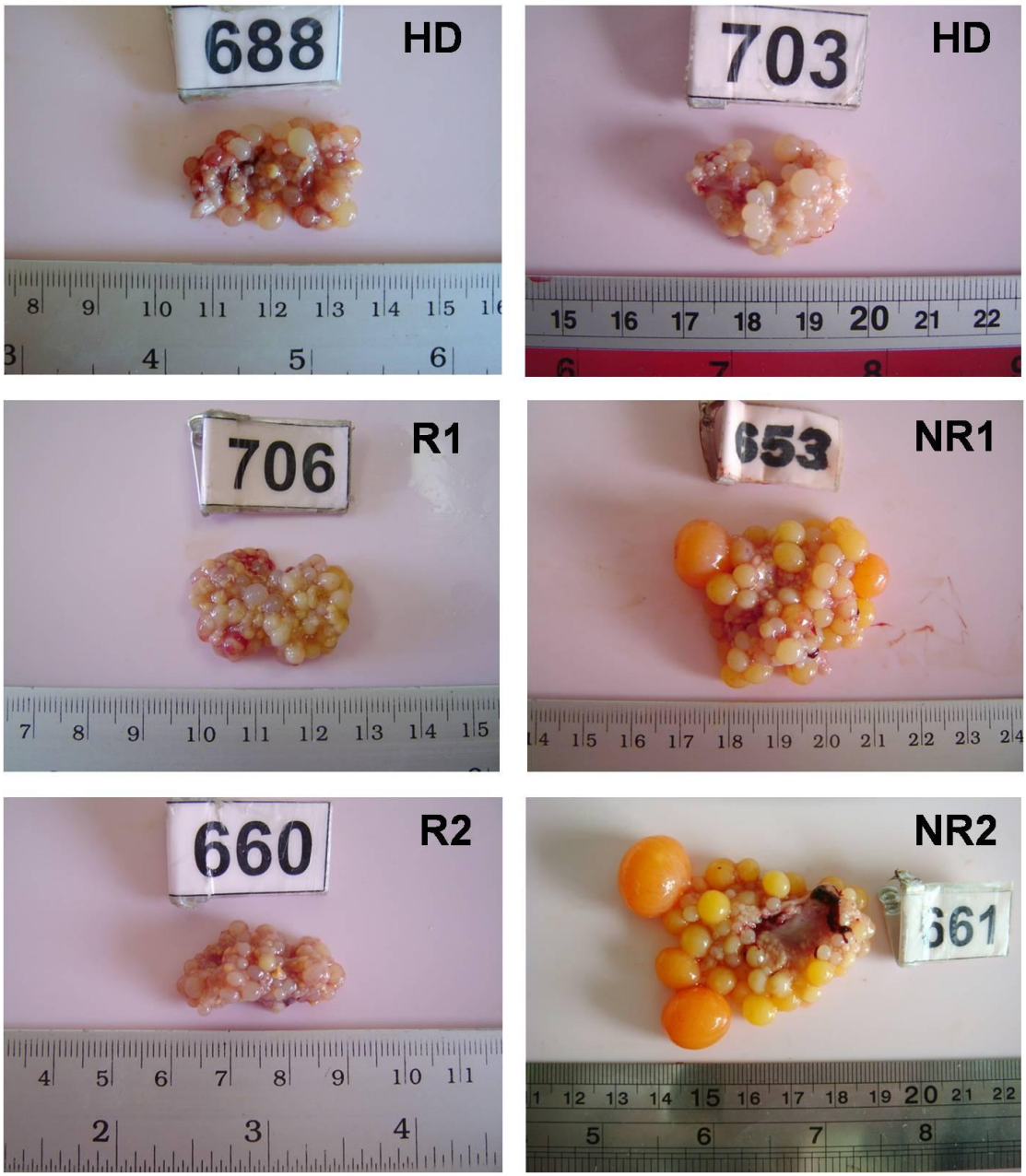

Figure 3.8 Photographs of the ovary of rearing (R) and non-rearing (NR) native Thai hens at day of hatch (HD) and different times of rearing or non-rearing their chicks.

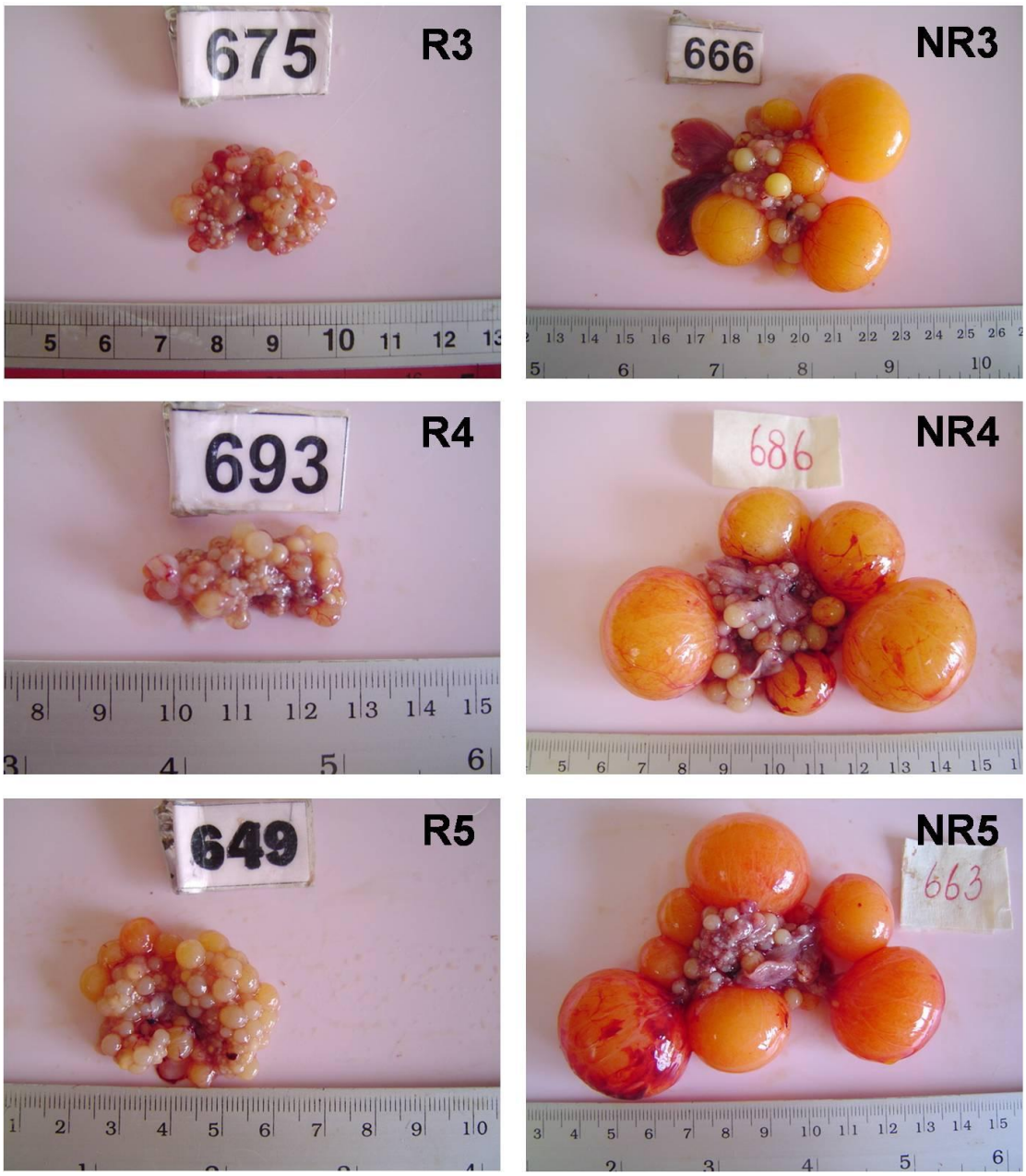

Figure 3.8 Photographs of the ovary of rearing (R) and non-rearing (NR) native Thai hens at different times of rearing or non-rearing their chicks (continued).

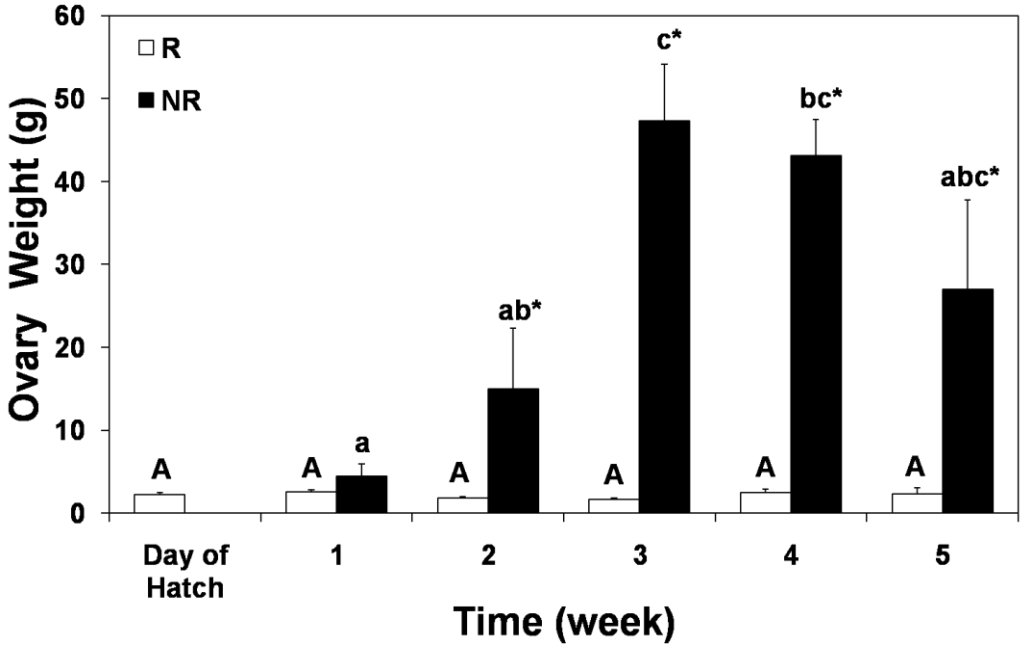

Figure 3.9 Changes in the ovary weights of rearing (R) and non-rearing (NR) native Thai hens. Values are presented as means ± SEM (n=5). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 3.8 Mean ± SEM of the ovary weight (g) of rearing (R) and non-rearing (NR) native Thai hens at different times of rearing or non-rearing their chicks (n=5). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Group	**Rearing Time (Week)**					
	Day of Hatch	**1**	**2**	**3**	**4**	**5**
R	2.25 ± 0.23^{A}	2.55 ± 0.21^{A}	1.85 ± 0.13^{A}	1.61 ± 0.18^{A}	2.45 ± 0.46^{A}	2.27 ± 0.73^{A}
NR	N/A	4.46 ± 1.49^{a}	$14.95 \pm 7.31^{ab*}$	$47.32 \pm 6.77^{c*}$	$43.12 \pm 4.30^{bc*}$	$26.99 \pm 10.79^{abc*}$

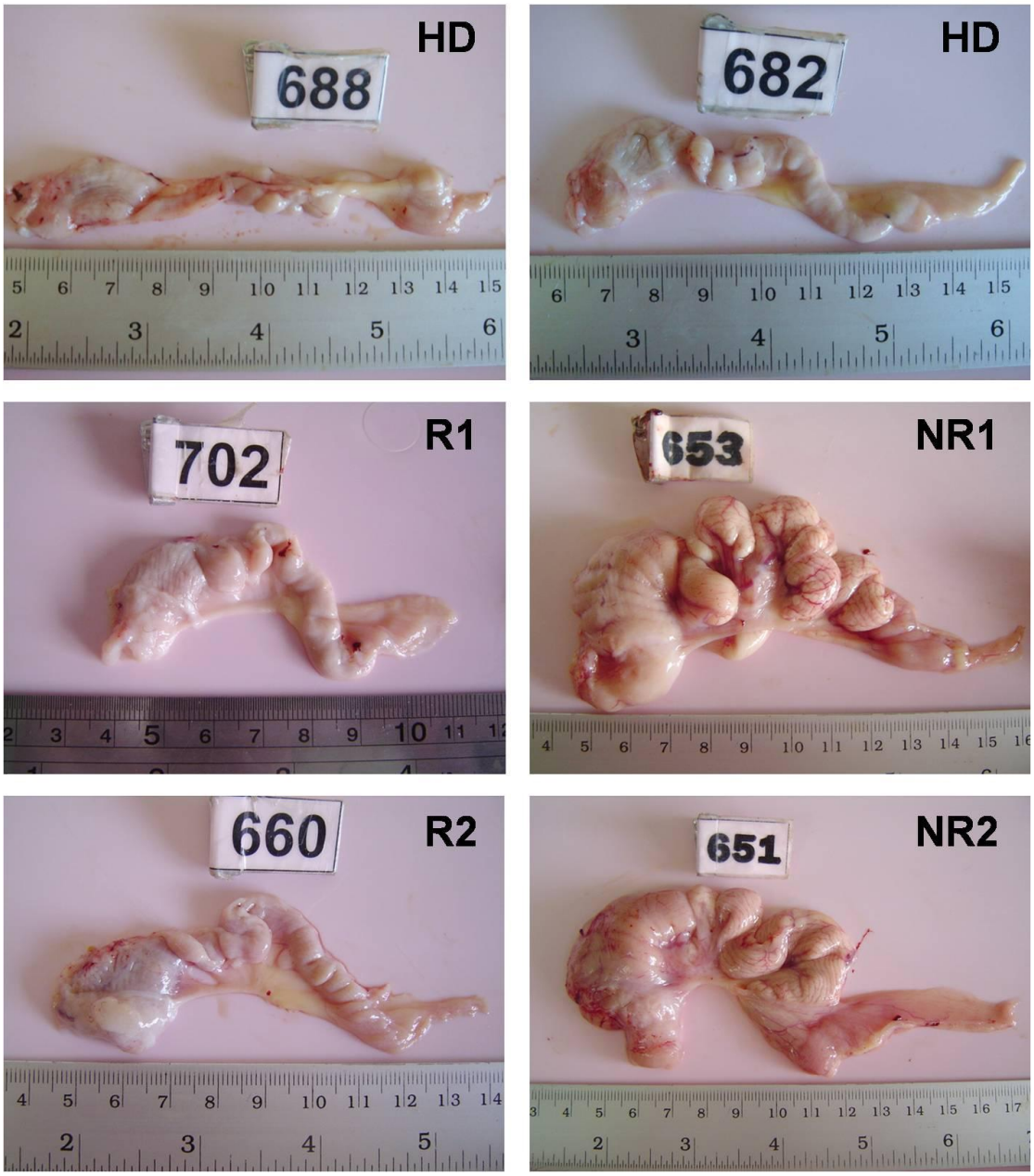

Figure 3.10 Photographs of the oviducts of rearing (R) and non-rearing (NR) native Thai hens at day of hatch (HD) and different times of rearing or non-rearing their chicks.

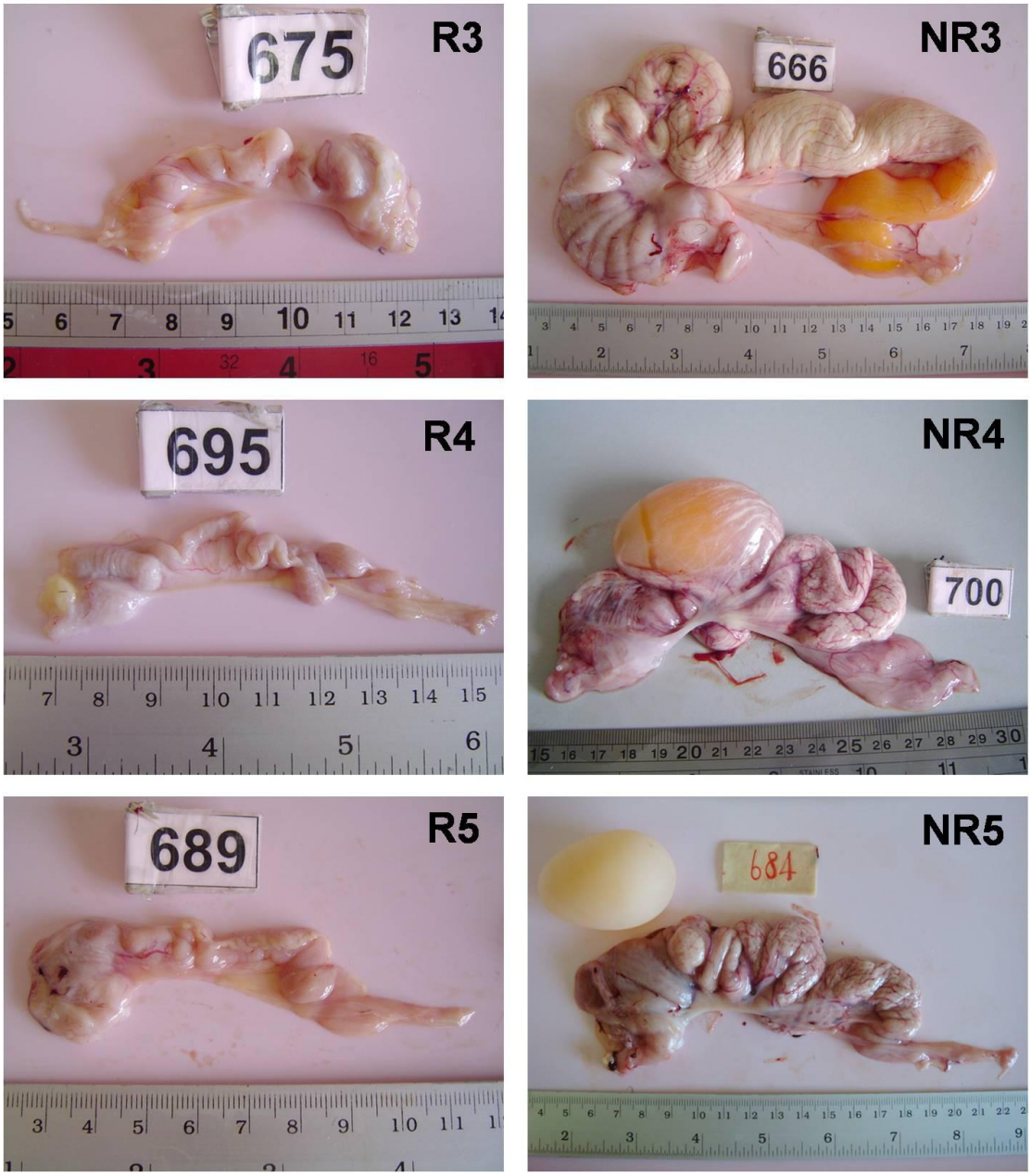

Figure 3.10 Photographs of the oviducts of rearing (R) and non-rearing (NR) native Thai hens at different times of rearing or non-rearing their chicks (continued).

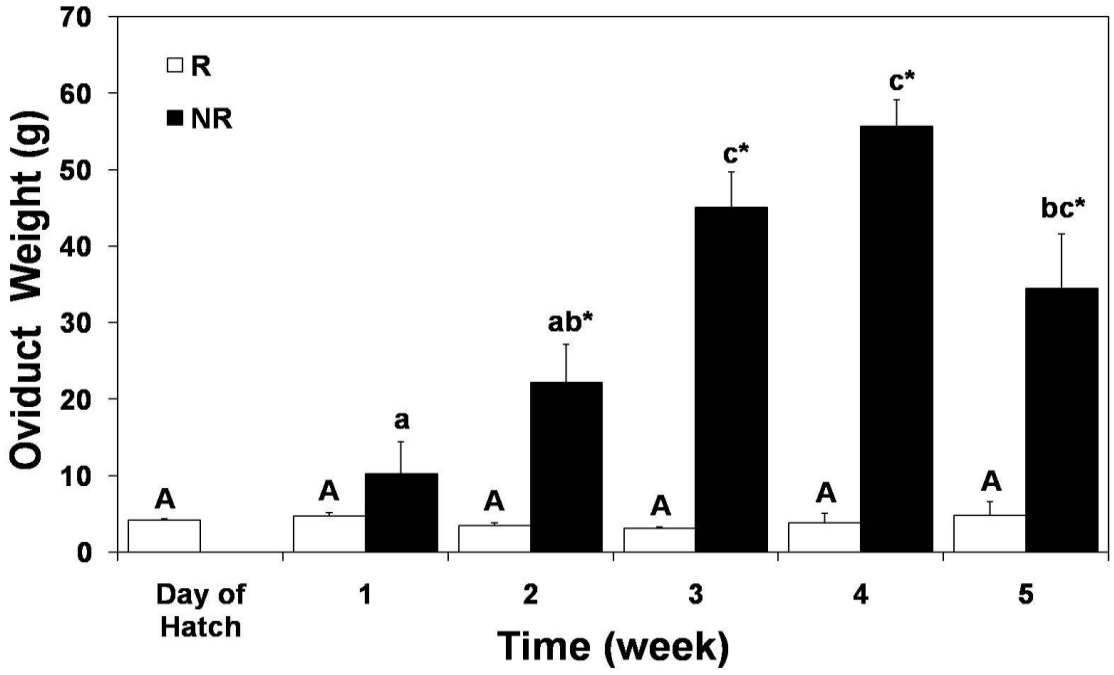

Figure 3.11 Changes in the oviduct weight of rearing (R) and non-rearing (NR) native Thai hens. Values are presented as means ± SEM (n=5). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 3.9 Mean ± SEM of the oviduct weight (g) of rearing (R) and non-rearing (NR) native Thai hens at different times of rearing or non-rearing their chicks (n=5). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Group	**Rearing Time (Week)**					
	Day of Hatch	**1**	**2**	**3**	**4**	**5**
R	4.15 ± 0.22^{A}	4.71 ± 0.48^{A}	3.51 ± 0.33^{A}	3.13 ± 0.23^{A}	3.87 ± 1.28^{A}	4.78 ± 1.87^{A}
NR	N/A	10.28 ± 4.18^{a}	$22.22 \pm 4.98^{ab*}$	$45.03 \pm 4.69^{c*}$	$55.66 \pm 3.54^{c*}$	$34.51 \pm 7.12^{bc*}$

3.5 Discussion

The results of the present study revealed that plasma PRL level is associated with rearing behavior in the native Thai chickens. During the eight weeks of the rearing period, plasma PRL concentrations remained at high levels on the day the chicks were hatched, and then rapidly decreased after the first week of hatching, and remained at low levels throughout the rearing period. Comparisons of plasma PRL levels between R and NR hens during the first five weeks of the rearing period were elucidated. The results showed that the levels of plasma PRL were decreased in hens that had their chicks removed and reached the lowest levels by the third week of separation from the chicks. In the R hens, plasma PRL levels were high when compared to those of the NR hens. Additionally, disruption of rearing behavior by removing their chicks increased the ovary and oviduct weights, the presence of ovarian follicles, and the number of egg laying hens. Therefore, changes in plasma PRL levels are associated with ovarian and oviduct recrudescence and initiation of a new laying cycle in the native Thai chickens.

In the present study, after the chicks were hatched, plasma PRL levels remained at high levels in the R hens. After the first week of rearing period, plasma PRL levels rapidly decreased and remained at low levels throughout the eight weeks of the rearing period. These results are consistent with previous findings that plasma PRL concentrations begin to fall immediately after hatching and continue to decline for about one week after the day of the chicks were hatched (Sharp et al., 1979; Lea et al., 1981; Zadworny et al., 1985; 1988; Leboucher et al., 1990; Kuwayama et al., 1992; Kosonsiriluk et al., 2008). As demonstrated in previous studies of gallinaceous species, changes in plasma PRL levels were observed across the reproductive cycle.

Plasma PRL levels were found to be low in non-laying hens, gradually increased in laying hens, continued to rise and reached the highest levels in incubating hens, and immediately declined to the basal levels when hens are caring for their young (El Halawani et al., 1984; Kosonsiriluk et al., 2008).

Circulating PRL levels of R hens were compared with those of NR hens during the first five weeks of the rearing period. The results showed differences in plasma PRL levels between R and NR hens. In the R hens, plasma PRL levels were higher than those of the NR hens, indicating the secretion of PRL in the R hens is facilitated by the presence of chicks and stimulates maternal behavior which, in turn, suppresses gonadal activity (Sharp et al., 1988; Richard-Yris et al., 1995). Furthermore, the hens that were allowed to rear their chicks returned to lay later than hens that were not allowed to rear the chicks, suggesting that physical contact familiar with auditory, and/or visual stimuli from chicks during the rearing period slows down the decrease of PRL secretion and inhibits gonadotropins and ovarian steroid hormones (Richard-Yris and Leboucher, 1987; Richard-Yris et al., 1987a; 1998; Leboucher et al., 1993). It has been further suggested that the presence of chicks had no effect on PRL secretion but tends to maintain its level (Sharp et al., 1988; Richard-Yris et al., 1995). Several studies provide evidence that PRL is not released at an increased rate while hens are caring for their young, but it is involved in the initiation and/or maintenance of maternal behavior and has an antigonadal role (Sharp et al., 1979; 1988; Bedrak et al., 1981; Kuwayama et al., 1992; Leboucher et al., 1993; Richard-Yris et al., 1995; Boos et al., 2007; Kosonsiriluk et al., 2008). In galliform birds, PRL is involved in maternal behaviors. The presence of chicks induces the emergence of specific maternal behaviors in many avian species (Maier, 1963;

Richard-Yris et al., 1983; Richard-Yris and Leboucher, 1987; Leboucher et al., 1990; 1993; Wang and Buntin, 1999). Replacement of the eggs by chicks induces maternal behaviors in incubating, non-incubating, and ovariectomized hens (Richard-Yris et al., 1987a; 1995; 1998; Leboucher et al., 1990; 1993; Lea et al., 1996). Physical contact with newly hatched chicks during brooding bouts slows down the decrease of PRL secretion and inhibits LH and estradiol release in maternal hens (Leboucher et al., 1993). Thus, it is possible that coexistence of newly hatched chicks may suppress LH secretion of the hen in the natural breeding cycle (Kuwayama et al., 1992). Furthermore, it is very well demonstrated that the presence of chicks inhibits the pituitary-ovarian axis (Richard-Yris et al., 1987a; Sharp et al., 1988) and non-brooders (Richard-Yris et al., 1983; 1987b). In some avian species, stimuli from the young or from parent-young interactions may promote or sustain elevated circulating PRL levels (Buntin, 1996). It has been suggested that a definite threshold in circulating PRL levels is necessary to promote and/or maintain post-hatching maternal behavior in precocial birds (Boos et al., 2007). Indeed, depending on the species, either a sharp decline or a slow decrease of PRL concentrations after hatching has been reported (Goldsmith and Williams, 1980; Dittami, 1981; Oring et al., 1986; 1988; Hall, 1987; Opel and Proudman, 1989; Richard-Yris et al., 1995; 1998; Setiawan et al., 2006). In those species, although PRL concentration during rearing period is lower than that of incubation period, the level remains higher than that of non-rearing ones (Boos et al., 2007), suggesting that PRL is likely involved in parental care after hatching (Criscuolo et al., 2002).

The presence of F1-F5 follicles, SYF, and SWF indicates the development of the reproductive system (Etches, 1993). In the present study, the R hens stopped

showing maternal behavior between 7 and 8 weeks after the chicks were hatched. This corresponded to the time when the hens came back to lay again after caring for their young. Other studies provide evidence demonstrating that when the chicks grow older and become fledged, brooding behavior progressively declines, leading to a drop in PRL levels (Richard-Yris et al., 1987a; 1989; 1998; Sharp et al., 1988; Boos et al., 2007). In good agreement with these results are findings in bantams, in which the hens stopped broody behavior between 4 to 10 weeks after the chicks were hatched, corresponding to the time when circulating LH levels had increased to the levels found in laying hens (Sharp et al., 1979). Whereas, the NR hens returned to lay at the third week after removal of the chicks. Similarly in bantam hens, Gifujidori hens and native Thai hens, the effects of removal of chicks from the care of hens induced ovarian recrudescence and egg laying between week 3 and 4 of separation from the chicks (Sharp et al., 1979; Kuwayama et al., 1992; Kosonsiriluk et al., 2008). On the other hand, rearing chick hens and subsequent hens that were removed from their chicks exhibit an abrupt increase in plasma LH levels concurrent with the decrease of plasma PRL levels (Leboucher et al., 1990). It has been reported that circulating LH and estradiol levels gradually increased after hatching or when chicks were present and reached peak levels immediately after removal of the chicks (Sharp et al., 1979; Richard-Yris et al., 1998; Kuwayama et al., 1992). In addition, the establishment of maternal behavior was accompanied by a significant long-term fall in the plasma levels of LH and gonadal steroids (Bedrak et al., 1981; Richard-Yris et al., 1988). Interestingly, LH secretion may also be inhibited by increased plasma PRL levels, and high levels of PRL have been suggested to be involved in the initiation and/or maintenance of broodiness and have an antigonaldal role in birds (Bedrak et al., 1981;

Sharp et al., 1988; Zadworny et al., 1988). Further evidence in support of these results indicates that immunization against PRL slows down ovarian follicular development and reduces hen egg laying performance, suggesting that PRL plays a stimulatory role in ovarian follicular development in chicken hens (Li et al., 2011).

In this present study, after removing the chicks from the hens, plasma PRL levels were decreased in the NR hens when compared with those of R hens and reached the lowest levels by the third week, which corresponded with an increase in ovary and oviduct weights at the time when hens returned to lay. These findings corresponded with the decreases in the number of VIP-ir neurons in IH-IN of nest-deprived native Thai hens, which paralleled the decreases in plasma PRL levels, and they came back into lay within 18 days of nest deprivation (Prakobsaeng et al., 2011). Further evidence supporting the above findings includes the demonstrated changes in the number of GnRH-I-ir neurons that were observed in the nucleus commissurae pallii (nCPa) of native Thai hens, which were correlated with the reproductive stages. The greatest number of GnRH-I-ir neurons was found in laying hens, then slightly decreased in incubating hens, and declined to the lowest levels during the rearing stage. Furthermore, studies in nest-deprived native Thai hens showed a significant increase in the number of GnRH-I-ir neurons in the nCPa (Sartsoongnoen et al., 2012), while VIP-ir neurons decreased and ovary and oviduct weights increased as hens started the new laying cycle (Prakobsaeng et al., 2011). Moreover, removal of the hens from their eggs resulted in an increase in LH secretion and hypothalamic contents of cGnRH-I mRNA (Dunn et al., 1996). These findings, taken together with the results of the present study, clearly demonstrate that PRL is involved in the

initiation and maintenance of rearing behavior. In addition, changes in this pituitary hormone affect reproductive characteristics of the native Thai chickens.

In summary, this present study indicates that plasma PRL levels are associated with rearing behavior in the native Thai chickens. The external cues such as physical contact with chicks and the presence of chicks are involved in the maintenance of plasma PRL levels and rearing behavior. Removal of chicks from the care of hens disrupts rearing behavior, decreases plasma PRL concentrations, induces ovarian recrudescence, increases ovary and oviduct weights, and finally induces the hens to come back to lay in the new cycle.

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CHAPTER IV

EFFECTS OF REARING BEHAVIOR ON NEUROENDOCRINE REGULATION OF THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKENS: ROLE OF VASOACTIVE INTESTINAL PEPTIDE

4.1 Abstract

It is well established that the initiation and maintenance of maternal behavior is correlated with plasma prolactin (PRL) levels. Vasoactive intestinal peptide (VIP), an octacosaneuropeptide, plays a pivotal role in the regulation of PRL secretion in birds, and is defined as the avian PRL-releasing factor. Changes in the number of hypothalamic VIP neurons within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) are directly correlated with circulating PRL levels throughout the reproductive cycle in native Thai chicken, a continuously breeding species found in the equatorial zone. Further studies indicated an association between VIP neurons in the IH-IN and dopaminergic neurons in the nucleus intramedialis (nI) and nucleus mamillaris lateralis (ML) with the expression of incubation behavior. To date, no data are available that describe the interrelationship and the functional aspect of the changes in VIPergic system during rearing behavior in the native Thai chicken. Therefore, this study was designed, utilizing an immunohistochemistry technique, to

compare the differential expression of VIP-immunoreactive (VIP-ir) neurons within the hypothalamic areas of rearing (R) native Thai hens with those of non-rearing (NR) ones. The results revealed that the hypothalamic VIP-ir neurons and fibers were not observed in the nucleus anterior medialis hypothalami, nucleus suprachaiasmaticus, pars medialis, nucleus periventricularis hypothalami, regio lateralis hypothalami, nucleus ventromedialis hypothalami, nI, and ML areas of both R and NR hens. The expression of VIP-ir neurons were found in the IH-IN in both groups. Changes in the number of VIP-ir neurons in R and NR hens were observed in the IH-IN. The greatest density of VIP-ir neurons was found in the IH-IN of R hens. The number of VIP-ir neurons in the IH-IN remained high after the day the chicks were hatched until day 7 of the rearing period then sharply decreased from day 10 to day 21. When the chicks were deprived from the hens, VIP-ir neurons counted were markedly decreased on day 4 and continued to be lower than those of R hens through day 21. The effects of removal of chicks from the care of hens induced ovarian and oviduct recrudescence and the initiation of a new laying cycle. Taken together, the present findings indicate an association between VIPergic system and rearing behavior in the native Thai chicken. The differential expression of VIP neurons in the IH-IN might play a regulatory role in year-round reproductive activity and subsequent PRL release in this equatorial bird. Disruption of rearing behavior by removing the chicks from the hens markedly decreases in the number of VIP-ir neurons in the IH-IN, suggesting that the VIPergic system in the IH-IN may be involved in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of rearing behavior in native Thai chickens. It is further indicated that the VIP/PRL system is not only a key regulator of incubation behavior as it is well

established but it may also be involved in the regulation of rearing behavior in the native Thai chicken. These findings suggest that the presence of chicks and/or physical contact with chicks induced maternal behavior in the native Thai hens as well. The relationship between hens and chicks while hens took care of their young might slow down the decreases in circulating PRL levels and the number of VIP-ir neurons in the IH-IN which, in turn, facilitate, stimulate or maintain maternal care behavior in the native Thai chickens.

4.2 Introduction

In avian species, it is well known that two major neuroendocrine systems play a pivotal role in the reproductive cycle. One system involves chicken gonadotropin releasing hormone-I (cGnRH-I or GnRH) and the subsequent secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH), known as GnRH/FSH-LH system (Peczely, 1989; Sharp et al., 1990; 1998). Another system involves vasoactive intestinal peptide (VIP) and the subsequent secretion of prolactin (PRL), also known as VIP/PRL system (El Halawani et al., 1997; 2001; Chaiseha and El Halawani, 2005). Both systems are influenced by dopaminergic (DAergic) neurotransmission (Bhatt et al., 2003; Chaiseha et al., 2003). Changes in LH and PRL concentrations during the avian reproductive cycle are well documented (Follett, 1984; El Halawani et al., 1988). Plasma PRL and LH levels are low in reproductively quiescent birds, while the levels are increased in reproductively active laying hens. During the incubating stage, circulating PRL levels are sharply elevated (El Halawani et al., 1984; Sharp et al., 1989; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008), while plasma LH levels are gradually suppressed (Lea et al., 1981; El Halawani and Rozenboim, 1993). In

birds, PRL has been implicated as a causative factor for the reduced circulating gonadotropin and ovarian regression, when birds shift from egg laying to incubation behavior in chickens, turkeys, pigeons, pheasants, mallard ducks, and cow birds (El Halawani et al., 1997). Similarly in gallinacous-temperate zone birds, hyperprolactinemia is associated with incubation behavior and ovarian regression in the native Thai chicken, a tropical non-seasonally breeding avian species (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008; Prakobsaeng et al., 2009; 2011). It is very well established that PRL secretion is tonically stimulated (Kragt and Meites, 1965; Bern and Nicoll, 1968; Ben-Jonathan et al., 1989) and VIP is the avian PRL releasing factor (PRF), which is secreted from neurons located in the infundibular nuclear complex (INF) of the caudo-medial hypothalamus (Sharp et al., 1989; El Halawani et al., 1997; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005).

VIP, an octacosapeptide, was first isolated from porcine duodenum (Said and Mutt, 1970). Subsequently, 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; Rosselin et al., 1982), with a high concentrations are 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 substances (Larsson et al., 1976; Marley and Emson, 1982; Lam, 1991). In mammals, VIP regulates the release of anterior pituitary hormones such as PRL (Kato et al., 1978; Rotsztejn et al., 1980; Frawley and Neill, 1981), growth hormone (Chihara et al., 1982; 1984), and adrenocorticotropic hormone (Oliva et al., 1982; White et al., 1982). It is well documented that VIP stimulates PRL release both *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Samson et al., 1980; Matsushita et al., 1983).

Several studies have been reported that VIP is a physiological PRL-releasing factor in many species of mammals (Frawley and Neill, 1981; Kato et al., 1984; Abe et al., 1985). In addition, VIP also regulates the amount of pituitary PRL mRNA and its protein expression (Ben-Jonathan et al., 1989; Maas et al., 1991). Furthermore, the regulatory effects on the circulatory, immune, reproductive, and gastrointestinal systems have been reported (Grossman, 1974; Andersson et al., 1982; Said, 1982; Gressens et al., 1993; Bakken et al., 1995; Gomariz et al., 2001).

In birds, VIP acts directly on the anterior pituitary gland to stimulate PRL secretion during the reproductive cycle (Lea and Vowles, 1986; Macnamee et al., 1986; Proudman and Opel, 1988; El Halawani et al., 1990; 1997; Kosonsiriluk et al., 2008). In turkeys, the relationship between VIP content in the median eminence (eminentia mediana; ME) and VIP mRNA steady-state levels occurring within the hypothalamus as well as of VIP levels in hypophysial portal blood and changes in circulating PRL levels are correlated with the reproductive stages (Youngren et al., 1996; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999). Immunocytochemical studies have shown that hypothalamic VIP-immunoreactive (VIP-ir) neurons in the INF and VIP-ir fibers in the ME correspond to the enhanced circulating PRL levels in turkeys and native Thai chickens (Mauro et al., 1989; Kosonsiriluk et al., 2008). Other studies have also shown increases in the number and cell size of VIP-ir neurons within this region in the domesticated pigeons and ring doves during periods of elevated circulating PRL levels (Peczely and Kiss, 1988; Cloues et al., 1990). 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).

Recently, it has been reported that VIP-ir neurons and fibers are extensively distributed throughout the brain of the native Thai chickens and are predominantly expressed in the diencephalon, where VIP-ir neurons are concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in the number of VIP-ir neurons within the IH and IN are directly correlated to plasma PRL levels throughout the reproductive cycle of native Thai chickens (Kosonsiriluk et al., 2008). The greatest number of VIP-ir neurons within the IH-IN found during incubation period, when the greatest plasma level of PRL observed (Kosonsiriluk et al., 2008; Prakobsaeng et al., 2009; 2011). Further studies in native Thai chicken indicates an association between VIP neurons in the IH-IN and DA neurons in the nucleus intramedialis (nI) and nucleus mamillaris lateralis (ML) areas with the degree of hyperprolactinemia during the incubating state, suggesting that the expression of incubation behavior in birds is regulated by the DAergic system which, in turn, stimulate VIP and subsequent PRL release (Prakobsaeng et al., 2011).

In contrast to the temperate zone seasonal breeding species, the native Thai chicken is a continuously breeding species found in the equatorial zone that produces eggs all year, independently on photoperiodic cues (Konsonsiriluk, 2007; Sartsoongnoen, 2007; Kosonsiriluk et al., 2008). To date, no data are available that describe the interrelationship and the functional aspect of the changes in VIPergic system during rearing behavior in the native Thai chickens. Therefore, the objectives of this study were to investigate changes in the number of VIP-ir neurons within the hypothalamic areas between rearing and non rearing native Thai hens. The findings of differential expression of VIP-ir neurons within the individual hypothalamic areas

may provide an insight of the role of VIP in the regulation of rearing behavior in the native Thai chickens.

4.3 Materials and Methods

4.3.1 Experimental Animals

Female and male native Thai chickens (*Gallus domesticus*), Pradoohangdam breed, 22-24 weeks old, were used. They were reared and housed (7-8 females : 1 male) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of darkness; 12L : 12D). Each hen was identified by wing band number. Feed and water were given *ad libitum*. The native Thai hens were randomly divided into two treatment groups; hens rearing chicks (R) and hens that had been removed from their chicks (NR). Hens in the R group were allowed to rear the chicks naturally. Hens in the NR group were disrupted from rearing behavior and not allowed to rear the chicks by removing them from their chicks to another pen. Egg production, nesting activity, and other behaviors were recorded daily throughout the experiments. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee.

4.3.2 Experimental Design

Seventy eight female and 10 male native Thai chickens, 22-24 weeks old, were used. The chickens were randomly divided into 10 floor pens (7-8 hens : 1 rooster) and observed for their daily behaviors. After hatching, the hens were divided into two groups; R and NR. The hens were then sacrificed at different time periods (day of hatch, day 4, 7, 10, 14, 17, and 21; n=6) after they started to rear their chicks

or after the chicks were removed. To visualize and localize VIP-ir neurons within the hypothalamic areas, the brain of each hen was fixed by pressure perfusion with 4% paraformaldehyde, sectioned with a cryostat, and then processed by immunohistochemistry (IHC). A postmortem examination of each hen was performed to confirm its reproductive status. The ovaries and oviducts were collected and weighed after the hens were sacrificed. 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 that contains a hierarchy of yellow yolky follicles with the diameter longer than 1 cm were identified as F1, F2, F3, F4, and F5 and several smaller follicles from which the large yolky follicles are recruited. The small follicles were classified according to their diameters as SYF (5-9 mm) and SWF (1-4 mm).

4.3.3 Processing of Tissues for Immunohistochemistry

Prior to perfusion, the hens were intravenously injected with 3,000 units of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA), and then euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). The head was removed and immediately fixed by pressure-perfusion via the carotid arteries with phosphate buffered saline (PBS, pH 7.4) 100 ml for 3-5 min, followed by a freshly prepared 4 % paraformaldehyde in 650 ml of 0.1 M PBS (pH 7.4) for 30 min according to the method described by Kosonsiriluk et al. (2008). The brain was then dissected intact from the skull, and soaked in 20 % sucrose in PBS at 4 °C for 48 hrs or until it is saturated for cryoprotection. The brain was then frozen in powdered dry ice for 1 hr, and stored at -35 °C until sectioned. Frozen brains were sectioned in

the coronal plane at a thickness of 16 µm using a cryostat (Leica CM1850, Leica Instruments GMbH, Nussioch, Germany). Sections were mounted on chrome alum-gelatin-coated glass slides with two sections per slide and stored desiccated at -20 °C until used. Four adjacent sections of each individual hypothalamic area were processed by IHC in order to visualize and analyze changes in the number of VIP-ir neurons. The stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988) was used to choose the sections containing the hypothalamic nuclei analyzed. For each area, the sections were chosen starting with the most rostral section that contained each nucleus and every subsequent section until the nucleus disappeared from view. The plane that expressed the greatest density of VIP-ir neurons was chosen to analyze. These planes of sections have been published in earlier reports (Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011).

4.3.4 Immunohistochemistry

Changes in the number of hypothalamic VIP-ir neurons of R and NR hens were determined by IHC according to the previously described method (Kosonsiriluk et al., 2008). The primary and secondary antibodies used for detecting VIP-ir neurons were VIP primary antibody (polyclonal anti-chicken VIP antiserum; VIP4-DYC8, generously provide by Dr. M.E. El Halawani, University of Minnesota, USA) and Cy^{TM}3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), respectively. Four adjacent sections from R and NR hens in the individual hypothalamic areas were thawed to room temperature prior to use. The sections were rehydrated in PBS for 30 min at room temperature. After removing from PBS, the sections were then incubated with 60 µl

of the primary antibody diluted with PBS (pH 7.4) containing 1 % bovine serum albumin and 0.3 % Triton-X 100 at 1:1000 dilution for overnight at 4 °C in a moist chamber. Subsequently, the sections were then washed three times with PBS (pH 7.4) for 5 min each. After washing, 60 µl of the secondary antibody at 1:500 dilution was applied onto the sections under dark condition. 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 and further analyzed.

4.3.5 Image Analysis

Microscopic images of the brain sections of the hens were visualized with a fluorescence microscope (Olympus IX71, Tokyo, Japan) using a cooled digital color camera (Olympus DP70, Tokyo, Japan). The images were captured and stored by DP70-BSW Software (Olympus, Tokyo, Japan). The differential expression of VIP-ir neurons in each individual area of the brain was visualized and analyzed. The number of VIP-ir neurons of four adjacent sections was counted manually to determine changes in the number of VIP-ir neurons. To aid in the documentation of neuroanatomical results, the nomenclature and schematic diagrams from the stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988) and the chicken hypothalamus (Kuenzel and van Tienhoven, 1982) were used to illustrate VIP immunoreactivity. The specificity of the anti-VIP antibody was tested by omission of the primary antibody during that step of immunohistochemistry. No immunostaining of VIP was observed in control sections.

4.3.6 Statistical Analysis

Significant differences in the number of VIP-ir neurons (means ± SEM) in the individual hypothalamic areas according to each treatment group were compared using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test. A probability less than 0.05 ($P<0.05$) indicated a significant difference. All statistical tests were analyzed employing the SPSS for Windows Software (version 13.0, SPSS Inc., Chicago, IL, USA).

4.4 Results

The distributions of VIP-ir neurons were determined across the hypothalamic areas; the nucleus anterior medialis hypothalami (AM), nucleus suprachaiasmaticus, pars medialis (SCNm), nucleus periventricularis hypothalami (PHN), regio lateralis hypothalami (LHy), nucleus ventromedialis hypothalami (VMN), IH, IN, ME, nucleus intramedialis (nI), and nucleus mamillaris lateralis (ML) areas (Figure 4.1). No VIP-ir neurons were observed in the AM, SCNm, PHN, LHy, VMN, nI, ML of R and NR hens. A dense accumulation of the VIP-ir fibers were located in the ME of both R and NR groups. The expression of VIP-ir neurons were found in the IH and IN areas in both groups (Figure 4.2). The greatest density of VIP-ir neurons was found in the IH-IN of R hens. The numbers of VIP-ir neurons were markedly lower when hens were removed from their chicks. The differential expression of VIP-ir neurons in the IH-IN of R and NR hens are shown in Figure 4.3. Changes in the number of VIP-ir neurons in the IH-IN of R and NR hens are shown in Figure 4.4 and Table 4.2. The results revealed that the number of VIP-ir neurons in the IH-IN remained high after the day the chicks were hatched until day 7 of the rearing period then sharply

decreased from day 10 to day 21. In addition, the greatest density of VIP-ir neurons was observed in the IH-IN of R hens, but the numbers were markedly lower. When the chicks were removed from the hens, VIP-ir neurons counted were markedly and significantly decreased ($P<0.05$) on day 4 and continued to be lower than those of R hens through day 21; day 4 (R4 vs NR4; 54.29 ± 2.84 vs 30.33 ± 2.91 cells), day 7 (R7 vs NR7; 32.17 ± 1.41 vs 13.42 ± 4.35 cells), day 17 (R17 vs NR17; 13.38 ± 1.56 vs 5.46 ± 0.84 cells), and day 21 (R21 vs NR21; 7.04 ± 0.96 vs 2.92 ± 0.76 cells). However, the number of VIP-ir neurons did not differ between R and NR groups at day 10 and day 14.

The reproductive characteristics of R and NR hens at different time periods were also recorded. The presence of F1-F5 follicles, SYF, and SWF (Figure 4.5) were observed in both groups. In the R hens, the presences of F1-F5 follicles, SYF, and SWF at different days of the rearing period are shown in Table 4.3. The results showed that F1-F5 follicles and SYF were not observed in the R hens that were rearing their chicks from day of hatch to day 21. However, the presence of SWF was found in all R hens. In contrast, the numbers of NR hens that exhibited the presence of F1-F5 follicles, SYF, and SWF are shown in Table 4.4. After the chicks were removed, the NR hens showed the presence of F1-F5 follicles within 17 days. Most of the NR hens exhibited the presence of SYF at day 14 after removing the chicks and all of them exhibited the presence of SWF. A number of NR hens started to lay, the new laying cycle, in day 21 after removal the chicks. Moreover, all NR hens came back into lay within 21 days after chick removal.

The ovaries of R and NR hens are shown in Figure 4.6. The ovary weights of R and NR hens are shown in Figure 4.7 and Table 4.5. When the hens were rearing

their young, the ovary weights showed no increase throughout 21 days of the rearing period. In contrast, the ovary weights in the NR hens displayed a significant increase ($P<0.05$) at days 7, 14, 17 and 21 when compared to those the R hens, and reached the highest weight at day 21 after removing the chicks ($P<0.05$; 46.82 ± 6.73 g).

The oviducts of R and NR hens are shown in Figure 4.8. The oviduct weights of R and NR hens are shown in Figure 4.9 and Table 4.6. Similarly, the oviduct weights of R hens showed no increase during 21 days of the rearing period. Whereas, in hens that had their chicks removed after hatching, the oviduct weights were significantly increased at day 7 after removal of chicks ($P<0.05$) and reached the highest weight at day 21 ($P<0.05$; 42.95 ± 3.60 g). In addition, after removal of chicks from the care of hens induced ovarian recrudescence within 7 days, appearance of yellow follicles by day 14, and egg laying by day 21. Whereas, the ovary and oviduct weights of hens were caring for their young did not differ at a different day of observation periods.

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).

AM	Nucleus anterior medialis hypothalami
SCNm	Nucleus suprachaiasmaticus, pars medialis
PHN	Nucleus periventricularis hypothalami
LHy	Regio lateralis hypothalami
VMN	Nucleus ventromedialis hypothalami
IH	Nucleus inferioris hypothalami
IN	Nucleus infundibuli hypothalami
ME	Eminentia mediana (Median eminence)
nI	Nucleus intramedialis
ML	Nucleus mamillaris lateralis
V III	Ventriculus tertius (Third ventricle)

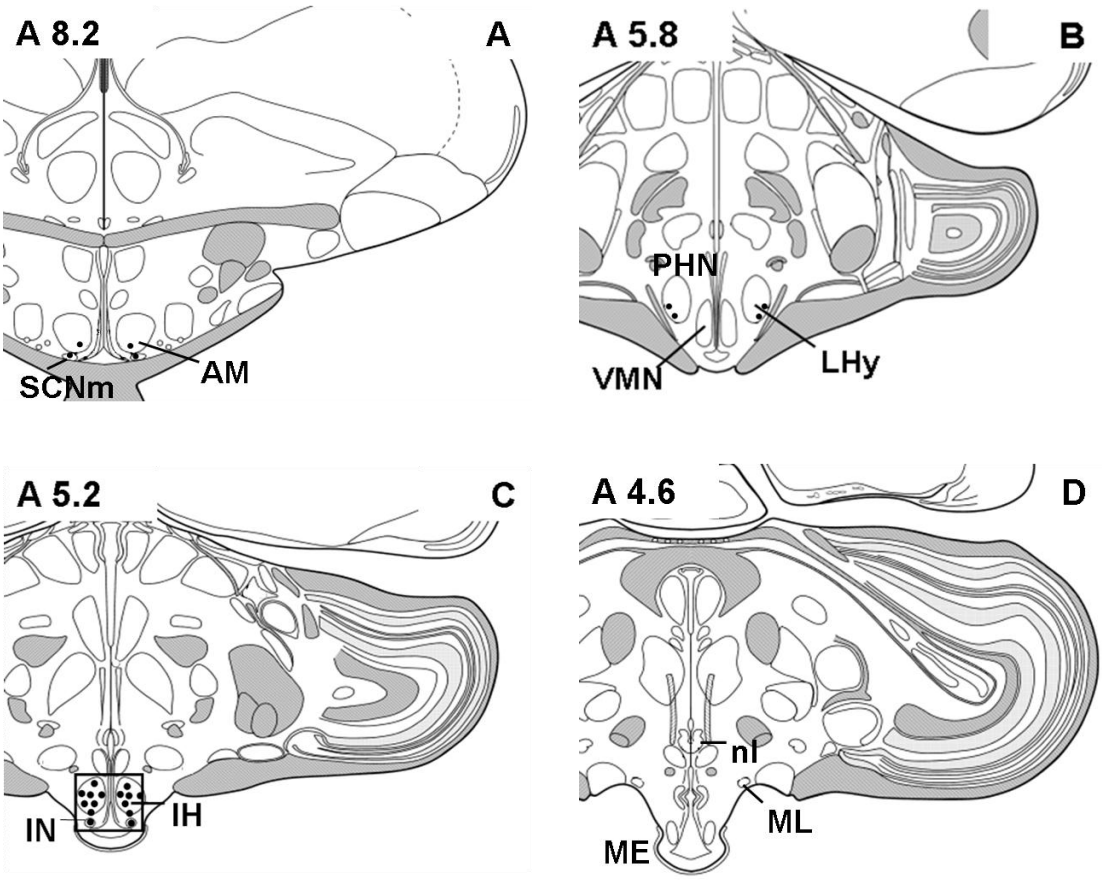

Figure 4.1 Schematic coronal brain sections showing the areas where the expression of VIP-ir (black dots) was observed (**A-D**). The sampling region for counting the number of VIP-ir neurons in the IH-IN (**C**) is represented by rectangles. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988).

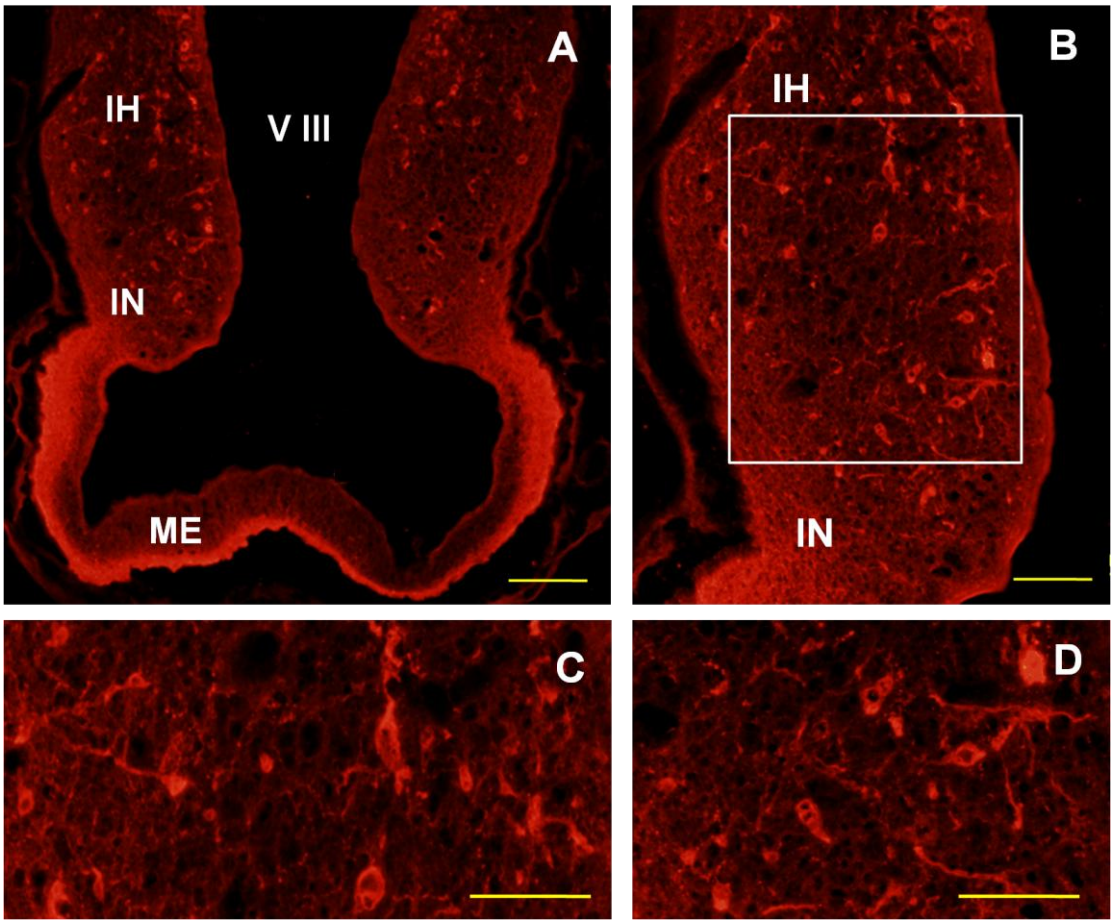

Figure 4.2 Photomicrographs illustrating the distributions of VIP-ir neurons and fibers in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) of the native Thai chicken (**A** and **B**). Rectangle indicates area from which the following photomicrographs were taken. Higher magnification of the VIP-ir neurons was demonstrated in the IH-IN area (**C** and **D**). Scale bar = 50 µm. For abbreviations, see Table 4.1.

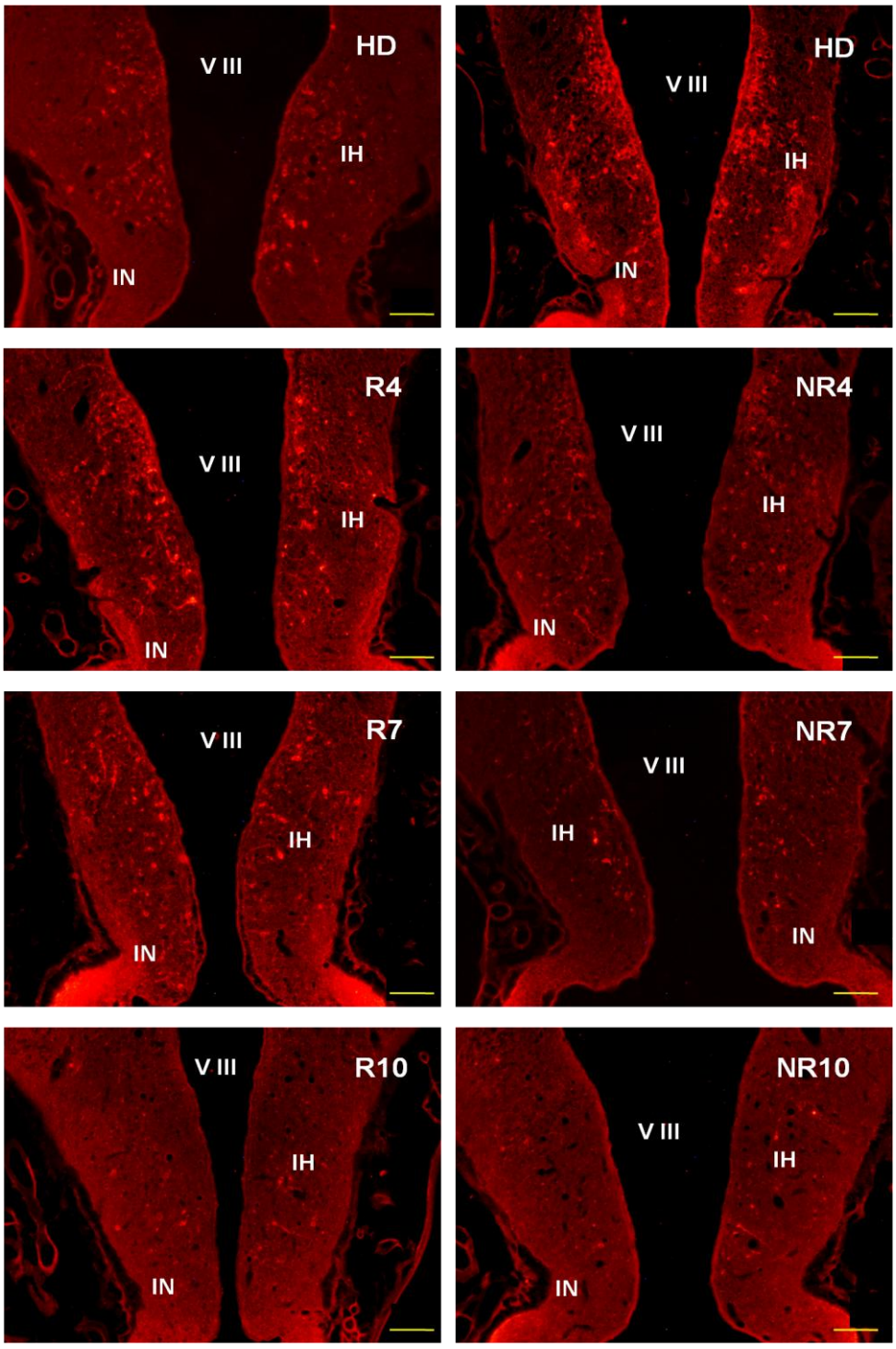

Figure 4.3 Photomicrographs showing the distributions of VIP-ir neurons and fibers in the IH-IN of rearing (R), and non-rearing (NR) native Thai hens on day of hatch (HD) and different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 μm.

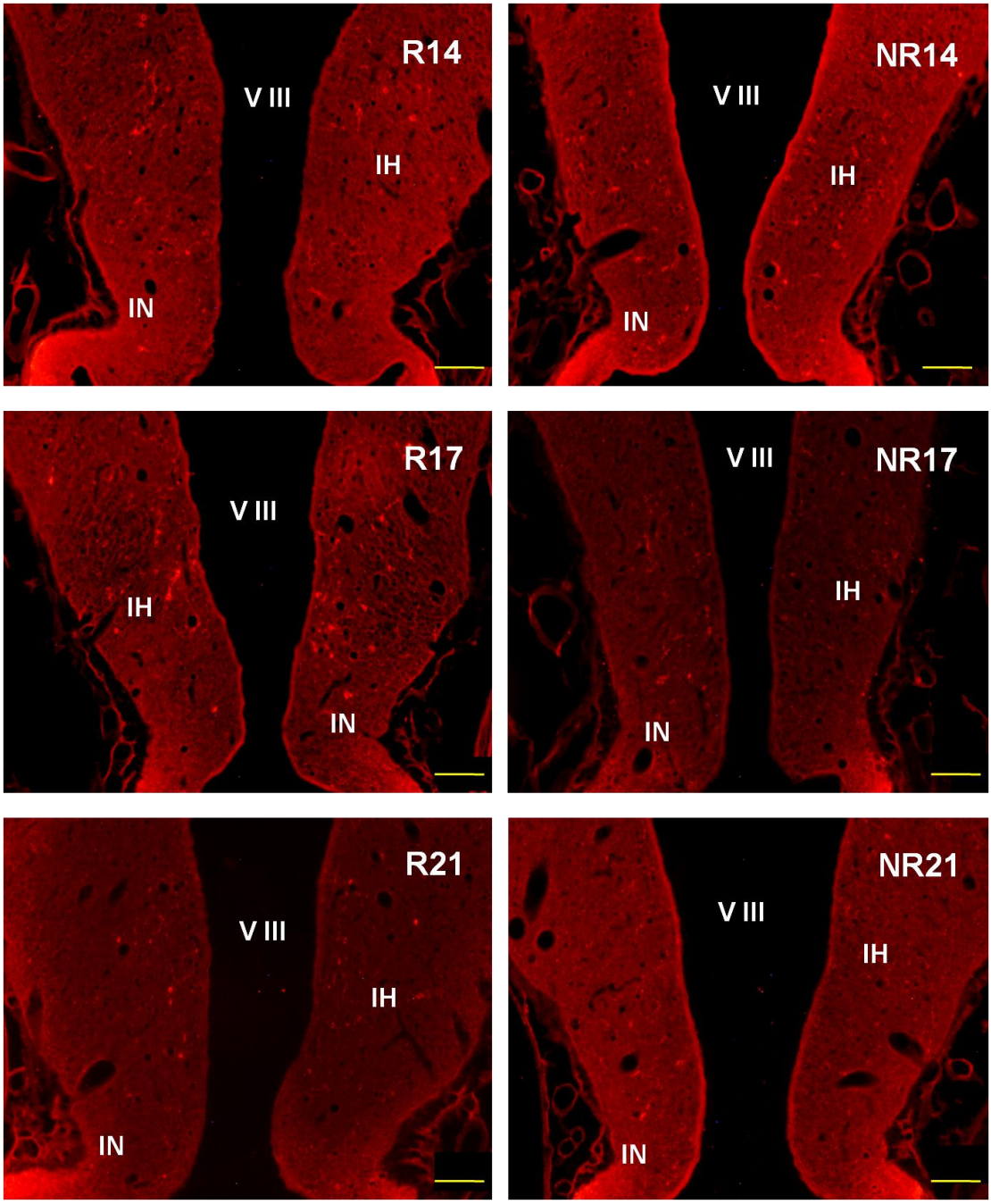

Figure 4.3 Photomicrographs showing the distributions of VIP-ir neurons and fibers in the IH-IN of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 µm (continued).

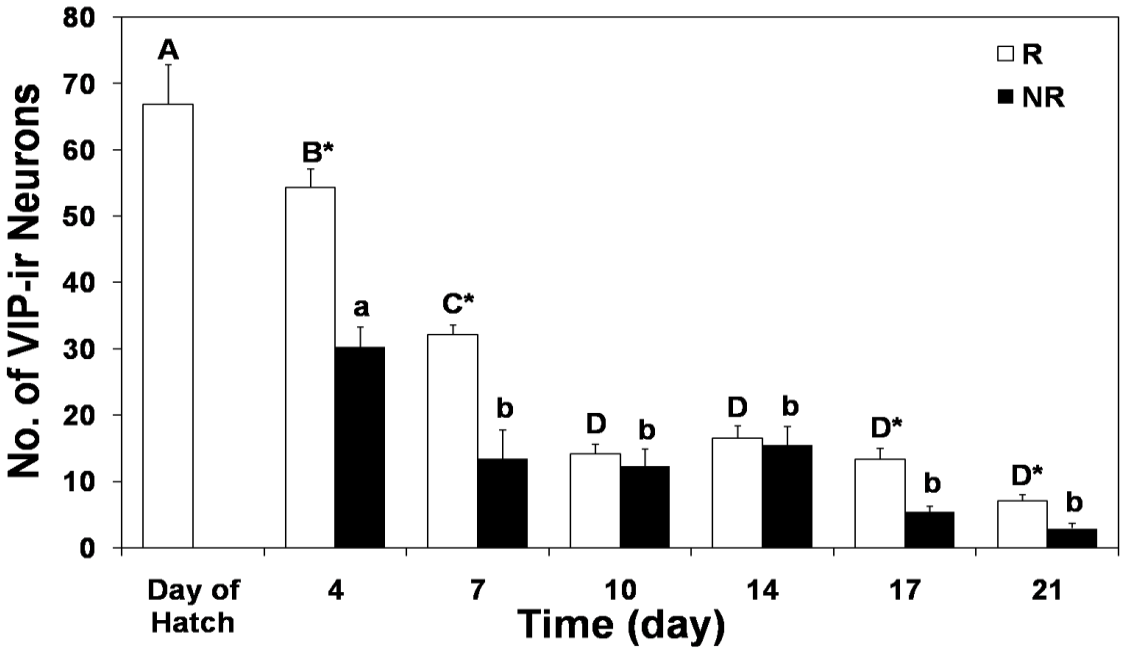

Figure 4.4 Changes in the number of VIP-ir neurons in the IH-IN of rearing (R) and non-rearing (NR) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 4.2 The number of VIP-ir neurons (Mean ± SEM) in the IH-IN of rearing (R) and non-rearing (NR) native Thai hens at different days of rearing or non-rearing their chicks (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Group	**Days Following Rearing or Non-Rearing Their Chicks**						
	Day of Hatch	**4**	**7**	**10**	**14**	**17**	**21**
R	66.88 ± 6.02^{A}	$54.29 \pm 2.84^{B*}$	$32.17 \pm 1.41^{C*}$	14.17 ± 1.40^{D}	16.50 ± 1.86^{D}	$13.38 \pm 1.56^{D*}$	$7.04 \pm 0.96^{D*}$
NR	N/A	30.33 ± 2.91^{a}	13.42 ± 4.35^{b}	12.29 ± 2.61^{b}	15.46 ± 2.83^{b}	5.46 ± 0.84^{b}	2.92 ± 0.76^{b}

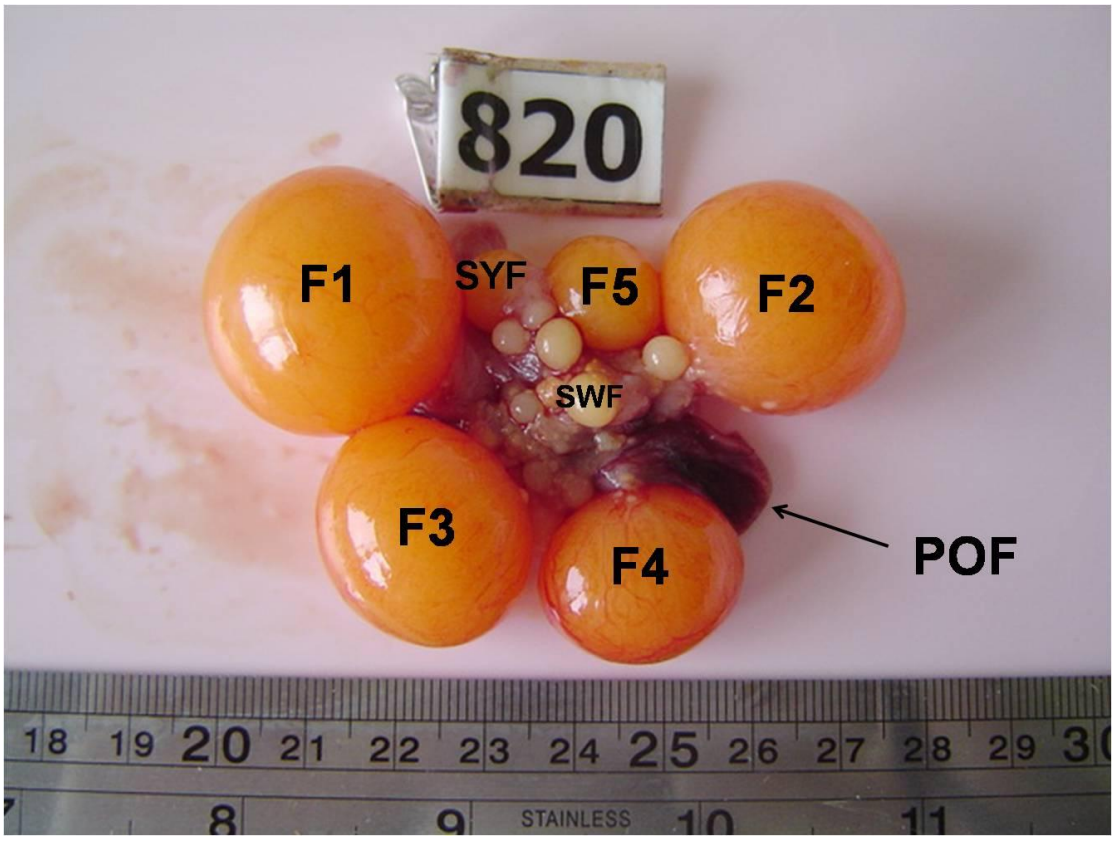

Figure 4.5 Photograph of the ovary of the native Thai hen during the egg laying period showing the F1-F5 follicles, small yellow follicles (SYF), small white follicles (SWF), and post-ovulatory follicles (POF).

Table 4.3 The number of native Thai hens that had the F1-F5 follicles, small yellow follicles (SYF), and small white follicles (SWF) at different days of rearing (n=6).

Follicles	Days Following Rearing Their Chicks						
	Day of Hatch	**4**	**7**	**10**	**14**	**17**	**21**
F1-F5	0	0	0	0	0	0	0
SYF	0	0	0	0	0	0	0
SWF	6	6	6	6	6	6	6

Table 4.4 The number of native Thai hens that had the F1-F5 follicles, small yellow follicles (SYF), and small white follicles (SWF) at different days of non-rearing and the number of hen came back to lay in each period (n=6).

Follicles	**Days Following Non-Rearing Their Chicks**						
	Day of Hatch	**4**	**7**	**10**	**14**	**17**	**21**
F1	0	0	0	0	0	6	6
F2	0	0	0	0	0	6	6
F3	0	0	0	0	0	6	6
F4	0	0	0	0	0	6	6
F5	0	0	0	0	0	6	6
SYF	0	0	0	0	5	6	6
SWF	6	6	6	6	6	6	6
Laying hens	0	0	0	0	0	0	6

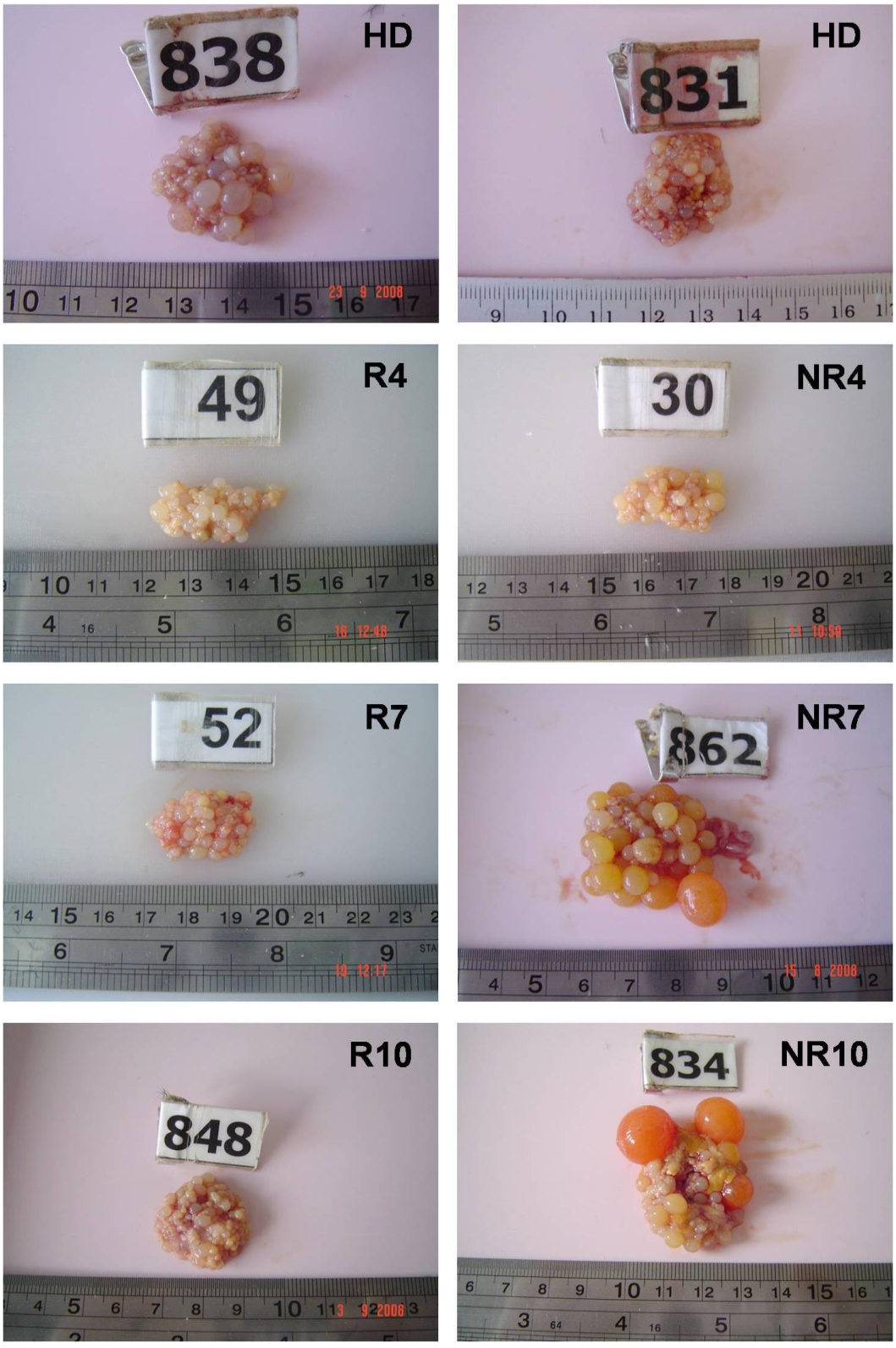

Figure 4.6 Photographs of the ovary of rearing (R) and non-rearing (NR) native Thai hens at day of hatch (HD) and different days of rearing or non-rearing their chicks.

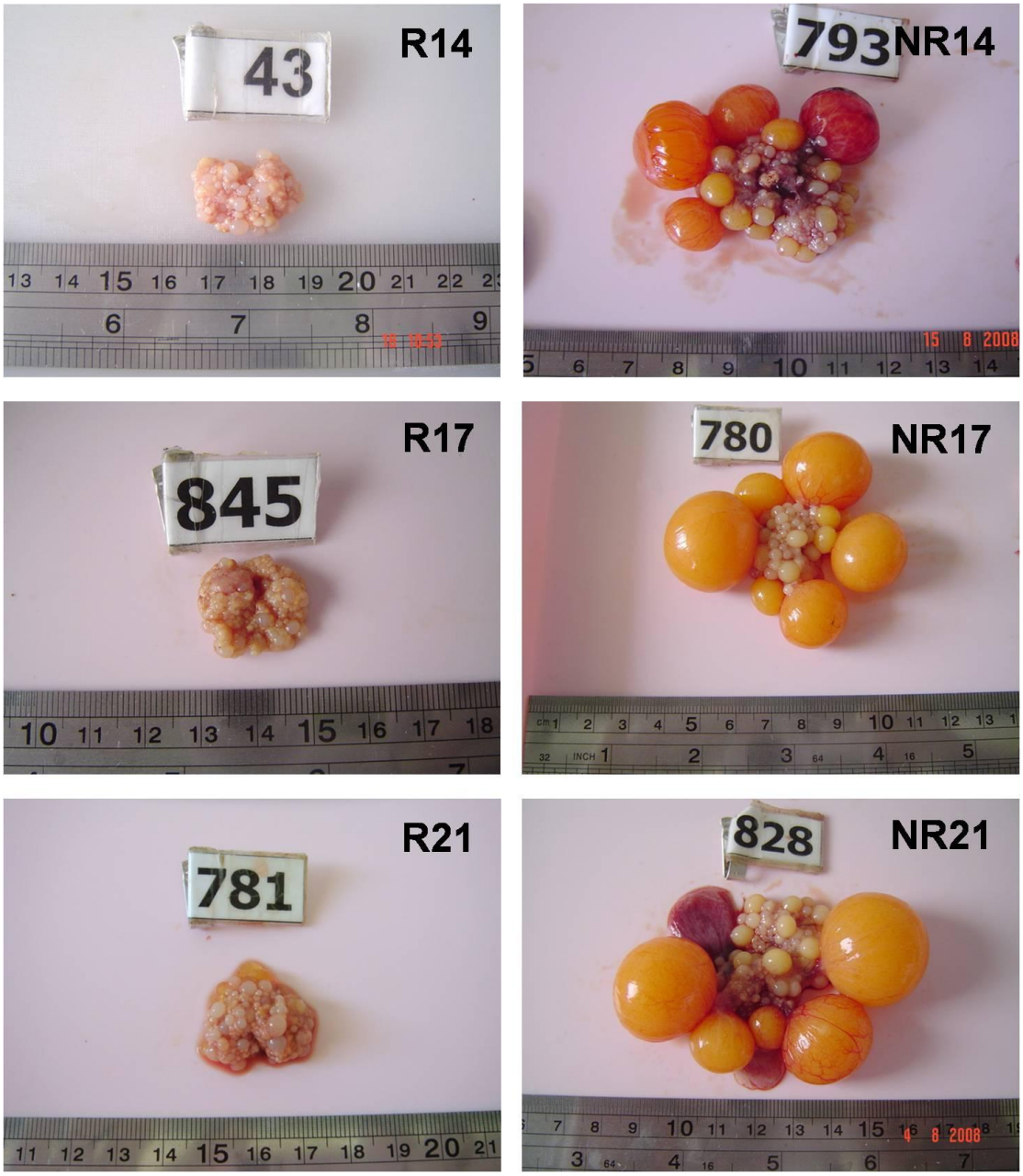

Figure 4.6 Photographs of the ovary of rearing (R) and non-rearing (NR) native Thai hens at different days of rearing or non-rearing their chicks (continued).

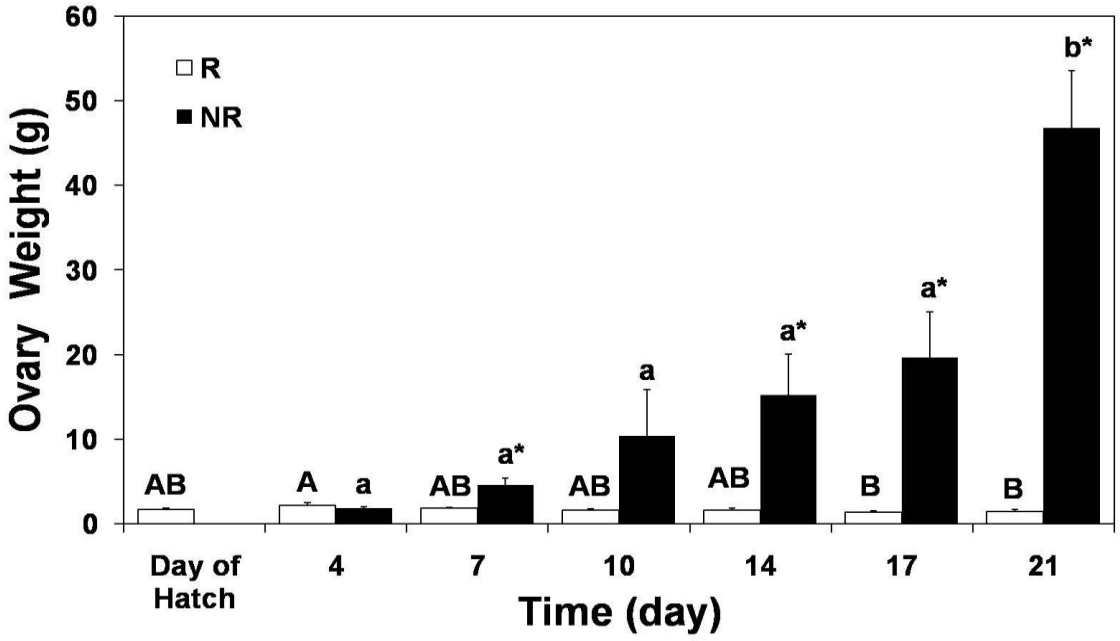

Figure 4.7 Changes in the ovary weights of rearing (R) and non-rearing (NR) native Thai hens. Values are presented as means ± SEM (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 4.5 Mean ± SEM of the ovary weight (g) of rearing (R) and non-rearing (NR) native Thai hens at different times of rearing or non-rearing their chicks (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Group	**Days Following Rearing or Non-Rearing Their Chicks**						
	Day of Hatch	**4**	**7**	**10**	**14**	**17**	**21**
R	1.73 ± 0.16^{AB}	2.22 ± 0.30^{A}	1.84 ± 0.13^{AB}	1.63 ± 0.15^{AB}	1.66 ± 0.21^{AB}	1.36 ± 0.16^{B}	1.48 ± 0.25^{B}
NR	N/A	1.89 ± 0.15^{a}	$4.62 \pm 0.80^{a*}$	10.39 ± 5.51^{a}	$15.26 \pm 4.83^{a*}$	$19.64 \pm 5.44^{a*}$	$46.82 \pm 6.73^{b*}$

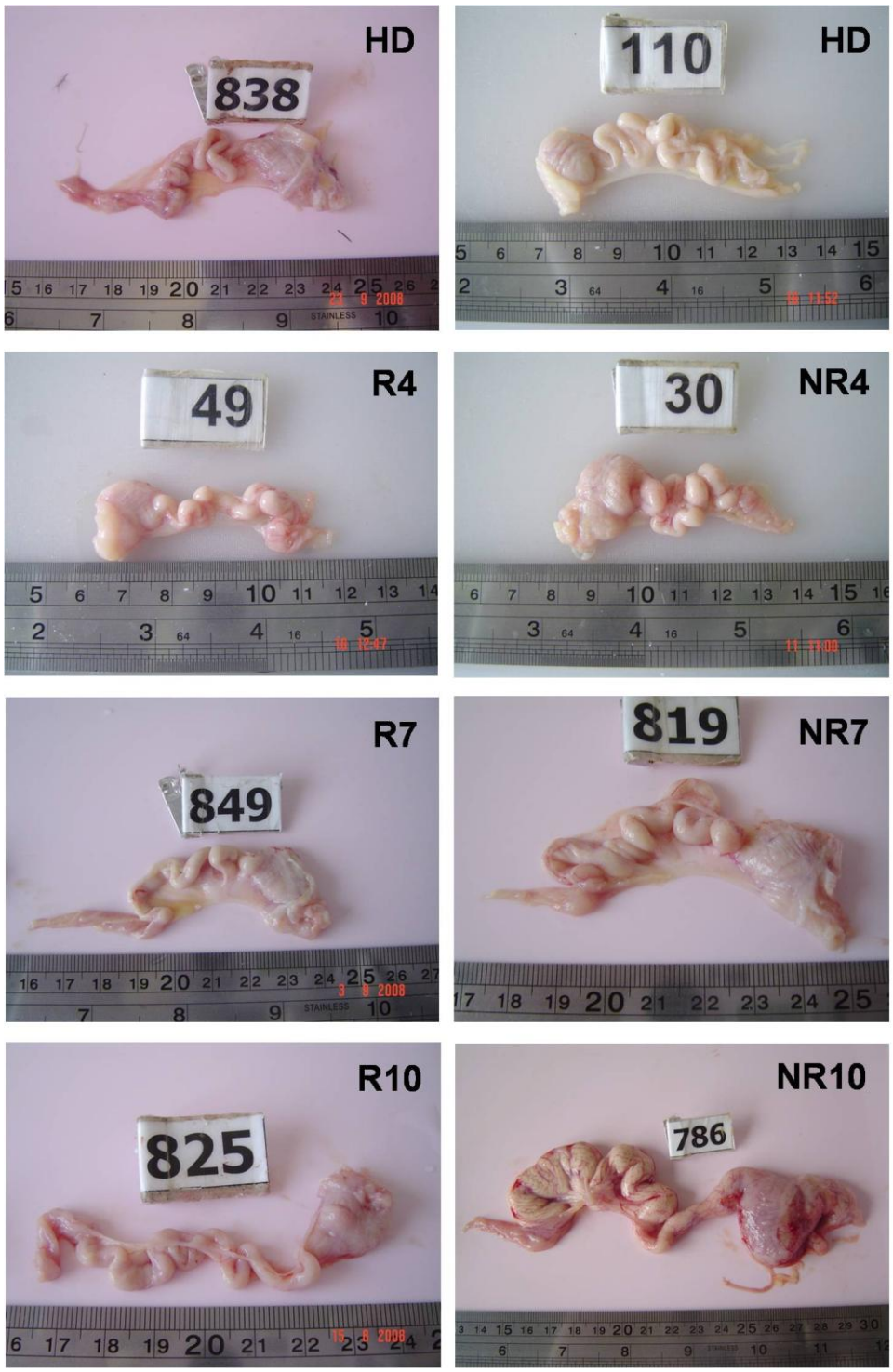

Figure 4.8 Photographs of the oviducts of rearing (R) and non-rearing (NR) native Thai hens at day of hatch (HD) and different days of rearing or non-rearing their chicks.

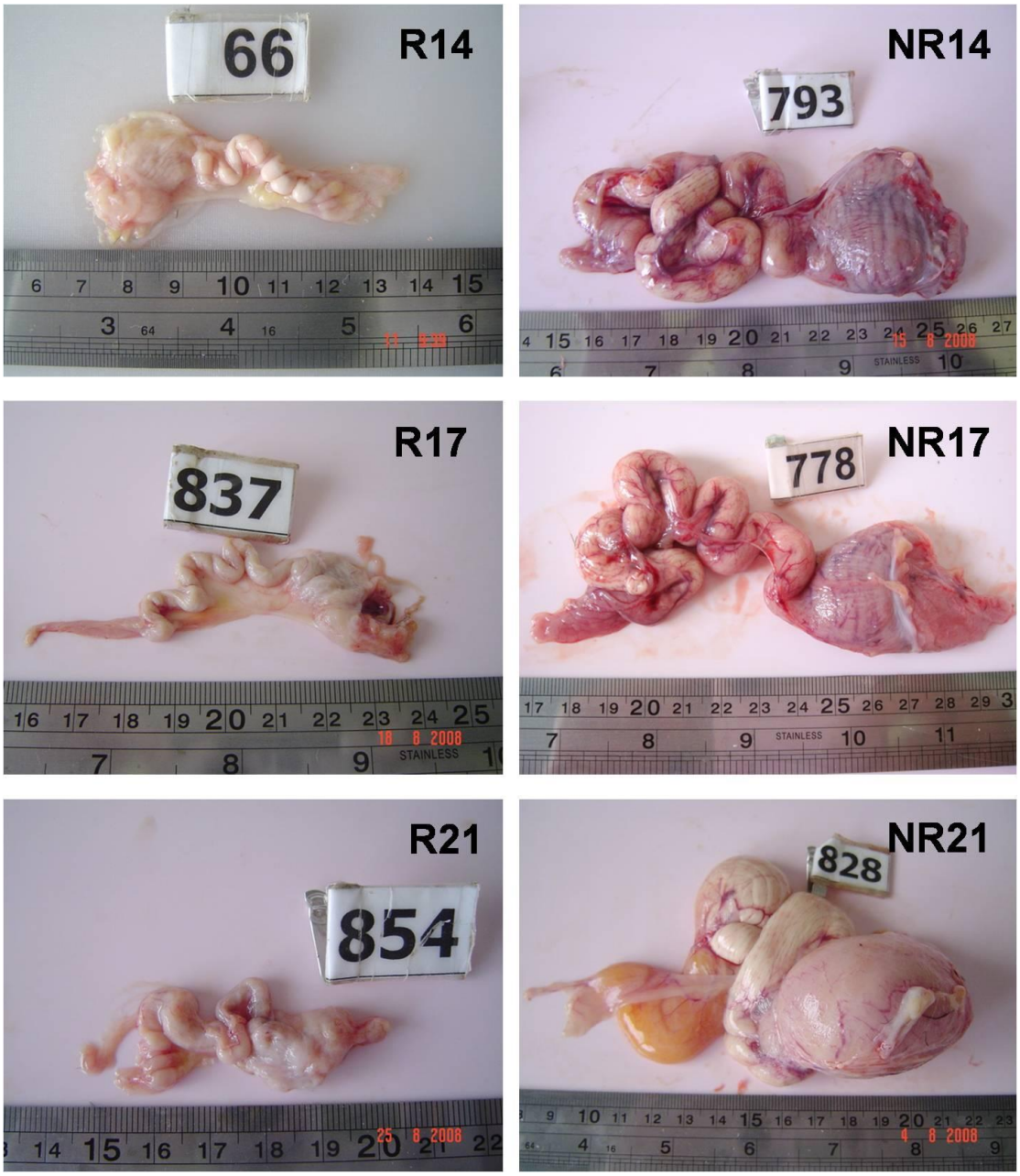

Figure 4.8 Photographs of the oviducts of rearing (R) and non-rearing (NR) native Thai hens at different days of rearing or non-rearing their chicks (continued).

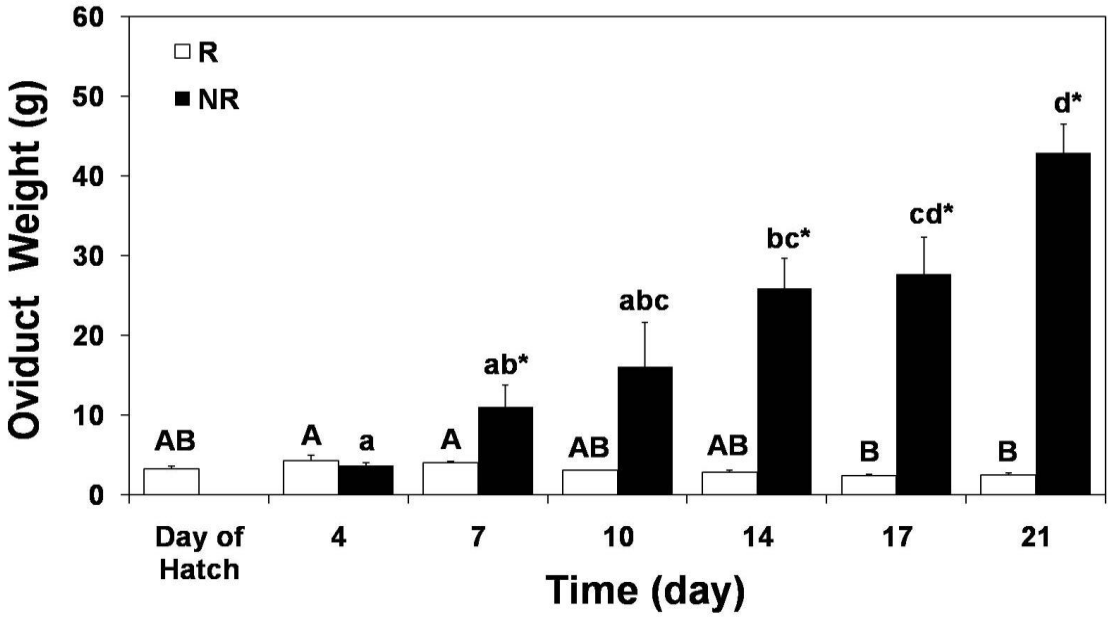

Figure 4.9 Changes in the oviduct weight of rearing (R) and non-rearing (NR) native Thai hens. Values are presented as means ± SEM (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 4.6 Mean ± SEM of the oviduct weight (g) of rearing (R) and non-rearing (NR) native Thai hens at different days of rearing or non-rearing their chicks (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Group	**Days Following Rearing or Non-Rearing Their Chicks**						
	Day of Hatch	**4**	**7**	**10**	**14**	**17**	**21**
R	3.24 ± 0.31^{AB}	4.28 ± 0.72^{A}	4.02 ± 0.19^{A}	3.06 ± 0.05^{AB}	2.83 ± 0.26^{AB}	2.36 ± 0.17^{B}	2.44 ± 0.30^{B}
NR	N/A	3.66 ± 0.34^{a}	$10.99 \pm 2.76^{ab*}$	16.07 ± 5.54^{abc}	$25.88 \pm 3.83^{bc*}$	$27.71 \pm 4.59^{bc*}$	$42.95 \pm 3.60^{d*}$

4.5 Discussion

The results from the present study revealed that the hypothalamic VIP-ir neurons and fibers were not observed in the AM, SCNm, PHN, LHy, VMN, nI, ML areas of both R and NR hens. The expression of VIP-ir neurons and fibers were found in the IH-IN area in both groups. Changes in the number of VIP-ir neurons within the IH-IN area were compared between R and NR hens. The greatest density of VIP-ir neurons was found in the R group and the neuronal densities were decreased in the NR group. The results of this present study clearly demonstrate the association between the hypothalamic VIPergic system and rearing behavior in native Thai chickens, revealing markedly differences in the number of VIP-ir neurons within the IH-IN between R and NR hens.

The number of VIP-ir neurons in the IH-IN remained high after the day the chicks were hatched until day 7 of the rearing period then sharply decreased from day 10 to day 21. When the chicks were removed from the hens, VIP-ir neurons were markedly decreased on day 4 and continued to be lower than those of R hens through day 21. In addition, the effects of removal of chicks from the care of hens induced ovarian and oviduct recrudescence and the initiation of a new laying cycle. The results of the present study provide evidence indicates a pivotal role of the VIPergic system in the regulation of rearing behavior in this equatorial bird.

In the current study, VIP-ir neurons and fibers were not observed in other hypothalamic areas except within the IH-IN of both R and NR hens. Furthermore, it has been reported that changes in the number of VIP-ir neurons within the IH-IN are correlated with changing concentrations of circulating PRL during the rearing period (Chaiyachet et al., 2010), suggesting that hypothalamic VIP expression in the IH-IN

and subsequent PRL release play a regulatory role in rearing behavior in the native Thai chickens. The results from this study are in good agreement with the previous findings that a large group of VIP-ir neurons found in the IH-IN and its differential expression may play a role in regulating the reproductive activity in birds (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; Kosonsiriluk et al., 2008; Prakobsaeng, 2010; Prakobsaeng et al., 2011). These findings are well supported by immunohistochemical studies illustrated changes in the number of VIP-ir neurons within the IH-IN are associated with the shifts in circulating PRL levels throughout the reproductive cycle of equatorial continuously breeding, non-photoperiodic birds (Kosonsiriluk et al., 2008). Furthermore, previous study reported that the expression of incubation behavior is activated by DA neurons in the nI and ML to VIP neurons in the IH-IN and subsequently stimulates PRL secretion, resulting in regression of the reproductive system (Prakobsaeng et al., 2011). In turkeys, a seasonal breeder, increases in the number of VIP-ir neurons within the INF paralleling increases in levels of plasma PRL. In addition, it has been reported that the group of VIP neurons in the INF stimulates the release of pituitary PRL 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; Chaiseha and El Halawani, 1999). Moreover, the increased VIP mRNA expression in the INF is correlated with increased levels of circulating PRL level and LH-β mRNA expression in the anterior pituitary (Bhatt et al., 2003). More evidence of hypothalamic VIP is associated with reproduction and PRL secretion have been studied in ring doves,

increases in number of VIP neurons and cell size were detected in the ventral infundibulum, anticipating the increases in plasma PRL levels (Cloues et al., 1990). The number of hypothalamic VIP-ir neurons were low in non-laying but the number of neurons and cell size were increased during laying and incubation stages. There were markedly increased during the incubating phase, then decreased during the rearing period (Mauro et al., 1989; Sharp et al., 1989; Cloues et al., 1990; Kosonsiriluk et al., 2008).

Changes in the number of VIP-ir neurons within the IH-IN of R and NR hens were compared and revealed that the numbers of VIP-ir neurons in the rearing hens were greater than those of hens not rearing their chicks, suggesting that the presence and physical contact with chicks induced maternal behavior, the larger number of VIP-ir neurons maintains circulating PRL levels which may, in part, necessary to facilitate/stimulate the expression of rearing behavior in the native Thai hens. It is well documented that, VIP is the avian PRF (El Halawani et al., 1997). In hens are caring for their young, tactile stimuli from the chicks or in combination with visual and/or auditory stimuli from newly hatched chicks maintain the circulating PRL levels and up regulate VIP expression (Sharp et al., 1988; 1989; Silver et al., 1988; Buntin et al., 1991; Leboucher et al., 1993). The increased number of VIP-ir neurons has been shown to be correlated with the up-regulation of VIP peptide contents and its mRNA expression and subsequently PRL concentrations (Mauro et al., 1989; 1992; Chaiseha and El Halawani, 1999).

In the present study, the numbers of VIP-ir neurons in the IH-IN of the hens at the day the chicks were hatched remained high until rearing day 7 then sharply decreased and continued to decline during the rest of observed rearing period. These

results are supported by the findings that the number of VIP-ir neurons within the IH-IN increased in the incubating phase and when the hens shifted from incubating eggs to rearing chicks, the number of VIP-ir neurons were sharply dropped, then returned to low level as found in non-laying hens (Kosonsiriluk et al., 2008). Similarly in ring doves, VIP-like immunoreactivity cells size and staining intensity in the ventral portion of the infundibular region in the brain were detected, VIP cell sizes were increased from about incubation day 14 to rearing day 14, and steady decreased during the remaining post-hatching period (Cloues et al., 1990). In addition, increases in the number and cell size of VIP-ir neurons within the medio-basal hypothalamus when PRL concentrations are high have been demonstrated in the domesticated pigeons and ring doves during the initiation of crop milk secretion and feeding the offspring (Peczely and Kiss, 1988; Cloues et al., 1990). Moreover, it has been well established that VIP in the caudo-medial hypothalamus might relate to the control of pituitary functions by projecting fibers to the external layer of the ME and influencing the pituitary PRL secretion (Mikami, 1986). Furthermore, in the current study, the R hens came back into lay later than the NR hens. In good agreement with these results are the findings in which incubating bantam hens were deprived of their nests by replacing eggs with chicks induced maternal behavior, resulting to start new laying cycle later than hens not allowed to rear their chicks (Sharp et al., 1988).

In the present study, after removing the chicks from the hens, the number of VIP-ir neurons in the IH-IN was sharply decreased from day 4 toward the end of observed times in the NR hens when compared with those of R hens and reached minimum values at day 21, which corresponded with an increase in ovary and oviduct weights at the time when hens returned to lay after removal of chicks. Similarly in

bantam hens, Gifujidori hens, and native Thai hens, the effects of removal of chicks from the care of hens induced ovarian recrudescence and egg laying between days 21 to 28 of separation from chicks (Sharp et al., 1979; Kuwayama et al., 1992; Kosonsiriluk et al., 2008). These findings are corresponded with the decreases in the number of VIP-ir neurons in IH-IN of nest-deprived native Thai hens paralleled the decreases in the plasma PRL levels, and they came back into lay within 18 days of nest deprivation (Prakobsaeng et al., 2011). Further evidence supported the above findings have been demonstrated that changes in the number of GnRH-I-ir neurons were observed in the nucleus commissurae pallii (nCPa) of native Thai hens, which correlated with the reproductive stages. The greatest number of GnRH-I-ir neurons found in laying hens, then slightly decreased in incubating hens, and declined to the lowest levels during the rearing stage (Sartsoongnoen et al., 2012). Further studies in nest-deprived native Thai hens showed a significant increase in the number of GnRH-I-ir neurons in the nCPa (Sartsoongnoen et al., 2012), decrease in VIP-ir neurons, accompanied by an increase in ovary and oviduct weights, following by the hens started the new laying cycle (Prakobsaeng et al., 2011). The increased neuroendocrine activity of the VIP/PRL system has been shown to suppress the GnRH/FSH-LH system (Sharp et al., 1998), reduce ovarian steroids secretion (Zadworny et al., 1988), terminate egg laying, and induce ovarian regression (Zadworny et al., 1988; Youngren et al., 1991). On the other hand, rearing chick hens and subsequent that hens had been removed their entire chicks exhibit an abrupt increase in plasma LH levels concurrent with the decrease of plasma PRL levels (Leboucher et al., 1990). It has been reported that circulating of LH and estradiol levels gradually increased after hatching or when chicks were present and reached peak values immediately after removal of chicks

(Sharp et al., 1979; 1988; Kuwayama et al., 1992; Richard-Yris et al., 1998). Thus, it is possible that PRL may also be importance in the increased the number of VIP-ir neurons in the IH-IN observed in rearing native Thai hens. These findings taken together with the results in the present study clearly implicate the enhanced activity of the VIP/PRL system in the initiation and maintenance of rearing behavior in native Thai hens. Indeed, in this present study, disruption of rearing behavior by removing the chicks from the hens markedly decreases in the number of VIP-ir neurons in the IH-IN.

In conclusion, the present findings indicate an association between the VIPergic system and rearing behavior in the native Thai chickens. The differential expression of VIP neurons in the IH-IN might play a regulatory role in year-round reproductive activity and subsequent PRL release in this equatorial bird, non photoperiodic species. Disruption of rearing behavior decreases the number of VIP-ir neurons in the IH-IN. Therefore, the VIPergic system in the IH-IN may be involved in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of rearing behavior in native Thai chickens. It is further indicated that the VIP/PRL system is not only a key regulator of incubation behavior as it is well established but it may also be involved in the regulation of rearing behavior in the native Thai chicken. These findings also suggest that the presence of chicks and/or physical contact with chicks induced maternal behavior in the native Thai hens. The relationship between hens and chicks while hens took care of their young might slow down the decreases in circulating PRL levels and the number of VIP-ir neurons in the IH-IN which, in turn, facilitate, stimulate or maintain maternal care behavior in this equatorial bird.

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CHAPTER V

EFFECTS OF REARING BEHAVIOR ON NEUROENDOCRINE REGULATION OF THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKENS: ROLE OF GONADOTROPIN RELEASING HORMONE

5.1 Abstract

There are two major neuroendocrine systems that play an important role in the avian reproductive cycle. One system involves gonadotropin releasing hormone-I (GnRH-I) and the subsequent release of follicle stimulating hormone (FSH) and luteinizing hormone (LH), named as GnRH/FSH-LH system. The other system involves the avian prolactin (PRL) releasing factor, vasoactive intestinal peptide (VIP) and the subsequent release of PRL, known as VIP/PRL system. The establishment of maternal behavior in birds is accompanied by a significant long term fall in plasma levels of LH and gonadal steroids, and the presence of chicks slows down the decrease in plasma PRL levels. The synthesis and secretion of FSH and LH are regulated by GnRH, a decapeptide which is secreted from hypothalamic neuronal cells. Changes in the number of hypothalamic GnRH-I neurons within the nucleus commissurae pallii (nCPa) are correlated with the reproductive cycle of native Thai chickens. Moreover, nest deprivation of incubating native Thai hens increases the

number of GnRH-I neurons in the nCPa. To date, there is no study describing the interrelationship and functional aspects of the GnRH system and rearing behavior in the native Thai chicken, non-temperate zone birds. Thus, to further understand the role of GnRH-I in the regulation of the reproductive cycle, especially the rearing behavior in this bird, native Thai chickens were divided into two groups; rearing (R) and non-rearing (NR) hens. The differential expression of GnRH-I-immunoreactive (GnRH-I-ir) neurons in the hypothalamus of R and NR hens were compared utilizing immunohistochemical techniques. Expression of hypothalamic GnRH-I-ir neurons within the nucleus anterior medialis hypothalami, nucleus suprachaiasmaticus, pars medialis, nCPa, nucleus septalis lateralis, nucleus paraventricularis magnocellularis, and regio lateralis hypothalami areas were observed in both groups. High expression of GnRH-I-ir neurons was found in the nCPa of NR hens, whereas fewer GnRH-I-ir neurons were observed in the nCPa of R hens. The number of GnRH-I-ir neurons in the nCPa was low in the R group and significantly increased by day 17 after removal of chicks from the hens. These findings indicate, for the first time, an association of the GnRH/FSH-LH system with rearing behavior in this non-photoperiodic, continuously breeding avian species. The expression of rearing behavior of native Thai chickens might be regulated, in part, by the differential expression of GnRH-I neurons in the nCPa. This study also confirms a pivotal role of GnRH-I in controlling of reproduction of this non-seasonally breeding, equatorial avian species.

5.2 Introduction

Avian reproductive is regulated by the integration of hormonal inputs by the hypothalamus, the pituitary, and the gonads. This system is referred as the hypothalamo-pituitary-gonadal (HPG) axis. It is very well documented that neurotransmitters, neurohormones, neuromodulators, and hormones of the HPG axis play an important role in the reproductive cycle of avian species. This HPG axis involves two major neuroendocrine systems controlling avian reproduction. These neuroendocrine systems include the gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH), and vasoactive intestinal peptide/prolactin (VIP/PRL) systems, and both systems are influenced by dopaminergic (DAergic) neurotransmission (Bhatt et al., 2003; Chaiseha et al., 2003; Chaiseha and El Halawani, 2005).

GnRH is a hypothalamic neuronal secretory decapeptide important for the regulation of reproduction in many vertebrates. GnRH was first isolated from porcine hypothalamus and has been sequenced (Peczely, 1989; Rivier, 2001). It consists of a family of at least 25 isoforms, and 15 isoforms are found in various vertebrate species (Gorbman and Sower, 2003; Kavanaugh et al., 2008). In avian species, three subtypes of GnRH and two subtypes of GnRH receptors have been found (Sun et al., 2001; Shimizu and Bedecarrats, 2006). Two distinct forms of GnRH have been isolated in chicken, cGnRH-I or GnRH-I and cGnRH-II (King and Millar, 1982; Miyamoto et al., 1982; Sherwood et al., 1988). The gene encoding cGnRH-I has been cloned and characterized (Dunn et al., 1993). GnRH-III is first demonstrated in lamprey and is also found in the brain of songbirds (Bentley et al., 2004).

In birds, cGnRH-I is responsible for the HPG axis, which is directly involved in controlling reproduction (Sharp et al., 1990). Growing evidence indicates that the three forms of GnRH influence avian gonadotropins secretion, but their abilities differ (Katz et al., 1990; Sharp et al., 1990; Guemene and Williams, 1992; Bentley et al., 2004; Stevenson and MacDougall-Shackleton, 2005; Proudman et al., 2006). Like in mammals, GnRH is synthesized by neurosecretory cells in the hypothalamus, released from the median eminence (ME) into the hypophysial portal vessels, and then transported to the pituitary gland, where it stimulates the secretion of the gonadotropins, FSH and LH. GnRH increases LH and FSH secretions of the anterior pituitary both *in vitro* and *in vivo* (Millar et al., 1986; Peczely, 1989). *In vivo* studies reveal that injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH concentrations in hens (Guemene and Williams, 1999; Proudman et al., 2006). Incubation of turkey anterior pituitary cells with GnRH results in an increase in LH-β-subunit mRNA expression and stimulates LH secretion (You et al., 1995). A pulsatile pattern of GnRH release is observed from the medial basal hypothalamus and the preoptic area (POA) *in vitro* (Li et al., 1994). In contrast, GnRH inhibits FSH-stimulated steroidogenesis in chickens but enhances LH-stimulated progesterone production (Hertelendy et al., 1982).

The egg laying period in birds is associated with relatively high levels of circulating FSH, LH, and gonadal steroids and is regulated by hypothalamic GnRH (El Halawani et al., 1988). cGnRH-I is the primary hypophysiotropic factor stimulating the release of LH, since active immunization against cGnRH-I causes a decline in plasma LH concentrations and complete regression of the reproductive system (Sharp et al., 1990). However, seasonal changes in the cGnRH-II-

immunoreactive neurons are noted, indicating an involvement of cGnRH-II in the control of reproduction (Teruyama and Beck, 2000; Stevenson and MacDougall-Shackleton, 2005). The various distributions of cGnRH-II and GnRH-III in avian brains suggest their functional significances. It is reported that cGnRH-II may act as a neurotransmitter (Jones, 1987; Katz et al., 1990), and GnRH-III may act as a potential mediator in transducing song-related stimuli to areas that control gonadotropin secretion (Bentley et al., 2004).

In birds, it has been reported that GnRH neuronal activity is regulated by photoperiod (Sharp and Blache, 2003). Photostimulatory inputs to GnRH neurons have the potential to increase GnRH mRNA transcription and GnRH release (Dunn and Sharp, 1999) as well as increase pituitary cell sensitivity to GnRH (Davies and Follett, 1975). The amount of hypothalamic GnRH increases during long day stimulation and decreases during photorefractoriness in many avian species (Dawson et al., 1985; Foster et al., 1987; Bluhm et al., 1991; Rozenboim et al., 1993a; Saldanha et al., 1994; Hahn and Ball, 1995; Dunn et al., 1996; Kang et al., 2006). In addition, gonadal steroid hormones, hypothalamic VIP, DA, and gonadotropin inhibitory hormone are thought to be involved in the regulation of GnRH secretion (Ramirez et al., 1984; Sharp et al., 1984; Deviche et al., 2000; Tsutsui et al., 2000). Moreover, active VIP immunoneutralization increases pituitary LH-β and FSH-β mRNA and is accompanied by a decline in PRL mRNA expression (Ahn et al., 2001).

In mammals, it is very well documented that GnRH regulates LH secretion in both spontaneous and induced ovulating species (Bakker and Baum, 2000). Similarly, GnRH plays a pivotal role in the control of avian reproduction as well. Changes in GnRH content are observed during the avian reproductive cycle. GnRH content of

discrete medial preoptic, infundibulum, and arcuate samples are higher in laying hens than that of non-laying hens (Advis et al., 1985). In turkeys, a temperate zone bird, it has been reported that GnRH-I mRNA is abundance within the nucleus commissurae pallii (nCPa), organum vasculosum, lamina terminalis, and nucleus septalis lateralis (SL) is greater in laying hens than in non-photostimulated and incubating hens, while lower mRNA expression is observed in photorefractory hens (Kang et al., 2006). Furthermore, an association between hypothalamic GnRH-I mRNA, GnRH-I peptide content, and pituitary gonadotropin secretion during the avian reproductive cycle has been documented (Millam et al., 1989; El Halawani et al., 1993; Rozenboim et al., 1993a; Dunn et al., 1996).

In mammals, GnRH perikarya axons terminate in the external layer of the ME, which is closed proximity to the terminals of tuberoinfundibular DA neurons (Ajika, 1979; Merchenthaler et al., 1984; Ugrumov et al., 1989). Like mammals, GnRH perikarya and fibers are more extensively distributed throughout the avian brains. GnRH-I localization studies have been conducted in the brains of many avian species, such as chickens (Kuenzel and Blahser, 1991), ducks (Bons et al., 1978), white-crowned sparrows (Blahser et al., 1989), Japanese quail (Teruyama and Beck, 2000), European starlings (Goldsmith et al., 1989), garden warblers (Bluhm et al., 1991), great tits and ring doves (Silver et al., 1992), turkeys (Millam et al., 1993), dark-eyed juncos (Saldanha et al., 1994), house sparrows (Hahn and Ball, 1995), cockerels (Sun et al., 2001), canaries (Bentley et al., 2004), and native Thai chickens (Sartsoongnoen et al., 2006; 2012). The main group of cGnRH-I cell bodies is located in the POA, with fibers extending along the third ventricle and then entering the ME, the area of GnRH secretion (Meddle and Follett, 1997). Furthermore, several studies have

reported the distribution of the cGnRH-I mRNA and its protein in the avian brain (Millam et al., 1989; Dunn and Sharp, 1999; Sun et al., 2001; Dawson et al., 2002; Kang et al., 2006). It has been reported that the greatest cGnRH-I mRNA expression occurs in the nCPa and around the organum vasculosum laminae terminalis (OVLT), which is more abundant in the nCPa, OVLT, and SL of the laying turkey hens than those of the non-photostimulated and incubating hens (Kang et al., 2006).

The differential expression of GnRH-I neurons has been reported in many temperate zone species, but little is known regarding neuroendocrine regulation in non-temperate zone gallinaceous birds. In contrast to the temperate zone seasonal breeding species, the native Thai chicken is a continuously breeding species found in the equatorial zone that produces eggs all year, independent of photoperiodic cues (Konsonsiriluk, 2007; Sartsoongnoen, 2007; Kosonsiriluk et al., 2008). Previous findings indicate that the distributions and the number of GnRH-I-ir neurons change across the reproductive cycle and during the incubation and nest deprivation periods of the native Thai chickens. The GnRH-I-ir neurons are distributed in a discrete region lying close to the third ventricle from the level of the POA through the anterior hypothalamus, with the greatest abundance found within the nCPa. Changes in the number of GnRH-I-ir neurons within the nCPa are correlated with the reproductive cycle of the native Thai chickens (Sartsoongnoen et al., 2012). Moreover, nest deprivation causes an increase in the number of GnRH-I-ir neurons in the nCPa of nest-deprived hens when compared with incubating hens, suggesting that GnRH-I expression is correlated with the reproductive stage of this non-seasonal breeding, equatorial species (Prakobsaeng et al., 2009; Sartsoongnoen et al., 2012).

To date, limited studies describe the interrelationship and functional aspects of the GnRH system and rearing behavior in the native Thai chickens. Therefore, the aim of this study was to investigate whether the differential expression of GnRH-I-ir neurons within the hypothalamic areas were associated with rearing behavior in the native Thai chickens. Differences in the number of GnRH-I-ir neurons within the hypothalamic areas of rearing hens with those of non-rearing hens were compared. The findings of differential expression of GnRH-I within individual hypothalamic areas may provide an insight into the mechanism(s) underlying the regulation of rearing behavior in the native Thai chickens.

5.3 Materials and Methods

5.3.1 Experimental Animals

Female and male native Thai chickens (*Gallus domesticus*), Pradoohangdam breed, 22-24 weeks old, were used. They were reared and housed (7-8 females : 1 male) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of darkness; 12L : 12D). Each hen was identified by wing band number. Feed and water were given *ad libitum*. The native Thai hens were randomly divided into two treatment groups; hens rearing chicks (R) and hens that had been removed from their chicks (NR). Hens in the R group were allowed to rear the chicks naturally. Hens in the NR group were disrupted from rearing behavior and not allowed to rear their chicks by removing them from the chicks to another pen. Egg production, nesting activity, and other behaviors were recorded daily throughout the experiments. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee.

5.3.2 Experimental Design

Seventy eight female and 10 male native Thai chickens, 22-24 weeks old, were used. The chickens were randomly divided into 10 floor pens (7-8 hens : 1 rooster) and observed for their daily behaviors. After hatching, the hens were divided into two groups; R and NR. The hens were then sacrificed at different time periods (day of hatch, day 4, 7, 10, 14, 17, and 21; n=6) after they started to rear their chicks or after the chicks were removed. To visualize and localize GnRH-I-ir neurons within the hypothalamic areas, the brain of each hen was fixed by pressure perfusion with 4 % paraformaldehyde, sectioned with a cryostat, and then processed by immunohistochemistry (IHC). A postmortem examination of each hen was performed to confirm its reproductive status.

5.3.3 Processing of Tissues for Immunohistochemistry

Prior to perfusion, the hens were intravenously injected with 3,000 units of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA), and then euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). The head was removed and immediately fixed by pressure-perfusion via the carotid arteries with 100 ml of phosphate buffered saline (PBS, pH 7.4) for 3-5 min, followed by 650 ml of a freshly prepared 4 % paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min according to a previously described method (Sartsoongnoen et al., 2012). 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. Frozen brains were sectioned in the coronal plane at a thickness of 16 µm using a cryostat (Leica

CM1850, Leica Instruments GMbH, Nussioch, Germany). Sections were mounted on chrome alum-gelatin-coated glass slides with two sections per slide and stored desiccated at -20 °C. Four adjacent sections of each individual hypothalamic area were processed by IHC in order to visualize and analyze changes in the number of GnRH-I-ir neurons. The stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988) was used to choose the sections containing the hypothalamic nuclei analyzed. For each area, the sections were chosen starting with the most rostral section that contained each nucleus and every subsequent section until the nucleus disappeared from view. The plane that expressed the greatest density of GnRH-I-ir neurons was chosen to analyze. These planes of sections have been published in earlier reports (Sartsoongnoen et al., 2012).

5.3.4 Immunohistochemistry

Changes in the number of hypothalamic GnRH-I-ir neurons of R and NR hens were determined by IHC according to a previously described method (Sartsoongnoen et al., 2012). The primary and secondary antibodies used for detecting GnRH-I-ir neurons were primary rabbit monoclonal antibody directed against GnRH-I (generously provide by Dr. J.R. Millam, University of California, Davis, USA) and Cy^{TM}3-conjugated AffiniPure donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.), respectively. Four adjacent sections from R and NR hens in the individual hypothalamic area were thawed at room temperature prior to use. The sections were rehydrated in PBS for 30 min at room temperature. After removing from PBS, the sections were then incubated with 60 µl of primary antibody at 1:1,000 dilution in PBS (pH 7.4) containing 1 % bovine serum albumin and 0.3 %

Triton-X 100 at 4 °C for overnight in a moist chamber, then washed three times with PBS (pH 7.4) for 5 min each. After washing, 60 µl of secondary antibody at 1:500 dilution in PBS was applied under dark conditions onto the sections. 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 and further analyzed.

5.3.5 Image Analysis

Microscopic images of the brain sections of the hens were visualized under a fluorescence microscope (Olympus IX71, Tokyo, Japan) using a cooled digital color camera (Olympus DP70, Tokyo, Japan). The images were captured and stored by DP70-BSW Software (Olympus, Tokyo, Japan). The differential expression of GnRH-I-ir neurons in each individual area of the brain was visualized and analyzed. The number of GnRH-I-ir neurons of four adjacent sections was counted manually to determine changes in the numbers of GnRH-I-ir neurons. To aid in the documentation of neuroanatomical results, the nomenclature and schematic diagrams from the stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988) and the chicken hypothalamus (Kuenzel and van Tienhoven, 1982) were used to illustrate GnRH-I immunoreactivity. The specificity of the anti-GnRH-I antibody was tested by omission of the primary antibody during that step of immunohistochemistry. No immunostaining of GnRH-I was observed in control sections.

5.3.6 Statistical Analysis

Significant differences in the number of GnRH-I-ir neurons (means ± SEM) in the individual hypothalamic areas according to each treatment group were compared utilizing one-way analysis of variance (ANOVA). Significant differences between treatment groups were computed utilizing Tukey's HSD Test. A probability less than 0.05 ($P<0.05$) indicated a significant difference. All statistical tests were analyzed employing the SPSS for Windows Software (version 13.0, SPSS Inc., Chicago, IL, USA).

5.4 Results

The distributions of GnRH-I-ir neurons and fibers were determined across the hypothalamic areas of R and NR hens; the nucleus anterior medialis hypothalami (AM), nucleus suprachaiasmaticus, pars medialis (SCNm), nCPa, SL, nucleus paraventricularis magnocellularis (PVN), and regio lateralis hypothalami (LHy) areas were analyzed (Figures 5.1). A high density of GnRH-I-ir neurons was found in the nCPa of NR hens (Figure 5.2), whereas less expression was observed in the nCPa of R hens. Some GnRH-I-ir fibers were observed in the AM, SCNm, SL, PVN, and LHy of both R and NR hens, but differences were not noted (Figure 5.3). The differential expression of GnRH-I-ir neurons in the nCPa of R and NR hens is shown in Figure 5.4. Changes in the number of GnRH-I-ir neurons in the nCPa of R and NR hens at different time periods are shown in Figure 5.5 and Table 5.2. In the comparison of R and NR groups, the number of GnRH-I-ir neurons in the nCPa was low in the R group. The number of GnRH-I-ir neurons in the nCPa was increased when the hens were deprived from their chicks, showing a significant difference ($P<0.05$) at day 17

(R17 vs NR17; 0.25 ± 0.13 vs 3.38 ± 1.28 cells), and day 21 (R21 vs NR21; 0.71 ± 0.30 vs 2.46 ± 0.67 cells) of observation.

Table 5.1 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988).

AM	Nucleus anterior medialis hypothalami
SCNm	Nucleus suprachaiasmaticus, pars medialis
nCPa	Nucleus commissurae pallii
SL	Nucleus septalis lateralis
PVN	Nucleus paraventricularis magnocellularis (Paraventricular nucleus)
LHy	Regio lateralis hypothalami

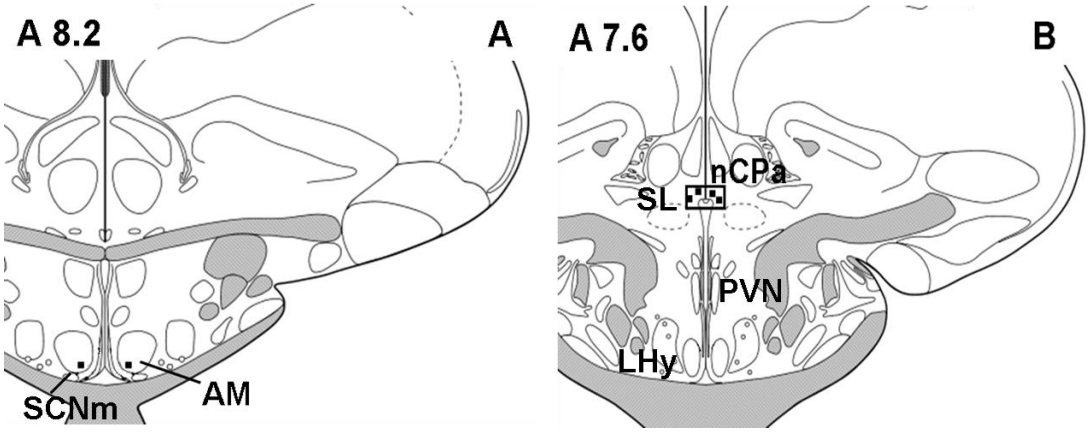

Figure 5.1 Schematic coronal brain sections showing the areas where the expression of GnRH-I-ir (black squares) was observed (**A-B**). The sampling regions for counting the number of GnRH-I-ir neurons in the nCPa (**B**) are represented by rectangles. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). For abbreviations, see Table 5.1.

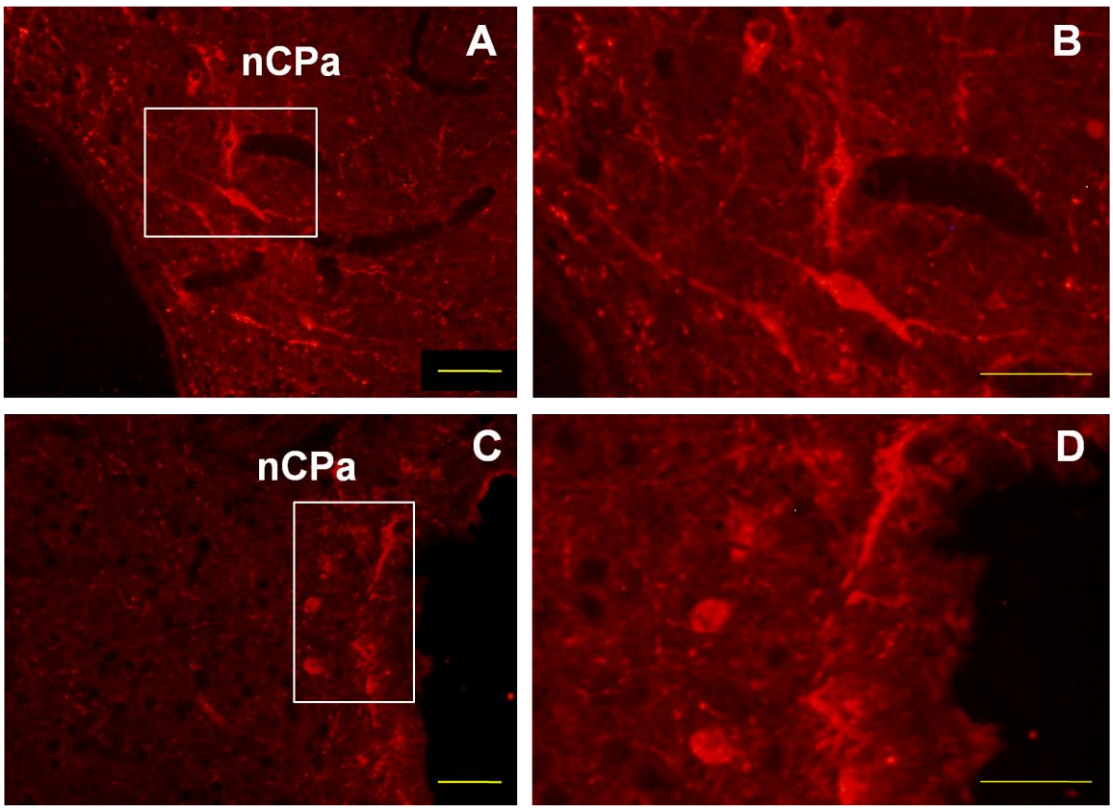

Figure 5.2 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the nucleus commissurae pallii (nCPa) of the native Thai chickens (**A, C**). Rectangle indicates area from which the following photomicrographs were taken. Higher magnification of the GnRH-I-ir neurons was demonstrated in the nCPa (**B, D**). For abbreviations, see Table 5.1. Scale bar = 50 µm.

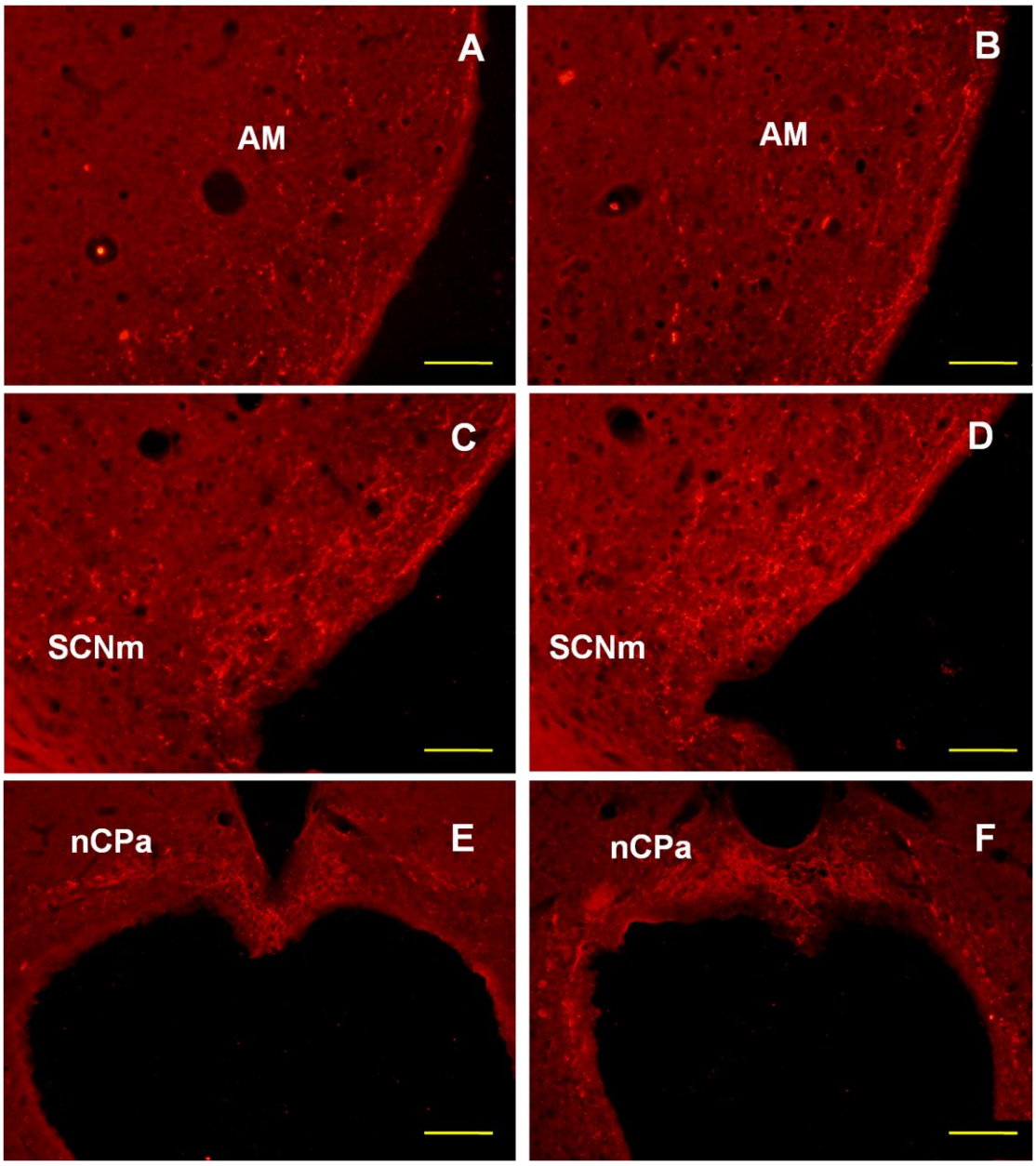

Figure 5.3 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the hypothalamic areas of rearing (**A, C, E, G, I, and K**) and non-rearing (**B**, **D**, **F**, **H, J, and L**) native Thai hens. For abbreviations, see Table 5.1. Scale bar = 100 µm.

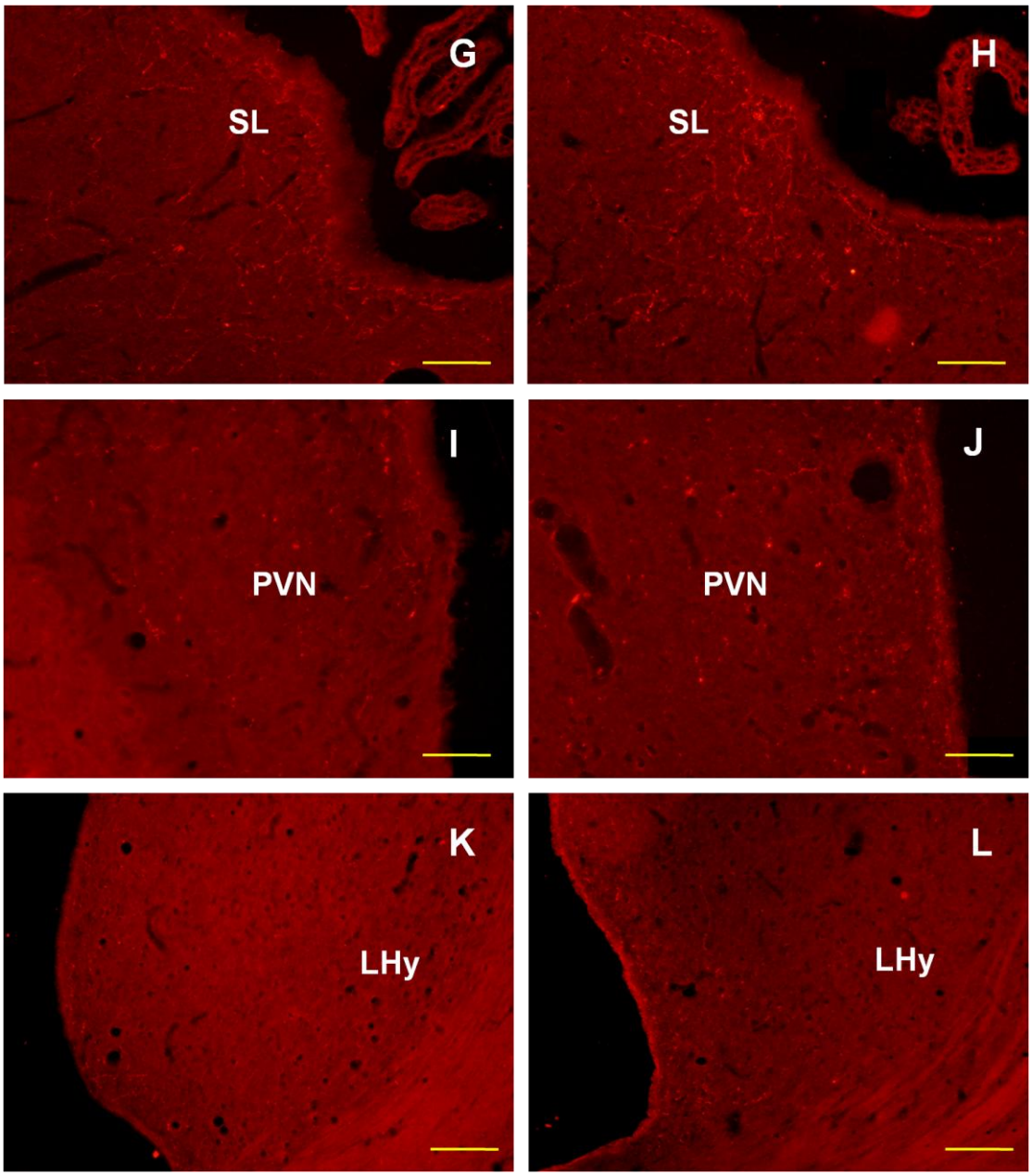

Figure 5.3 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the hypothalamic areas of rearing (**A, C, E, G, I, and K**) and non-rearing (**B**, **D**, **F**, **H, J, and L**) native Thai hens. For abbreviations, see Table 5.1. Scale bar = 100 µm (continued).

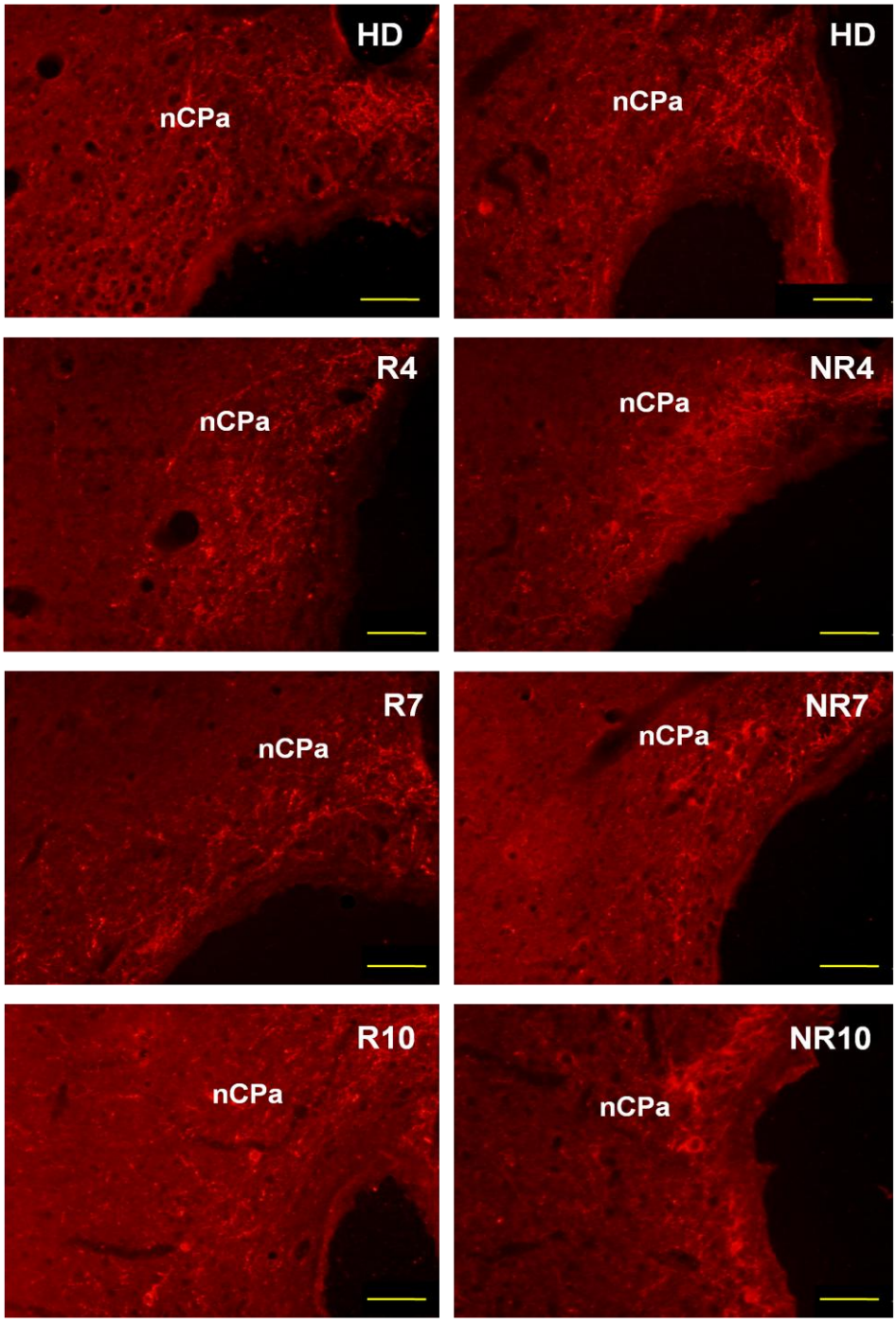

Figure 5.4 Photomicrographs showing the distributions of GnRH-I-ir neurons and fibers in the nucleus commissurae pallii (nCPa) of rearing (R), and non-rearing (NR) native Thai hens on day of hatch (HD) and different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 5.1. Scale bar = 100 µm.

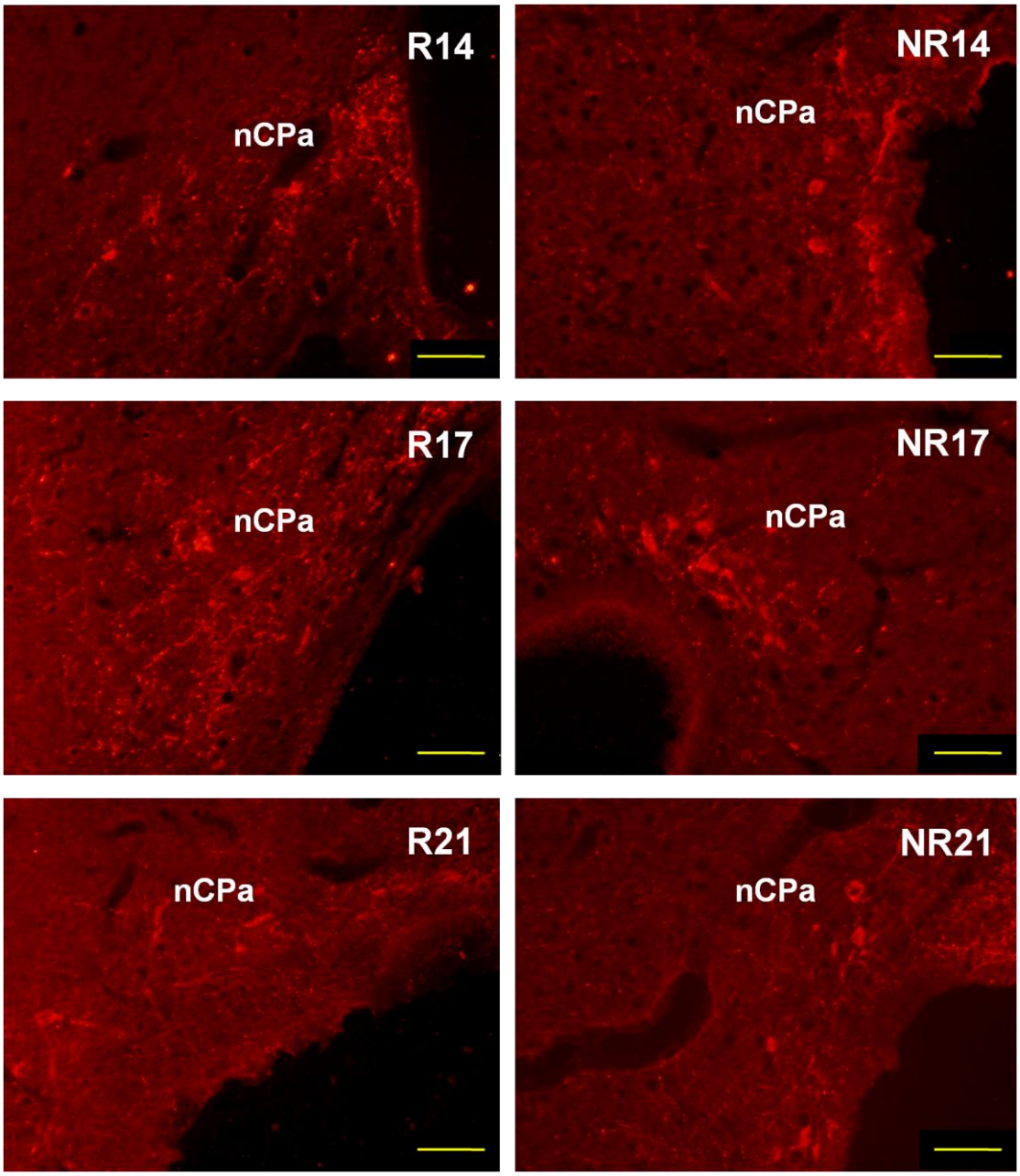

Figure 5.4 Photomicrographs showing the distributions of GnRH-I-ir neurons and fibers in the nucleus commissurae pallii (nCPa) of rearing (R), and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 5.1. Scale bar = 100 µm (continued).

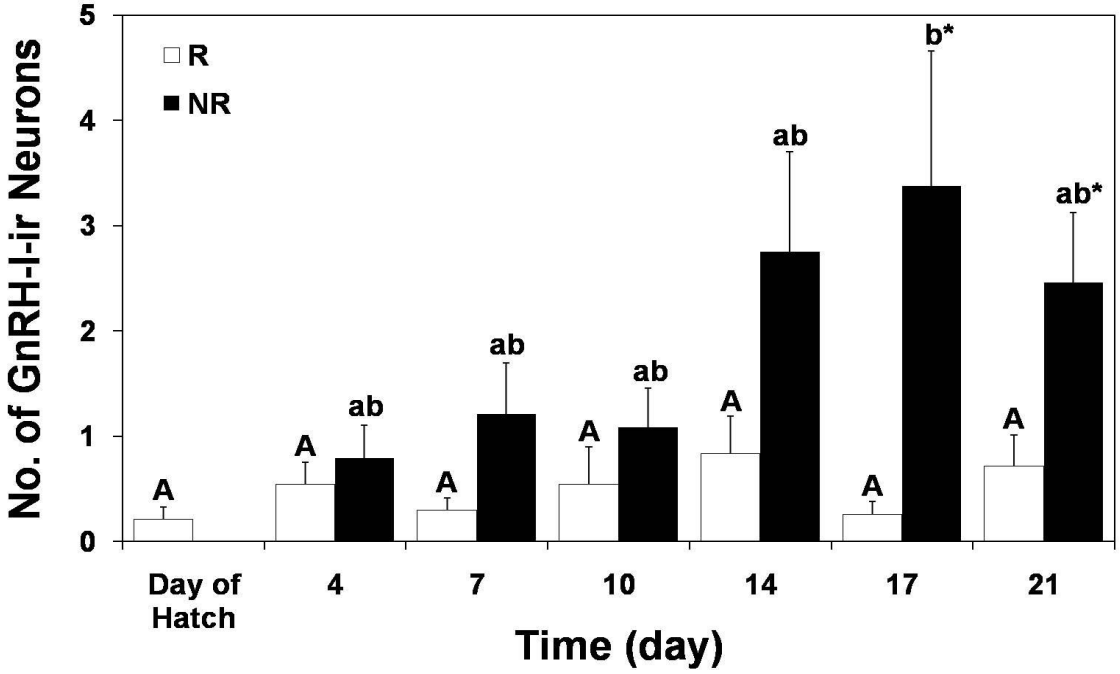

Figure 5.5 Changes in the number of GnRH-I-ir neurons in the nCPa of rearing (R) and non-rearing (NR) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 5.2 The number of GnRH-I-ir neurons (Mean ± SEM) in the nCPa of rearing (R) and non-rearing (NR) native Thai hens at different days of rearing or non-rearing their chicks (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Group	**Days Following Rearing or Non-Rearing Their Chicks**						
	Day of Hatch	**4**	**7**	**10**	**14**	**17**	**21**
R	0.21 ± 0.12^{A}	0.54 ± 0.21^{A}	0.29 ± 0.12^{A}	0.54 ± 0.36^{A}	0.83 ± 0.36^{A}	0.25 ± 0.13^{A}	0.71 ± 0.30^{A}
NR	N/A	0.79 ± 0.31^{ab}	1.21 ± 0.48^{ab}	1.08 ± 0.37^{ab}	2.75 ± 0.95^{ab}	$3.38 \pm 1.28^{b*}$	$2.46 \pm 0.67^{ab*}$

5.5 Discussion

The results from the present study demonstrate that GnRH-I-ir neurons and fibers were distributed throughout the hypothalamic areas of R and NR hens and were predominantly expressed in the nCPa area. GnRH-I-ir fibers were observed within the AM, SCNm, SL, PVN, and LHy of both R and NR hens. A high accumulation of GnRH-I-ir neurons was found in the nCPa of NR hens. Changes in the number of GnRH-I-ir neurons within the hypothalamic areas of R and NR hens were also observed in the nCPa. The number of GnRH-I-ir neurons in the nCPa was low in the R hens and significantly increased by day 17 after the chicks were removed from the hens. These findings indicate, for the first time, an association of the GnRH/FSH-LH system with rearing behavior in this non-photoperiodic, continuously breeding avian species.

In the present study, the distributions of GnRH-I-ir neurons and fibers were observed throughout the hypothalamus of R and NR hens, with the greatest density found within the nCPa area and the differential expression of GnRH-I neurons observed in both groups. High expression of GnRH-I-ir neurons was found in the nCPa of NR hens, whereas lower numbers of them were observed in the nCPa of R hens, suggesting that GnRH-I expression in this hypothalamic area may be involved in the reproductive regulatory system of the native Thai chickens. The obvious group of GnRH-I-ir neurons and fibers found in the nCPa in this study corresponded with previous studies in the native Thai chickens, demonstrating that the majority of GnRH-I-ir neurons and fibers are distributed primarily in the preoptic region throughout the caudal end of the hypothalamus, where the greatest density of GnRH-I-ir neurons was found within the nCPa (Sartsoongnoen, 2007; Prakobsaeng et al.,

2009; Prakobsaeng, 2010; Sartsoongnoen et al., 2012). Further evidence supporting these findings are studies in Japanese quails, domestic chickens, and turkeys, indicating that the greatest numbers of GnRH-I-ir neurons and GnRH-I mRNA expression are found in the nCPa (Teruyama and Beck, 2000; Kang et al., 2006; Kuenzel and Golden, 2006). The extensive distribution of GnRH-I immunoreactivity in the present study is consistent with the important role played by this hypothalamic neuropeptide in gonadotropin secretion and in the control of reproductive activity, as previously reported in other avian species (Mikami et al., 1988; Katz et al., 1990; Silver et al., 1992; Millam et al., 1993; van Gils et al., 1993; Dawson and Goldsmith, 1997; Sharp et al., 1998).

Changes in the number of GnRH-I-ir neurons were observed in the nCPa of R and NR hens, and the numbers of GnRH-I-ir neurons were compared. The number of GnRH-I-ir neurons in the nCPa was low in the R hens and did not differ at different times during observation of the rearing period. These results correspond with the changes of these hypothalamic neurons in the nCPa throughout the reproductive cycle of the native Thai chickens. It was reported that the greatest number of GnRH-I-ir neurons in the nCPa is found in laying hens, which then decreases in incubating and non-laying birds, with the lowest numbers found during the rearing stage. Moreover, nest deprivation of incubating native Thai hens increases the number of GnRH-I-ir neurons in the nCPa (Sartsoongnoen et al., 2012). Furthermore, changes in GnRH synthesis and secretion are correlated with the reproductive stage of other birds (Sharp et al., 1990; Rozenboim et al., 1993a; 1993b; Dunn et al., 1996; Kang et al., 2006). It has been reported that birds at the peak level of reproductive activity have more GnRH-I-ir neurons and fibers than those of sexually inactive or photorefractory

birds (Sharp et al., 1990; Sharp, 1993; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998; Stevenson and MacDougall-Shackleton, 2005; Stevenson and Ball, 2009). Hypothalamic GnRH mRNA expression is greater in laying hens than that of incubating hens (Dunn et al., 1996; Kang et al., 2006) and is lowest in photorefractory hens (Kang et al., 2006). In addition, GnRH peptide contents of discrete medial preoptic, infundibulum, and arcuate samples are higher in laying hens than those of non-laying hens (Advis et al., 1985). Recently, it has been suggested that changes in the number of GnRH-I neurons in the nCPa are key regulators of the avian reproductive cycle, whether birds are photoperiodic or equatorial breeders (Sartsoongnoen et al., 2012).

Disruption of rearing behavior by removing the chicks from the hens results in an immediate increase in plasma LH and estradiol concentrations and a marked decrease in plasma PRL levels (Sharp et al., 1979; Leboucher et al., 1990; Kuwayama et al., 1992; Chaiyachet et al., 2010). In the present study, the numbers of GnRH-I-ir neurons in the nCPa of the NR hens were gradually increased at day 4 and significantly increased by day 17 when compared with the R hens during observation periods. These results are supported by the studies indicating that after rearing chicks hens and subsequent that hens had been removed their entire chicks exhibit an abrupt increase in plasma LH levels concurrent with a decrease in plasma PRL levels (Leboucher et al., 1990). Indeed, it has been reported that the secretion of LH and estradiol are gradually increased after hatching or the presence of chicks and reach peak values immediately after removal of chicks (Sharp et al., 1979; 1988; Kuwayama et al., 1992; Dunn et al., 1996; Richard-Yris et al., 1998). This increase in plasma LH levels is associated with a significant increase in hypothalamic cGnRH-I

mRNA (Dunn et al., 1996; Dunn and Sharp, 1999). Additionally, it also has been reported that ovary mass of birds is positively correlated with GnRH-I mRNA levels in the brain of sexually mature passerine songbirds (Ubuka and Bentley, 2009).

Removal of chicks from the care of hens results in an increase in the number of GnRH-I-ir neurons in the nCPa (this study) and a decrease in VIP-ir neurons in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) and a marked decline in plasma PRL levels (Chaiyachet et al., 2010). In addition, disruption of rearing behavior increases ovary and oviduct weights, and hens resume egg laying within 21 days after removing of chicks. Therefore, the number of GnRH-I-ir neurons in the nCPa, which decrease in the R hens but increase in the NR hens, may be involved in the regulation of PRL and LH secretions and rearing behavior in the native Thai chickens. These findings are further supported by studies in nest-deprived native Thai hens showing a significant increase in the number of GnRH-I-ir neurons in the nCPa (Sartsoongnoen et al., 2012), a decrease in VIP-ir neurons in the IH-IN, and a precipitous decline in plasma PRL levels, which are accompanied by an increase in ovary and oviduct weights following the start of the new laying cycle (Prakobsaeng et al., 2011).

In avian species, it has been suggested that the presence of newly hatched chicks may suppress LH secretion and prevent decreases in plasma PRL levels, and depressed LH secretion is associated with a decrease in the expression of hypothalamic GnRH-I. This decrease is thought to be regulated by the inhibitory effect of PRL (Sharp et al., 1988; Kuwayama et al., 1992; Dunn et al., 1996). Moreover, PRL acts concomitantly with VIP to inhibit LH by means of reduction of GnRH content in the hypothalamus (Rozenboim et al., 1993b). Immunoneutralization

of VIP increases pituitary LH-β and FSH-β mRNAs and is accompanied by a decline in PRL mRNA expression (Ahn et al., 2001). The neuronal interactions between the DAergic, VIPergic and GnRH-I neuronal systems in the regulation of reproductive activities in birds have been well established. The relationship between the DAergic and the GnRH-I systems has been demonstrated by photostimulation in turkeys, revealing that during the photoinducible phase, the number of activated DA neurons in the nucleus premamillaris and GnRH-I neurons in the nCPa are increased, as well as an up-regulation of GnRH-I mRNA expression (Thayananuphat et al., 2007). It has been reported that GnRH perikarya axons terminate in the ME, which is in close proximity to the terminals of tuberoinfundibular DA neurons (Ajika, 1979; Merchenthaler et al., 1984; Ugrumov et al., 1989). It has also been proposed that DA from the tuberoinfundibular area may be one of the putative neurotransmitters responsible for the increased activity of GnRH within the ME of chicks showing precocious puberty (Fraley and Kuenzel, 1993). Furthermore, DA axons and terminals are found intermingled with VIP neurons in the infundibular nuclear complex, GnRH neurons in the POA, and with both VIP and GnRH terminals in the external layer of the ME (Contijoch et al., 1992; Fraley and Kuenzel, 1993). Moreover, it has been suggested that the interactions between the VIP and the GnRH systems are innervated by VIP-ir fibers and GnRH-I-ir neurons in the lateral septal organ in the nucleus accumbens, the caudal-most septal area, and the ME (Teruyama and Beck, 2001). Activation of DAergic neurons in the nucleus mamillaris lateralis is associated with the activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006). Immunohistochemical studies of the native Thai chickens suggested that the expression of VIP neurons in the IH-IN following hatching of the young may, in

part, account for the difference in reproductive neuroendocrine responses of this equatorial bird (Kosonsiriluk et al., 2008). It has been further demonstrated that the increase in GnRH-I-ir neurons caused by nest deprivation is related to the DAergic system (Sartsoongnoen et al., 2012). It has been reported that DA-ir neurons in the nucleus intramedialis increase significantly during the incubating stage (Sartsoongnoen et al., 2008), and nest deprivation results in decreased DA-ir neurons in this area, paralleling decreased VIP-ir neurons in the IH-IN (Prakobsaeng et al., 2011). These data well support an association of the neuronal interactions between GnRH-Iergic, VIPergic, and DAergic systems in the regulation of reproductive activity in the native Thai chickens.

In summary, this present study clearly demonstrates that disruption of rearing behavior by removing the chicks from the hens increases the number of GnRH-I-ir neurons in the nCPa, suggesting that GnRH-I neurons in this hypothalamic area may be involved in the suppression of rearing behavior in the native Thai chickens. Furthermore, the initiation of maternal care behavior in the native Thai chickens might be, in part, regulated by the differential expression of GnRH-I neurons in the nCPa. Alternatively, the changes noted may be restricted to the reinitiation of reproductive activity and egg laying that follows removal of chicks from the hen.

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CHAPTER VI

CONCLUSION

Native Thai chickens highly express maternal behaviors including incubation behavior and broodiness or rearing behavior. These behaviors affect the production of the native Thai chickens because they reduce the number of eggs produced. Therefore, in order to improve the productivity of this chicken, it is very important to understand the basic neuroendocrinology influencing its maternal behaviors, especially the rearing behavior of the native Thai chickens. The avian reproductive system is regulated by the hypothalamo-pituitary-gonadal axis. The two neuroendocrine systems that play a pivotal role in the avian reproductive system are the gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) and vasoactive intestinal peptide/prolactin (VIP/PRL) systems. Both systems are influenced by dopaminergic neurotransmission. Thus, the aims of this dissertation were to investigate the roles of PRL, VIP, and GnRH on the neuroendocrine regulation of rearing behavior in native Thai chickens.

The results revealed that plasma PRL concentrations remained at high levels on the day the chicks were hatched, then rapidly decreased after the first week of hatching and then remained at low levels throughout the eight weeks of the rearing period (Figure 6.1A). The ovary and oviduct weights of hens rearing their chicks were significantly decreased since the chicks were hatched then showed a significant increase at week 8 of the rearing period (Figures 6.1B and C). During five weeks of

the rearing period, plasma PRL levels of hens rearing chicks (R) were compared with those of non-rearing chicks hens (NR). The levels of plasma PRL were markedly decreased in hens that had their chicks removed and reached the lowest levels by the third week of separation from chicks. In the R hens, plasma PRL levels were high when compared to those of the NR hens during the different observation periods (Figure 6.2A). Disruption of rearing behavior by removing their chicks increased the ovary and oviduct weights, the presence of ovarian follicles, and the number of egg laying hens (Figures 6.2B and C).

The decrease of plasma PRL concentrations in the NR hens paralleled the decline in the number of VIP-immunoreactive (VIP-ir) neurons in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN; Figure 6.3A). The number of VIP-ir neurons in the IH-IN remained high after the day the chicks were hatched until day 7 of the rearing period and then sharply decreased from day 10 to day 21. When the chicks were deprived from the hens, VIP-ir neurons were markedly decreased on day 4 and continued to be lower than those of R hens through day 21 (Figure 6.3A). In contrast with the levels of plasma PRL and the numbers of VIP-ir neurons in the IH-IN, the number of GnRH-I-ir neurons in the nucleus commissurae pallii (nCPa) was low in the R group and significantly increased by day 17 after removal of chicks from the hens (Figure 6.3B). In addition, the effects of removal of chicks from the care of hens induced ovarian and oviduct recrudescence within 7 days, appearance of yellow follicles by day 14, and the initiation of a new laying cycle by day 21 (Figures 6.3C and D).

In summary, the present study indicated an association between plasma PRL levels, VIP neurons in the IH-IN, and GnRH-I neurons in the nCPa, suggesting that

the expression of rearing behavior in the native Thai chickens might be, in part, regulated by the differential expression of VIP neurons in the IH-IN and GnRH-I neurons in the nCPa. The relationship between hens and chicks while hens took care of their young might slow down the decreases in circulating PRL levels and the number of VIP-ir neurons in the IH-IN which, in turn, facilitate, stimulate or maintain rearing behavior in the native Thai chickens. Taken together, the present findings indicate that disruption of rearing behavior by removing the chicks from the hens markedly decreases plasma PRL levels and the number of VIP-ir neurons in the IH-IN, and is accompanied by an increase in the number of GnRH-I-ir neurons in the nCPa, suggesting that the VIPergic system in the IH-IN and the GnRH system in the nCPa may be involved in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of rearing behavior in native Thai chickens. It is further indicated that the VIP/PRL system is not only a key regulator of incubation behavior as it is well established, but it may also be involved in the regulation of rearing behavior in the native Thai chicken. These findings further suggest that the presence of chicks and/or physical contact with chicks induced maternal care behavior in the native Thai hens as well. These findings also indicate, for the first time, an association of GnRH/FSH-LH system with rearing behavior in this non-photoperiodic, continuously breeding avian species. This confirms a pivotal role of hypothalamic GnRH-I in controlling of avian reproduction of this equatorial species.

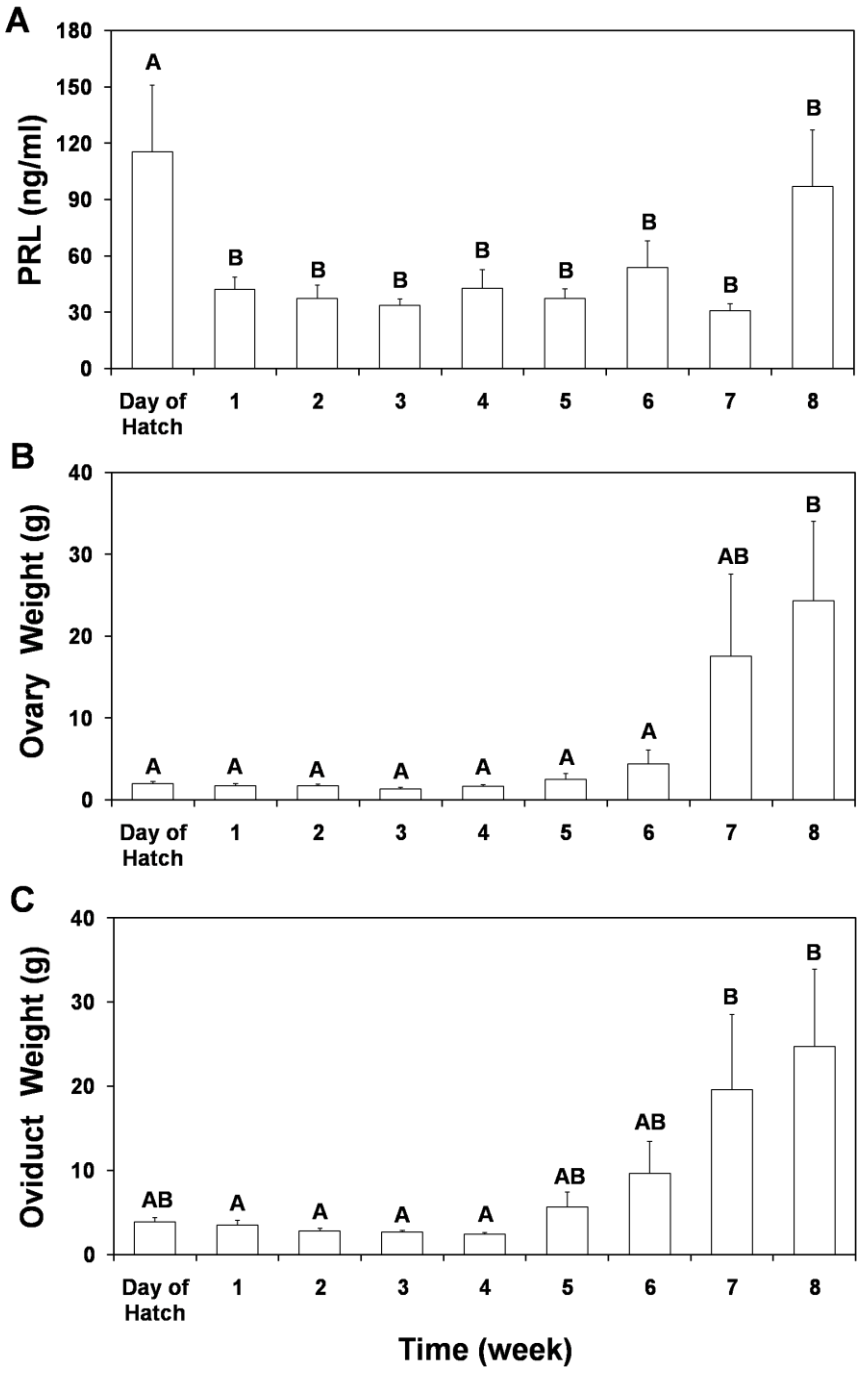

Figure 6.1 Changes in: **A**, plasma PRL concentration; **B**, ovary; **C**, oviduct weights of native Thai hens rearing chicks during the eight weeks of rearing period. Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$).

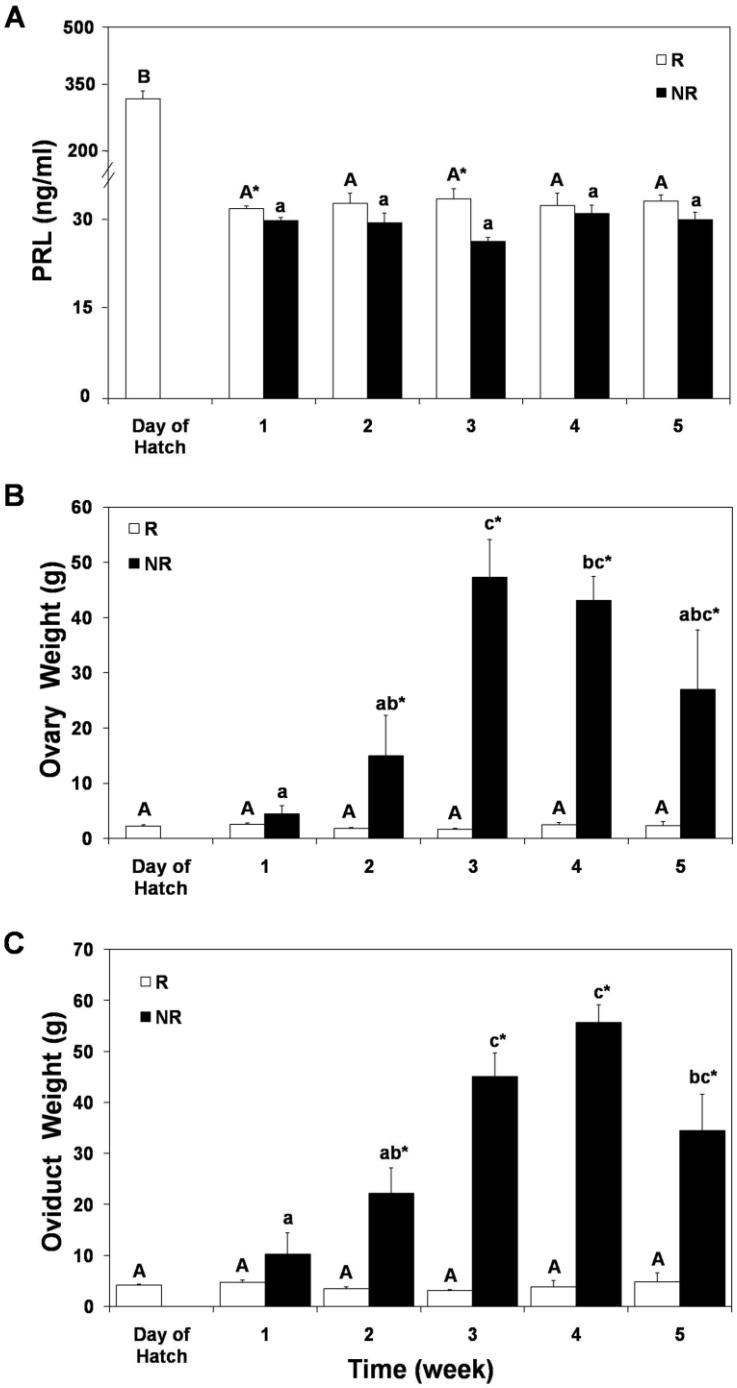

Figure 6.2 Changes in: **A**, plasma PRL concentration; **B**, ovary; **C**, oviduct weights of rearing (R) and non-rearing (NR) native Thai hens. Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

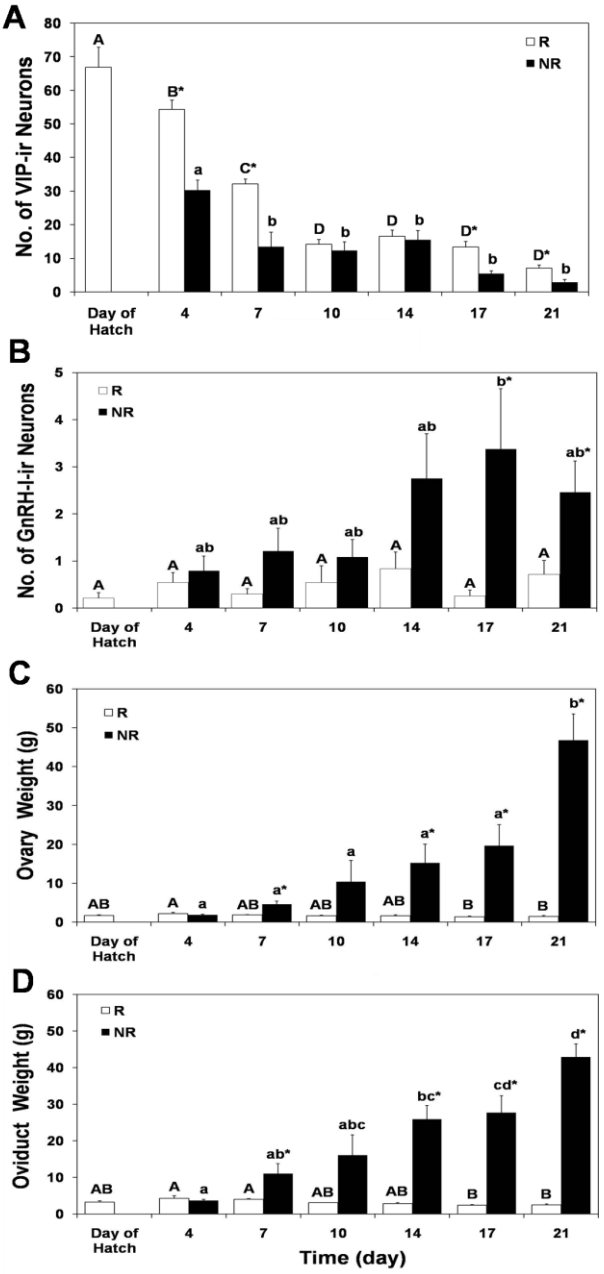

Figure 6.3 Changes in: **A**, the number of VIP-ir neurons in the IH-IN; **B**, the number of GnRH-I-ir neurons in the nCPa; **C**, ovary; **D**, oviduct weights of rearing (R) and non-rearing (NR) native Thai hens. Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

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