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EFFECTS OF INCUBATION BEHAVIOR UPON THE NEUROENDOCRINE REGULATION OF THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKENS

Nattiya Prakobsaeng

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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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การเพิ่มขึ้นของระดับโปรแลคตินมีความสัมพันธ์กับพฤติกรรมการฟักไข่ในไก่พื้นเมืองไทย การแสดงออกของพฤติกรรมดังกล่าวก่อให้เกิดปัญหาประสิทธิภาพการผลิตไข่ลดลง ซึ่งความสัมพันธ์ของโปรแลคติน วาโซแอคทีฟอินเทสทินอลเปปไทด์ โกนาโดโทรปินรีลิสซิงฮอร์โมน และโดปามีนกับการควบคุมพฤติกรรมการฟักไข่โดยระบบประสาทและระบบต่อมไร้ท่อในไก่พื้นเมืองไทยได้มีการศึกษาโดยการเปรียบเทียบการเปลี่ยนแปลงของจำนวนเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ เซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมน และเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสของไก่ที่ฟักไข่และไก่ที่ถูกพรากจากรังโดยการใช้เทคนิคอิมมูโนฮิสโตเคมิสทรี โดยไทโรซีนไฮดรอกซีเลสได้ถูกใช้เป็นตัวบ่งชี้ถึงเซลล์ประสาทที่ผลิตโดปามีน ระดับโปรแลคตินในพลาสมาถูกตรวจสอบโดยการใช้เทคนิคเอนไซม์ลิงค์อิมมูโนซอร์เบนท์แอสเสย์ ผลการศึกษาพบว่าโปรแลคตินมีระดับสูงในช่วงของการฟักไข่และลดลงอย่างมีนัยสำคัญภายในหนึ่งวันหลังจากการพรากไก่จากรัง จำนวนเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์บริเวณนิวเคลียสอินเฟอริโอริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไมมีจำนวนมากระหว่างช่วงของการฟักไข่และลดลงอย่างมีนัยสำคัญในวันที่หกของการพรากไก่จากรัง จำนวนเซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนบริเวณนิวเคลียสคอมมิสซูรีพาลลิไอมีจำนวนต่ำในไก่ที่ฟักไข่และเพิ่มขึ้นอย่างมีนัยสำคัญในวันที่หกของการพรากไก่จากรัง จำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสบริเวณนิวเคลียสอินทราเมดิเอลิสและนิวเคลียสแมมิลลาริสแลเทอราลิสมีจำนวนมากระหว่างช่วงของการฟักไข่และลดลงในวันที่สิบและวันที่หกของการพรากไก่จากรังตามลำดับ การขัดขวางพฤติกรรมการฟักไข่โดยการพรากไก่จากรังส่งผลให้น้ำหนักของรังไข่และท่อนำไข่ จำนวนฟอลลิเคิลในรังไข่ และจำนวนไก่ไข่เพิ่มมากขึ้น ผลการศึกษาบ่งชี้ว่าสิ่งแวดล้อมจากภายนอกอันได้แก่ การปรากฏของรังและไข่ มีส่วนร่วมในการกระตุ้นการหลั่งของโปรแลคตินและการดำรงอยู่ของพฤติกรรมการฟักไข่ในไก่พื้นเมืองไทย การพรากไก่ที่กำลังฟักไข่ออกจากรังทำให้ระดับโปรแลคตินลดลง ซึ่งสอดคล้องกับการลดลงของจำนวนเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์บริเวณนิวเคลียสอินเฟอริโอริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไม การเพิ่มขึ้นของจำนวนเซลล์ประสาทที่ผลิต

โกนาโดโทรปินรีลิสซิงฮอร์โมนบริเวณนิวเคลียสคอมมิสซูรีพาลลิไอ และควบคู่ไปกับการลดลงของจำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซิเลสบริเวณนิวเคลียสอินทราเมดิเอลิสและนิวเคลียสแมมิลลาริสแลเทอราลิส แสดงให้เห็นถึงความเชื่อมโยงระหว่างเซลล์ประสาทวาโซแอคทีฟอินเทสทินอลเปปไทด์บริเวณนิวเคลียสอินเฟอริโอริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไม เซลล์ประสาทโกนาโดโทรปินรีลิสซิงฮอร์โมนบริเวณนิวเคลียสคอมมิสซูรีพาลลิไอ และเซลล์ประสาทโดปามีนบริเวณนิวเคลียสอินทราเมดิเอลิสและนิวเคลียสแมมิลลาริสแลเทอราลิสกับการเพิ่มขึ้นของระดับโปรแลคติน เซลล์ประสาทโดปามีนบริเวณนิวเคลียสอินทราเมดิเอลิสและนิวเคลียสแมมิลลาริสแลเทอราลิสอาจจะมีอิทธิพลต่อเซลล์ประสาทวาโซแอคทีฟอินเทสทินอลเปปไทด์บริเวณนิวเคลียสอินเฟอริโอริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไมและเซลล์ประสาทโกนาโดโทรปินรีลิสซิงฮอร์โมนบริเวณนิวเคลียสคอมมิสซูรีพาลลิไอในการควบคุมการหลั่งโปรแลคตินและพฤติกรรมการฟักไข่ของไก่ที่อาศัยอยู่ในแถบเส้นศูนย์สูตรและสืบพันธุ์ได้ทุกฤดูกาลชนิดนี้

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ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NATTIYA PRAKOBSAENG : EFFECTS OF INCUBATION BEHAVIOR UPON THE NEUROENDOCRINE REGULATION OF THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKENS.
THESIS ADVISOR : ASST. PROF. YUPAPORN CHAISEHA, Ph.D.
398 PP.

DOPAMINE/GONADOTROPIN RELEASING HORMONE/INCUBATION/ NATIVE THAI CHICKEN/PROLACTIN/TYROSINE HYDROXYLASE/ VASOACTIVE INTESTINAL PEPTIDE

Hyperprolactinemia has been known to be associated with incubation behavior in native Thai chickens. The expression of such behavior is a costly problem, resulting in substantial loss of potential egg production. The association of prolactin (PRL), vasoactive intestinal peptide (VIP), gonadotropin releasing hormone-I (GnRH-I), and dopamine (DA) with the neuroendocrine regulation of incubation behavior were investigated in the native Thai chickens. The changes in the numbers of VIP-immunoreactive (VIP-ir), GnRH-I-immunoreactive (GnRH-I-ir), and tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the brain of incubating hens (INC) with those of nest-deprived hens (ND) were compared using immunohistochemistry. TH was used as a marker for DA neurons. Plasma PRL levels were determined by enzyme-linked immunosorbent assay. The results revealed that plasma PRL levels were high during incubating period and significantly decreased within a day of nest deprivation. The numbers of VIP-ir neurons in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) were high during incubating period and

significantly declined by day 6 of nest deprivation. The number of GnRH-I-ir neurons in the nucleus commissurae pallii (nCPa) was low in the INC group and significantly increased by day 6 of nest deprivation. The numbers of TH-ir neurons in the nucleus intramedialis (nI) and nucleus mamillaris lateralis (ML) were high during incubating period and significantly decreased by day 10 and day 6 of nest deprivation, respectively. Disruption of incubation behavior by nest deprivation increased the ovary and oviduct weights, the presence of ovarian follicles, and the number of egg laying hens. These results indicate that external cues including the presence of the nest and eggs are involved in the stimulation of PRL secretion and maintenance of incubation behavior in the native Thai chickens. Nest deprivation of incubating chicken reduces circulating PRL levels and is associated with a reduction in the number of VIP-ir neurons in the IH-IN, an increase in the number of GnRH-I-ir neurons in the nCPa, and a parallel decrease in the number of TH-ir neurons in the nI and ML areas, suggesting an association between VIP neurons in the IH-IN, GnRH-I neurons in the nCPa, and DA neurons in the nI and ML with the degree of hyperprolactinemia. DA neurons in the nI and ML may influence the VIP neurons in the IH-IN and GnRH-I neurons in the nCPa in the regulation of PRL secretion and the incubation behavior of this non-seasonally breeding, equatorial species.

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Student's Signature_____

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CHAPTER I

INTRODUCTION

1.1 Rational of the Study

Native Thai chicken (*Gallus domesticus*), belongs to genus Gallus of the family Phasianidae. It is a small domestic animal that probably originated from one of the several wild jungle fowls, which still found wildly distributed throughout Southeast Asia and was domesticated approximately 3,000 years ago. They have been raised in the countryside of Thailand for many generations. The main objectives for raising native Thai chickens are for consumption, sport competition, and recreation. It is not only a main animal protein food source, but it also can be sold for supplemental income for families as well. Its meat is firm 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 the good opportunity for production in industrial scale. Recently, the native Thai chicken has become the new economic domestic animal of Thailand with presently growing demand and relatively high price. The market price of native Thai chickens is two to three times higher than those of broilers. Therefore, there are no market problems concerning the native Thai chicken prices. To date, there are about 62 millions native Thai chickens in Thailand which are raised by 2.8 millions farmer's family and this raised animal is one of the exported goods that gained income about 2.2 millions baht per year. Moreover, the native Thai chickens are easy to raise, resistant to diseases, and acclimatized to the

local environments. It can be raised under poor environmental conditions in the backyard with local feeds. 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 tend to focus on "mixed farming" that is the strategies for helping rural farmers to increase self-sufficiency. The native Thai chicken is one of the significant resources of Thailand which need to be developed. However, the native Thai chickens have low productivity. The reproductive performance of native chicken is much lower than those of cross breeds and hybrids, especially egg-laying performance. The number of egg per hen is limited for producing the chicks. Generally, the native Thai hen lays eggs 3-4 times per year rather than laying all year long, and it produces 4-17 eggs per clutch. Thus, it produces about 30-92 eggs per year which is significantly lower than that of the imported hen which produces eggs all year long (240-270 eggs per year).

One of the main causes of low reproductive performance in native Thai chicken is the incidence of maternal behaviors such as incubation behavior. The onset of incubation behavior affects the number of egg production because it terminates egg laying. These cause the problem in order to be produced them commercially in poultry industry in Thailand. At present, market demands of native Thai chickens cannot be met by supplies due to their low productions. Thus, in order to increase the production of the native Thai chicken in Thailand, it is very important to understand the basic neuroendocrinology influencing its reproductive activities, especially incubation behavior. Although the native Thai chicken has been domesticated in Thailand for long time but there are only a limited number of researchers studying the neuroendocrine regulation of reproduction, especially incubation behavior.

Native Thai chicken is the domesticated chicken without genetic selection. It always expresses high maternal behaviors which is a heritable trait from the wild jungle fowl. Maternal behaviors are hormonal dependent and initiated with the onset of incubation behavior and continue through the period when the young are taking care by parent (broody/rearing behavior). As mentioned above, these behaviors constrain the number of egg produced. Parental behavior is defined as the behavior of the parents that contribute to the survival of their offspring. In some vertebrate species, mature male appear to have positive effects on infant development, growth, well-being, or survival knowing as paternal care. Most mothers display maternal behavior after parturition and serve the immediate provision of care and defense for their offspring. Maternal care in birds includes incubation and brooding or rearing behaviors. The term incubation refers to the maternal care of unhatched eggs and the taking care of chicks after hatching is known as brooding. One or both parents must incubate the egg until hatching and then provide post-hatching care.

The onset of incubation behavior is characterized by regression of the ovary and oviduct and is associated with declining plasma levels of gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), and follicle stimulating hormone (FSH), and greatly increasing of circulating prolactin (PRL) levels. PRL, an anterior pituitary hormone, has been implicated as a causative factor in the onset and maintenance of incubation behavior in birds. During reproductively quiescent stages of the cycle, plasma PRL levels are very low. During laying and incubating stages, circulating PRL levels increase dramatically and this rising in PRL levels has been implicated as the cause for cessation of ovulation, ovarian regression, and induction of incubation behavior. PRL is widely thought to play a role in parental behaviors by mediating

incubation behavior, crop milk production and secretion, feeding of the young, and nest defense. The expression of incubation behavior is a costly problem, resulting in substantial loss of potential egg production. In commercial flocks, nest and egg deprivation is a traditionally procedure used to disrupt incubation behavior. The decreasing of plasma PRL level and increasing of plasma LH level are observed in nest-deprived hens. Therefore, in order to increase the production of native Thai chickens in Thailand, it is deeming essential to understand the neuroendocrine regulation of the incubation behavior.

The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying, egg laying, incubating eggs, and rearing chicks. The primary components of the integrated female reproductive system are the brain, especially the hypothalamus, the pituitary, and the ovary. This integrated system is referred to as the hypothalamic-pituitary-gonadal axis (HPG axis). It is very well established that neurotransmitters, neuromodulators, neurohormones, and hormones of this axis play a pivotal role in the reproductive cycle of avian species. However, the neuronal and hormonal substances regulating reproduction in birds remain vaguely elucidated. In birds, there are two neuroendocrine systems that play a significant role in the reproductive cycle. One system involves chicken gonadotropin releasing hormone-I (cGnRH-I or GnRH) and the subsequent release of FSH and LH (GnRH/FSH-LH system) and the other system involves vasoactive intestinal peptide (VIP) and the subsequent release of PRL (VIP/PRL system). Both systems are influenced by dopaminergic (DAergic) neurotransmission.

It has been very well documented that gonadotropins and cGnRH-I are essential regulators of the reproductive cycle in several avian species. Gonadotropins,

FSH and LH, produced from anterior pituitary, are responsible for ovarian follicular growth, maintenance of the hierachical size of the follicles, and triggering ovulation, respectively. Subsequently, as the follicles increase in size, their production of steroid hormones, including progesterone (P) and estradiol (E) increase. In addition, there are evidences suggesting that P and E play an important role in modulation of gonadotropins secretion via feedback effect. In birds, physiological functions of these steroid hormones are correlated with ovulation and female secondary sex characteristics. It is very well established that the gonadotropins synthesis and secretion are under stimulatory control of the hypothalamic releasing factor, GnRH-I.

GnRH is a hypothalamic neuronal secretory decapeptide that play an important role in controlling of reproduction in vertebrates. GnRH regulates secretion of gonadotropins through binding to its specific receptor on the pituitary gonadotrops. To date, three forms of GnRH have been elucidated in the avian brain, cGnRH-I, cGnRH-II, and GnRH-III. It has been reported that cGnRH-I and cGnRH-II can differential stimulate the release of FSH and LH. However, cGnRH-I is thought to be the main hypophysiotropic factor stimulating the release of LH since immunization against cGnRH-I, but not cGnRH-II, causes a decline in the plasma LH concentrations and complete regression of the reproductive system. It has been reported that GnRH-I is the form that is directly involved in controlling of reproduction in avian species such as chickens, mallards, King penguins, turkeys, and cockatiels. This decaneuropeptide increases LH and FSH secretion. Ovarian development is found to be correlated with plasma LH levels and the amount of GnRH-I content, indicating that GnRH-I expression is important for maintaining of pituitary-ovarian function in chickens. Birds that display the reproductive activities have more GnRH

immunoreactive cells and fibers when compared with the sexually inactive ones. Moreover, in temperate zone birds, the stimulatory effect of long day usually appears to be associated with an increased hypothalamic GnRH content, while reproductively inactive photorefractoriness is correlated with a decreased GnRH content. There are increasing evidences indicating the involvement of hypothalamic DA in the regulation of cGnRH-I and the secretion of LH and FSH. Changes in the number of GnRH-I-immunoreactive (GnRH-I-ir) neurons in the nucleus commissurae pallii (nCPa) across the reproductive cycle of the native Thai chickens have been reported. The highest number of GnRH-I-ir neurons is observed in the nCPa of the laying hens, when compared with other reproductive stages.

As mentioned above, native Thai hens express high maternal behaviors such as incubation and rearing behaviors. Expression of incubation behavior affects the number of egg production because it terminates egg laying. There are several lines of evidence indicating that hormones play an important role in the reproductive cycle of avian species, including incubation behavior. PRL 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 incubation in birds. It is also well documented that PRL is under stimulatory control of hypothalamic VIP, the avian PRL-releasing factor (PRF). Moreover, some evidences suggest that DA plays an intermediary role in PRL secretion.

VIP, an octacosapeptide, has been found to be extensively distributed in the central and peripheral nervous systems with its high concentrations are found in the hypothalamus in mammals. It is considered to function as a neurotransmitter and

neuroendocrine substance. In birds, VIP plays a pivotal role in the regulation of PRL secretion. VIP meets the classical criteria for defining that it acts as the hypophysiotrophic PRF in birds. These criteria include; 1) the presence of VIP-immunoreactive (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-specific receptors on anterior pituitary cells, 5) the ability of VIP in regulating of to regulate anterior pituitary lactotrophs, and 6) the alteration of pituitary function, due to antagonism of VIP. It is very well documented that variations in hypothalamic VIP immunoreactivity, VIP contents, VIP mRNA steady-state levels, VIP mRNA expression in the infundibular nuclear complex, VIP receptor mRNA in the pituitary, and VIP concentrations in hypophysial portal blood are correlated with the changes in circulating PRL levels in many avian species. VIP neurons are found extensively throughout the avian hypothalamus. 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 of the infundibulum. Changes in numbers of VIP-ir neurons within the IH-IN area are directly correlated with changing of plasma PRL levels throughout the reproductive cycle. These findings suggested that hypothalamic VIP expression in the IH-IN of the native Thai chicken plays a regulatory role in year-round reproductive activity. The abundance of VIP neuronal networks in the hypothalamus of the native Thai chickens suggested its importance in the regulation of reproductive activities in this equatorial bird.

DA is found in both central and peripheral nervous systems of many species and has several important physiological functions involved in a wide variety of behaviors and reproduction. In mammals, the regulation of PRL secretion is under the inhibitory control of hypothalamic tuberoinfundibular DAergic neurons, which releases DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs. Removal of this DAergic inhibition results in an increased PRL release and hyperprolactinemia. This is not the case in birds, while removal of hypothalamic inputs results in the completed cessation of PRL secretion. In birds, it has been documented that PRL secretion is tonically stimulated by the PRF, VIP. At present, unlike the mammalian DAergic strategy for PRL control, the role of DA in the regulation of avian PRL secretion is unclear. DA neurons are found throughout the hypothalamus and have been shown to be immunoreactive for VIP. DA has been measured and visualized in various avian species including domestic fowls, quails, pigeons, zebra finchs, chickens, budgerigars, collared doves, turkeys, canaries, and native Thai chickens. Unlike mammals, it has been established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending on multiple DA receptor subtypes. 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. It has been established that dynorphin, serotonin, 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. Some other evidences suggest an inhibitory role of DA on GnRH release in mammals as well as in birds. Further evidence for the involvement of DA in correlating with GnRH is

derived from the occurring of dense concentration of tyrosine hydroxylase (TH; the rate limiting enzyme for DA synthesis) and GnRH-containing processes which are located in the lateral and mediobasal portion of the external layer of the hen median eminence. This result provides an opportunity for synaptic interaction between GnRH and DA. Activation of DAergic cells in the nucleus mamillaris lateralis is associated with the activation of GnRH-I and VIP neurons and the release of LH and PRL. Recently, the differential expression of hypothalamic TH-immunoreactive (TH-ir) neurons are compared across the reproductive cycle of the native Thai chickens. TH-ir neurons and fibers are found throughout the brain and are predominantly located within the diencephalon and mesencephalon. Interestingly, changes in the number of TH-ir neurons in the nucleus intramedialis (nI) are observed across the reproductive cycle and directly correlated with variations in circulating PRL levels. The population of TH-ir neurons in the nI increase significantly during the egg incubation period, when circulating PRL levels are the greatest. These findings indicate that an association exists between DA neurons 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 this tropical non-seasonally breeding avian species.

This dissertation was proposed to investigate the neuroendocrine regulation of incubation behavior in the female native Thai chickens. The results from this study will provide an insight into the neuroendocrine mechanism(s) underlying the regulation of the incubation behavior of native Thai chickens, which has never been studied. The knowledge gained from this dissertation can be then applied

commercially in poultry industry to increase egg production of native Thai chickens in Thailand.

1.2 Research Objectives

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

1.2.2 To study the differential expressions of VIP, GnRH-I, and DA that associated with the neuroendocrine regulation of incubation behavior in the female native Thai chickens.

CHAPTER II

LITERATURE REVIEW

2.1 Native Thai Chicken

Native Thai chickens or Thai indigenous chickens (*Gallus domesticus*), belongs to genus Gallus of the family Phasianidae, originated from the wild jungle fowl which still found wildly distributed throughout Southeast Asia (Austic and Nesheim, 1990). It is domesticated by village people approximately 3,000 years ago. Some characteristics of native Thai chickens are inherited from the wild jungle fowl and still expressed are maternal behaviors (incubation and rearing behaviors; Beissinger et al., 1998). Historically, native Thai chickens have long been in the countryside of Thailand. The main objectives of raising native Thai chickens are for consumption, sport competition, and recreation. It is not only a main animal protein food source, but it can be sold for supplemental income for families as well. The native Thai chickens are easy to raise, resistant to diseases, and acclimatized to the local environments. Generally, the native Thai chicken has a slower growth rate than that of the 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 advantage over native Thai chicken in term of weight gain when treated with local feeds (Leotarakul and Pimkamlia, 1999). Moreover, native Thai chicken is well adapted to the poor condition of small farm or simple rural environment. Its resistance to diseases and hot

climate is considerable higher than that of high performance or hybrid breeds, resulting in high potential for raising native Thai chickens in rural areas (Kajaroen et al., 1989). Chickens can adapt to high heat and imported broilers are less tolerant to the high heat than that of Thai indigenous chickens crossbred and Thai indigenous chickens (Aengwanich, 2008).

In Thailand, there are about 62 millions native Thai chickens or 22 % of total chicken production which are broilers 62 % and layers 16 % (Department of Livestock Development, 2010). Native Thai chickens provide high quality meat with low fat and good taste, resulting in high demand by consumers. It has been reported that the characteristics of the indigenous chicken meat are similar to spent hen meat but are much different from imported broiler meat (Chuaynukool et al., 2007). The indigenous chicken muscles contain higher protein and collagen contents but lower fat contents than those of broiler muscles. Moreover, the shear values of indigenous chicken muscles either raw or cooked are higher than those of broiler muscles (Wattanachant et al., 2004; 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) has been 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 breeds or genotypes, 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). Not only the firm and low fat meat, free of drug residues such

as antibiotics also make consumer 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 that of the commercial broilers in Thailand, Hong Kong, China, and Japan (Chotesangasa and Gongruttananun, 1999a; Jaturasitha et al., 2008).

2.1.1 The Production of Native Thai Chicken

Native Thai chicken is suited to the small farm raising system but the improving of the supply of chicks for fattening need to be developed as well (Haitook et al., 2003). The reproductive performance of native chicken is much lower than those of cross breeds and hybrids, especially egg-laying performance is critical to secure a sufficient number of chicks for fattening (Chotesangasa et al., 1994b). In commercial system, hatchability is not the problem for producing the chicks, if the number of egg per hen is not limited. Normally, the native Thai hen lays eggs 3-4 times per year, 4-17 eggs per clutch rather than lays 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 time, the peak production are 38.0 % and 75.5 %, respectively (Chotesangasa et al., 1994b). The total number of egg 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 commercial hen (Chotesangasa et al., 1994b). The low potential in egg production of the native Thai chicken 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). In addition, growth rate of the native Thai chicken is significantly slower than that of the imported chicken.

Thus, improving the efficiency of native Thai chicken production would benefit to poultry industry in Thailand.

2.1.2 The Reproduction of Native Thai Chicken

The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying, egg laying, incubating eggs, and rearing chicks (Figure 2.1; Kosonsiriluk, 2007). It has been reported that progesterone and prolactin (PRL) plasma levels are related to reproductive cycle of the native Thai chicken (Katawatin et al., 1997; Sangkaew, 1999; Kosonsiriluk et al., 2008). The circulating levels of progesterone and estradiol are higher in hen that has hen-day egg production recorded more than 80 % than those of layer with its egg production recorded below 25 % and the hen which laid no egg or non-layer (Chotesangasa et al., 1994a). However, plasma luteinizing hormone (LH) levels do not change during reproductive stages (Kosonsiriluk et al., 2007). Changes in the number of vasoactive intestinal peptide (VIP)-immunoreactive (ir), tyrosine hydroxylase (TH)-ir (as a marker for dopamine; DA), and gonadotropin releasing hormone-I (GnRH-I)-ir neurons in the hypothalamus of the native Thai chicken are observed across the reproductive cycle and correlated directly with variations in plasma PRL levels (Sartsoongnoen et al., 2006; 2008; Kosonsiriluk et al., 2008). In addition, the effects of photoperiod on growth, carcass quality, reproductive development, laying performance, and reproductive efficiency have been reported (Chotesangasa and Santipong, 1994; Chotesangasa and Gongruttananun, 1996a; 1996b; 1999a; 1999b; Kosonsiriluk, 2007; Sartsoongnoen, 2007). The egg production is higher in native Thai chickens raised under short photoperiod (8L : 16D) and then long photoperiod (15L : 9D) lighting

regimen during growing and laying periods, respectively (Chotesangasa and Santipong, 1994). Moreover, hens that raised under long day photoperiod (16L : 8D) show higher in the ovary and oviduct weights and the numbers of ovarian hierarchical follicles than those of other groups (Kosonsiriluk, 2007). Thus, in order to increase the production of the native Thai chicken in Thailand, it needs to be improved in both growth and reproductive performances. As mentioned above, it is very important to understand the basic neuroendocrinology influencing its reproductive activities, especially incubation behavior.

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).

2.2 Parental Behavior

Parental behavior is defined as the behavior of the parent that contributes to the survival of its offspring. The term maternal refers to the mother and paternal refers to the father. In mammals, the significant characteristics of parental behavior are; 1) coincident in the onset of birth, lactation, and maternal care, 2) rapid formation of an attachment of the mother to offspring, 3) occurrence in the behavioral interaction between the mother and the young during their development until weaning, and 4) the significance of the mother-offspring unit as the basis of social organization/interaction.

In birds, there is the period of egg incubation in the nest previous hatching of the young, in addition to the aforementioned characteristics of mammals, but the mother-offspring unit is not the basis for social organization (for review, see Rosenblatt, 2003). Furthermore, the factors that may influence male parental behaviors and hormonal changes are stimuli from the pregnant female and stimuli from the newborn pups, whereas maternal behaviors are influenced by the maternal hormones of the female and stimuli from the pups (Ziegler, 2000). PRL has long been known to play a significant role in maternal care. Besides, it is also important in species which father contributes to parental care and found to be connected with paternal care in fish, birds, and mammals (Schradin and Anzenberger, 1999; Ziegler, 2000; Wynne-Edwards and Timonin, 2007).

2.2.1 Paternal Behavior

Paternal care is termed as the behaviors that perform by mature male which appear to have positive influences on infant development, growth, well-being, and

survival of the offspring (Fernandez-Duque et al., 2009). Paternal care can be divided into two patterns; direct and indirect. Direct paternal care is included all activities that father do for their young that exert an immediate physical influence on them and thought to increase their survival rate such as feeding, warning, and playing. Indirect paternal care is included the activities that father do for the young by the independently of the presence of the young but it is advantage for them. For example, father defenses of a territory to maintain critical resources (Schradin and Auzenberger, 1999). The neuronal and hormonal control of paternal behavior has been extensively investigated, there are many listed hormones associated with paternal care such as PRL, sex steroids, glucocorticoids, oxytocin, and vasopressin (Moore, 1992; Schradin and Anzenberger, 1999; Ziegler, 2000; Bales et al., 2004; Pedersen et al., 2006; Wynne-Edwards and Timonin, 2007).

2.2.2 Maternal Behavior

Most mother display maternal behavior after parturition and serve the immediate provision of care and defense for their offspring (Brunton and Russell, 2008). In fact, the maintenance of the life of the species is dependent on the presence of precise maternal care in the period that the child is dependent on the mother (Swain et al., 2007). In mammals, maternal behavior can be classified into two main patterns. First, maternal behavior shown by mammals that build the nests for their altricial young and those that only shortly establish a birth site for the precocial young. Second, the patterns are quite similar, centering around nursing, and weaning follows a similar course in different species (Rosenblatt, 1980). The mechanisms underlying the control of maternal behavior may be derived from the processes involving in birth

or the regulation of lactation in mammals including changes in circulating levels of progesterone, estrogen, oxytocin, and PRL (Ziegler, 2000). These hormones increase activities in the medial preoptic area (POA) of the hypothalamus, the area that important for the expression of maternal behaviors (Featherstone et al., 2000). Moreover, in ewe, maternal experience helps her to recognize her lamb depending on vaginocervical feedback to the brain to stimulate an interest in lamb odors (Keverne et al., 1993).

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, hens develope maternal behavior gradually in four stages; brooding, titbitting, clucking, and normal broody behavior (Ramsay, 1953). The incidence of maternal behavior concurs with a pause in laying and a decrease in plasma gonadal steroid levels (Richard-Yris et al., 1983). It has been reported that, 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). In Japanese quails, females brood their chicks for longer time than males (Ruscio and Adkins-Regan, 2004).

2.2.3 Parental Behavior in Mammals

The most parental behavior in mammals is the maternal behavior with exception in some species such as California mice, wolves, and callitrichids (marmosets and tamarins; Gubernick et al., 1993; Ziegler et al., 1996; Jochle, 1997). The hormonal basis of maternal behavior in rats is the ovarian hormones (estrogen and progesterone), the anterior pituitary hormones (beta-endorphin and PRL), and the posterior pituitary hormone (oxytocin), which is secreted by several hypothalamic nuclei and associated brain regions (Rosenblatt et al., 1988; Kendrick, 2000). It has been reported that the onset of nest building in sows is associated with a decline in plasma progesterone levels, an increase in PRL plasma levels and a sharp rise in plasma prostaglandin F2 alpha levels at the day before parturition (Algers and Uvnas-Moberg, 2007). In addition, the changes in plasma concentrations of estradiol, progesterone, and PRL are correlated with the activities of the maternal nest building in rabbits (Gonzalez-Mariscal et al., 1996; Gonzalez-Mariscal, 2001). PRL is required for the ovarian hormones to be effective in stimulating maternal behavior (Rosenblatt et al., 1988). The females primed with gonadal steroids display a pattern of PRL responses to pups as observed in lactating rats but not in males, demostrating a longer capability of parental response in male rats than virgin female rats (Samuels and Bridges, 1983).

Under appropriate physical environmental conditions, the presence of the male mice increases pup care and may help maternal behavior (Wright and Brown, 2000). During lactation period, stimulating from the piglets affects the release of several hormones which not only regulate the milk-let down effect, but regulate the metabolism and mammary milk production as well (Algers and Uvnas-Moberg,

2007). In California mice, both males and females show the same amount of parental care except the lactation of females. For example, they build the nests, carry the young, lick the young, and warming the pups (Gubernick et al., 1993). In wolves, they display a communal breeding system, not only the mother and the father but other females and males care for the young as well (Jochle, 1997). The socially monogamous cotton-top tamarin monkey is a cooperative breeder with the breeding male providing extensive parental care shortly after birth (Ziegler et al., 1996). It has been reported that the father tamarins have elevated levels of circulating PRL before the birth of infants, suggesting that the environmental cues from the pregnant females are very important (Ziegler, 2000).

2.2.4 Parental Behavior in Birds

In birds, one or both parents must incubate their eggs until hatching and then provide post-hatching care. The extent of parental care for their eggs and chicks is depended upon the developmental maturity of the hatchling such as precocial and altricial chicks. Care of the young ranges from guarding and guiding in the most precocial species such as Anseriformes and Galliformes to provisioning of all food and intensive brooding for thermoregulation such as Passeriformes and Psittaciformes (for review, see Vleck, 1998). However, some avian species express the most precocial chicks which require no post-hatchicng care. In megapodes such as Australian brush-turkeys show no parental care, they lay eggs in underground nests and these eggs are incubated by external heat sources and then the chicks dig out of their nests by themselves and live independently of their parents and their siblings (Goth, 2002; Goth and Vogel, 2003). Moreover, the common cuckoos and the brown-

headed cowbirds, the parasitic species, they lay their eggs in the nests of other species and let them to raise their young (Winfree, 1999; Kruger, 2007). Likely, in some duck species such as Goldeneye ducks show intraspecific brood parasitism, the females lay their eggs in the nest of other females (Andersson and Eriksson, 1982).

It has been established that PRL is necessary to promote and/or to maintain post-hatching parental care in birds (Boos et al., 2007). Generally, circulating PRL levels decrease rapidly after the chicks hatch in species with precocial young, and the presence of the chicks can modify the rate of decreased levels (Dittami, 1981; Opel and Proudman, 1989). In species with altricial young, circulating PRL levels decrease gradually, suggesting that parental brooding is required for survival of the chicks. PRL concentrations often begin to decline only after the chicks complete thermal independence and do not require constant brooding (Goldsmith, 1991). In addition, PRL concentrations are usually higher in female than that of in male birds, whether or not the males participate in the incubation (Dawson and Goldsmith, 1982; Hiatt et al., 1987). Moreover, it has been suggested that nest attendance and provisioning may alternately influence the formation of the adult phenotype and effect an individual changes of survival of Florida scrub jays (Rensel et al., 2010). Birds that deposited a large amount of maternal androgens in their eggs and the concentration of these yolk androgens is related to the social environment of the mother and can affect offspring survival, behavior, morphology, physiology, immune function, growth, and sex determination (Groothuis et al., 2005; Goth et al., 2008; Muller et al., 2009). Furthermore, it has been suggested that the presence of a maternal hen influences the distribution of activity in young domestic chicks (Wauters et al., 2002).

2.3 Incubation Behavior

2.3.1 Physiological and Behavioral Correlates of Incubation

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 gonadotropin and ovarian steroid levels, ovarian regression and 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).

In bantam hens, the onset of incubation 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 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 them to guarantee that they are all 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 start after hens accumulated a full clutch of eggs. Bantam hens have accumulated about 10-20 eggs per clutch. However, turkeys 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).

In temperate zone birds such as turkeys, an exogenous PRL administration induces ovarian regression (Hargis et al., 1987), and ovarian regression also induces

by a reduction in day length (short day photoperiod). Follicular atresia begins with the larger preovulatory follicles and proceeds down the follicular hierarchy until all preovulatory follicles become atretic. Follicular atresia occurs after termination of egg laying and normally is extensive five days after the last egg is laid (Porter et al., 1987). Moreover, ovarian regression seems to be very extensive in less than seven days after follicular atresia occurs. These phenomenal concomitant with the decrease in circulating LH levels and increase nesting preceding full expression of incubation behavior following exposure of laying hens to reduced day length (Porter et al., 1987), suggesting that ovarian collapsed may result from decrease of LH secretion.

It has been documented that most avian species that incubate 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, so called brood patch. This brood patch develops prior to initiation of the incubation and functions to facilitate heat transfer from the hen to the eggs as well as the transmission of tactile stimuli to the hen (for review, see El Halawani et al., 1988a). In turkeys, tactile stimuli at the brood patch appear to be mediated the suppression of plasma PRL levels 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 concentrations of PRL (Hall and Goldsmith, 1983). Further evidence suggests that the formation of brood patch begins about five days prior to the initiation of incubation behavior (Lea et al., 1981). Administration of estrogen accompanied with PRL results in the development of the brood patch in canaries (Steele and Hinde, 1963) and white crowned sparrows (Bailey, 1952). However, an exogenous PRL induction of

incubation behavior does not result in enhanced brood patch formation in turkeys (Hargis et al., 1987).

It has been reported that birds eat and drink very little and lose their weights during the incubation period. Weight loss during incubation period has been reported in 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 hatch but may persist for a prolonged period if the nest contains unhatched eggs. Many species of wild birds that incubate infertile eggs persists for about 50 % longer than that of normally require to hatch them (Skutch, 1962). During extended incubation in bantam hens, the 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 (Bertrand, 1994).

2.3.2 Neuroendocrine Regulation of Incubation Behavior

The onset of incubation behavior is correlated with declining plasma levels of LH and gonadal steroids (estrogen and progesterone) and increasing plasma levels 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 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 level begins to rise 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). PRL level during rearing period is lower than that of incubation period, but this PRL level remains higher than that of the non-rearing ones (Boos et al., 2007), indicating that PRL is most likely involved in parental care after hatching (Criscuolo et al., 2002). PRL is involved in many aspects of reproductive physiology and behaviors. It is thought to play a pivotal role in maternal behaviors by mediating 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). It has been well established that the increased in PRL concentrations maintains incubation behavior (Sharp et al., 1988). In turkeys, incubation behavior is facilitated by the combined action of estradiol, progesterone, and PRL (El Halawani et al., 1986). Administration of PRL into laying turkey hens causes ovarian regression (Opel and Proudman, 1980; Hargis et al., 1987) and inhibits the exogenous gonadotropins stimulated secretion of gonadal steroids (Camper and Burke, 1977). Stimulus of nesting maintains high PRL levels in incubating hens. Removal of incubation turkeys and native Thai chickens 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). In doves, PRL secretion do not increase at the onset of incubation as occurs in other avian species, but it increases when the crop sacs are proliferating and producing crop milk for feeding the young (Goldsmith et al., 1981). Plasma concentrations of LH are higher in male doves than that of in females and higher during courtship than that of during incubation and brooding periods (Goldsmith et al., 1981). It has been further reported that ovarian hormones suppress LH release during incubation 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 dependent upon rearing conditions in turkey hens (Bedecarrats et al., 1997). In addition, the peripheral nervous input acts on the onset of incubation behavior (Book et al., 1991). The areas of the brain that involved in the expression of incubation behavior are the nucleus tuberis, nucleus preopticus medialis, nucleus ovoidalis, and paleostriatum primitivum (Georgiou et al., 1995). The genetic control of incubation behavior in domestic hens also has been studied. Romanov et al. (2002) reported that incubation behavior is not controlled by major genes on the Z chromosome. At least two autosomal genes are involved in causing and inhibiting the behavior with equal influence.

2.3.3 Disruption of Incubation Behavior

Nest deprivation results in the disruption of incubation behavior, increases in plasma LH and estradial concentrations, and decreases in plasma PRL levels (El Halawani et al., 1980; Sharp et al., 1988; Dunn et al., 1996; Richard-Yris et al., 1998). The changes of plasma concentrations of LH and PRL are reversed when hens re-nested (Sharp et al., 1988). Depriving the hens from their eggs results in an increase in LH secretion and hypothalamic contents of cGnRH-I mRNA (Dunn et al., 1996). Pituitary PRL mRNA levels are correlated directly with plasma PRL concentrations which the level is higher in incubating hens than that of in laying hens and rapidly decrease when birds are deprived of their nests (Talbot et al., 1991). Incubation behavior can be prevented by using passive immunization against PRL (Crisostomo et al., 1997). Furthermore, passive immunoneutralization of VIP increases pituitary PRL contents, which may effect the decreased of PRL secretion (Talbot et al., 1991).

Moreover, maternal responses and variations in plasma PRL and testosterone levels have been studied. In broody hens, the secretion of PRL is facilitated by the presence of chicks (Sharp et al., 1988). After exposed to stimulation by chicks, incubating hens show complete maternal behavior and decreasing in plasma levels of testosterone as same as plasma PRL levels increase as they abandone their nests (Richard-Yris et al., 1987a; Leboucher et al., 1990). Moreover, it has been well established that nest deprivation of incubating hens results in a precipitous decline in plasma PRL levels, programmed cell death of lactotrophs, disappearance of mammosomatotrophs, increased proliferative activity of pituitary cells, and recruitment of somatotrophs arising primarily from mitosis of non-somatotrophic cells (Ramesh et al., 2001).

Plasma PRL levels are high during incubation period and rapidly decrease on the day of hatching. In the other hand, plasma LH levels are low during incubation period, gradually increase after hatching, and reach a peak after removal of the chicks (Kuwayama et al., 1992). It has been reported that incubating hens leave their nests while adapt newly hatched chicks and come back into lay later than the hens that are not allowed to rear the chicks (Richard-Yris and Leboucher, 1986; Richard-Yris et al., 1987b). The decreasing of plasma PRL levels is not observed in hens that can only see and hear but not touch the poults. As indicated above, only physical contact between hens and poults causes the changes of plasma PRL levels in incubating hens (Opel and Proudman, 1988a). In addition, the hens that are partially separated from their chicks cause a decline of the clucking rate (Richard-Yris and Leboucher, 1986). Moreover, parent ring doves exposed to squab show more fos immunoreactivity in the POA and lateral hypothalamus than those of squab-deprived parents (Buntin et al., 2006).

2.4 Neuroendocrine Regulation of the Avian Reproductive Cycle

The control of avian reproductive system involves the interaction of external stimuli with endocrine mechanisms. Avian reproductive cycle is regulated by the integration of the hypothalamus, the pituitary, and the gonads (testis and ovary). 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. The HPG axis involves two major neuroendocrine systems for controlling avian reproduction. These neuroendocrine systems are the chicken GnRH/follicle stimulating hormone (FSH)-LH; GnRH/FSH-LH and VIP/PRL neuroendocrine systems. Both systems are influenced by dopaminergic (DAergic) neurotransmission (Bhatt et al., 2003; Chaiseha et al., 2003b). In addition, in temperated zone birds, both systems depend on the photoperiod and the transduction of photoperiodic information, resulting in either gonad recrudescence or it associated sexual activity or gonad regression and the termination of reproductive activity. The final common pathway regulating the 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 (ME; Chaiseha and El Halawani, 2005). The hypothalamic GnRH stimulates pituitary gonadotrophs to secrete FSH and LH, which in turn responsible for ovarian follicular growth and ovulation at the period of egg laying. On the contrary, at the period of egg incubation, VIP stimulates pituitary lactotrophs to synthesize PRL and stimulates PRL secretion and then regression of the gonads. Moreover, GnRH and VIP can directly affect the gonads via the appropriate gonadal receptors (Asem and Novero, 1993; Johnson, 2000; Sun et al., 2001).

It has been studied and 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., 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. At the onset of nesting activity, circulating LH levels begin to increase continuously and reach a peak amount at about 8 to 2 hrs before ovulation (Mashaly et al., 1976). Unlike LH, plasma concentration of chicken FSH (cFSH) is low throughout the ovulatory cycle, but a significant decrease in cFSH occurs right before the preovulatory LH surge and a significant increase occurs during 3 hrs prior to oviposition as plasma LH levels decrease (Krishnan et al., 1993). There after, LH levels continue to drop during 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 this rising PRL level that causes the cessation of ovulation, ovarian regression, and induction of incubation behavior. 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). High levels of PRL may inhibit LH secretion (Zadworny and Etches, 1987). 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 (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 levels by reducing GnRH levels in the hypothalamus (Rozenboim et al., 1993b) and the abundance of LH-β subunit and PRL mRNAs shows an inverse relationship in photostimulated/laying and incubating turkey hens (Wong et al., 1992b). Administration of ovine PRL 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).

In incubating chickens (Sharp et al., 1979), ducks (Goldsmith and Williams, 1980), and swans (Goldsmith, 1982) that have been allowed to hatch and rear the young have shown that PRL levels decline at the end of the incubation period, suggesting that this decline may occur on or before the day of hatching in the ducks (Goldsmith and Williams, 1980). Wentworth et al. (1983) suggests that this decline PRL levels could be related to the pipping and hatching of eggs, and the subsequent transit to maternal behavior. Little is known about the mechanism(s) that down regulate PRL secretion at the end of incubation. The possibilities include loss of stimuli from the nest of eggs when the eggs are hatched, receipt of stimuli from the hatching, or a combination of these factors. That loss of stimuli from the nest of eggs

is involved and is supported by observations that egg removal in grouses (Etches et al., 1979) or removal of incubating turkey hens from their nests (El Halawani et al., 1980; Proudman and Opel, 1981) evokes a sharp fall in plasma PRL levels. Involvement of stimuli from the hatchings is reported in ducks (Goldsmith and Williams, 1980) and turkeys (Wentworth et al., 1983), suggesting that PRL levels in the incubating hens may begin to fall as early as pipping of the eggs. It has been reported that tactile stimuli from poults cause PRL levels fall abruptly in singly caged hens that exhibit incubation behavior without access to the nest or eggs (Opel and Proudman, 1988a). At this reproductive stage, GnRH levels decrease due to an increase in inhibitory neuronal input to the GnRH neurons.

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

It is very well documented that gonadotropins (FSH and LH) secretion is govern by the central nervous system (CNS) through the hypothalamus. The hypothalamus synthesizes GnRH which in turn stimulates the synthesis and release of the pituitary gonadotropins (Ulloa-Aguirre and Timossi, 2000; Shalev and Leung, 2003). Once environmental stimuli are transduced by the specific receptors, they influence the secretion of GnRH located in hypothalamic regions in both birds and mammals. In birds, the egg laying period is associated with relatively high levels of circulating FSH, LH, and gonadal steroids and is regulated by hypothalamic GnRH (El Halawani et al., 1988b). GnRH increases LH and FSH secretion of the anterior pituitary both *in vitro* and *in vivo* (Peczely, 1989). In *in vivo* study, injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH concentration in hens

(Guemene and Williams, 1999). GnRH agonists may imitate the native hormone and induce an endogenous LH surge (Shalev and Leung, 2003). In contrast, GnRH inhibits FSH-stimulated steroidogenesis in birds as well as in mammals but enhances LH-stimulated progesterone production (Hertelendy et al., 1982). cGnRH-I does not affect circulating FSH concentrations but stimulates LH secretion when administrated to 3 weeks old cockerels (Krishnan et al., 1993). GnRH release occurs episodically from the mammalian hypothalamus, and the frequency and amplitude of GnRH release determine the pattern of gonadotropins secretion (Levine and Ramirez, 1982; Moenter et al., 1992). In birds, a pulsatile pattern of GnRH-I release is observed from the medial basal hypothalamus (MBH) and POA *in vitro* (Li et al., 1994). Changes in pituitary responsiveness to GnRH are negatively correlated to changes in the circulating LH levels (Balthazart et al., 1980). Moreover, it has been established that adrenergic stimulation at the hypothalamic level can release GnRH and thereby increase gonadotropins secretion (Yu et al., 1991).

2.4.2 Vasoactive Intestinal Peptide/Prolactin System

In birds, PRL has been implicated as a causative factor in the onset and maintenance of incubation behavior (El Halawani et al., 1997). It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968) and that principal PRL-releasing factor (PRF) is VIP (El Halawani et al., 1997; 2001; Chaiseha and El Halawani, 1999; 2005). It has been very well established that VIP is associated with the reproductive cycle in birds (El Halawani et al., 1997). 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). Variations in VIP immunoreactivity, VIP contents, and VIP mRNA steady-state levels occurring within the hypothalamus are mirrored with changes in PRL concentrations throughout the turkey reproductive cycle (Mauro et al., 1989; Chaiseha and El Halawani, 1999). Changes in pituitary VIP receptor mRNA is also observed across the reproductive stages in turkeys. Increased VIP receptor mRNA in the pituitary is observed in turkey hens with normal (laying) or high PRL secretion (incubating), while much less VIP receptor mRNA is observed in the pituitary of hypoprolactinemic non-photostimulated and photorefractory turkey hens (Chaiseha et al., 2004). These results are in good agreement with studies indicating variations in VIP immunoreactivity and VIP contents in the infundibular nuclear complex (INF) and ME, VIP mRNA steady-state levels in the INF (Mauro et al., 1989; Chaiseha and El Halawani, 1999), where VIP acts as the PRF (El Halawani et al., 1997), and VIP concentrations in turkey hypophysial portal blood (Youngren et al., 1996a). This suggests that the VIP receptors located in the INF may involve in avian PRL secretion and indicates that PRL secretion is principally regulated by VIP receptors at the pituitary level (Chaiseha et al., 2004). Recently, changes in the number of VIP-ir within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) are observed across the reproductive cycle of the native Thai chickens and correlated directly with variations in PRL levels (Kosonsiriluk et al., 2008), suggesting that hypothalamic VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity of this equatorial bird.

In response to long day length, the VIP/PRL secretion is increased gradually, and progressively. Both their release and gene expression are up-regulated (Wong et al., 1991; El Halawani et al., 1996; Tong et al., 1997; Chaiseha et al., 1998).

Activation of the GnRH/FSH-LH system in photosensitive female turkeys initiates the reproductive activity. When gonadotropins stimulate estrogen secretion and induce sexual receptivity (Wineland and Wentworth, 1975; El Halawani et al., 1986), they also prime the VIP/PRL system to enhance PRL secretion (El Halawani et al., 1983).

2.5 Gonadotropins: Structure, Function, and Regulation of Secretion

Gonadotropins are the member of glycoprotein hormones that secrete by gonadotroph cells of the anterior pituitary gland and are derived from the family that includes LH, FSH, thyroid stimulating hormone (TSH), and placental chorionic gonadotropin (CG). Two principal gonadotropins in vertebrates are LH and FSH. Moreover, CG is found only in primates and equine species. These gonadotropins are heterodimers, consisting of two different subunits including α- and β-subunits, which are encoded from different genes. The α-subunits are identical among all glycoprotein hormones and contain 92 amino acids. The β-subunits are different and determine the hormonal specificity and species specificity (Pierce and Parsons, 1981). In mamamals, it is predicted that the α- and β-subunits of these glycoprotein hormones are evolved from a common ancestral gene (Dayhoff, 1978; Fontaine and Burzawa-Gerard, 1977; Licht et al., 1977), but the mechanism(s) of evolution of the subunits of these four hormones remains to be further investigated. In addition, the β-subunit confers specificity of its biological action by mediating interaction with its specific receptor. It has a pivotal role involving in a wide variety of physiological functions in vertebrates. In general, FSH functions concurrently with LH to promote growth and differentiation of the gonads, control gametogenesis, and regulate gonadal endocrine functions (Moyle and Campbell, 1996). The cellular localization of gonadotropins has

been determined in a variety of species. The majority of gonadotrophs in each species contains both FSH and LH. In pigs (Dacheux, 1984) and lizards (Naik et al., 1980), all gonadotrophs contain both FSH and LH. In humans, at least two thirds of gonadotrophs contain both hormones (Pelletier et al., 1976). However, it has been indicated that FSH and LH reside in separate populations of gonadotrophs in chicken pituitary (Proudman et al., 1999) and bovine pituitary (Bastings et al., 1991). FSH-immunopositive cells are much less numerous than that of LH positive cells and FSH is largely absented from the outer margin of the chicken pituitary. In Japanese quails, pituitary cells that are bound anti-chicken FSH serum also bound anti-chicken LH serum (Mikami, 1983).

2.5.1 The Structure of Follicle Stimulating Hormone

FSH has a molecular weight (MW) about 30 kiloDaltons (kDa), which protein dimer contains two polypeptide, α- and β-subunits. FSH-β-subunit is first isolated and characterized from the human pituitary (Shome and Parlow, 1974) and consists of 118 amino acids with a predominant proportion of molecules having 108 residues due to microheterogeneity at the NH_2 and COOH termini. The NH_2-terminal portion up to 32 residues shows homology with the β-subunits of other glycoprotein hormones including LH, human CG (hCG), and TSH. Nevertheless, the amino acid sequence differs a great deal from others in the rest of the molecule and confirms the specificity of the β-subunits of these hormones (Shome and Parlow, 1974). It have been well documented that complementary DNAs (cDNAs) of the FSH-β-subunits have been cloned in humans (Jameson et al., 1988), monkeys (Schmidt et al., 1999), sheep (Mountford et al., 1989), rats (Maurer, 1987), mice (Kumar et al., 1995), pigs (Kato,

1988), bovines (Esch et al., 1986), and opossums (Lawrence et al., 1997). In addition, FSH molecules have been purified in sea turtles (Licht and Papkoff, 1985) and bullfrogs (Hayashi et al., 1992), a non-mammalian species. FSH molecules have also been purified in avian species such as chickens (Sakai and Ishii, 1980; Krishnan et al., 1992) and ostriches (Koide et al., 1996). Furthermore, the cDNA encoding precursor molecule of FSH-β-subunit has cloned and characterized in Japanese quails (Kikuchi et al., 1998), chickens (Shen and Yu, 2002), Japanese Crested ibis (Kawasaki et al., 2003), and ducks (Shen et al., 2006). The amino acid sequence of chicken FSH-β-subunit shows 98 % homology with Japanese quail and 93 % with ostrich, whereas a lower homology (66 to 70 %) is observed when compared with mammalian FSH-β-subunit. The amino acid sequences of FSH-β-subunit in different species are shown in Figure 2.2.

FSH acts by binding to its specific receptors localized exclusively in the gonads. FSH receptor (FSHR) belongs to the family of guanine nucleotide-binding protein (G-protein)-coupled receptors, the complex transmembrane proteins which characterized by seven hydrophobic helices inserted into the plasma membrane and by intracellular and extracellular domains of variable dimensions depending on the type of ligands (Gudermann et al., 1995). Signals initiated by binding to the FSHR are tranduced within the cells by the second messenger system, 3'-5'-cyclic adenosine monophosphate (cAMP). The intracellular portion of the FSHR is bind to a G stimulatory-(Gs) protein, and upon the receptor activation by hormonal interaction with the extracellular domain, triggeres the cascade of events that finally leads to the specific physiological effects. The first sequence of a putative FSHR DNA fragment is reported (Parmentier et al., 1989). To date, the FSHR sequences are well

characterized in humans, primates, equines, ovines, swines, bovines, chickens, and reptiles (for review, see Simoni et al., 1997). In birds, cDNA corresponding to chicken FSHR (cFSHR) has been cloned and characterized (You et al., 1996).

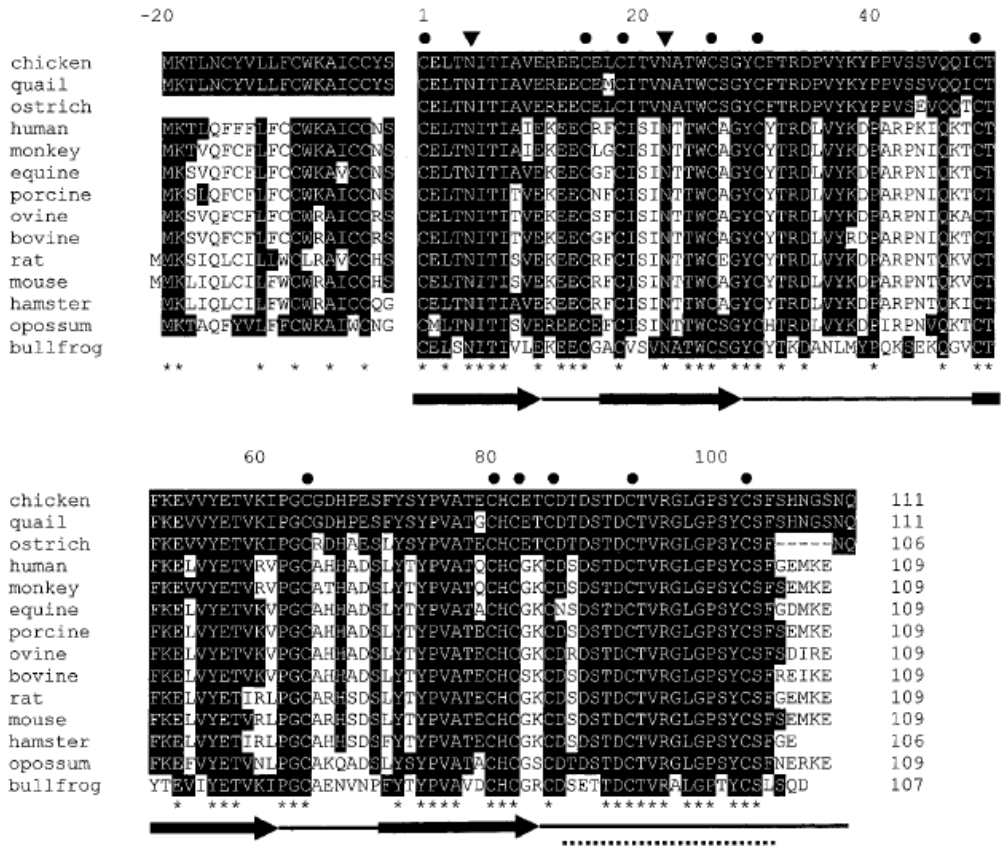

Figure 2.2 Multiple sequence alignments of FSH-β-subunit of different species. Residues identical to chicken FSH-β are presented in white letters. The conserved cysteines are denoted by ●, the putative N-linked glycosylation sites by ▼, and * under sequences indicates conserved residues. Arrows represent β-strands; single lines are loops. Line with dots corresponds to the "seat-belt" region in crystal structure of human FSH (Shen and Yu, 2002).

The overall sequence of 693 amino acids is determined and considered more homologous to the rat FSHR (71.8 %) and bovine FSHR (72.2 %) than that of the characterized chicken LH receptor (cLHR; Johnson et al., 1996a). The nucleic acids and amino acid sequence of cFSHR are 60.1 % and 49.4 % identical to the respective cLHR sequence. The FSHR gene expression is highly gonad- and cell-specific, underlying its importance for the oogenesis and spermatogenesis. Receptor-binding studies suggest that FSH binds specifically to receptors located on the membrane of sertoli cells (Fritz, 1978; Kangasniemi et al., 1990). FSH binding has been also localized in the granulosa cells (Richards and Midgley, 1976; Richards, 1980) and ovarian tissues (granulosa, theca, and stromal cells), but not in the oviduct, adrenal gland, liver, muscle, or brain (You et al., 1996) in chickens. Several studies confirm that granulosa cells are the only cell type expressing-FSHR. However, this finding is in contrast to the expression pattern observed for the LH receptor (LHR) and TSH receptor (TSHR). LHR expression can be demonstrated in a variety of organs and tissues (Simoni et al., 1997).

2.5.2 The Structure of Luteinizing Hormone

Like FSH, LH is a heterodimer consisting of α- and β-subunits. A common α-subunit contains 92 amino acids (MW of 13.5 kDa) coupled to a unique β-subunit of 121 amino acids (MW of 14.5 kDa), each subunit is encoded by different genes. The β-subunit contains the same amino acid sequence as the β-subunit of hCG and both stimulates the same receptor. However, both LH and hCG are different in the composition of their sugar moieties, the hCG-β-subunit contains an additional 24 amino acids. The biological half-life of LH is 20 minutes, shorter than that of FSH

and hCG which their biological half-lives are 3-4 hrs and 24 hrs, respectively. cDNAs and deduced peptide sequences of the LH-β-subunit molecule are available for mammalian species such as bovines, sheep, goats, pigs, giant pandas, rats, mice, dogs, hamsters, and rhesus monkeys, Furthermore, cDNA encoding precursor molecule of the LH-β-subunit of birds has been reported in chickens, Japanese quails, and turkeys (Noce et al., 1989; Ando and Ishii, 1994; You et al., 1995a). It has been reported that turkey and chicken LH-β-subunit sequences share approximately 92 % and 93 % nucleotide and amino acid sequence similarities, respectively (You et al., 1995a). The sequence homology among LH-β-subunit of different species is also shown in Figure 2.3. The alignments of the amino acid sequences of signal peptide and apoprotein of the putative LH-β-subunit of different species are shown in Figures 2.4a and b, respectively.

LH confers its specific biological actions and is responsible for interaction with the LHR. LHR is a member of the subfamily of glycoprotein hormone receptors within the superfamily of G protein-coupled receptors. LHR is also named luteinizing hormone/choriogonadotropin receptor (LHCGR) or lutropin/choriogonadotropin receptor (LCGR), since it interacts with both LH and hCG. LHR consists of 674 amino acids which size 80-90 kDa of mature LHR (Dufau, 1990) and occupies seven membrane-spanning domains (Dufau, 1998). The extracellular domain of the LHR receptor is heavily glycosylated. This transmembrane domain consists of two highly conserved cysteine residues, building disulfide bonds to stabilize the receptor structure. With LH binded, the receptor shifts conformation and thus mechanically activates the G proteins, and then activates the adenylate cyclase to produce cAMP (Ryu et al., 1998). The LHR consists of 674 amino acids. It has been reported that

LHR cDNAs are cloned and characterized from human ovarian libraries as well as the testes of pig, rat, and mouse (for review, see Dufau, 1998). In birds, partial LHR cDNAs have been isolated in chickens and Japanese quails (Akazome et al., 1994; Johnson et al., 1996a). It seems to have two different LHR isoforms in chicken, one of which has 86 bp insert located in the extracellular domain of the molecule. Three different, alternatively spliced, LHR cDNA isoforms have been characterized in chickens and turkeys (You, 1997; You et al., 2000). The amino acid sequence of the cLHR shares 73.2 % and 74.2 % homology with the rat and porcine LHR sequences, respectively, with the highest homology occurring within the seven transmembrane spanning regions (86-88 % identity vs. mammalian sequences). In mammalian LHRs, there are some evidences indicating that after binding to the LHR, the second messenger signaling mechanisms in the ovary include the activation of adenylate cyclase/protein kinase A and phospholipase C/phosphatidylinositol cadcases (Mcneilly et al., 1982; El Halawani et al., 1988a).

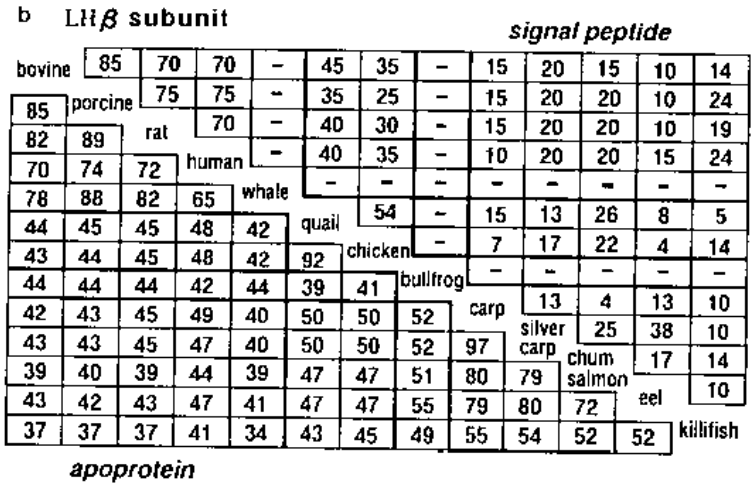

Figure 2.3 The percentage of homology sequence among LH-β-subunit of different species (Ando and Ishii, 1994).

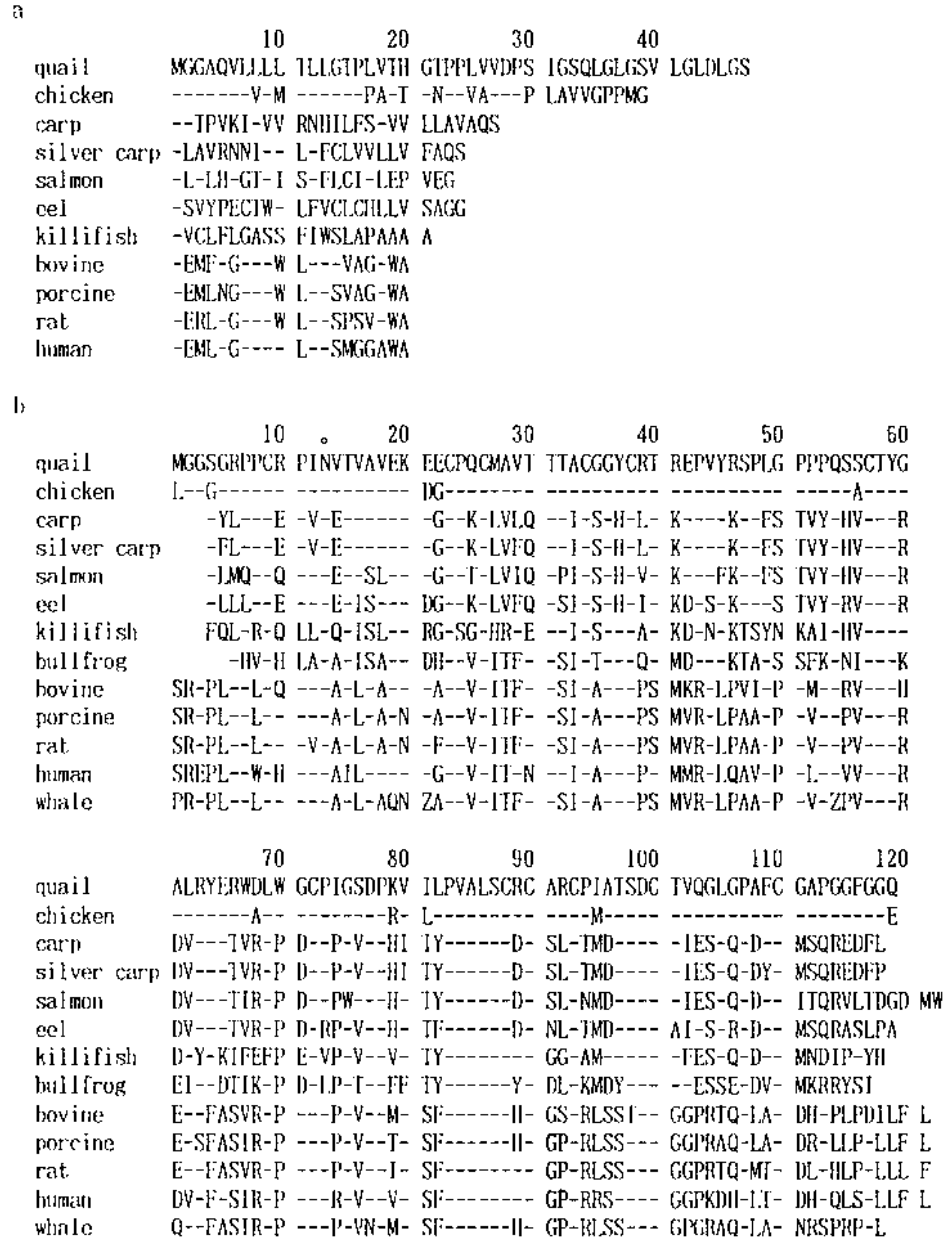

Figure 2.4 The amino acid sequence alignments of signal peptide (a) and apoprotein (b) of the putative LH-β-subunit in different species. Dashes indicate amino acid residues which are indicated to those in the Japanese quail sequences (Ando and Ishii, 1994).

2.5.3 The Function of Gonadotropins in Mammals

The biological actions of gonadotropins in mammals include stimulation of the maturation and function of the gonads and the regulation of gametogenesis and steridogenesis. According to the “two cells, two gonadotropins” theory (Fevold, 1941; Greep et al., 1942; Kobayashi et al., 1990; Hillier et al., 1994; Moyle et al., 1994), both FSH and LH are necessary for ovarian follicular maturation and the synthesis of ovarian steroid hormones. In deed, reproduction in mammals depends on the pulsatile release of gonadotropins, acting concurrently to regulate gonadal functions. First, FSH primes the initial phase of follicogenesis with an increase in gene transcription encoding growth factors to induce LHRs on granulosa cell membranes, and then promotes estradiol secretion. It has been established that FSH and estradiol are required for the acquisition of LHR by granulosa cells, whereas the synthesis of androgen which is a precursor for estradiol is controlled by LH (Hsueh et al., 1984). LH promotes the production of androgens including dehydroepiandrosterone, androstenedione, and testosterone from cholesterol and pregnenolone, by stimulating 17α-hydroxylase activity in the thecal cells. Then, the androgens diffuse to the granulosa cells, whereas FSH stimulates the expression of the cytochrome P450 aromatase, which converts the androgens to estrogens (Erickson et al., 1985; Richards, 1994). In females, LH is responsible for follicular maturation, ovulation, and transformation of follicles into corpora lutea and the maintenance of luteal activity. An LH surge is then participated in oocyte meiosis and consequent ovulation after the initial phase of ovarian follicular growth. The LH surge triggers ovulation by promoting the rupture of the preovulatory follicle and the release of the ovum. LH also enhances the subsequent stages of follicular development and steroidogenesis in

granulosa and luteal cells. In males, LH acts through plasma membrane receptors on the Leydig cells to maintain general metabolic processes and steridogenic enzymes and regulate the production and secretion of androgens (Levi-Setti et al., 2004).

2.5.4 The Function of Gonadotropins in Birds

FSH and LH are responsible for many reproductive physiological functions. In fact, among the most important endocrine, paracrine, and autocrine factors that mediate follicular growth and differentiation are the gonadotropins and growth factors. The ovarian follicles of chicken are probably an excellent model to study follicular selection. The stages of follicular development can easily be determined by the size of the follicles. Chicken ovaries contain thousands of cortical follicles (less than 1 mm in diameter), hundreds of small white follicles (SWF; 1-5 mm in diameter), five or six small yellow follicles (SYF; 5-8 mm in diameter), and five or six preovulatory follicles (10-35 mm in diameter) arranged in a hierarchy, which a single follicle is selected each day from the pool of SYF to join the exclusive group of preovulatory follicles destined for ovulation (for review, see Johnson, 1993).

As the follicles increase in size, the granulosa and theca externa cells produce amounts of steroid hormones (Porter et al., 1989). The three-cell model of steroidogenesis in avian follicles that takes the differential steroidogenic activities of the theca externa, theca interna, and granulosa cell layers into account has been proposed (Porter et al., 1989; Nitta et al., 1991; Velazquez et al., 1991; Kato et al., 1995). This theory is based on the facts that two kinds of steroidogenic cells are presented in the theca layers, which are testosterone and estrogen (Kato et al., 1995). Progesterone, testosterone, and estrogen in the avian follicles are synthesized in the

granulosa, theca interna, and theca externa layers, respectively. Granulosa tissue of F1-F4 preovulatory follicles contains significantly more progesterone than does theca tissues (Etches and Duke, 1984; Kato et al., 1995).

It is very well documented that FSH induces mainly ovarian follicular growth in birds (Chaudhuri and Maiti, 1998; Rose et al., 2000) and maintains the hierarchical size of the follicles. Daily injection of FSH increases the numbers of SWFs, SYFs, and preovulatory follicles without disrupting the hierarchy in mature hens (Palmer and Bahr, 1992). The primary targets of FSH are the granulosa cells of SYF and the sixth (F6) to the third (F3) largest follicles since FSHR, FSH mRNA, and FSH-stimulated adenylate cyclase activity is found in this layer (Calvo et al., 1981; Calvo and Bahr, 1983; Bahr and Calvo, 1984; Ritzhaupt and Bahr, 1987; You et al., 1996; Zhang et al., 1997). Other studies indicate that the levels of LHR mRNA are increased, while the levels of FSH mRNA are decreased in granulosa cells of the mature follicles (Johnson et al., 1996b; You et al., 1996; Zhang et al., 1997; Yamamura et al., 2001). Relatively low levels of FSH binding have been determined within ovarian stroma, theca cells, and granulosa cells, and this binding generally decreases during the follicular development (Etches and Cheng, 1981; Ritzhaupt and Bahr, 1987). Beside the function of FSH to regulate the follicular growth, it has been well established that FSH can induce production of steroids from the follicles. FSH stimulates progesterone production in granulosa cells from the F6-F3 follicles (Hammond et al., 1981). There is also evidence that FSH can induce modest, but significant progesterone, androgen, and estrogen production from the theca layers of the prehierarchical follicles *in vivo* (Kowalski et al., 1991) and prevents granulosa cells from undergoing apoptosis *in vitro* (Johnson et al., 1996b).

LH stimulates progesterone production by the largest follicle (F1), leading to ovulation (Pollock and Orosz, 2002) and also promotes progesterone secretion by the granulosa cells of the primary F3 to F1 follicles (Hammond et al., 1981). It has been reported that the primary target for LH is the granulosa layer of the preovulatory F1 follicles (Calvo et al., 1981; Calvo and Bahr, 1983; Bahr and Calvo, 1984). cLHR mRNA transcript in the granulosa cells is found to be expressed only within the preovulatory follicles (Johnson et al., 1996b). A preovulatory release of LH by the anterior pituitary induces ovulation during the ovulatory cycle (Etches and Cunningham, 1976; Mashaly et al., 1976; Cunningham, 1987; Etches, 1990). The frequency of preovulatory LH surges is an important determinant of ovulation and oviposition rates in birds. Ovulation of the F1 follicle occurs 6-8 hrs after the preovulatory surge of LH (Mashaly et al., 1976; Proudman et al., 1984). After that, oviposition of completely formed eggs occurs about 25 hrs after ovulation (Wolford et al., 1964). The preovulatory surges of LH are associated with surges of progesterone produced form the F1 follicles (Kappauf and van Tienhoven, 1972; Mashaly et al., 1976; Bahr et al., 1983; Etches and Duke, 1984). Injection of exogenous LH (Fraps et al., 1942; Neher and Fraps, 1950; Opel and Nalbandov, 1961) or progesterone (Fraps and Dury, 1943; Tanaka et al., 1987; Nakada et al., 1994) can induce single or multiple ovulations of the hierarchical follicles *in vivo*. In contrast, FSH and estrogen are relatively constant during the ovulatory cycle (Krishnan et al., 1993; Yang et al., 1997; Liu et al., 2001; Bacon et al., 2002). Significant decline in FSH occurs prior to the preovulatory LH surge and increase during the 12 hrs prior to oviposition (Krishnan et al., 1993).

2.5.5 The Neuroendocrine Regulation of Gonadotropins Secretion

The neroendocrine control of the gonadotropins synthesis and secretion is the complex ones and involved the interplay amoung the gonads, pituitary gland, hypothalamus. The synthesis and secretion of FSH and LH are regulated mainly by the hypothalamic decapeptide hormone, GnRH. In deed, the synthesis and secretion of these gonadotropins are regulated by the pulsatile release of GnRH which occurs episodically from the hypothalamus. The frequency and amplitude of GnRH release from hypothalamic neuronal cells is a critical and rate-limiting step for the control and maintenance of gonadotropins secretion from pituitary gonadotrophs. It has been reported that pulse of GnRH initiates pulsatile secretion of FSH and LH from the anterior pituitary gland (Clarke and Cummins, 1982; Levine et al., 1985; Levine and Duffy, 1988). More evidences indicate that changes in GnRH pulse frequency throughout the ovulatory cycle determine changes in the relative amounts of LH to FSH release (Wildt et al., 1981). GnRH is released in discrete pulses at intervals ranging from about 30 min to a few hrs. High-frequency GnRH pulses (one pulse every 30 min) favor LH release, whereas low-frequency pulses (one pulse every 120 min) favor FSH release (Paschke et al., 1994; Kaiser et al., 1997). Furthermore, both LH and FSH are then released in a pulsatle manner into the systemic circulation, and in turn control the processes of folliculogenesis, ovulation, gametogenesis, and steroidogenesis.

As aforementioned, it has been very well documented that gonadotropins secretion are associated with the reproductive cycle in several avian species. It has been suggested that PRL is associated with the regulation of gonadotropins secretion in both birds and mammals. Manipulations of PRL levels affect the changes in

circulating LH levels. Systemic administration of PRL decreases hypothalamic GnRH-I and GnRH-II contents and plasma LH levels (Rozenboim et al., 1993b), while incubation of anterior pituitary cells with PRL inhibits LH-β-subunit gene expression (You et al., 1995b). Moreover, it has been indicated that PRL inhibits the steroidogenic activity of LH in turkeys (Camper and Burke, 1977). An injection of PRL antiserum is associated with an increase in plasma LH levels in incubating chickens (Lea et al., 1981). Exogenous PRL administration suppresses the increase in LH secretion which occurs after ovariectomy (El Halawani et al., 1991), or when incubating chickens are nest-deprived (Sharp et al., 1988).

Gonadal steroids act on the hypothalamus and/or pituitary to regulate either positively or negatively of the synthesis and secretion of gonadotropins. It has been reported that gonadal steroids may regulate gene expression of gonadotropins subunits (for review, see Burger et al., 2004). Circulating LH level is directly related to gonadal activity and the regulation of steroidogenesis (Robinson et al., 1988). Estrogen is the intraovarian regulators of preantral follicular growth (Goldenberg et al., 1972; Richards, 1979), granulosa cell proliferation (Williams, 1940), and LHR expression (Knecht et al., 1985).

More evidences have been reported that adrenergic stimulation at the hypothalamic level can release GnRH and thereby increase gonadotropins secretion (Yu et al., 1991). The inhibitory effect of serotonin (5-HT; Sharp et al., 1984; 1989b) and the stimulatory effect of norepinephrine (NE; Knight et al., 1984) on GnRH secretion have been reported. Microinjections of 5-HT into the caudal ventromedial nucleus (VMN) of the turkey hypothalamus remarkably impede the PRL release effected by electrical stimulation in the POA (Youngren and El Halawani, 2002).

Electrical stimulation in the POA also activates GnRH-ir and VIP-ir neurons as indicated by c-fos mRNA expression in the POA and INF areas, respectively (El Halawani et al., 2004). Furthermore, immunoneutralization of VIP reveals that VIP acts as an antagonist toward both FSH and LH secretion in the turkey hens (Ahn et al., 2001).

In addition, inhibins (INHs) are also found to regulate the HPG axis. The correlation between FSH and INH-B levels has been reported (Tilbrook et al., 1993; Anawalt et al., 1996; Jensen et al., 1997). In mammalian species, active immunization against INH neutralizes endogenous INH and increase rates of ovulation (Brown et al., 1990; Wrathall et al., 1990; Scanlon et al., 1993). However, no significant increase in egg production is observed in INH-immunized hens (Ahn et al., 2001). Moreover, it has been reported that a variety of growth factors such as insulin-like growth factors and bone morphogenetic proteins are expressed throughout the development of follicles and oocytes and interact with gonadotropins to control the maturation of follicles in mammals (for review, see Webb et al., 2007).

In seasonally temperate zone avian species, gonadotropins secretion and their gene expression are stimulated by long day length (Nicholls et al., 1988; Dawson et al., 2001) and require the functional integrity of the GnRH neuronal system (Katz et al., 1990; Sharp et al., 1990). Plasma concentrations of FSH and LH increase in juvenile female chickens after photostimulation (Dunn et al., 1990; Lewis et al., 1994; 1998; 1999; 2001; Sreekumar and Sharp, 1998; Dunn and Sharp, 1999). It is suggested that photostimulation may advance sexual maturation by increasing gonadotropins secretion, especially FSH, which in turn, stimulates ovarian follicular development (Palmer and Bahr, 1992).

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

2.6.1 The Structure of Gonadotropin Releasing Hormone

Hypothalamic GnRH, referred to as type one mammalian GnRH (mGnRH), is first isolated and sequenced from porcine hypothalamus (Peczely, 1989; Rivier, 2001). Later, a second molecular form of GnRH has been 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). It is a hypothalamic neuronal secretory decapeptide that important for the control of reproduction in vertebrates. Todate, it has been reported that GnRH consists of a family of at least 24 isoforms, 14 isoforms are found in various vertebrate species (Gorbman and Sower, 2003). All these forms consist of 10 amino acids with conserved amino acids in position 1, 2, 4, 9, and 10 (Figure 2.5; Powell et al., 1994), 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 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 the domestic chickens (Sharp et al., 1990).

	1	2	3	4	5	6	7	8	9	10
SEA BREAM	pGLU-	HIS-	TRP-	SER-	TYR-	GLY-	LEU-	SER-	PRO-	GLY-NH2
MAMMAL	pGLU-	HIS-	TRP-	SER-	TYR-	GLY-	LEU-	ARG-	PRO-	GLY-NH2
CHICKEN-I	pGLU-	HIS-	TRP-	SER-	TYR-	GLY-	LEU-	GLN-	PRO-	GLY-NH2
CATFISH	pGLU-	HIS-	TRP-	SER-	HIS-	GLY-	LEU-	ASN-	PRO-	GLY-NH2
SALMON	pGLU-	HIS-	TRP-	SER-	TYR-	GLY-	TRP-	LEU-	PRO-	GLY-NH2
CHICKEN-II	pGLU-	HIS-	TRP-	SER-	HIS-	GLY-	TRP-	TYR-	PRO-	GLY-NH2
DOGFISH	pGLU-	HIS-	TRP-	SER-	HIS-	GLY-	TRP-	LEU-	PRO-	GLY-NH2
LAMPREY-III	pGLU-	HIS-	TRP-	SER-	HIS-	ASP-	TRP-	LYS-	PRO-	GLY-NH2
LAMPREY-I	pGLU-	HIS-	TYR-	SER-	LEU-	GLU-	TRP-	LYS-	PRO-	GLY-NH2

Figure 2.5 Amino acid sequences of the identified GnRH peptides (Powell et al., 1994).

GnRH regulates gonadotropins secretion through binding to the specific receptors on the surface of pituitary gonadotrophs. GnRH receptors have been cloned from several mammalian species (for review, see Ramakrishnappa et al., 2005). It has revealed that GnRH receptor is 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, causing an increase in phospholipase C activity and generates inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca^{2+} from intracellular stores, whereas DAG activates protein kinase C. These events lead to the synthesis and release of gonadotropins (Stojilkovic and Catt, 1995). In mammals, the discovered of type II GnRH receptor (Millar et al., 2001; Neill et al., 2001) is shown to be highly selective for GnRH-II and is widely expressed in reproductive tissues and the CNS. The

expression of GnRH-II receptor in the majority of gonadotrophs suggests its role in the regulation of gonadotropins 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 known that each GnRH form has its unique locations within the brain, suggesting 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 the terminals of them have been observed in the hypophysial portal plexus. However, neurons expressing GnRH-II reside in the midbrain and the major terminal field for these neurons is the medial habenula. In contrast to forebrain GnRH-I fibers, few GnRH-II fibers are visible in the ME (for review, see Bakker and Baum, 2000).

Like in mammals, GnRH perikarya and fibers are more widely distributed throughout the avian brain. A number of 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 tit 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; Sartsoongnoen, 2007). There are three major groups of immunoreactive (ir)-cGnRH-I 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 cells 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 perikarya are found including the olfactory bulb, olfactory tubercle, lobus parolfactorius, nucleus accumbens, 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, the basal hypothalamus, the anterior pituitary gland, and the testes of cockerels (Sun et al., 2001). 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 (Foster et al., 1987; Meddle and Follett, 1997). Specific GnRH-I-ir neurons are found in several hypothlalamic regions including the POA, nucleus preopticus medialis (POM), anterior hypothalamus (AM), paraventricular nucleus (PVN), and nucleus commissurae pallii (nCPa). Additional scattered neurons are also found in the nucleus septalis lateralis (SL) and around the organum vasculosum laminae terminalis (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). It has been indicated that cGnRH-I mRNA expressions are the greatest in the nCPa and around the OVLT. This study reports that cGnRH-I mRNAs abundance within the nCPa, OVLT, and SL are greater in laying than that of in non-photostimulated and incubating turkey hens (Kang et al., 2006).

The presence of cGnRH-II in the ME has been studied but its involvement in the control of reproduction is remained controversial. A large group of cGnRH-II ir-neurons in the midbrain in the oculomotor region and basal lateral hypothalamus are observed in turkeys and Japanese quails (Millam et al., 1993; 1995; 1998; Teruyama and Beck, 2000). cGnRH-II fibers are found in the ME of quails and chickens (Clerens et al., 2003; Teruyama and Beck, 2000; van Gils et al., 1993). cGnRH-II-ir fibers are found prominently in the POA, lateral septum, 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 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 both brain and other tissues. The absent of GnRH-II-immunoreactivity has 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 which includes hippocampus and song control areas (Bentley et al., 2004).

2.6.3 The Function of Gonadotropin Releasing Hormone in Mammals

GnRH is the key neuropeptide controlling reproductive functions in all vertebrate species. It is very well documented that GnRH is the primary hypothalamic regulator of LH release in both spontaneous and induced ovulators. In spontaneously ovulating species such as rats, mice, guinea pigs, sheep, monkeys, and women, ovarian steroids secreted from mature ovarian follicles induce a pulsatile pattern of GnRH release in the ME that, in turn, stimulates a preovulatory LH surge. In contrast, the preovulatory release of GnRH and the resultant preovulatory surge of LH is induced by the receipt of genital somatosensory stimuli during mating of the induced ovulating species including rabbits, ferrets, cats, and camels (for review, see Bakker and Baum, 2000). GnRH that produced in specific hypothalamic and preoptic neurons is secreted into the portal circulation to act on the gonadotrophs, where its specific receptors are presented (Stojilkovic and Catt, 1995). In deed, GnRH release occurs episodically from the mammalian hypothalamus. The release of GnRH is strictly episodic in males and throughout the female reproductive cycle (Moenter et al., 2003). It has been reported that the frequency and amplitude of GnRH release from hypothalamic neurons is critical and rate-limiting step for the control and maintenance of gonadotropins secretion. It has been demonstrated that pulse of GnRH initiates pulsatile secretion of the FSH and LH (Clarke and Cummins, 1982; Levine et al., 1985; Levine and Duffy, 1988; Moenter et al., 1991). Changes in the GnRH pulse

frequency throughout the ovulatory cycle determine changes in the relative amounts of FSH and LH release (Wildt et al., 1981) which high-frequency GnRH pulses stimulate LH release, whereas low-frequency pulses stimulate FSH release (Kaiser et al., 1997). FSH and LH are then released in a pulsatile manner into the circulation to regulate the gametogenesis and steroidogenesis (Conn and Crowley, 1994; Stojilkovic and Catt, 1995).

Not only the hypothalamus and the pituitary gland are the principal sources and target sites for GnRH, respectively. It has been reported that extra-hypothalamic GnRH as well as extra-pituitary GnRH receptors have been found across different type of tissues such as ovaries, placenta, endometria, oviducts, testes, prostrate gland, and mammary glands (for review, see Ramakrishnappa et al., 2005). GnRH in these tissues is considered to act by autocrine or paracrine manner eliciting a variety of responses depending on the type of target tissues and physiological conditions. GnRH has been shown to cause a direct stimulatory effect on steriodogenesis and an inhibitory effect on gonadotropin-stimulated androgen biosynthesis in male gonads (Hsueh and Jones, 1982). Moreover, GnRH has been shown to elicit a mixed effect of both inhibitory and stimulatory responses affecting ovarian functions (Sharp, 1982; Janssens et al., 2000). GnRH agonist administration at a lower dose for short-term duration is shown to stimulate testosterone secretion in adult male rats, (Sharpe et al., 1982), whereas the effect is opposite when the agonist is administered at higher dose or for long-term duration (Arimura et al., 1979; Hsueh and Erickson, 1979). The functions of GnRH involving in the process of fertilization are also observed. GnRH agonist can increase the cleavage rate of bovine oocyte *in vitro* (Funston and Seidel, 1995). Furthermore, since GnRH mRNA has been found in the mammary gland of the

pregnant and lactating rats, suggesting that PRL may regulate GnRH expression (Palmon et al., 1994; Ikeda et al., 1995). GnRH is suggested to be involved in inducting follicular atresia and programmed cell death in the ovary. It has been hypothesized that GnRH mRNA plays an autocrine or paracrine regulatory role in the growth of reproductive tissue tumors such as ovarian carcinomas and endometrial carcinomas (Ramakrishnappa et al., 2005).

2.6.4 The Function of Gonadotropin Releasing Hormone in Birds

Like in mammals, GnRH also plays a pivotal role in the control of avian reproduction. At the peak level of reproductive activity, birds have more GnRH-ir cells and fibers than those of sexually inactive or photorefractory birds (Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998; Sharp et al., 1990). 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 (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 that of in laying hens (Dunn et al., 1996). GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples are higher in laying hens than that of in non-laying hens (Advis et al., 1985). In turkeys, it has been reported that GnRH-I mRNA abundance within the nCPa, organum vasculosum, lamina terminalis, and nucleus septalis lateralis is greater in laying hens than that of in non-

photostimulated and in incubating hens, 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 GnRH mRNA in the hypothalamus (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 transported to the pituitary gland, where it stimulates the secretion 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 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 concentrations 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 levels (Guemene and Williams, 1999).

Up 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 decline in the plasma LH concentrations and complete regression of the reproductive system. The absent of cGnRH-II-immunoreactivity in the ME of Japanese quails, chickens, and turkeys has 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 intracerebroventricular (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 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).

2.6.5 The Regulation of Gonadotropin Releasing Hormone Secretion

In birds, it has been reported that photoperiod is the main regulator of the GnRH neuron activities (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 pituitary sensitivity to GnRH (Davies and Follett, 1975). In deed, there are growing evidences that photoperiod is associated with GnRH system. The stimulatory effect of long day photoperiod appears to be associated with an increased GnRH contents or increased immunoreactivity for GnRH in the hypothalamus and ME in avian species (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 stimulation 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 high (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 reproductive quiescent turkeys maintained under a short day lighting program (Kang et al., 2006). Consistency with this finding, the nCPa respond 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 cells 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 which is associated with a decrease in FSH and LH secretion is related to a decrease in hypothalamic

GnRH-I (Dawson et al., 2001; 2002; Hua, 2001). Another study reveals that the reduction in hypothalamic GnRH in photorefractory birds is associated with a reduction in GnRH precursor and proGnRH-GTPase 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 GnRH neurons activity (Bruggeman et al., 1998; Ciccone et al., 2006; Deviche et al., 2006; Moore et al., 2006). The HPG axis is 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 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 and its peptide content in the POA and cGnRH-I receptor in the pituitary cells are suppressed by estrogen.

Hypothalamic VIP is also thought to inhibit the expression of GnRH (Deviche et al., 2000). It is very well documented that VIP receptor mRNA and its peptide co-exist with cGnRH-I cells and fibers in the lateral septum 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 in an electron microscopy study (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). Immunoneutralization of VIP significantly increases pituitary content of LH-β and FSH-β mRNAs and is accompanied by a decline in PRL mRNA expression (Ahn et al., 2001). PRL acts concomitantly with VIP to inhibit LH by means of reduction of GnRH at the hypothalamic level (Rozenboim et al., 1993b).

It is well known that adrenergic stimulation at the level of the hypothalamus can release GnRH and thereby 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 perikaya axons are terminated in the external layer of the ME, which is closed proximity to the terminals of tuberoinfundibular DA (TIDA) neurons (Ajika, 1979; Merchenthaler et al., 1984; Ugrumov et al., 1989), but little is know about these GnRH cell 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). Thus, it is reasonable to consider whether any regional specificity exists in those DA neurons that are neuroendocrine in nature, for example, controlling the release and expression of VIP/PRL and GnRH/FSH-LH systems.

Another factor affecting GnRH secretion is a gonadotropin-inhibitory hormone (GnIH; Tsutsui et al., 2000). GnIH inhibits LH and FSH synthesis and their

release *in vitro* (Ciccone et al., 2004). The expression of GnIH receptor mRNA is found in the quail diencephalon, in the ME close to cGnRH-I fiber terminal (Bentley et al., 2006b) as well as in the pituitary gland (Yin et al., 2005). In addition, an increase in melatonin (MEL) levels during a short day photoperiod induces an increase in GnIH gene expression (Bentley et al., 2003; Ciccone et al., 2004). It has been reported that MEL seems to act directly since GnIH neurons are equipped with MEL receptors (Ubuka et al., 2005). More details of GnIH are discussed briefly in the next section.

2.7 Gonadotropin Inhibiting Hormone: Structure and Function

2.7.1 The Structure of Gonadotropin Inhibiting Hormone

It is well established that the neuropeptide control of gonadotropins secretion is primarily through the stimulatory control of the hypothalamic decapeptide, GnRH. To date, it has been established that the hypothalamus contains a novel hypothalamic GnIH, and this dodecapeptide inhibits gonadotropins release. GnIH is discovered and first isolated from the brain of the Japanese quails. This isolated peptide contains a C-terminal -Arg-Phe-NH_2 sequence (RFamide) and is shown to have the sequence Ser-Ile-Lsy-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH_2 (Tsutsui et al., 2000). Subsequently, this peptide is demonstrated to be located at the hypothalamo-hypophysial system and to decrease gonadotropins release, but not PRL release from cultured anterior pituitary cells in a dose-dependent manner. Thus, it is termed GnIH.

The cDNA sequence of GnIH has been cloned in Japanese quails (Satake et al., 2001), domestic chickens (NCBI accession number AB120325), and white-crowned sparrows (Osugi et al., 2004). Similar peptides are also presented in

amphibians, fish, and mammals (including humans; Bentley et al., 2006b). The amino acid sequence of chicken GnIH differs from Japanese quail GnIH at position 3, where arginine conservatively substitutes lysine. The amino acid sequence of white-crowned sparrow GnIH differs from Japanese quail GnIH at position 5, 6, and 7 (Osugi et al., 2004) and the homology is about 66 %. In addition, the GnIH precursor polypeptide is cleaved into three separate mature peptides in birds (GnIH-related peptide 1 or -RP-1, and GnIH-RP-2) and possibly two peptides in mammals (RFamide-related peptides 1 and 3; Bentley et al., 2006b).

A GnIH receptor has been first identified in Japanese quails (Yin et al., 2005). GnIH receptor is a member of G protein-coupled receptors and specifically binds to GnIH in a dose-dependent manner. Reverse-transcriptase-mediated polymerase chain reaction products reveals the expression of GnIH receptor mRNA in the pituitary gland and several brain regions (Yin et al., 2005). It is possible that GnIH may act at the level of hypothalamus via GnIH receptors. Furthermore, other brain regions such as cerebrum, mesencephalon, and spinal cord, also contain GnIH receptor mRNA, suggesting multiple regulatory functions of GnIH in the avian brain (Yin et al., 2005; Bentley et al., 2006b).

2.7.2 The Localization of Gonadotropin Inhibiting Hormone in the Brain

GnIH localization in the brain is deeming essential in order to understand its physiological functions. GnIH localization in the brain of the Japanese quails has been reported (Tsutsui et al., 2000; Ubuka et al., 2003; Ukena et al., 2003). The localizations of GnIH have been elucidated in many avian species such as song sparrows, house sparrows, Gambel's white-crowned sparrows (Bentley et al., 2003;

Osugi et al., 2004), cardueline finches, and tropical sparrows (Bentley et al., 2006b). Clusters of dense GnIH-ir neurons are observed in the PVN regardless of sex and species. GnIH-ir neurons in the PVN are parvocellular neurons with bipolar or tripolar in the ventral portion of PVN, which show no immunoreaction with the antibodies against vasotocin and mesotocin (Ukena et al., 2003). *In situ* hybridization study confirms the cellular localization of GnIH mRNA in the PVN of Japanese quails and sparrows (Ukena et al., 2003; Osugi et al., 2004). Some scattered small GnIH-ir cells are also located in the septal area. In contrast to the highly localized cluster of cell bodies, GnIH-ir fibers are widely distributed in the diencephalic and mesencepahlic brain regions. Dense networks of GnIH-ir fibers are observed in the ventral paleostriatum, septal area, POA, hypothalamus, and optic tectum. The most prominent fibers are found in the ME and the dorsal motor nucleus of the vagus in the medulla oblongata.

Thus, the presence of GnIH-ir fibers in the ME supports a role for GnIH in gonadotropins regulation. The distributions of GnIH-ir fibers also are observed outside the hypothalamic area, suggesting the role of GnIH in participating not only in neuroendocrine functions, but also in behavioral and autonomic mechanisms (Ukena et al., 2003). The presence of GnIH in the PVN appears to be a conserved property among several avian species (Tsutsui et al., 2005; 2007). GnIH fibers are located in extremely close proximity to GnRH neurons in the POA in birds (Bentley et al., 2003). Taken together with the findings that GnIH fibers extend to the ME terminals suggests that GnIH may influence the GnRH system at the neuron and fiber terminal levels. It is thus possible that GnIH acts at the level of hypothalamus to regulate gonadotropins release as well as at the pituitary level (Kriegsfeld et al., 2006).

To date, the presence of GnIH has been investigated extensively in mammals, particularly in rodent species such as Syrian hamsters, rats, and mice (Ukena and Tsutsui, 2001; Kriegsfeld et al., 2006). GnIH cell bodies are observed occupiedly a location slightly caudal to that found in birds and confined to the rostral-caudal extent of the dorsomedial hypothalamus. GnIH fibers form an extensive network extending along a midventral and dorsal continuum from the tenia tectum to the hindbrain. GnIH fibers found in mammals are not detected in the external layer of the ME, but are detected in the internal layer, suggesting that GnIH might not regulate gonadotropins via the traditional pathway of pituitary as it likely does in birds (Bentley et al., 2006b).

2.7.3 The Function of Gonadotropin Inhibiting Hormone

As aformentioned, GnIH is discovered and named because it inhibits gonadotropins secretion. 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 Japanese quails is indicated in *in vitro* study. Incubation of GnIH with anterior pituitary cells decreases plasma and mRNA levels of LH and FSH in dose-dependent manner, but does not change plasma PRL levels (Tsutsui et al., 2000). GnIH also inhibits circulating LH *in vivo*. Intraperitoneal administration of GnIH into Japanese quails via osmotic pump results in significantly reduced plasma LH levels (Ubuka et al., 2006). GnIH injected simultaneously 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). Administration of GnIH via ICV infusion to the third ventricle

induces a sharp decrease of plasma LH levels in photostimulated female white-crowned sparrows (Bentley et al., 2006a; 2006b). Moreover, it has been illustrated that GnIH also inhibits gonadotropins common α- and β-subunits production as well as their release (Ciccone et al., 2004; Tsutsui et al., 2005; 2006; Ubuka et al., 2006). GnIH effects on plasma LH levels are found to be similar in Syrian hamsters (Kriegsfeld et al., 2006), providing additional evidence for properties of the physiological action of GnIH.

The GnIH distributions in the highly photoperiodic songbird species such as sparrows suggest that GnIH might play a significant role in the termination of breeding season in these species (Bentley et al., 2006b). GnIH mRNA expression levels are higher in reproductively inactive and incubating hens than that of in laying hens, but administration of GnIH into nest-deprived incubating hens fails to suppress plasma LH levels (Ciccone et al., 2004). It has been proposed that the GnIH expression is photoperiodically controlled and increased under short day photoperiod (Ubuka et al., 2005), when the nocturnal duration of MEL secretion increases (Cockrem and Follett, 1985). Mel_{1c}, MEL receptor subtype, is co-expressed in GnIH-ir neurons in the PVN (Ubuka et al., 2005). These findings raise the indication that MEL may act directly on GnIH neurons via its receptor to induce GnIH expression. Chronic GnIH treatment decreases plasma testosterone levels and gonadotropins synthesis and release in a dose-dependent manner in mature birds, (Ubuka et al., 2006). However, in immature birds, chronic treatment with GnIH suppresses normal testicular growth and plasma testosterone levels (Ubuka et al., 2006). Taken together, these results reveal that GnIH inhibits gonadal development and maintenance by inhibiting gonadotropins synthesis and release. Thus, GnIH is likely an important

neuropeptide for the regulation of avian reproduction.

The presence of GnIH recptor mRNA in extra-hypothalamic regions such as cerebrum, mesencephalon, and spinal cord suggests multiple regulatory functions of GnIH in the avian brain as well (Yin et al., 2005; Bentley et al., 2006b). It has been reported that GnIH stimulates feeding behavior in chicks (Tachibana et al., 2005) and inhibits female sexual behaviors in white-crowned sparrows (Bentley et al., 2006b). These physiological evidence support a role of GnIH in the regulation of reproduction in birds and the role of neurotransmitters regulating GnIH secretion is inconclusive and requires further elucidation.

2.8 Prolactin: Structure, Function, and Regulation of Secretion

2.8.1 The Structure of Prolactin

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

The major form of PRL found in the pituitary gland is 23 kDa and is consisted of 5 exons and 4 introns (Cooke et al., 1981; Truong et al., 1984). Variants of PRL isoform have been characterized in many mammals. PRL variants can be the results of alternative splicing of the primary transcript, proteolytic cleavage, phosphorylation, glycosylation, and other posttranslational modifications, thus altering its physiological functions (Sinha, 1995). PRL is synthesized as a preprohormone consisting of 227

amino acids in most mammalian species (Miller and Eberhardt, 1983). The mature hormone contains 194-199 amino acids which MW of 23 kDa, depending on species. Hormone structure is stabilized by three intramolecular disulfide bonds. The primary structure of PRL is first illustrated in the ovine (Li et al., 1970). The complete amino acid sequences of PRLs of more than 25 species have been determined (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 acid residues seem to be conserved among different species (Watahiki et al., 1989). The homology sequences of PRLs among different species and their primary structures are shown in Figures 2.6 and 2.7, respectively.

PRL is one of 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 evolved from a common ancestral gene by gene duplication (Niall et al., 1971) about 500 millions years ago. It has been demonstrated that PRL is also synthesized by a number of extra-pituitary 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., 2003b), but its physiological function is poorly understood and need to be further investigated. PRL is synthesized and secreted by a broad range of other cells in the body including the most prominently various immune cells, mammary epithelium, placenta, the deciduas of the pregnant uterus, and brain (Ben-Jonathan et al., 1996). Moreover, PRL synthesis is also found in lacrimal gland, adrenal gland,

corpus luteum, prostate gland, testis, and pancreas (Ben-Jonathan et al., 1996; Freeman et al., 2000). Up 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.

PRL receptor (PRLR), a single membrane-bound protein transmembrane receptor, is a member of Class I cytokine receptor superfamily that includes the receptor of GH, leptin, erythropoietin, and interleukins (Bazan, 1989; 1990; Kelly et al., 1991). PRL, PL, and primate GH, binds the PRLR. PRL and GH receptors share some structural and functional features despite their low sequence homology (30 %; Goffin and Kelly, 1996). The receptor is activated by the binding of a single ligand to the receptor to dimerizing two identical receptor subunits, leading to activation of Jak2-kinase asscociated with the cytoplasmic domain which subsequently activates a number of signaling cascade 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 termed signal transducers and activators of transcription (Stats). Not only the Jak2-Stat cascade is the major signaling pathway of the PRLR, other transducing pathways are also involved in signal transduction by this receptor as well. Activation of mitogen-activated protein kinases (MAPK) 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 reported 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 give 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 observed in the mammary gland and ovary, the best characterized sites of PRL 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). In addition, tissue distributions of PRLR mRNA have been characterized 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, PRLR is found in the CNS of the rats and a wide range of peripheral organs including 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 is found in the CNS, choroid plexus, bed nucleus of the stria terminalis, amygdala, central gray of the midbrain, thalamus, hypothalamus, cerebral cortex, and olfactory bulb. PRLR is 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 crop sac, brood patch, thyroid gland, liver, kidney, leg, skin, intestine, adipose tissue, adrenal gland, thymus, spleen, heart, brain, pineal gland, ovary, testis, and oviduct (Tanaka et al., 1992; Chen and Horseman, 1994; Zhou et al., 1996; Ohkubo et al., 1998; Pitts et al., 2000). Moreover, 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 2.6 The percentage of homology sequence of PRLs among different species (Sinha, 1995).

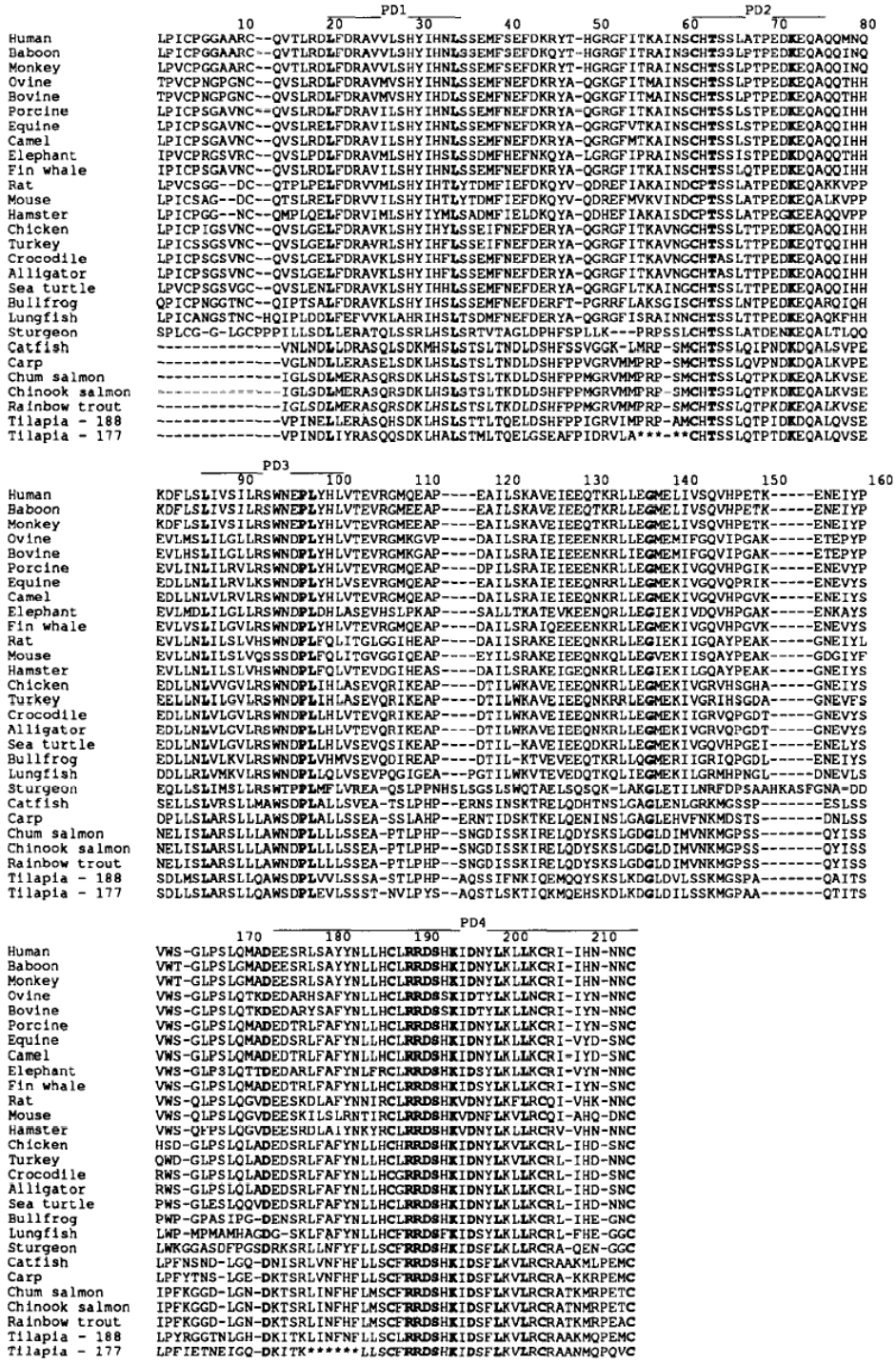

Figure 2.7 Primary structures of PRLs of different species. (**-**) indicates positions left blank to optimize alignment of amino acid sequences. (*) indicates absence of residues from a genetic variant of tilapia PRL. PD is PRL domain. PDI-PD4 indicates the four highly conserved domains of the PRLs (Sinha, 1995).

2.8.2 The Function of Prolactin in Mammals

Well documentedly, PRL interacts with its specific receptors in a broad variety of target tissues to affect physiological functions that have been broadly grouped into those that effect reproduction, water and electrolyte balance, brain and behavior, growth and development, endocrinology and metabolism, osmoregulation, metabolism, behaviors such as migration, the nurturing of the young in different vertebrate species, and immunoregulation (Saeki and Tanabe, 1955; Houdebine, 1983; Bole-Feysot et al., 1998; Harris et al., 2004). PRL seems to be an omnipotent hormone, but it is best known for its role in milk production. PRL has an essential role for lactation in mammals involving in the development of mammary gland (Bern and Nicoll, 1968). The effects of PRL on the mammary gland such as growth and development of mammary gland (mammogenesis), synthesis of milk (lactogenesis), and maintenance of milk secretion (galactopoiesis) have been well established. Hypophysectomy during pregnancy prevents subsequent lactation, suggesting that lactogenesis requires pituitary PRL. Replacement of PRL to hypophysectomized rabbits fully restores lactation, while hypophysectomy of rats and mice stops lactation. Moreover, aqueous extracts of anterior pituitary gland which containing PRL can initiate lactation in pseudopregnant rabbits (for review, see Freeman et al., 2000). It has been suggested that the initiation and maintenance of lactation following parturition is dependent on the mitogenic effects of PRL upon mammary cell development and its regulation of transcription and translation of milk proteins (Ben-Jonathan et al., 1989). PRL, cortisol, and insulin act together to stimulate transcription of the genes that encode milk proteins and appear to modulate ovulation since

elevated physiological or pathological levels results in the cessation of cyclicity (Nicoll, 1974).

PRL also plays a critical role in the maintenance of corpus luteum and progesterone secretion in some mammals, especially in rodents (Risk and Gibori, 2001) by maintaining the structural and functional integrity of corpus luteum for 6 days after mating (Morishige and Rothchild, 1974). In addition, it induces transcription of estrogen receptor (Frasor and Gibori, 2003) and 3β-hydroxysteroid dehydrogenase that involved in progesterone synthesis (Feltus et al., 1999). In contrast, there is evidence in the rats that PRL induces programmed cell death in the corpora lutea (Kanuka et al., 1997), suggesting that it may be luteolytic as well (Malven and Sawyer, 1966; Wuttke and Meites, 1971). PRL seems to be important in several non-lactational aspects of reproduction as well. PRL is necessary for maintainance of corpora lutea in some species (Morishige and Rothchild, 1974). It also affects other behaviors related to reproduction such as mating and maternal behaviors (Dutt et al., 1994).

PRL also plays a significant role in reproduction, maternal care, and parental behaviors in mammals. PRL is required for the ovarian hormones to be effective in stimulating maternal behaviors (Rosenblatt et al., 1988). It has been reported that the changes in the plasma concentrations of PRL is correlated with the activities of the maternal nest building in rabbits (Gonzalez-Mariscal et al., 1996; Gonzalez-Mariscal, 2001). Enhancement of endogenous PRL secretion in response to DA antagonism has been reported to have no effect on mating behavior in females (Sodersten et al., 1983). Suppression of the spontaneous release of PRL with DA agonists when the rats are sexually receptive in the afternoon of proestrus, dramatically decreases sexual

receptivity (Mena and Grosvenor, 1972). In addition, PRL and its receptors are expressed in human and rat prostate epithelial cells, where their levels are increased by androgen treatment (Nevalainen et al., 1997). Hyperprolactinemia has a direct effect on hyperplasia of the prostate gland, suggesting the role of PRL on cellular proliferation. In addition of its actions, PRL also plays a role in maintaining constancy of the internal environments by regulation of the immune system, osmotic balance, and angiogenesis (for review, see Freeman et al., 2000). PRL has been found to stimulate proliferation of oligodendrocyte precursor cells and these cells differentiate into oligodendrocytes which are responsible for myelin-coating on axons in the CNS (Gregg et al., 2007).

2.8.3 The Function of Prolactin in Birds

It has been studied and well documented that PRL are associated with the reproductive cycle in several avian species (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 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 levels 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). In addition, PRL is involved in many aspects of reproductive physiology and behaviors. It is widely 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 in birds (Lea and Vowles, 1986; Macnamee et al., 1986; Pedersen, 1989; Buntin et al., 1991; Youngren et al., 1991). These results supports that PRL regulates the onset and maintenance of incubation behavior in galliform birds.

Some evidence suggests that PRL plays a role in terminating egg laying, therefore regulates clutch size in species that lay more than two eggs per clutch. Cessation of egg laying is associated with an increase plasma PRL concentrations (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 depress LH secretion (Zadworny and Etches, 1987; El Halawani et al., 1993; Sharp et al., 1998). Administration of exogenous PRL suppresses plasma gonadotropins in turkeys (El Halawani et al., 1991) and domestic fowls (Sharp et al., 1988). It is suggested that PRL acts centrally to reduce LH levels by reducing hypothalamic GnRH levels (Rozenboim et al., 1993b). In incubating birds, suppression of gonadotropins secretion involves in a mechanism independent of increase PRL secretion (Sharp et al., 1988; 1989a; Lea and Sharp, 1989; Lea et al., 1996). In addition, PRL may also directly inhibit ovarian steroidogenesis (Rozenboim et al., 1993b), leading to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct.

2.8.4 The Regulation of Prolactin Secretion

In mammals, PRL secretion is regulated by both stimulatory and inhibitory hypothalamic factors. Its mainly regulation is 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 MBH known as the TIDA and serves as the physiological inhibitor of PRL secretion (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). DA released from TIDA neurons acts directly upon 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). In addition, DA and its agonists inhibit the release and gene expression of PRL and proliferation of lactotrophs (Birge et al., 1970; Shaar and Clemens, 1974; Pawlikowski et al., 1978; Maurer, 1981), suggesting 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 physiological stimulus such as suckling (Chiocchio et al., 1979; Selmanoff and Wise, 1981; Demarest et al., 1983) is necessary for PRL release (Grosvenor et al., 1980). Similarly, in *in vitro* studies indicate that pituitary PRL release is stimulated after short term exposure of DA (Fagin and Neil, 1981; Denef et al., 1984). These studies demonstrate the physiological relevance of DA as the PIF. On the other hand, 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 G-proteins depending on its specific receptor subtypes (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994).

VIP has been shown to involve in the regulation of PRL secretion from the pituitary gland (Kato et al., 1978; Rotsztejn et al., 1980; Reichlin, 1988). VIP is 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) are found. It has been reported that VIP can stimulate PRL release both *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Shaar et al., 1979; Enjalbert et al., 1980; Samson et al., 1980; Matsushita et al., 1983). In addition, 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). Moreover, the amount of pituitary PRL mRNA and PRL synthesis are appears to be regulated by VIP (Ben-Jonathan et al., 1989; Maas et al., 1991). In rats, VIP mRNA is increased during the lactation period (Gozes and Shani, 1986). An increase in the concentrations of VIP in the hypophysial portal blood is relative to the peripheral blood (Said and Porter, 1979; Shimatsu et al., 1981). Furthermore, 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 indicated above, the data purposes VIP as the mammalian PRF.

Thyrotropin-releasing hormone (TRH) also acts as the hypothalamic PRF in mammals. It has been established that TRH stimulates PRL release both *in vivo* (Grosvenor and Mena, 1980; de Greef and Visser, 1981; Laverriere et al., 1988; Lafuente et al., 1994) and *in vitro* (Maas et al., 1991) and also PRL gene transcription (Potter et al., 1981; Laverriere et al., 1988). The release 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 contradictive results 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 control of PRL secretion in birds involves the interaction of external stimuli with endocrine mechanisms. Critical environmental stimuli include sensory information concerning photoperiod, ambient temperature, and the presence of eggs and offspring. These external stimuli as well as the 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).

The regulation of PRL secretion and gene expression are under the inhibitory control of TIDA neurons in the hypothalamus in mammals (Ben-Jonathan and Hnasko, 2001). This is not the case in birds, where removal of hypothalamic inputs results in the complete cessation of PRL secretion (Tixier-Vidal et al., 1966; Chadwick et al., 1978; Hall et al., 1986). It has been 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 PRL gene expression is influenced by hypothalamic VIP, the PRF in avain species (El Halawani et al., 1997; 2001; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005). In the past six decades, several studies support the pivotal role of VIP as the only avian PRF. Immunoneutralization of VIP prevents an increase in circulating PRL that follows photostimulation, prevents the induction of incubation behavior, up regulates 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., 1989a; El Halawani et al., 1995; 1996; Dawson and Sharp, 1998; Ahn et al., 2001). Furthermore, it has been reported that 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 apparent contrast with mammals, DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptor subtypes (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003a; 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., 2003a). The stimulatory effect of DA on PRL secretion is regulated via 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 through D_2 DA receptors (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003a; Al Kahtane et al., 2003). In addition, changes in DAergic activity during the turkey reproductive cycle paralleled the changes in plasma PRL levels, number of VIP-ir neurons, VIP peptide contents and its mRNA expression within the INF (El Halawani et al., 1980; 1984b; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003a; 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, recent evidences indicate that dynorphin, 5-HT, DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ opioid, serotonergic, 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).

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

2.9.1 The Structure of Vasoactive Intestinal Peptide

VIP, an octacosapeptide, consists of 28 amino acids. It is 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 is considered to function 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 hypophysial portal blood (Said and Porter, 1979; Shimatsu et al., 1981; Brar et al., 1985; Mutt, 1988) led to the hypothesis that VIP participates in the regulation of anterior pituitary functions.

VIP is a neuropeptide of 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 are mediated via the adenylate cyclase and the production of cAMP (Hokfelt et al., 1980; Couvineau et al., 1990; Lutz et al., 1995). The peptides of this family are probably the results of exon duplication coupled to gene duplication. It has been ducumented that VIP gene contains 7 exons, each exon encoding 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; Yamagami et al., 1988; Giladi et al., 1990). To date, mammalian VIP cDNAs (Itoh et al., 1983; Nishizawa et al., 1985) and chicken and turkey VIP cDNAs (McFarlin et al., 1995; Talbot et al., 1995; You et al., 1995b) have been cloned. The open reading frame of mammalian VIP is comprised of 165 amino acids. It has been reported that chicken VIP is different from mammalian VIP in its amino acid sequence at position 11, 13, 26, and 28, but the number of amino acid residue is the same (Nilsson, 1975). In addition, 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. In contrast, the short form is found only in the turkey hypothalamus and comprises 4-6 % of all VIP transcripts (You et al., 1995b). The amino acid sequence of VIP and the member in VIP/glucagon/secretin family are shown in Figure 2.8.

	1 5 10 15 20 25
p/b VIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN[a]
c VIP	HSDAVFTDNYSRFRKQMAVKKYLNSVLT[a]
p PHI	HADGVFTDDFSRLLGQLSAKKYLESLI [a]
p SECRETIN	HSDGTFTSELSRLRDSARLQRLLQGLV [a]
m GLUCAGON	HSQGTFTSDYSKYLDSRRQDFVQWLMNT
p GIP	YAEGTFISDYSIAMDKIRQQDFVNWLLA...

Figure 2.8 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 have 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 receptor; VIP1 and VIP2 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. VIP receptors are the members of G protein-coupled family, which biological actions are mediated via interaction with specific receptors that are coupled to adenylate cyclase and the production of cAMP (Gourdji et al., 1979; Bjoro et al., 1987; Couvineau et al., 1990; Lutz et al., 1995). There have reported that VIP receptor is presented in several organs including 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). Moreover, a single VIP receptor is also expressed and functioned in non-mammalian species (Kansaku et al., 2001). Avian VIP receptors have been cloned and characterized in 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).

2.9.2 The Function of Vasoactive Intestinal Peptide in Mammals

Originally, VIP is considered to be a gastrointestinal hormone in mammals (Grossman, 1974) and is believed to be presented in endocrine cells of mammalian

and avian species (Polak et al., 1974). VIP has been found to be distributed in the central and peripheral nervous systems (Larsson et al., 1976; Said and Rosenberg, 1976; Giachetti et al., 1977; Hokfelt et al., 1982; Rosselin et al., 1982). Significant concentrations of VIP are detected in the gastrointestinal tract, heart, lung, thyroid gland, kidney, immune system, urinary bladder, and genital organs. The widespread distributions of VIP throughout the CNS and pheripheral organs are correlated with its involvement in a wide variety of physiological effects. It plays roles in smooth muscle relaxation, stimulation the secretion of water into pancreatic juice and bile, inhibition of gastric acid secretion and absorption from the intestinal lumen, cell proliferation, and increasing of gastric motility (for review, see Gozes et al., 1999; Gozes and Furman, 2003). Various physiological functions of VIP have been reported such as vasodilation (Bakken et al., 1995), broncodilation (Tam et al., 1990), exocrine secretions (Alonso et al., 1994; Nassar et al., 1995; Rodriguez-Lopez et al., 1995), increasing blood flow (Shimizu and Taira, 1979; Bloom and Edwards, 1980; Heistad et al., 1980; Andersson et al., 1982), energy metabolism, especially the enzymatic breakdown of glycogen to glucose (Magistretti et al., 1981), stimulation of thyroid hormones secretion (Ahren et al., 1980), bone resorption (Hohmann et al., 1983), and controlling the homeostasis of the immune system such as immunosuppression and antiinflammatory (Gomariz et al., 2001). The presence of VIPergic nerve fibers are shown in both central and peripheral lymphoid organs (Bellinger et al., 1996). These VIP-containing nerve terminals establish the anatomical link between the CNS and the immune system. VIP appears to modulate maturation of specific populations of effector cells, T cell recognition, antibody production, and homing capabilities.

High concentrations of VIP have been detected in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 1991). VIP is also detected in the cerebral cortex, hippocampus, corpus striatum, and vagal centers of the medulla oblongata (Gozes et al., 1999). VIP acts as a neurotransmitter and/or neuromodulator (Said and Rosenberg, 1976) to promote neuronal survival, induce neuronal differentiation, modulate neurotransmitter synthesis, and influence neuronal excitability (Klimaschewski, 1997). It has been reported that VIP regulates the release of anterior pituitary hormones such as PRL (Kato et al., 1978; Rotsztejn et al., 1980; Frawley and Neill, 1981; Reichlin, 1988), GH (Chihara et al., 1982), and adrenocorticotropic hormone (ACTH; Oliva et al., 1982; White et al., 1982). VIP can stimulate PRL release both *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Samson et al., 1980; Matsushita et al., 1983). Administration of VIP antiserum inhibits PRL release induced by stress, 5-HT, or suckling (Shimatsu et al., 1984; Abe et al., 1985; Ohta et al., 1985). VIP also regulates the amount of pituitary PRL mRNA and its proteins (Ben-Jonathan et al., 1989; Maas et al., 1991). Hypothalamic VIP mRNA is increased during lactation in rats (Gozes and Shani, 1986). Moreover, VIP receptors in the pituitary cells (Gourdji et al., 1979; Bjoro et al., 1987) as well as VIP promotes the entry of extracellular calcium ions into the PRL-secreting pituitary cells (Bjoro et al., 1987; Prysor-Jones et al., 1987) have been reported. These data confirm VIP as the PRF in mammals. Furthermore, VIP also regulates neuroendocrine inhibition of LH (Stobie and Weick, 1989), stimulates oxytocin and vasopressin release from the neurohypophysis (Ottesen et al., 1984), stimulates male sexual behaviors (Gozes et al., 1989), maintains the neuronal survival (Brenneman and Eiden, 1986), and modulates circadian rhythms (Moore, 1983; Yuwiler, 1983; Card

and Moore, 1984).

2.9.3 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). Several lines of evidence support VIP as the most important PRF in birds (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). VIP meets the classical criteria for defining it as the hypophysiotrophic PRF in birds. These criteria include; 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-specific 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 evidence have been provided by the findings that immunoneutralization of endogenous VIP reduces levels of circulating PRL and pituitary PRL mRNA and totally blocks the PRL release affected by electrical stimulation of the medial preoptic nucleus (MPOA; El Halawani et al., 1990b; Youngren et al., 1994) as well as 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 upon 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 secretion in birds. VIP regulates PRL gene expression by enhancing the transcription rate of PRL and up-regulating PRL mRNA stability (Tong et al., 1998). It is very well documented that 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 anti-VIP 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 and prevents the expression of incubation behavior in female turkeys (El Halawani et al., 1996; 2001). It is suggested that the stimulatory action of VIP occurs via specific binding sites located on anterior pituitary cell membranes, which changes throughout the reproductive cycle of the turkeys (Rozenboim and El Halawani, 1993; Chaiseha et al., 2004). As mentioned above, it is supported the role of VIP as the avian PRF.

Elevated hypothalamic VIP peptide and its mRNA 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 (Ahn et al., 2001) and delays the onset of photorefractoriness and molt in starling (Dawson and Sharp, 1998). Even though the functional significance of these findings remains to be clarified, they imply that VIP also exerts an inhibitory influence on the gonadotropin system. These indicate that VIP has a central inhibitory influence on GnRH/FSH-LH release in birds (Pitts et al., 1994).

2.9.4 The Regulation of Vasoactive Intestinal Peptide Secretion

Since this research dissertation is conducted in the native Thai chickens, this section will discuss only the regulation of VIP secretion in birds. Indeed, the regulation of mammalian VIP is very well documented worldwide. It has been suggested that VIP mediates the effects of photoperiod on PRL secretion in the turkey (El Halawani et al., 1996) and quantification of hypothalamic VIP reveal an increased VIP content following photostimulation (Mauro et al., 1992), and it has been demonstrated that VIP 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). This result lends 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 result also implies 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 located in the INF region of the hypothalamus (Mauro et al., 1989). 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 area. 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, turkey melanopsin (tOPN4x) is found in DA-MEL co-localized neurons in the nucleus premamillaris (PMM) and is implicated as an important component of the photoreceptive system regulating reproductive activity in temperate zone birds (Kang et al., 2010).

It has also been reported that VIP is also inhibited by high concentration of circulating PRL. 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 also reduces hypothalamic VIP contents and the number of anterior pituitary VIP binding sites (Rozenboim et al., 1993a), suggesting that PRL may act directly at the pituitary level. Moreover, PRL binding sites have been found within the avian hypothalamus (Buntin and Ruzycki, 1987; Buntin and Walsh, 1988) and PRL receptor mRNA is also detected in the brain of chicken (Tanaka et al., 1992) and the hypothalamus of turkey (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 PRL receptors lining the third ventricle, thereby decreasing the number of hypothalamic VIP-containing neurons (Saldanha and Silver, 1995).

Immunoneutralization of VIP 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, and extends the duration egg laying period, but does not prevent spontaneous gonadal regression and molting (Sharp et al., 1989a; El Halawani et al., 1995; 1996; Dawson and Sharp, 1998; Ahn et al., 2001). Despite the well established antigonadotropic effects of PRL, it seems that the high circulating PRL levels of laying and non-incubating birds is not the primary cause of GnRH/gonadotropins suppression and the termination of reproduction (Juss, 1993; Dawson and Sharp, 1998).

2.9.5 The localization of Vasoactive Intestinal Peptide in the Avian Brain

It is very well documented that the distributions of VIP-containing neurons have been conducted in the brain of 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). 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), especially in the

areas of the MPOA, medial hypothalamus, AM, hypothalamus pars lateralis (LHy), and INF (den Boer-Visser and Dubbeldam, 2002). In general, three types of VIP-ir neurons and fibers are described. The first consists of a large number of spindle or bipolar neurons that connected the third ventricle to the external layer of the ME. A second set of VIP-ir fibers extends from the infundibular nucleus to the ME. The third type of VIP-ir neurons terminates upon small capillaries within the hypothalamus. 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. Depriving incubating birds from their nests are found to reduce circulating PRL levels and hypothalamic VIP immunoreactivity (Mauro et al., 1989). Fluctuations in hypothalamic VIP immunoreactivity and expression within the INF parallel fluctuations in circulating PRL concentrations (Chaiseha and El Halawani, 1999). The number, area, and density of hypothalamic VIP-ir neurons are greater in incubating than that of in laying hens (Sharp et al., 1989a). In addition, in the domesticated pigeons, increase in the number and size of VIP-ir neurons within this region following the periods of elevated circulating PRL has been reported (Peczely and Kiss, 1988; Cloues et al., 1990). 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). Moreover, lesions in the INF can prevent the PRL increase after

photostimulation (Youngren et al., 1989).

All these data indicate that the VIP neurons in the INF are an important factor in the stimulation of PRL secretion. Moreover, it has been established that VIP axon terminals have been found in close apposition to GnRH neurons in the lateral septal organ and POA (Teruyama and Beck, 2001) and an inverse relationship between VIP in the INF and GnRH in the POA has been reported (Deviche et al., 2000). It has been indicated that a subset of VIP-ir neurons within the medial basal hypothalamus 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 tOPN4x in the hypothalamic PMM DA-MEL neurons acts as an important component of the photoreceptive system regulating reproductive activity in temperate zone birds (Kang et al., 2010).

2.10 Dopamine: Structure, Function, and Regulation of Secretion

2.10.1 The Structure of Dopamine

DA is discovered (Carlsson and Hillarp, 1956; Benes, 2001) and found in both central and peripheralnervous systems of many species. DA is a neurotransmitter/neuromodulator which chemical name is 4-(2-aminoethyl) benzene-1,2-diol and the formula is $C_6H_3(OH)_2$-CH_2-CH_2-NH_2. It belongs to a group of catecholamines (CA) and functions as classical neurotransmitters in the brain, therefore they communicate between neurons and act within the anatomically confined space of the synapses. It has several significant physiological functions involving in a wide variety of behaviors and reproduction. DA is a precursor of NE and then epinephrine (E) in the biosynthetic pathway for these neurotransmitters. CA

and indolamines such as 5-HT are referred to as monoamine, a water soluble molecule that is decarboxylated derivatives of amino acids. CA has distinctive structure, which are the single amine group, a nucleus of catechol (a benzene ring with two adjacent hydroxyl groups), and a side chain of ethylamine or one of its derivatives (Wood-Gush, 1973).

Tyrosine is the precursor for DA synthesis. The majority of circulating tyrosine originates from dietary sources. However, small amounts of tyrosine are derived from hydroxylation of phenylalanine by phenylalanine hydroxylase from the liver (Missale et al., 1998). Tyrosine enters the neurons by an energy-dependent uptake process. It is then converted to DA by two enzymes that act in sequence, which are TH and 1-aromatic amino acid decarboxylase (AADC), these enzymes are named dihydroxyphenylalanine decarboxylase (DDC). TH is considered to be the rate-limiting enzyme in this biosynthetic pathway. It converts tyrosine into 3,4-dihydroxyphenylalanine (L-DOPA) and then L-DOPA is catalyzed by AADC to produce DA. DA is further processed into NE by DA beta-hydroxylase (DBH) in some neurons. Those neurons also contain phenylethanolamine N-methyl transferase (PNMT) that converts NE to E. The biosynthetic pathway of CA is shown in Figure 2.9.

TH activity is the most critical enzyme that regulates DA synthesis. In humans, TH gene is localized at chromosome 11p and encodes a single form of TH that can be alternatively spliced (Powell et al., 1984). Targeted disruption of the TH gene results in perinatal lethality, which can be rescued by L-DOPA administration (Kobayashi et al., 1995). The mature enzyme is composed of four subunits of approximately 60 kDa each (Kumer and Vrana, 1996) and each monomer is

comsisted of an inhibitory regulatory domain at the N terminus and a catalytic domain at the C terminus. The catalytic domain contains protein binding region and a putative leucine zipper at the C terminus that participates in intersubunit binding.

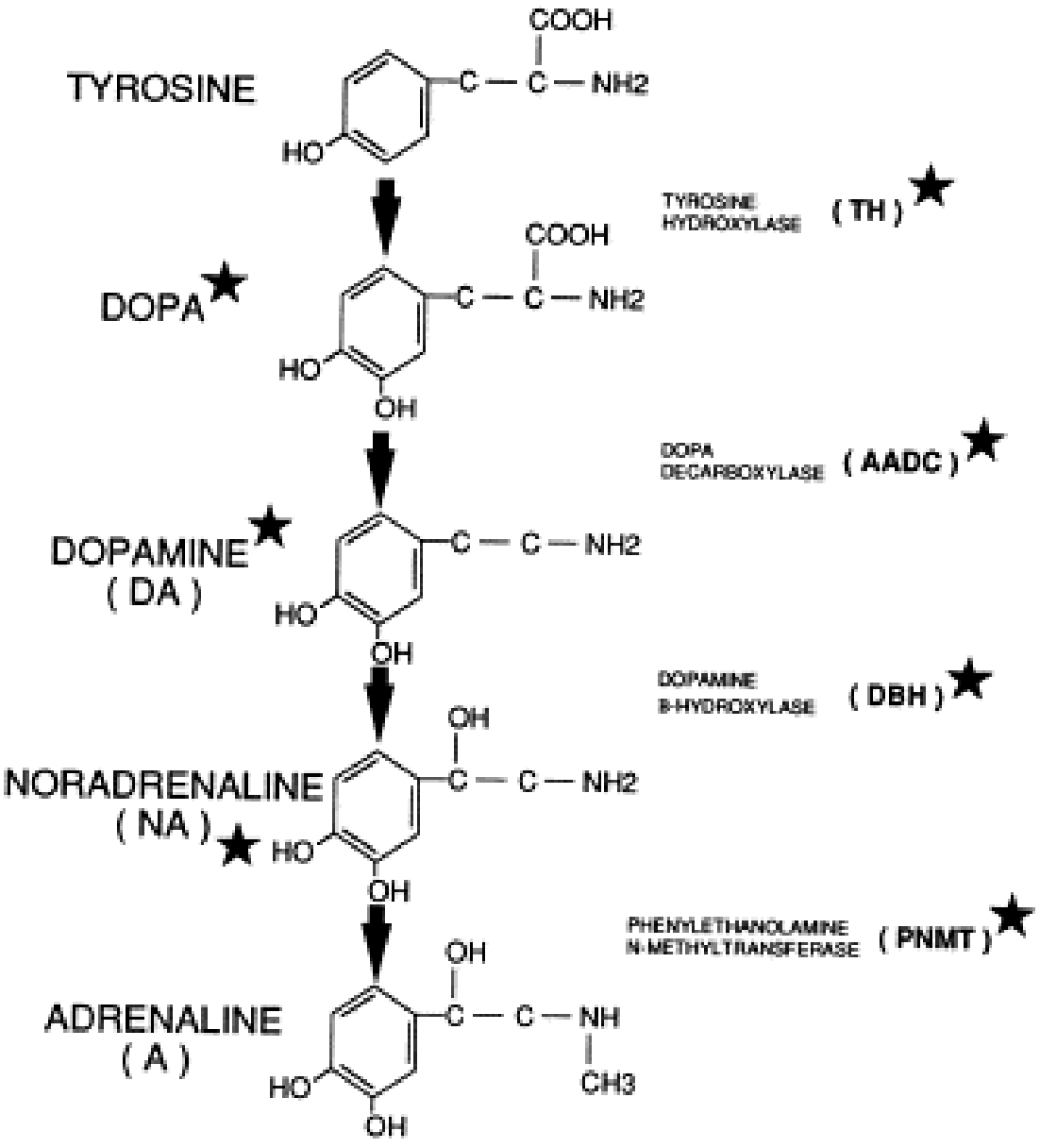

Figure 2.9 Biosynthetic pathway of catecholamines and available antisera as indicated by asterisks (Smeets and Gonzalez, 2000).

2.10.2 The Dopamine Receptors

DA exerts its biological actions by binding to its specific receptors, which belongs to the G protein-coupled receptors family. Five distinct subtypes of DA receptors (D_1-D_5) are prominent in the CNS of the vertebrates (Contreras et al., 2002). These types of receptor have been isolated, characterized, and subdivided into two families based on the basis of their stimulatory or inhibitory activities on adenylate cyclase (Kebabian and Calne, 1979). The D_1-like DA subfamily comprises of D_1 and D_5 DA receptors and termed the D_{1A} and D_{1B} DA receptors by some researchers (Monsma et al., 1990; Sibley, 1991). The D_2-like DA receptor includes D_2, D_3, and D_4 DA receptors. Activation of the D_1-like DA receptors promotes adenylate cyclase activity via $G_{s\alpha}$ subunit, while activation of the D_2-like DA receptors inhibits adenylate cyclase activity via $G_{i\alpha}$ subunit. However, the G_o and G_q proteins, which are associated with ion channels and phosphoinositide cascade, are also involved (Stoof and Kebabian, 1984; Sidhu and Niznik, 2000). Characterization of cDNAs for five receptor subtypes shows that the D_1 and D_5 DA receptors share high homology in their transmembrane sequences and also the transmembrane sequences of D_2, D_3, and D_4 DA receptors are conserved in the three receptor subtypes (Missale et al., 1998).

The distributions of DA receptor subtypes have been well elucidated in mammals. The five subtypes of DA receptors have distinct localization within the brain and are expressed in a tissue-specific manner in the periphery (Sunahara et al., 1993; Contreras et al., 2002). Generally, D_1 and D_2 DA receptors are the most widespread and expressed at the highest levels (Dearry et al., 1990; Fremeau et al., 1991; Missale et al., 1998; Vallone et al., 2000). The D_1 DA receptor is mainly expressed in the caudate putamen, nucleus accumbens, olfactory tubercle, cerebral

cortex, and amygdala (Mansour et al., 1990; Jackson and Westlind-Danielsson, 1994). The D_2 DA receptors mRNA is highly expressed in the substantia nigra (SN), ventral tegmental area (VTA), hippocampus, and in both anterior and intermediate lobes of the pituitary gland, whereas the amygdale contains low levels of D_2 DA mRNA (Meador-Woodruff et al., 1989; Mansour et al., 1990; Bouthenet et al., 1991; Weiner et al., 1991). The D_3 DA receptor has been found in the SN and VTA, but it is expressed in a minority of DAergic neurons when compared with the D_2 DA receptor (Diaz et al., 1994; 1995). The D_4 DA receptor appears to be highly expressed in the frontal cortex, amygdale, hippocampus, hypothalamus, and mesencephalon (Van Tol et al., 1991; O'Malley et al., 1992). The D_5 DA receptor is poorly expressed and restricted to the hippocampus, lateral mamillary nucleus, and parafascicular nucleus of the thalamus, where the D_1 DA receptor is not significantly expressed (Tiberi et al., 1991; Meador-Woodruff et al., 1992). In the peripheral tissues, the low expression of D_1 and D_4 DA receptors in the kidney and D_5 DA receptor in the heart have been reported (Chio et al., 1994).

In birds, there are three D_1 DA receptor subtypes (D_{1A}, D_{1B}, D_{1D}) have been cloned in chickens (Demchyshyn et al., 1995). Cloning of cDNAs from brain encoding D_1 and D_2 DA receptors has been reported in turkeys (Schnell et al., 1999a; 1999b). Moreover, the nucleotide sequence of the avian D_2 DA receptor demonstrates 75 % homology to the known mammalian D_2 DA receptor. The D_1-like DA receptor has been found in the brain of pigeons (Richfield et al., 1987; Dietl and Palacios, 1988), European starlings (Casto and Ball, 1994), quails (Ball et al., 1995), chicks (Schnabel et al., 1997; Sun and Reiner, 2000), and turkeys (Schnell et al., 1999a; Chaiseha et al., 2003a). On the other hand, the D_2-like DA receptor has been mapped

in the brain of pigeons (Richfield et al., 1987), quails (Levens et al., 2000), and turkeys (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). The distributions of D_2 DA receptor mRNA has been found widespread throughout the brain, pineal gland, cortex, cerebellum, and also in the pituitary gland of the turkeys. The presence of hypothalamic D_1 DA and pituitary D_2 DA receptor mRNAs is found to increase in correlating with the reproductive stages (Chaiseha et al., 2003a). The D_1 DA receptor subtype has been classically described as being stimulatory (Bates et al., 1990; Civelli et al., 1991; Sibley and Monsma, 1992; Jarvie and Caron, 1993; Jaber et al., 1996; Strange, 1996), thus it is suggested that activation of the D_1 DA receptors on pituitary lactotrophs could stimulate PRL secretion (Schnell et al., 1999a).

2.10.3 The Localization of Dopamine

In mammals, DA is synthesized primarily in the CNS. Limited production of DA occurs in the adrenal medulla and also non-neuronal tissues such as pancreas and anterior pituitary gland (Ben-Jonathan and Hnasko, 2001). The mammalian brain comprises of several anatomically distinct DA neuronal systems that differ in their neurochemical characteristics and physiological functions. The distribution of CA-containing cells is first described in the brain of rats. The CA neurons in the brain are organized into 12 groups, named A1 to A12 from caudal to rostral (Dahlstrom and Fuxe, 1964). These cells are located mainly in the arcuate and the anterior periventricular nuclei of the hypothalamus. Studies by utilizing IHC methods with antibodies against the various biosynthetic enzymes including TH, DBH, and PNMT to identify CA cell groups have been reported (Hokfelt et al., 1984a; 1984b). To date, the CA systems in the brain of other mammalian species have been reported

worldwide.

According to the name of CA cell groups in the CNS of the rats (Dahlstrom and Fuxe, 1964), there are 17 DAergic/NEergic (A1-A17) and three adrenergic (C1-C3) cell groups. Two distinct CA cell groups are recognized in the caudal rhombencephalon; a ventrolateral tegmental (A1, C1) and a dorsomedial group (A2, C2) in the nucleus tractus solitarri/area postrema complex. The A3 cell group is found within the dorsal accessory inferior olive. The C3 adrenergic group lies along the midline within and dorsal to the medial longitudinal fascicle. In the pons, NEergic cells are classified into four groups (A4, A5, A6, A7). Amoung these cell groups, the A6 (locus coeruleus) is the most prominent one. The CA cells in the midbrain are classified into three groups, A8 (retrorubral), A9 (SN), and A10 (VTA), on the basis of their localizations. At least five distinct CA cell groups (A11-A15) are recognized in the diencephalon of the rats. The numbers of DA-containing neurons in the diencephalon are comparable to those in the SN and VTA, which are generally considered to be the major loci of DA neurons in the brain (Lookingland and Moore, 2005). The A11 (caudal diencephalic group) is located in the periventricular gray matter of the thalamus, hypothalamus, and rostral midbrain. These neurons project their axons to the spinal cord (Skagerberg and Lindvall, 1985), suggesting a role in sensory and nociceptive processing as well as sensorimotor integration of these neurons (van Dijken et al., 1996; Levant and McCarson, 2001). The A12 (TIDA) neurons are observed throughout the arcuate nucleus (ARC) and in the adjacent part of the periventricular nucleus of the MBH. Sexual difference in the number of TH-ir neurons in the dorsomental and ventrolateral subdivition of the ARC has been reported (Cheung et al., 1997). This neurons group is implicated in the regulation of

pituitary hormone secretion (Moore, 1987). The regulation of PRL secretion is under the inhibitory control of TIDA neurons (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). These neurons release DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). The A13 incertohypothalamic DA neurons are clustered in the rostral portion of the medial zona incerta, whereas the A14 DA neurons are located in the periventricular nucleus. The A15 can be divided into two groups; A15d, a compact dorsal group locates in the ventral portion of the bed nucleus of the stria terminalis, and more caudally, ventral to the anterior commissure, and A15v, the ventrolateral neurons find above the optic chiasm and within the supraoptic nucleus. These neurons are prominent in the ventrolateral hypothalamus of seasonal breeding species such as sheep (Tillet and Thibault, 1989) and are believed to mediate steroid hormones suppression of gonadotropins secretion during anestrus in ewes (Gayrard et al., 1994; Lehman et al., 1996). The most rostral DA cell bodies in the brain are found in the olfactory bulb (A16) and retina (A17).

In avian species, the anatomical distribution of the avian DAergic system obviously resembles to that of mammals (Moons et al., 1994; Reiner et al., 1994). DA has been measured and visualized in many avian species including domestic fowls (Knigge and Piekut, 1985), quails (Ottinger et al., 1986; Balthazart et al., 1992; 1998; Bailhache and Balthazart, 1993; Absil et al., 2001), pigeons (Kiss and Peczely, 1987; Berk 1991; Divac et al., 1994; Durstewitz et al., 1998), zebra finches (Barclay and Harding, 1990; Bottjer, 1993; Mello et al., 1998), chickens (Contijoch et al., 1992; Moons et al., 1994; 1995), budgerigars (Roberts et al., 2001), collared doves (den Boer-Visser and Dubbeldam, 2002), turkeys (Al-Zailaie and El Halawani, 2000), and canaries (Appeltants et al., 2001). DA neurons are found throughout the avian

hypothalamus (Kiss and Peczely, 1987; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been shown to be immunoreactived for VIP (Mauro et al., 1989; 1992; Hof et al., 1991) and VIP mRNA (Kuenzel et al., 1997; Chaiseha and El Halawani, 1999). The localizations of DA-ir neurons in the chicken hypothalamus and hindbrain have been reported (Smeets and Gonzalez, 1990; Kuenzel et al., 1992). Moreover, several DA neuronal groups have been observed in the preoptic hypothalamic areas of the turkeys (Al-Zailaie and El Halawani, 2000; Al-Zailaie, 2003) including the POM, AM, suprachiasmatic nucleus (SCN), nucleus ventrolateralis thalami (VLT), PVN, LHy, VMN, nucleus dorsomedialis hypothalami (DMN), nucleus mamillaris medialis (MM), and PMM. The distributions of TH-ir positive and DBH negative cells are found in the hypothalamus of turkeys and other avian species (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Reiner et al., 1994; den Boer-Visser and Dubbeldam, 2002). In addition, TH-ir neurons are predominantly located within the diencephalon and mesencephalon. The changes in the number of TH-ir neurons are observed in the nucleus intramedialis (nI) across the reproductive cycle of the native Thai chickens (Sartsoongnoen et al., 2008). The existence of DAergic fibers in the ME has been reported in quails (Bailhache and Balthazart, 1993), chickens (Moons et al., 1994), and turkeys (Al-Zailaie, 2003). Given their widespread distributions, the findings that DA axons and terminals are found intermingled with VIP neurons in the INF, GnRH neurons in the POA, and with both VIP and GnRH terminals in the external layer of the ME (Contijoch et al., 1992; Fraley and Kuenzel, 1993), it is reasonable to consider whether any regional specificity exists in those DA neurons that are neuroendocrine in nature, i.e., controlling the release and expression of VIP/PRL and GnRH/FSH-LH systems.

Recent findings demonstrate that the presence of DA-MEL neurons in the PMM of the turkey hypothalamus, where DA and MEL are synthesized and co-localized. It is suggested that the pattern of serotonin/catecholamine neuronal distributions and their variable interaction with PMM DA-MEL neurons during different reproductive stages may offer a significant neuroanatomical basis for understanding the control of avian reproductive seasonality and may constitute a critical cellular process involved in the generation and expression of seasonal reproductive rhythms and suggests a previously undescribed mechanism(s) by which light signals gain access to neural targets in seasonally breeding temperate zone birds (Al-Zailaie et al., 2006; Kang et al., 2007; 2009; 2010; Thayananuphat et al., 2007a; 2007b; El Halawani et al., 2009).

2.10.4 The Function of Dopamine in Mammals

DA is a neurohormone released by the hypothalamus and has the main function to inhibit the release of PRL from the anterior pituitary gland as the principle PIF. The neurons in the ARC produce DA and secrete into the hypothalamo-hypophysial blood vessels to regulate the secretion of PRL from the pituitary gland. It has been established that the concentrations of DA in hypophysial portal blood are maintained at the physiologically active levels (Ben-Jonathan et al., 1977; Gibbs and Neill, 1978; Ben-Jonathan et al., 1980) and the pituitary lactotrophs contain DA receptors (Caron et al., 1978; Cronin et al., 1978; Goldsmith et al., 1979). During proestrus or following a suckling stimulus, hypophysial portal blood DA concentrations decrease in association with increase circulating PRL (Plotsky and Neill, 1982). DA and its agonists attenuate PRL secretion, PRL gene expression, and lactotrophs proliferation (Birge et al., 1970; MacLeod and Lehmeyer, 1974; Shaar and

Clemens, 1974; Lamberts and MacLeod, 1990). Moreover, PRL levels increase after treatment with DA antagonists (Smalstig et al., 1974; MacLeod and Lamberts, 1978). One signal for PRL release among other endocrine factors is the dissociation of DA from its receptors. Thus, the removal of DA appears to play an important physiological role in the regulation of PRL secretion.

It is well established that DA which is released from the hypothalamic TIDA neurons serves as the physiological inhibitor of PRL secretion (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001) and is mediated through the D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). Removal of this DAergic inhibition results in an increase in PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). However, several studies have been reported that DA at low concentrations stimulates PRL secretion both *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 suggest that all lactotrophs have the potential to respond to the inhibitory and stimulatory effects of DA (Kineman et al., 1994) or that a subpopulation of lactotrophs sensitive to the stimulatory effect of DA exists (Burris et al., 1992; Burris and Freemen, 1993) and the two opposite effects of DA upon PRL secretion may be mediated by distinct G proteins (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994). In rats, the stimulation of pituitary PRL secretion may mediate through the D_1 and/or D_5 DA receptors (Porter et al., 1994). These data support the role of DA as the PRF. Another possibility is that the various PRL-releasing and -inhibiting factors which are known to exert their effects at the pituitary level may also interact at the hypothalamic level to control PRL secretion (Moog and Samson, 1990).

It is well recognized that DA plays a major role in the control of various aspects of reproduction including the secretion of gonadotropins and activation of male and female sexual behaviors. DA neurons are found prominently in the ventrolateral hypothalamus of seasonal breeding species such as sheep (Tillet and Thibault, 1989), and are believed to mediate steroid hormones suppression of gonadotropins secretion during anestrus in ewes (Gayrard et al., 1994; Lehman et al., 1996). DA exerts both stimulatory and inhibitory effects in the control of GnRH and LH secretion. It has been reported that GnRH axons are terminated in the external layer of the ME, which is closed proximity to terminals of TIDA neurons (Ajika, 1979; Ugrumov et al., 1989). These neurons are mediated hyperprolactinemia-induced suppression of LH secretion (Selmanoff, 1981). Furthermore, DA can also inhibit GH and TSH release via direct actions under normal baseline conditions (Lookingland and Moore, 2005).

DA participates in several physiological functions in mammals; for example food and water intake, body homeostasis, behaviors and cognition, motor activity, regulation of milk secretion, sleep, mood, attention, learning, and reproductive regulation (Bertolucci-D'Angio et al., 1990; Cooper and Al-Naser, 1993; Wilson et al., 1995; Velasco and Luchsinger, 1998; Ben-Jonathan and Hnasko, 2001; Hull et al., 2004; Wellman, 2005). Some findings link DA to putative drive systems for hunger and thirst. The effects of DA on blood pressure, cardiac output, and regional blood flow have been reported since the DA receptor subtypes have been found in the peripheral organs such as in blood vessels, adrenal gland, kidney, and heart. DA also has several physiological functions related to vasodilatation, regulation of CA release, sodium reabsorption, renin and aldosterone secretion, and vasopressin action.

The effects of DA on motor activity have been extensively studied (for review, see Clark and White, 1987; Jackson and Westlind-Danielsson, 1994). The important roles of DA in the control of movements have been demonstrated in Parkinson's disease. This disease is characterized by strong reduction of circulating DA due to the degeneration of DAergic neurons. The impairment of emotional processes in neurologic and psychiatric pathologies involving the DAergic system such as Parkinson disease, schizophrenia, autism, attention-deficit hyperactivity disorder, Huntington disease, frontal lobe lesions, and the influence that administration of DAergic agonist/antagonists exert on the processing of emotion, suggests a role for DA in emotional process (for review, see Salgado-Pineda et al., 2005). In domesticated farm animals such as cattle, there is a growing literature which implicates CA as important neuroendocrine regulators. These include studies of thermoregulation, hormonal secretion, feeding behavior, physiological/psychological indicators of stress, animal well-being, and the etiology of some disorders caused by grazing endophyte-infected fescue (Leshin et al., 1995).

2.10.5 The Function of Dopamine in Birds

In birds, the role of DA in the regulation of PRL secretion is still large obscure for comparing it to the mammalian DAergic strategy for PRL control. It has been reported and well established that DAergic influences are involved in stimulating and inhibiting avian PRL secretion. DA inhibits pituitary PRL release *in vitro* (Harvey et al., 1982; Hall and Chadwick, 1984; Hall et al., 1986; Xu et al., 1996). DA or its agonist, apomorphine, reduces PRL secretion in pigeons and chickens. However, this effect is reversed by the DA receptor antagonist, pimozide (Hall and Chadwick,

1983). In chickens, DA inhibits the release of PRL stimulated by TRH, hypothalamic extract, or by previous exposure of the pituitary gland to estrogen (Hall and Chadwick, 1984). Moreover, ICV infusion of DA in laying turkey hens can either stimulate or inhibit PRL secretion depending upon the concentrations used (Youngren et al., 1995). Thus, both stimulatory and inhibitory effects of on avian PRL secretion are depended upon multiple DA receptors (Youngren et al., 1996b). This action is confirmed by the presence of both D_1 and D_2 DA receptor mRNAs in the turkey brain and the pituitary cells (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). It is suggested that the stimulatory effect of DA on PRL secretion is regulated via D_1 DA receptors residing in the INF, where the VIP neurons are located. In the contrary, DA inhibits PRL release and synthesis at the pituitary level through D_2 DA receptors by blocking the action of VIP (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003a; Al Kahtane et al., 2003). It has been reported that DA also activate hypothalamic VIP gene expression in the INF (Bhatt et al., 2003). In addition, it has been demonstrated that the signalling mechanism(s) underlying the interaction between VIP and DA in the regulation of PRL secretion involved with protein kinase A (Kansaku et al., 1998), Ca^{2+} (Hall et al., 1985; Al Kahtane et al., 2003; 2005) and protein kinase C (Sun and El Halawani, 1995).

There are some other evidences suggesting an inhibitory role for DA on GnRH release in mammals as well as in birds (Ramirez et al., 1984; Sharp et al., 1984). Several DA neuronl groups have been identified in the preoptic-hypothalamic areas (Kiss and Peczely, 1987; Reiner et al., 1994). Exogenous DA activates hypothalamic VIP gene expression and this increased expression is limited exclusively to the avian INF and the increased VIP mRNA in the INF is correlated with increased levels of

circulating PRL and LH-β mRNA in the anterior pituitary (Bhatt et al., 2003). Further evidence suggests the involvement of DA in correlating with GnRH is derived from a dense concentration of TH (the rate-limiting enzyme for DA synthesis) and GnRH-containing processes which locate in the lateral and mediobasal portion of the external layer of the hen ME (Contijoch et al., 1992). This result provides an opportunity for synaptic interaction between GnRH and DA. DAergic neurons inhibit GnRH release through presynaptic inputs at the ME level, as has been demonstrated in the chicken (Contijoch et al., 1992; Fraley and Kuenzel, 1993). Activation of DAergic cells in the nucleus mamillaris lateralis (ML) is associated with the activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006). The relationship of DAergic system in the PMM and GnRH-I system in the nCPa during the photo-induction reproductive activity has been reported. c-fos mRNA expressions within the PMM are differentially activated by light and corresponded with a rhythm of photosensitivity (Thayananuphat et al., 2007a; 2007b). It is suggested that DA in the PMM that proposed to be the DA A11 group, is suggested its function in controlling the reproductive seasonality in the temperate zone birds. To date, DA-MEL co-localized neurons have been found in the PMM and shown to cycle rhythmically with photoperiodic changes (Kang et al., 2007; 2010). Hypothalamic DA-MEL neurons may constitute a critical cellular process involved in the generation and expression of seasonal reproductive rhythms (El Halawani et al., 2009) through tOPN4x, an important component of the photoreceptive system (Kang et al., 2010). Moreover, it has been reported that clock gene in the PMM can be induced by long photoperiod and light during the daily photosensitive phase, thus promote reproductive activity (Leclerc et al., 2010).

Like in mammals, DA plays a role in many aspects of sexual activities and reproduction. It has been reported that DA in the medial POM facilitates male sexual behaviors (Hull et al., 1995; Dominguez and Hull, 2005; Bharati and Goodson, 2006). Administration of D_1 DA agonist demonstrates an increase the aspects of sexual behaviors in quails (Balthazart et al., 1997). It is hypothesized that DA within the posterior hypothalamus, particularly from the nI may be play a role in the onset of puberty (Fraley and Kuenzel, 1993). DA neurons located in the PVN and ML might be possible to influence gonadal maturation (Kuenzel, 2000). In addition, it has been suggested that the rostral A11 DA neurons of the caudal hypothalamus are involved in courtship singing in songbirds such as zebra finches (Bharati and Goodson, 2006). Moreover, DA also involves in motor functions (Rieke, 1980; 1981) and the regulation of food and water intake (Deviche, 1984; Ravazio and Paschoalini, 1992) in birds.

2.10.6 Dopamine Regulation of Prolactin Secretion

In mammals, the regulation of PRL secretion by DA is involved in VIP and 5-HT. VIP fibers are found intermingle with DAergic neurons in the ARC and periventricular nucleus, which VIP2 receptors are located on a soma and proximal dendrites of these DA-containing neurons. It is suggested that VIP may regulate PRL secretion in mammals by controlling the delivery of DA to the anterior pituitary gland (Gerhold et al., 2001). More evidences suggest that DA and 5-HT appear to have a complementary interaction regarding PRL secretion. DA and 5-HT are co-localized within neurons in the baboon hypothalamus (Kiss and Halasz, 1986). DA antagonist inhibits an increase in serotonergic activity (King et al., 1985). In addition,

intraventricular injections of 5-HT in rats reduce DA levels in portal blood (Pilotte and Porter, 1981). Infact, the primary function of TIDA neurons is to suppress the pituitary secretion of PRL. However, it has been found that the synthesis and release of α-MSH and multiple acetylated form of β-endorphin from the melanotrophs is tonically inhibited by DA acting on inhibitory D_2 DA receptors located directly on these cells (Cote et al., 1982; Tilders et al., 1985).

In birds, it is well documented that DA plays an intermediary role in PRL secretion in birds, requiring an intact VIPergic system in order to release PRL (Youngren et al., 1996b). This finding is supported with several studies. Intracranial infusions of DA are ineffective in releasing PRL in turkeys actively immunized against VIP, suggesting that DA affects PRL secretion by stimulating the release of VIP. The infusion of VIP into the turkey pituitary affects a rapid and substantial increase in circulating PRL, an increase that is completely suppressed when DA is infused in conjunction with VIP (Youngren et al., 1998). Co-expression of D_2 DA receptor mRNA seen in VIP expressing neurons within the LHy and INF have been reported (Chaiseha et al., 2003a). In addition, it has been found that D_2 DA receptor agonist, puinpirole, inhibits VIP-stimulated PRL secretion and PRL mRNA levels when incubated with turkey anterior pituitary cells (Xu et al., 1996). These results support that DA blocks the VIP-stimulated release of PRL release by activating D_2 DA receptors. It is suggested that the inhibitory effects of DA on VIP-induced PRL gene transcription may result from DA suppression of the transcriptioning fraction of Pit-1 (Al Kahtane et al., 2003). A conserved consensus Pit-1-biding site has been proposed in the avian and teleost PRL/GH gene family (Ohkubo et al., 1998). Pit-1 cDNA has been cloned in turkeys and chickens (Tanaka et al., 1991; Wong et al.,

1992a; Kurima et al., 1998). It is also known that the secretion of avian PRL also requires an intact serotonergic system (El Halawani et al., 1988c, Chaiseha et al., 2010). Exogenous DA activates hypothalamic VIP gene expression and this increased expression is limited exclusively to the avian INF. The increased VIP mRNA in the INF is correlated with increased levels of circulating PRL in the anterior pituitary (Bhatt et al., 2003). To date, it is concluded that dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a 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).

2.11 References

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CHAPTER III

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

3.1 Abstract

Prolactin (PRL) is a pituitary hormone that plays a significant role in reproduction, maternal care, and parental behavior in many vertebrate species. In birds, the rising of PRL levels has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior. The objective of this study was to investigate the circulating PRL levels in non-laying (NL), laying (L) and incubating (INC) hens as well as to compare the changes in plasma PRL levels of INC hens with those of nest-deprived hens (ND). Native Thai hens were divided into 2 groups; INC hens which were allowed to incubate their eggs and ND hens which were not allowed to incubate their eggs by depriving of the nests. Blood samples were collected in NL, L, INC, and ND hens for determining PRL levels by enzyme-linked immunosorbent assay. The ovaries and oviducts were collected, weighed, and recorded the presence of follicles after the hens were sacrificed. The results revealed that plasma PRL levels were low in NL and L hens and reached the highest levels in INC hens. Plasma PRL levels were increased during incubating period and declined to

the same levels of that of NL hens at hatching day. When hens were deprived from their nests, plasma PRL concentrations were decreased within a day of nest deprivation and remained low throughout the period of nest deprivation. Disruption of incubation behavior by nest deprivation increased the ovary and oviduct weights, the presence of ovarian follicles, and the number of egg laying hens. This study indicates that incubation behavior in the native Thai chicken is regulated by PRL. The external cues such as nests and eggs are involved in the maintenance of plasma PRL levels and incubation behavior in this equatorial non-photoperiodic continuous breeder bird.

3.2 Introduction

Prolactin (PRL), a polypeptide hormone, is synthesized and secreted from the lactotrophs, the specialized cells of the anterior pituitary gland (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000). In mammals, PRL plays a significant role in reproduction, maternal care, and parental behaviors. It has an essential role for lactation since it involved in the development of mammary gland (Bern and Nicoll, 1968), synthesis of milk, and maintenance of milk secretion. In birds, PRL is widely thought to play a pivotal role in parental behaviors by mediating an increase 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 in birds (Lea and Vowles, 1986; Macnamee et al., 1986; Pedersen, 1989; Buntin et al.,

1991; Youngren et al., 1991).

The important factor for successful reproduction is not only sexual activity, but also the successful of caring the young. Maternal behaviors are crucial to the survival of fertilized eggs or offspring (Thayananuphat, 2007). PRL is believed to function in hormonal control of maternal behaviors in various species. The role of PRL in the induction and maintenance of maternal care has been extensively investigated. It has been reported that PRL is associated with incubation behavior in many avian species such as pigeons, pheasants, cowbirds, turkeys, mallard ducks, and chickens (Riddle et al., 1935; Breitenbach and Meyer, 1959; Hohn, 1959; Burke and Dennison, 1980; Goldsmith and Williams, 1980; El Halawani et al., 1988; Sharp et al., 1988; Youngren et al., 1991; 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. On the contrary, during the periods of laying and incubating, circulating PRL levels increase dramatically (El Halawani et al., 1984; 1997; Kosonsiriluk et al., 2008). It is this rising PRL level that causes the cessation of ovulation, ovarian regression, and induction of incubation behavior. The onset of incubation behavior is correlated with decreasing plasma luteinizing hormone (LH) levels and gonadal steroids (Cogger et al., 1979; Burke and Dennison, 1980; Lea et al., 1981; Rozenboim et al., 1993). An elevated level of circulating PRL has a negative effect on the reproductive performance, resulting in decreased egg production and initiation of incubation. PRL levels increase at the onset of incubation behavior and are maintained at high levels during incubation phase in the pituitary gland (Saeki and Tanabe, 1955) as well as in the circulation (Sharp et al., 1979; Burke and Dennison, 1980; Proudman and Opel,

1988) and decline when incubation behavior is terminated (El Halawani et al., 1980; Wentworth et al., 1983). 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 bantams, canaries, chickens, cowbirds, ducks, mallard ducks, pheasants, pigeons, ring doves, spotted sandpipers, turkeys, white-crowned sparrows, wild starlings, and native Thai chickens (Sharp et al., 1977; Burke and Dennison, 1980; Goldsmith and Hall, 1980; Goldsmith et al., 1981; 1984; Dawson and Goldsmith, 1982; Bluhm et al., 1983; El Halawani et al., 1984; 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 suppress LH levels by reducing hypothalamic gonadotropin releasing hormone (GnRH) contents (Rozenboim et al., 1993) and the abundance of LH-β subunit and PRL mRNA shows an inverse relationship in incubating turkey hens (Wong et al., 1992).

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 concentrations (Etches et al., 1979; Burke and Dennison, 1980; Lea et al., 1981; Bluhm et al., 1983; Hall and Goldsmith, 1983; Silverin and Goldsmith, 1983). Several studies have been suggested that an increase in plasma PRL levels during incubating period may depress LH secretion (Zadworny and Etches, 1987; El Halawani et al., 1993; Sharp et al., 1998). The elevated PRL levels and depressed LH levels of incubating hens are maintained by tactile stimuli from the nests and eggs (El Halawani et al., 1980; 1986; Opel and Proudman, 1988) and can be reversed by nest deprivation (El Halawani et al., 1980; Proudman and Opel, 1981; Zadworny and Etches, 1987; Sharp et al., 1988)

or the introduction of chicks (Leboucher et al., 1990). Nest deprivation results in the disruption of incubation behavior, increases in plasma LH and estradial concentrations, and decreases in plasma PRL levels (El Halawani et al., 1980; Sharp et al., 1988; Dunn et al., 1996; Richard-Yris et al., 1998). The changes of plasma LH and PRL concentrations are reversed when hens are re-nested (Sharp et al., 1988). Pituitary PRL mRNA levels are correlated directly with plasma PRL concentrations which higher in incubating birds than that of in laying birds and rapidly decrease when birds deprived of their nests (Talbot et al., 1991).

The regulation of avian PRL secretion and PRL gene expression is influenced by hypothalamic vasoactive intestinal peptide (VIP), the avian PRL-releasing factor. (El Halawani et al., 1997; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005). It is very well documented that variations in hypothalamic VIP immunoreactivity, VIP contents, VIP mRNA steady-state levels, VIP mRNA expression in the infundibular nuclear complex (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; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999) chickens (Sharp et al., 1989), and doves (Cloues et al., 1990). Moreover, immunoneutralization of VIP prevents an increase in circulating PRL that follows photostimulation. It also prevents the induction of incubation behavior, up-regulates LH-β- and follicle stimulating hormone (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). It has been reported that dopaminergic (DAergic) influences are involved in both stimulating and

inhibiting avian PRL secretion. DA stimulates PRL secretion acting centrally via D_1 DA receptors in the hypothalamus. DA also inhibits PRL secretion by activating via D_2 DA receptors at pituitary level, antagonizing the effect of VIP (Youngren et al., 1996; 1998; 2002; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). In addition, It has been indicated that dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a 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).

The control of avian PRL secretion also involves in the interaction of external stimuli such as the presence of nests, eggs, and offspring with endocrine mechanisms. Removal of these stimuli induces a significant increase in LH and decrease in PRL levels (El Halawani et al., 1980; Goldsmith et al., 1984; Richard-Yris et al., 1987b; 1998; Sharp et al., 1988; Lea and Sharp, 1989; Mauro et al., 1989; Opel and Proudman, 1989; Leboucher et al., 1993; Dawson and Sharp, 1998) following induce ovarian recrudescence and resume egg laying (Huang et al., 2008; Kosonsiriluk et al., 2008).

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, which is independent on photoperiodic cues (Kosonsiriluk, 2007; Kosonsiriluk et al., 2008). The native Thai hens highly express maternal behaviors including incubation behavior and broodiness. There are limited data about the neuroendocrine regulation of incubation behavior in this gallinaceous bird from the non-temperate zone. It has been known for a long time that incubation behavior is associated with the VIP/PRL and GnRH/FSH-LH systems. These systems are affected

by light information that reaches the specific area of the brain. Since light does not seem to affect the reproductive cycle of the native Thai hens, the established neuroendocrinology of incubation behavior may not be fully applied to this species of birds. Recently, plasma PRL and LH levels across the reproductive cycle of the native Thai chicken 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 of plasma PRL levels throughout the reproductive cycle. These findings suggest that hypothalamic VIP expression in the IH-IN of the native Thai chicken plays a regulatory role in year-round reproductive activity (Kosonsiriluk, 2007; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008).

This present study was designed to further investigate the role of PRL in the regulation of the incubation behavior in the native Thai chickens. The changes in plasma PRL levels as well as ovary and oviduct weights were compared between incubating and nest-deprived native Thai hens. The findings gained from this study will provide the information of neuroendocrine regulation of incubation behavior in the native Thai chicken which could help to improve the productivity of the native Thai chickens.

3.3 Materials and Methods

3.3.1 Experimental Animals

Female native Thai chickens (*Gallus domesticus*), Pradoohangdam breed, were used. They were reared and housed with mature roosters (5-8 females : 1 male) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of dark; 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; incubating eggs (INC) and non-incubating or nest deprivation (ND). Hens in the INC group had stopped laying and had been sitting on the nests for three to four times per day showing incubating behavior. They were allowed to incubate their eggs naturally. Hens in the ND group were disrupted from incubating behavior and not allowed to incubate their eggs by removing them from their nests 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

Ten female and 2 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 2 floor pens (5 hens : 1 rooster) and observed their behaviors daily. Blood samples were collected from the brachial vein of non-egg laying (NL; hens had never laid eggs), egg laying (L; hens were in their first laying cycle and had been laying for 7 days), and incubating hens (INC; hens had stopped laying and had been exhibiting incubating behavior) at day 3. After that, hens were divided into two groups; INC and ND. Blood samples were collected again at day 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 after they started to incubate their eggs or after nest deprivation. 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

Sixty five female and 8 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 8 floor pens (8-9 hens : 1 rooster) and observed their behaviors daily. Hens were divided into two groups; INC and ND. Blood samples were collected from the brachial vein of each hen prior to euthanize with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France) at different time periods (day 3, 6, 8, 10, 14, 18, and 21) of INC or ND. 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 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 (5-10 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 ELISA. Egg production, nesting activity, and other behaviors were recorded daily throughout the experiment.

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 at the dilution of 1:2,000. The plates were then incubated at 4 °C for overnight and blocked with blocking solution (100 µl of 0.4 % casein, 0.01 % thimerosal, 1 mM EDTA), 25 µl of anti-PRL (1:20,000, kindly provide by Dr. John Proudman, USDA, USA), and 25 µl of β-PRL tracer (1:50,000) were added into the reaction, then incubated at 4 °C for overnight. The reactions were measured the absorbent at 405 nm. The assay of plasma PRL levels in native Thai chickens was validated as follows. Pooled plasma samples of native Thai chickens produced a dose-response curve that paralleled with a chicken PRL standard curve. Plasma samples were determined in duplicate within a single assay. The intra-assay 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 level in individual INC and ND hen are shown in

Figures 3.1 and 3.2, respectively. The concentrations of plasma PRL in native Thai chickens are shown in Figure 3.3. Plasma PRL levels were low in NL (26.51 ± 3.59 ng/ml) and L (25.31 ± 2.67 ng/ml) birds. When hens incubated their eggs, plasma PRL levels increased immediately and remained high throughout the incubating stage ($P<0.05$). The PRL levels started to decline at day 20 of incubation. The lowest PRL level was found at day 22 of incubation or the day when the chicks were hatched. Disruption of incubation behavior by nest deprivation was accompanied by a precipitous decline in plasma PRL levels ($P<0.05$) within 24 hrs of nest deprivation (day 4, 26.46 ± 2.93 ng/ml), equaling that of NL birds. Plasma PRL levels remained low as long as the hens were deprived of their nests.

3.4.2 Experiment II

The plasma PRL concentrations of INC and ND hens that incubating eggs or deprive of their nests at different time periods are shown in Figure 3.4 and Table 3.1. In INC group, plasma PRL levels tended to increase at day 10 (442.77 ± 52.33 ng/ml) or in the middle phase of incubation and then immediately declined at late incubation period. Plasma PRL concentrations significantly decreased by day 21 of incubation ($P<0.05$; 65.23 ± 35.94 ng/ml). When hens were deprived of their nests, plasma PRL levels significantly decreased by day 6 of nest deprivation ($P<0.05$; 34.14 ± 6.55 ng/ml) and remained at the same levels throughout day 6 to day 21 of nest deprivation.

The reproductive characteristics of INC and ND hens at different time periods were also recorded. The presence of F1-F5 follicles, SYF, and SWF (Figure 3.5) were observed in both groups. In INC hens, the presence of F1-F5 follicles, SYF, and SWF

at different days of incubation are shown in Table 3.2. The results showed that F1-F5 follicles were not observed in INC hens that incubated eggs for 6-21 days. A few numbers of INC hens that incubated eggs for 3-8 days exhibited the presence of SYF. However, the presence of SWF is found in all of INC hens. In contrast, the number of ND hens that exhibited the presence of F1-F5 follicles, SYF, and SWF are shown in Table 3.3. More than 50 % of ND hens showed the presence of F1-F5 follicles after 10 days of nest deprivation. Most of ND hens exhibited the presence of SYF and all of them exhibited the presence of SWF. The number of ND hens that started to lay, the new laying cycle were found in day 14 of nest deprivation. Moreover, on day 18 of nest deprivation, 70 % of ND hens started to lay (Table 3.3).

The ovaries of INC and ND hens are shown in Figure 3.6. The ovary weight of INC and ND hens are shown in Figure 3.7 and Table 3.4. The ovary weight of INC hens is decreased since the hens started to incubate their eggs. In ND group, the ovary weights gradually increased and reached the highest weight at day 18 of nest deprivation (38.04 ± 23.08 g).

The oviducts of INC and ND hens are shown in Figure 3.8. Similarly, the oviduct weight of INC and ND hens are shown in Figure 3.9 and Table 3.5. The oviduct weight of INC hens significantly decreased ($P<0.05$) by day 18 of incubation. In contrast, when hens were deprived of their nests, the oviduct weight of INC hens significantly increased by day 18 of nest deprivation ($P<0.05$; 42.33 ± 19.90 g). When compared between both groups, both ovary and oviduct weights of the ND hens were significantly increased by day 8 of nest deprivation and were higher than those of INC hens throughout day 8 to day 21 of observation periods.

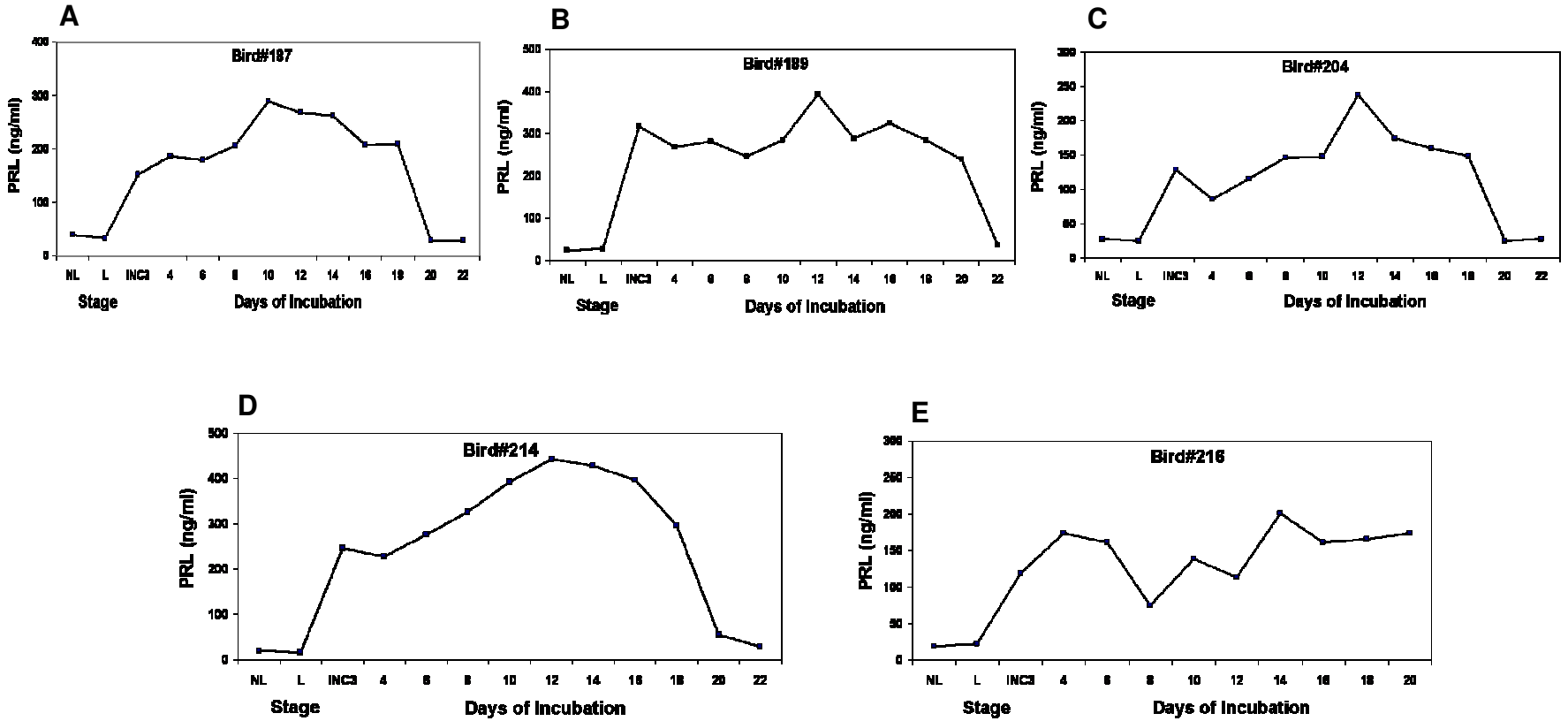

Figure 3.1 Plasma PRL levels of incubating (INC) native Thai hens; birds #187 (**A**), #189 (**B**), #204 (**C**), #214 (**D**), and #216 (**E**).

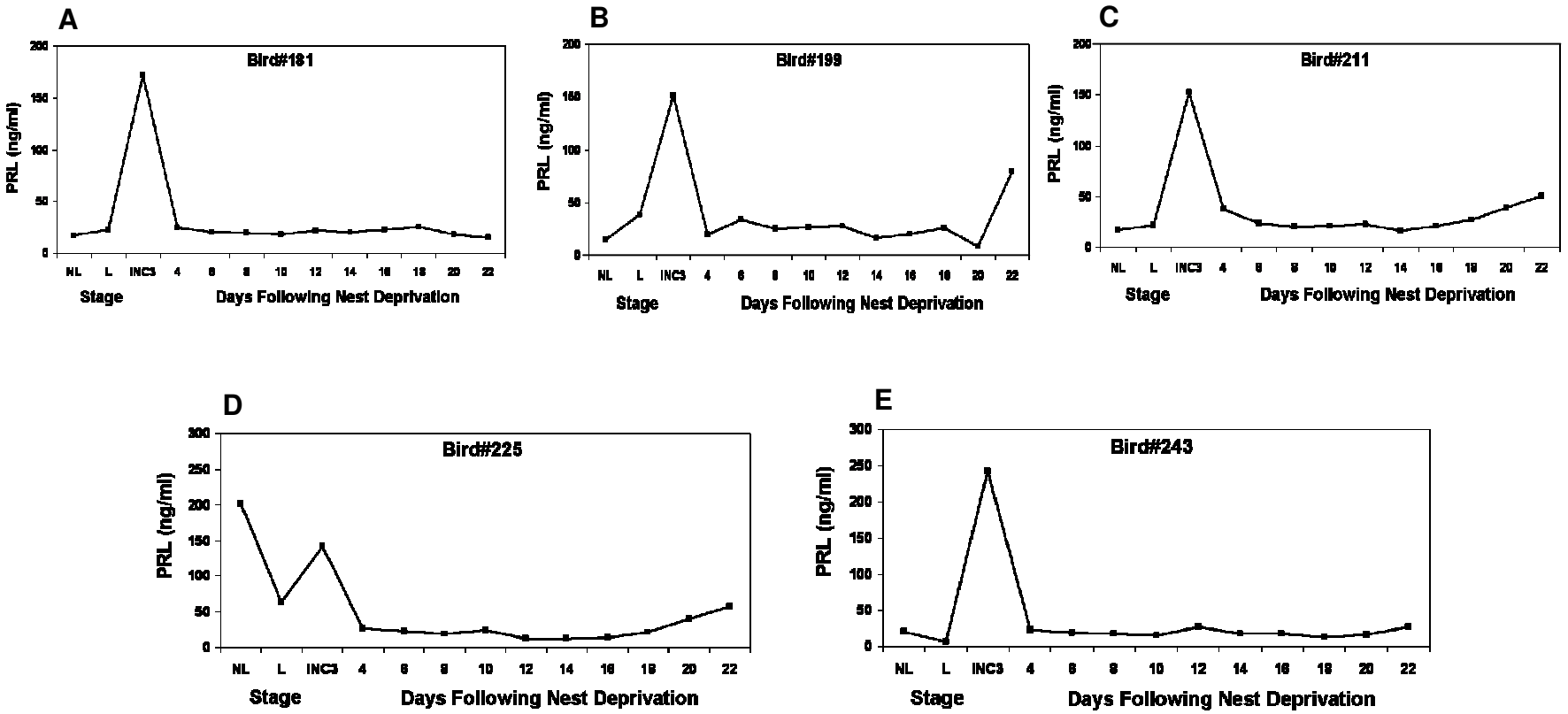

Figure 3.2 Plasma PRL levels of nest-deprived (ND) native Thai hens; birds #181 (**A**), #199 (**B**), #211 (**C**), #225 (**D**), and #243 (**E**).

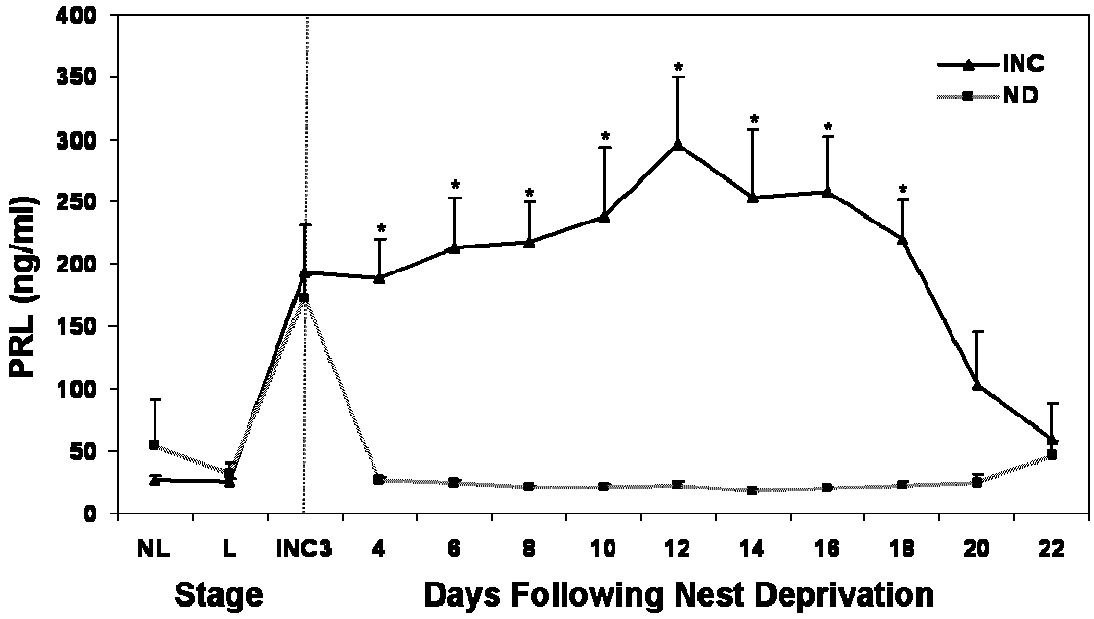

Figure 3.3 Changes in plasma PRL concentrations (mean ± SEM) before and after initiation of incubation and nest deprivation of native Thai chickens. Hens were divided into two groups after day 3 of incubation (INC3); one group continued to incubate their eggs (INC; n=5) and birds in the second group were nest-deprived (ND; n=5). Blood samples were collected prior to egg laying (NL), during egg laying (L), and following incubation and nest deprivation for determination of plasma PRL levels. *P<0.05 for a comparison between groups at a given time point.

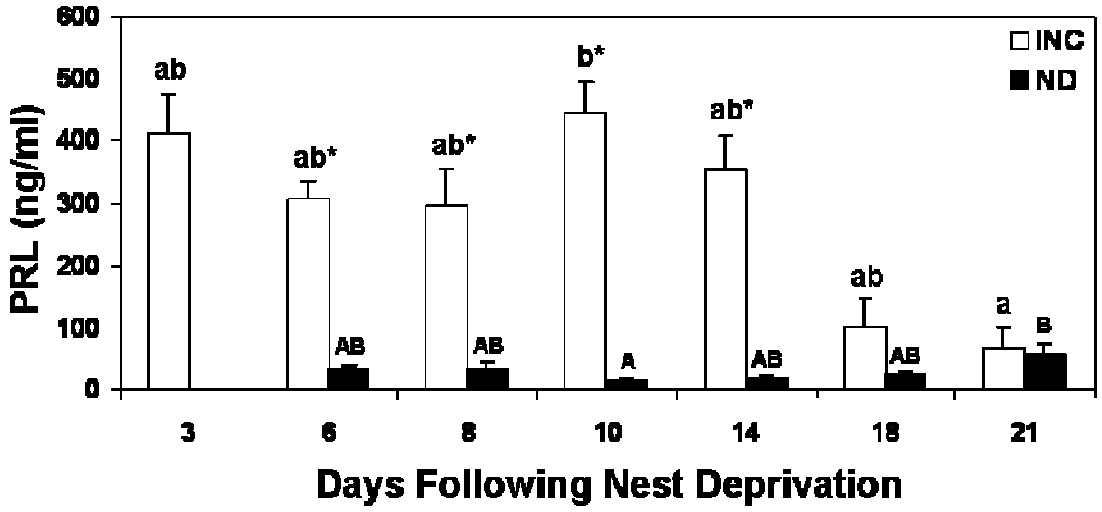

Figure 3.4 Changes in plasma PRL concentrations of incubating (INC; n=5) and nest-deprived (ND; n=5) native Thai hens. Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 3.1 Mean ± SEM of the plasma PRL concentrations (ng/ml) of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation (n=5) or nest deprivation (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	**Days Following Nest Deprivation**						
	3	**6**	**8**	**10**	**14**	**18**	**21**
INC	412.13 ± 63.53^{ab}	307.94 ± 27.14^{ab*}	296.07 ± 60.66^{ab*}	442.77 ± 52.33^{b*}	352.13 ± 58.36^{ab*}	270.94 ± 173.63^{ab}	65.23 ± 35.94^{a}
ND	N/A	34.14 ± 6.55^{ab}	33.34 ± 12.16^{ab}	15.39 ± 1.40^{a}	20.24 ± 2.02^{ab}	24.75 ± 3.78^{ab}	56.92 ± 15.86^{b}

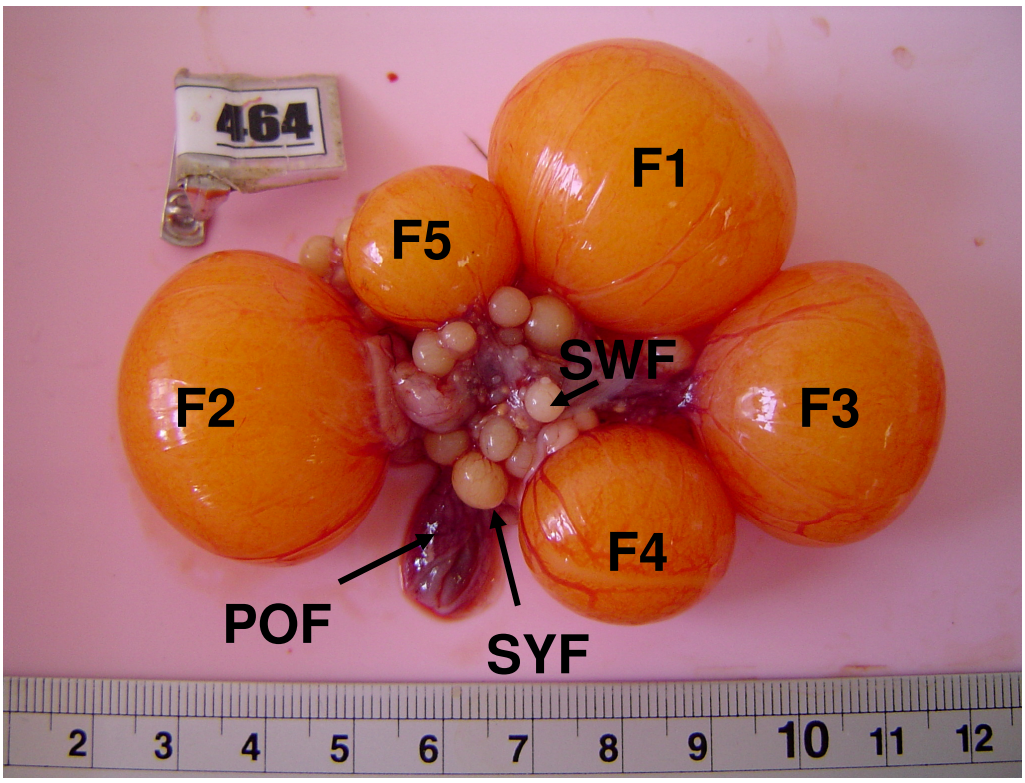

Figure 3.5 Photograph of the ovary of the native Thai hen showing the F1-F5 follicles, small yellow follicles (SYF), small white follicles (SWF), and post-ovulatory follicles (POF).

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 days of incubation (n=10).

Follicles	**Days of Incubation**						
	3	**6**	**8**	**10**	**14**	**18**	**21**
F1-F5	1	0	0	0	0	0	0
SYF	4	4	3	0	0	0	1
SWF	10	10	10	10	10	10	10

Table 3.3 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 nest deprivation and the number of hen came back to lay in each period (n=10).

Follicles	**Days Following Nest Deprivation**					
	6	**8**	**10**	**14**	**18**	**21**
F1	2	7	9	8	8	8
F2	0	4	7	5	8	8
F3	0	2	7	5	7	8
F4	0	1	4	5	7	7
F5	0	0	3	4	7	5
SYF	9	9	10	9	10	9
SWF	10	10	10	10	10	10
Laying hens	0	0	0	3	7	7

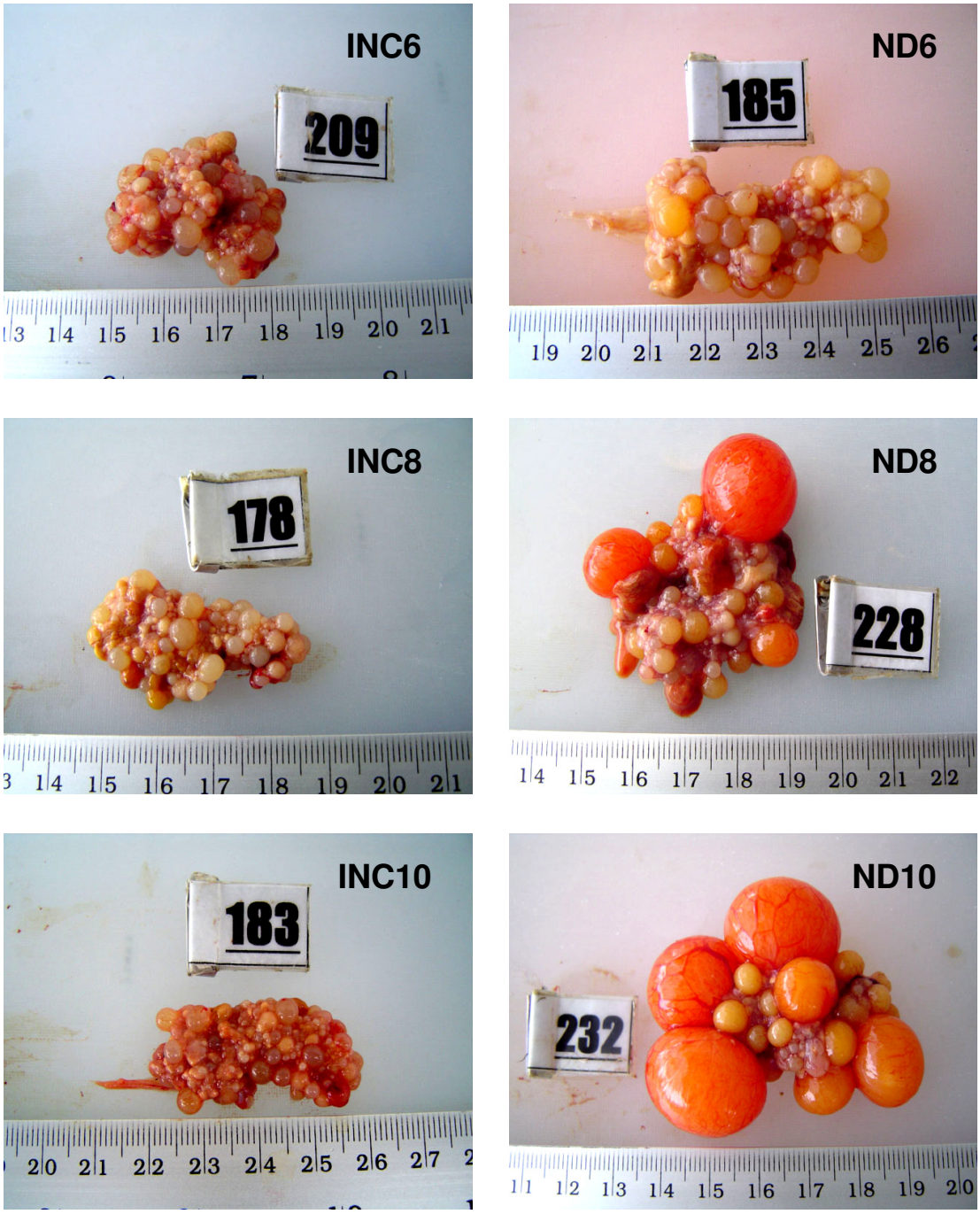

Figure 3.6 Photographs of the ovary of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation.

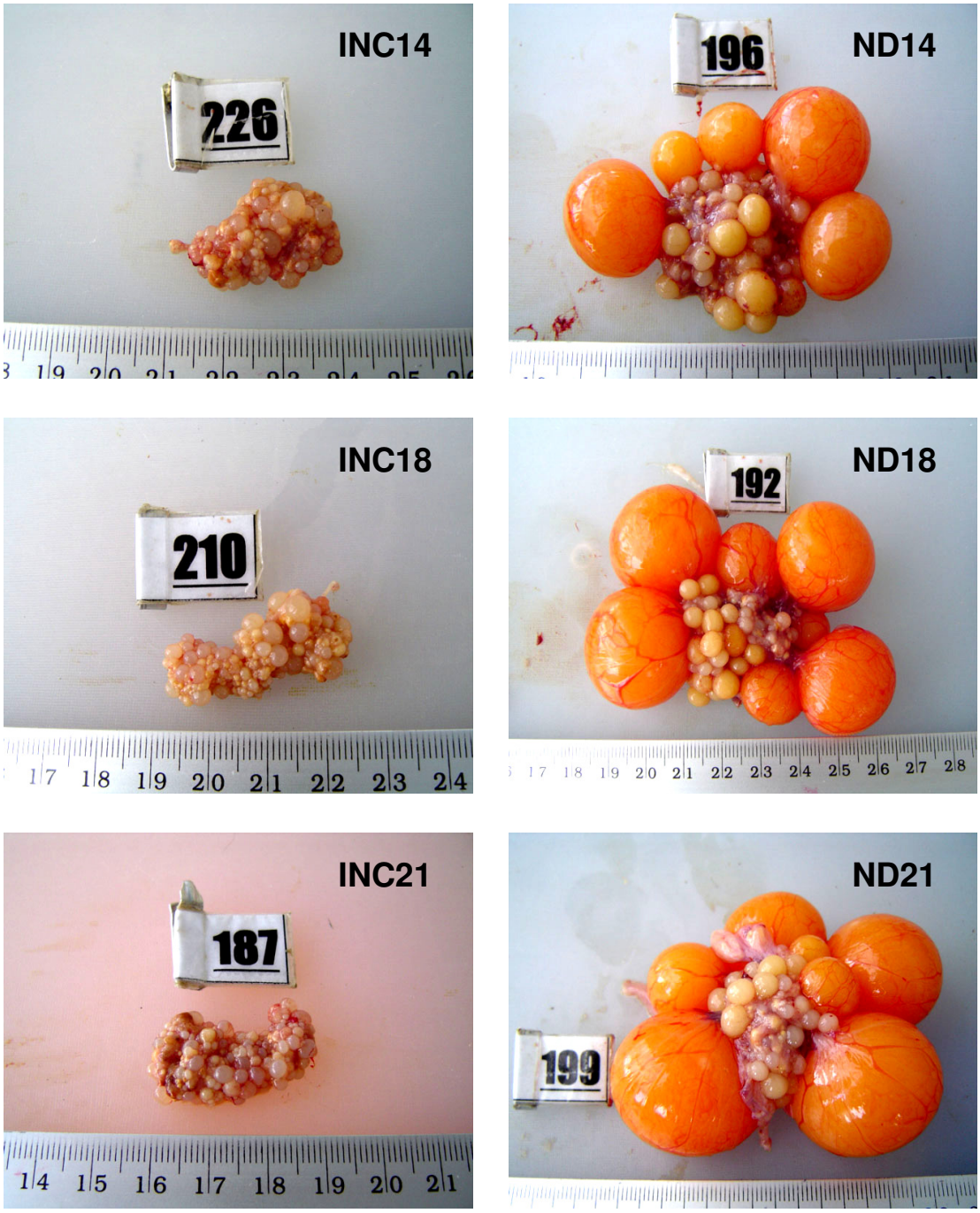

Figure 3.6 Photographs of the ovary of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation (continued).

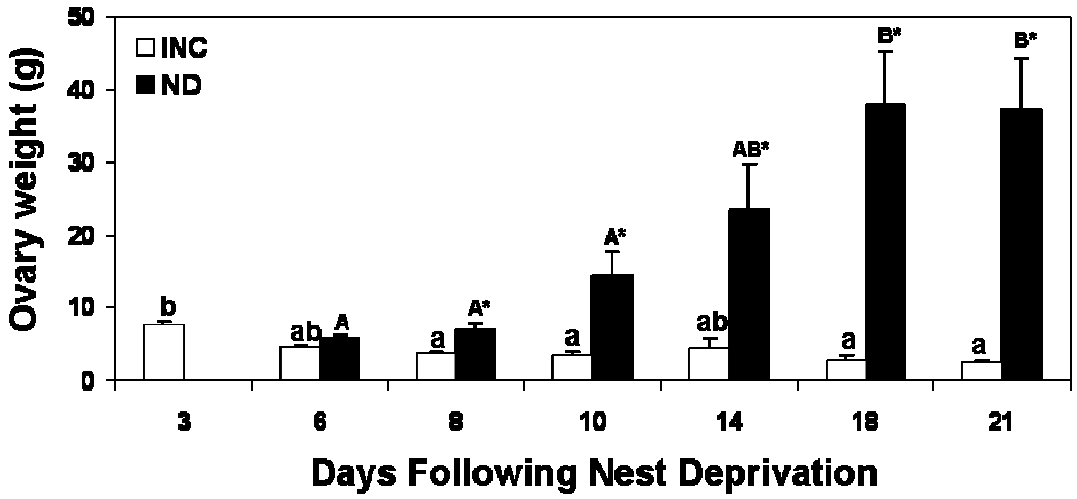

Figure 3.7 Changes in the ovary weights of incubating (INC) and nest-deprived (ND) native Thai hens. Values are presented as means ± SEM (n=10). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 3.4 Mean ± SEM of the ovary weight (g) of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation (n=10). 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 Nest Deprivation						
	3	**6**	**8**	**10**	**14**	**18**	**21**
INC	7.67 ± 0.55 [b]	4.70 ± 0.71 [ab]	3.64 ± 0.77 [a*]	3.54 ± 1.10[a]*	4.46 ± 4.35 [ab]	2.86 ± 2.16 [a*]	2.58 ± 0.55 [a*]
ND	N/A	5.81 ± 1.73 [a]	6.98 ± 3.25 [a]	14.60 ± 10.61 [a]	23.42 ± 20.28 [ab]	38.04 ± 23.08 [b]	37.34 ± 22.42 [b]

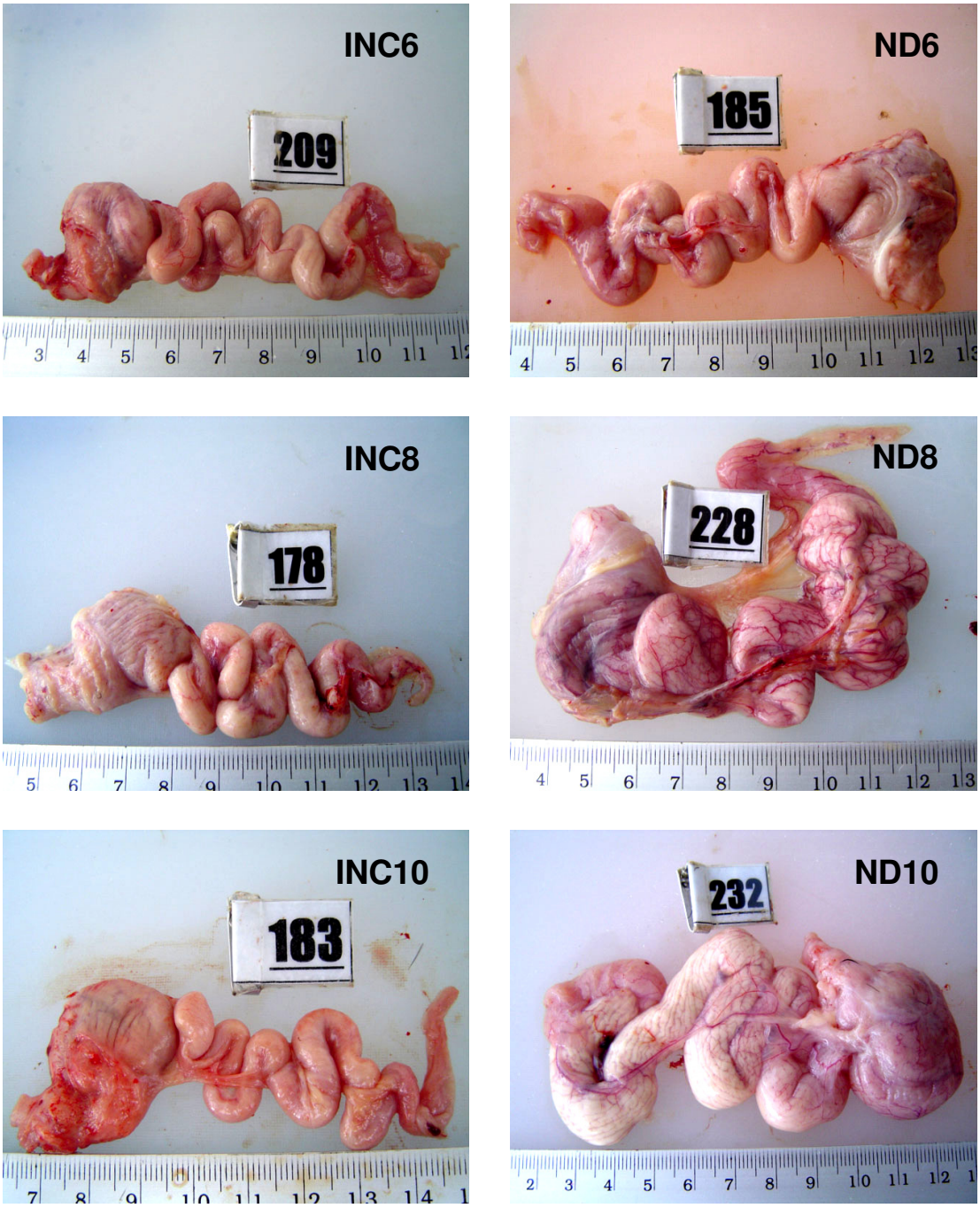

Figure 3.8 Photographs of the oviducts of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation.

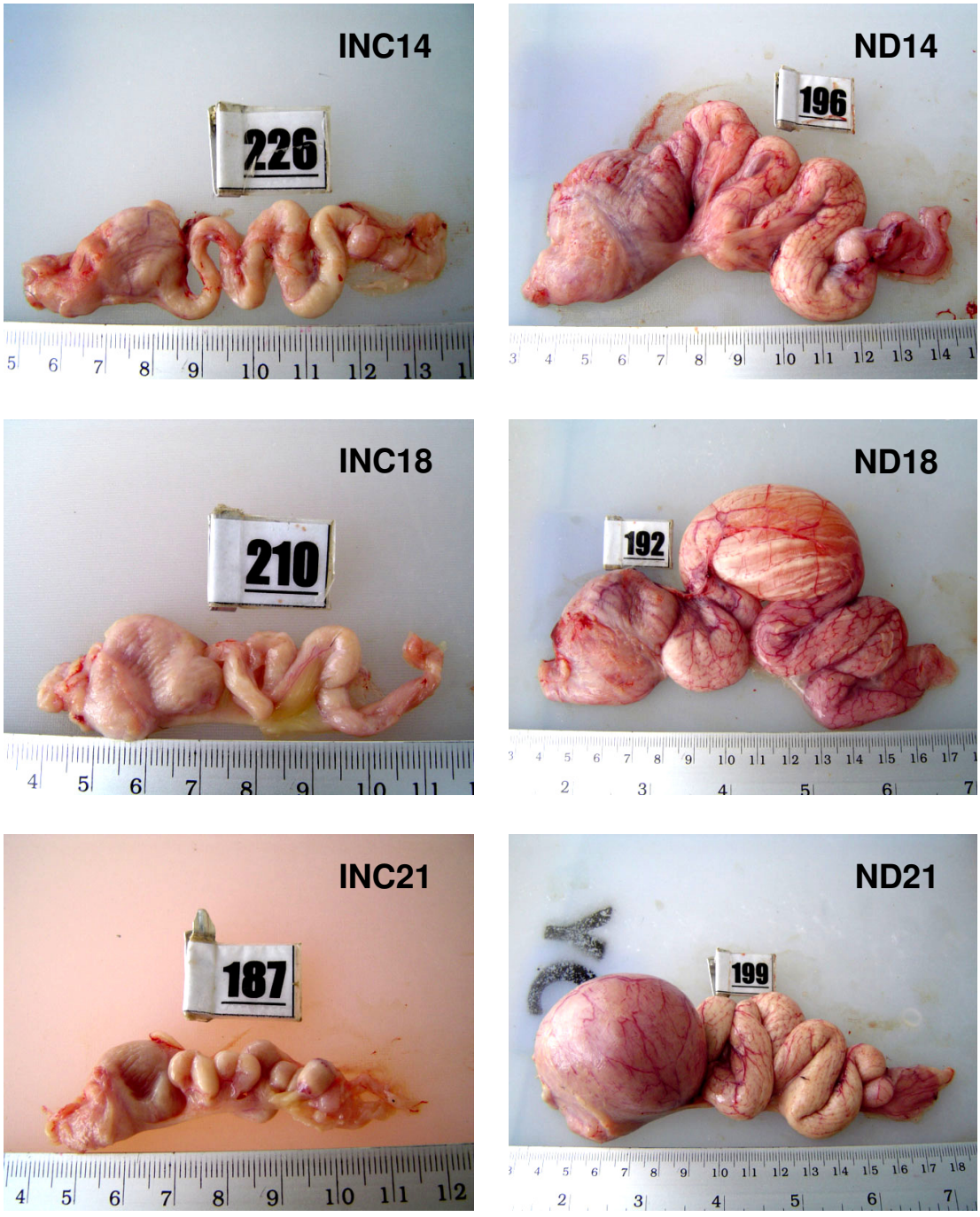

Figure 3.8 Photographs of the oviducts of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation (continued).

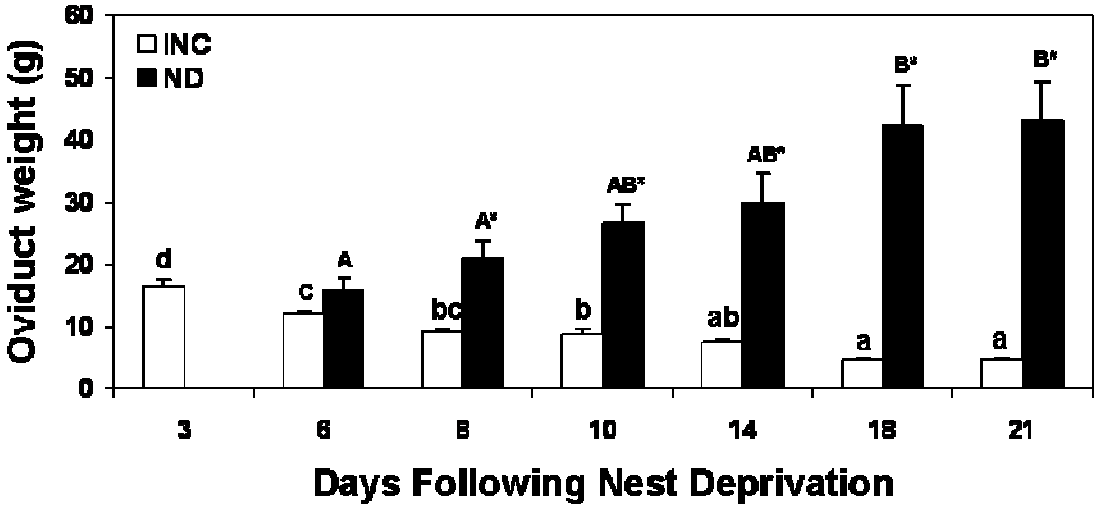

Figure 3.9 Changes in the oviduct weight of incubating (INC) and nest-deprived (ND) native Thai hens. Values are presented as means ± SEM (n=10). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 3.5 Mean ± SEM of the oviduct weight (g) of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation (n=10). 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 Nest Deprivation						
	3	**6**	**8**	**10**	**14**	**18**	**21**
INC	16.23 ± 1.29 ^{d}	11.81 ± 1.89 ^{c}	8.97 ± 1.82 ^{bc*}	8.76 ± 2.32 ^{b*}	7.35 ± 1.88 ^{ab*}	4.48 ± 0.91 ^{a*}	4.62 ± 0.84 ^{a*}
ND	N/A	15.80 ± 6.14 ^{a}	20.90 ± 8.57 ^{a}	26.35 ± 10.54 ^{ab}	29.75 ± 15.39 ^{ab}	42.33 ± 19.90 ^{b}	43.17 ± 19.10 ^{b}

3.5 Discussion

The results of the present study showed that incubation behavior of the native Thai chickens were associated with plasma PRL levels. Plasma PRL levels were low in NL and L hens and reached the highest levels when hens incubated eggs. During incubation period, plasma PRL levels were high throughout the early and middle phase of incubation and started to decline at the late incubation period to the same levels of NL birds when the chicks were hatched. Interestingly, nest deprivation of incubating hens reduced circulating PRL concentrations within a day of nest deprivation. The plasma levels of PRL remained low throughout the period of nest deprivation. In addition, disruption of incubation behavior by nest deprivation increased the ovary and oviduct weights, the presence of ovarian follicles, and the number of egg laying hens. Thus, the changes of plasma PRL levels were associated with the ovarian and oviduct recrudescence and initiation of a new laying cycle in the native Thai chickens.

It has been reported that changes in plasma PRL concentrations are observed across the reproductive cycle of the native Thai chickens (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). Similarly, the changes of plasma PRL levels at different reproductive stages were also observed in this study. These findings correspond with the studies in temperate zone birds such as chickens and turkeys that changes in pituitary PRL gene expression and its plasma PRL levels are highly correlated with the reproductive cycle (Knapp et al., 1988; El Halawani et al., 1990; Talbot et al., 1991; Wong et al., 1991; You et al., 1995; Tong et al., 1997). In incubating native Thai hens, plasma PRL levels reached the highest levels. In turkeys, circulating PRL concentrations increased dramatically during incubating period (El Halawani et al.,

1984) and associated with the decreased in the plasma LH concentrations and ovarian steroids as well as the regression of the ovary and oviduct (Sharp, 1980) and this rising of PRL level has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior. In contrast with this present result, PRL secretion does not increase at the onset of incubation in doves as occurs in other avian species, it increases when the crop sacs are proliferating and producing milk for feeding the young (Goldsmith et al., 1981). PRL stimulates crop sac development and its levels are not attained in adults until after the young have hatched (Goldsmith, 1983).

In native Thai chickens, plasma PRL levels are low in non-laying, gradually increased in laying, high during incubation, and rapidly decrease on the day of hatching (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008; present study). After hatching, plasma PRL levels sharply decrease from the peak levels during incubating eggs to the basal levels in rearing chicks (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). The changes of plasma PRL concentrations during the transition of incubation to rearing periods are also reported in native chicken of the Sudan (Eltayeb et al., 2010). Moreover, a sharp decrease in circulating PRL levels are also found in birds that their eggs are replaced with poults during the transition from incubating eggs to brooding of the young (Opel and Proudman, 1989). Incubating hens leave their nests while adapt newly hatched chicks and come back into lay later than the hens that not allowed to rear chicks (Richard-Yris and Leboucher, 1986; Richard-Yris et al., 1987b). However, the decreasing of plasma PRL levels is not found in hens that could only see and hear but not touch the poults. Only the physical contact between the hens and poults cause the changed plasma PRL levels in incubating hens (Opel

and Proudman, 1988). These data suggested that the physical contact of chicks involved in the maintenance of plasma PRL levels and incubation behavior.

In this study, the disruption of incubation behavior by removing native Thai hens from their nests was accompanied by a precipitous decline in plasma PRL levels. This method of nest deprivation results in the expression of similar behaviors associated with the disruption of incubation and decreased in plasma PRL concentrations, whereas plasma LH levels are increased in turkeys (El Halawani et al., 1980; Mauro et al., 1989; Ramesh et al., 2001), domestic hens (Richard-Yris et al., 1987a; 1998; Leboucher et al., 1993), ring doves (Lea and Sharp, 1989), bantams (Sharp et al., 1988), canaries (Goldsmith et al., 1984), common eider (Criscuolo et al., 2002), and Magang geese (Huang et al., 2008). These changes can be reversed when birds return to the nests (Goldsmith et al., 1984; Sharp et al., 1988). Other evidences demonstrate that the introduction of chicks or poults to incubating hens stop incubation, abandon the nests, and show maternal behavior as well as induce a decline in plasma PRL levels in turkeys (Opel and Proudman, 1989), native Thai chickens (Kosonsiriluk et al., 2008), bantams (Sharp et al., 1988), and domestic hens (Richard-Yris et al., 1998; Leboucher et al., 1993). In yellow-eyed penguins, the tactile and visual stimuli of artificial eggs increase PRL levels, brood patch width, and frequently in sit prone on their nests (Massaro et al., 2007). Active immunization against recombinant-derived PRL reduces the incidence, delays the development (March et al., 1994), or prevents the occurrence of incubation behavior in birds. Taken together, with this the present study, the evidences support the role of PRL in the regulation and maintenance of incubation behavior in birds. The lost of direct stimuli from nests, eggs, and tactile stimuli from chicks disrupt incubation behavior and induce a

decrease in plasma PRL secretion in birds. It has been reported that disruption of broodiness in INC hens is accompanied by a precipitous decline in plasma PRL levels. The numbers of VIP-ir neurons in the IH-IN are high during incubation period and decrease when hens are deprived of their nests, indicating an association between VIP neurons in the IH-IN with the degree of hyperprolactinemia, suggesting that the expression of incubation behavior in the native Thai chicken might be, in part, regulated by the differential expression of VIP neurons in the IH-IN and subsequent PRL release (Prakobsaeng et al., 2009). In addition, plasma PRL levels and the numbers of VIP-ir neurons in the IH-IN of native Thai hens rearing chicks are compared with those of non-rearing chicks. When hens are not allowed to rear chicks, the number of VIP-ir neurons decrease as compared to their respective hens rearing chicks and these decreased VIP-ir neurons are accompanied by a precipitous decline in plasma PRL levels. These findings suggest that the VIP/PRL system is not only a key regulator of incubation behavior but it may also be involved in the regulation of rearing behavior in gallinaceous avian species (Chaiyachet et al., 2010).

The presence of F1-F5 follicles, SYF, and SWF indicates the development of the reproductive system (Etches, 1993). In this study, when hens were deprived from their nests, the ovarian recrudescence was induced, the ovary and oviduct weights significantly increased, and the hens came back to lay within 18 days of nest deprivation. In good agreement with these present results, the study in Magang geese reveals that having the terminated incubation, the geese resumed to lay in 24 days following recruitment of large white follicles into hierarchical development (Huang et al., 2008). In addition, it has been reported that forced molting and incubation behavior depress ovarian steroids production during gonadal regression (Porter et al.,

1991a; 1991b). The reinitiation of reproductive activity and egg laying in response to nest deprivation of incubating birds do not appear to be attributable only to its suppressive effect on PRL secretion, but also to its associated increase in the activity of the GnRH/FSH-LH system (El Halawani et al., 1980; Sharp et al., 1988; Mauro et al., 1989; Ramesh et al., 2001, Sartsoongnoen et al., 2006). High levels of PRL during the incubation period directly inhibit hypothalamic secretion of GnRH, which in turn reduces pituitary secretion of LH and leads to regression of the gonads (Curlewis, 1992; El Halawani and Rozenboim, 1993). Moreover, removal the hens from their eggs results an increase in LH secretion and hypothalamic contents of cGnRH-I mRNA (Dunn et al., 1996). It is well established that PRL is an antigonadotropin that reduces circulating FSH and LH (Lea et al., 1981; 1986; El Halawani and Rozenboim, 1993; El Halawani et al., 1997), induces and maintains ovarian regression, and initiates incubation behavior (El Halawani et al., 1997). The elevated PRL levels and depressed LH levels of incubating hens are maintained by tactile stimuli from the nests and eggs (El Halawani et al., 1980; 1986; Opel and Proudman, 1988) and can be reversed by nest deprivation (El Halawani et al., 1980; Proudman and Opel, 1981; Zadworny and Etches, 1987; Sharp et al., 1988) or the introduction of chicks (Leboucher et al., 1990). These findings taken together with the results in the present study clearly implicate the enhanced activity of PRL in the initiation and maintenance of incubation behavior and regression of the reproductive system in the native Thai chickens.

In summary, this present study indicates that incubation behavior of the native Thai chicken is regulated by PRL. The external cues such as nests and eggs involve in the maintenance of plasma PRL levels and incubation behavior. The lost of stimuli

from nests and eggs terminates incubation behavior, reduce PRL secretion, induce ovarian recrudescence, increase ovary and oviduct weights, and finally induce the hens to come back to lay in the new cycle.

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CHAPTER IV

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

4.1 Abstract

The onset of incubation behavior is correlated with increasing plasma prolactin (PRL) levels. Vasoactive intestinal peptide (VIP), an octacosaneuropeptide that plays a pivotal role in the regulation of PRL secretion in birds, is defined as the PRL-releasing factor (PRF). In temperate zone birds, PRL secretion is under stimulatory control by VIP which is secreted from neurons located in the infundibular nuclear complex (INF) of the hypothalamus. This study was designed, utilizing an immunohistochemistry technique, to compare the differential expression of VIP-immunoreactive (VIP-ir) neurons in the brain of incubating (INC) native Thai hens with those of nest-deprived (ND) ones. The results revealed that the hypothalamic VIP-ir neurons and fibers were observed across the nucleus anterior medialis hypothalami (AM), nucleus suprachaiasmaticus, pars medialis (SCNm), nucleus periventricularis hypothalami (PHN), regio lateralis hypothalami (LHy), nucleus ventromedialis hypothalami (VMN), nucleus inferioris hypothalami (IH), nucleus

infundibuli hypothalami (IN) areas of INC and ND hens. Significant differences in the number of VIP-ir neurons within the AM, SCNm, PHN, LHy, and VMN areas were not observed between INC and ND hens. The greatest density of VIP-ir neurons was found in the IH-IN of INC hens. Changes in the number of VIP-ir neurons in the hypothalamus of INC and ND hens were observed in the IH-IN area. The number of VIP-ir neurons was high during incubating period and significantly declined by day 6 of nest deprivation. The number of VIP-ir neurons in ND hens was lower than those of INC hens throughout day 21 of nest deprivation. The present finding indicates an association between VIP and incubation behavior, confirming the role of VIP as the PRF in this equatorial bird. 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 the native Thai chickens, the non-photoperiodic species. Nest deprivation of incubating chickens decreases the number of VIP-ir neurons in the IH-IN. It is suggested that the VIPergic system in the IH-IN of the hypothalamus may involve in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of incubation behavior in this equatorial bird.

4.2 Introduction

It is well known that two major neuroendocrine systems play important roles in the avian reproductive cycle. The first system involves gonadotropin releasing hormone-I (GnRH-I) and the subsequent release of luteinizing hormone (LH) and follicle stimulating hormone (FSH; Sharp et al., 1998), known as the GnRH/FSH-LH system. The other system involves the avian prolactin (PRL) releasing factor (PRF), vasoactive intestinal peptide (VIP), and the subsequent release of PRL (El Halawani

et al., 1997; Chaiseha and El Halawani, 2005), which is named as the VIP/PRL system. 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. Throughout incubation, circulating PRL levels are sharply elevated (El Halawani et al., 1984; Sharp et al., 1989; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008), whereas plasma LH levels are gradually suppressed (Lea et al., 1981; El Halawani and Rozenboim, 1993). In addition, pituitary PRL mRNA and its protein are strongly correlated with the avian reproductive cycle (Wong et al., 1991). In birds, 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, and cow birds (El Halawani et al., 1997). Like Gallinacous-temperate zone birds, hyperprolactinemia has been 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).

VIP, an octacosapeptide, functions as a neurotransmitter and neuroendocrine substance (Larsson et al., 1976; Marley and Emson, 1982). 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 the high concentrations are found in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 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), and adrenocorticotropic hormone (Oliva et al., 1982; White et al., 1982). It is well documented that VIP can stimulate PRL release both *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Samson et al., 1980; Matsushita et al., 1983). 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, it has been established for sometimes that PRL secretion is tonically stimulated (Kragt and Meities, 1965; Bern and Nicoll, 1968), and VIP is the avian 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). It is very well documented that variations in hypothalamic VIP immunoreactivity, VIP contents, VIP mRNA steady-state levels, VIP mRNA expression in the INF, VIP receptor mRNA in the pituitary gland, 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., 1996; Chaiseha et al., 1998; 2004; Chaiseha and El Halawani, 1999), chickens (Sharp et al., 1989), doves (Cloues et al., 1990) and native Thai chickens (Kosonsiriluk et al., 2008). In temperate zone birds such as the turkeys, expression and secretion within the VIP/PRL system are activated by an escalating photoperiod which stimulates the gonad development.

Hypothalamic VIP mRNA steady-state levels and VIP contents in the median eminence (ME) increase following photostimulation and are closely correlated with the increasing level of PRL secretion (Chaiseha et al., 1998). Furthermore, VIP also exerts an inhibitory influence on the gonadotropin system. Elevated hypothalamic VIP peptide and mRNA 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 (Ahn et al., 2001) and delays the onset of photorefractoriness and molt in starling (Dawson and Sharp, 1998). Other studies have also demonstrated increases in the number and 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).

VIP regulates PRL gene expression by enhancing the transcription rate of PRL and up-regulating PRL mRNA stability (Tong et al., 1998). Passive immunization with anti-VIP serum decreases plasma PRL and pituitary mRNA levels and terminates incubation behavior (Talbot et al., 1991). In addition, active immunoneutralization of endogenous VIP reduces levels of circulating PRL and pituitary PRL mRNA and totally blocks the PRL release affected by electrical stimulation of the medial preoptic nucleus as well as blocks the hormonal and behavioral characteristics of incubating hens (El Halawani et al., 1990; 1995; 1996; 2001; Youngren et al., 1994).

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 areas are directly correlated to plasma PRL levels of the native Thai chickens (Kosonsiriluk et al., 2008). The greatest number of VIP-ir neurons within the IH-IN area is found during incubation period, when the greatest plasma level of PRL is observed (Kosonsiriluk et al., 2008; Prakobsaeng et al., 2009). Furthermore, plasma PRL levels and the numbers of VIP-ir neurons in the IH-IN of native Thai hens rearing chicks are compared with those of non-rearing chicks. The results reveal that when hens are not allowed to rear chicks, the number of VIP-ir neurons decrease as compared to their respective hens rearing chicks and these decreased VIP-ir neurons are accompanied by a precipitous decline in plasma PRL levels (Chaiyachet et al., 2010).

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; Kosonsiriluk et al., 2008; Sartsoongnoen, 2007). There are a limited number of studies providing data about the neuroendocrine regulation in this gallinaceous bird living in the non-temperate zone. Importantly, there is no study delineating the anatomical distribution and functional aspect of the VIPergic system with incubation behavior in the native Thai chickens. The objectives of this study were to investigate whether the differential expression of VIP-ir neurons within the hypothalamic areas are correlated with incubation behavior in the native Thai chickens. Changes in the number VIP-ir neurons within the hypothalamic areas of incubating hens with those of nest-deprived hens were compared, particularly within the IH-IN area. The findings of the differential expression of VIP-ir neurons in the IH-IN area with the degree of

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

4.3 Materials and Methods

4.3.1 Experimental Animals

Female native Thai chickens (*Gallus domesticus*), Pradoohangdam breed, were used. They were reared and housed with mature roosters (8-9 females : 1 male) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of dark; 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; incubating eggs (INC) and non-incubating or nest deprivation (ND). Hens in the INC group had stopped laying and showed incubating behavior by sitting on the nests for three to four times per day. They were allowed to incubate their eggs naturally. Hens in the ND group were disrupted from incubating behavior and not allowed to incubate their eggs by removing them from their nests 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

4.3.2.1 Experiment I

Twelve female and 2 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 2 floor pens (6 hens : 1 rooster) and observed their daily behaviors. Hens were divided into two groups; INC and ND.

The hens were sacrificed at day 10 after they started to incubate their eggs or after nest deprivation. The brains were pressure-perfused, sectioned with a cryostat, and processed by immunohistochemistry (IHC) to localize and identify VIP-ir neurons in the brain. The reproductive stages were identified by behavioral observation and confirmed by postmortem examination at the end of the experiment.

4.3.2.2 Experiment II

Seventy eight female and 10 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 10 floor pens (7-8 hens : 1 rooster) and observed their daily behaviors. Hens were divided into two groups; INC and ND. The hens were then sacrificed at different time periods (day 3, 6, 8, 10, 14, 18, and 21; n=6) after they started to incubate their eggs or after nest deprivation. The brain of each hen was pressure-perfused, sectioned with a cryostat, and processed by IHC to visualize and analyze the changes in the number of VIP-ir neurons in the IH-IN area. The reproductive stages were identified by behavioral observation and confirmed by postmortem examination at the end of the experiment.

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 brain area were processed by IHC in order to visualize and analyze the changes in the number of VIP-ir neurons.

4.3.4 Immunohistochemistry

Changes in the number of VIP-ir neurons in the hypothalamus of INC and ND hens were conducted 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 INC and ND 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 in the IH-IN. 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 per section (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. The 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

4.4.1 Experiment I

Changes in the number of hypothalamic VIP-ir neurons were observed at day 10 of incubation and nest deprivation. The number of VIP-ir neurons was compared across 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 (Figures 4.1, 4.2 and 4.3). The greatest density of VIP-ir neurons was found in the IH-IN of INC hens (Figure 4.2). The numbers of VIP-ir neurons were markedly lower when hens were nest-deprived. Significant differences in the number of VIP-ir neurons within the AM, SCNm, PHN, LHy, and VMN areas were not observed between INC and ND hens. A dense accumulation of the VIP-ir fibers and scattered VIP-ir neurons were seen in the LHy, whereas very few VIP-ir neurons were found in the AM, SCNm, and VMN in both groups. No VIP-ir neurons were observed in the PHN, nI, and ML. A

dense accumulation of the VIP-ir fibers were located in the ME of both INC and ND groups.

4.4.2 Experiment II

The differential expression of VIP-ir neurons in the IH-IN area of INC and ND hens are shown in Figure 4.4. The changes in the number of VIP-ir neurons in the IH-IN of INC and ND hens are shown in Figure 4.5 and Table 4.2. In INC group, the number of VIP-ir neurons in the IH-IN remained high from day 3 through day 21 of incubation. When hens were deprived from their nests, counted VIP-ir neurons were markedly and significantly decreased ($P<0.05$) by day 6 (INC6 vs ND6; 75.17 ± 6.10 vs 42.15 ± 4.61 cells) and persisted lower than those of INC hens throughout day 21 of nest deprivation ($P<0.05$; INC8 vs ND8; 73.79 ± 7.71 vs 42.40 ± 7.58, INC10 vs ND10; 81.79 ± 9.69 vs 25.38 ± 4.10, INC14 vs ND14; 86.88 ± 8.60 vs 25.83 ± 3.68, INC18 vs ND18; 76.64 ± 9.19 vs 25.00 ± 4.50, INC21 vs ND21; 79.26 ± 10.53 vs 28.70 ± 4.87 cells). In addition, the number of VIP-ir neurons did not differ among INC and ND groups at different day of observation. The distribution patterns of VIP-ir neurons in the IH-IN area were consistent in every INC hens. When the hens were nest-deprived, the number of VIP-ir neurons decreased in the same patterns.

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)

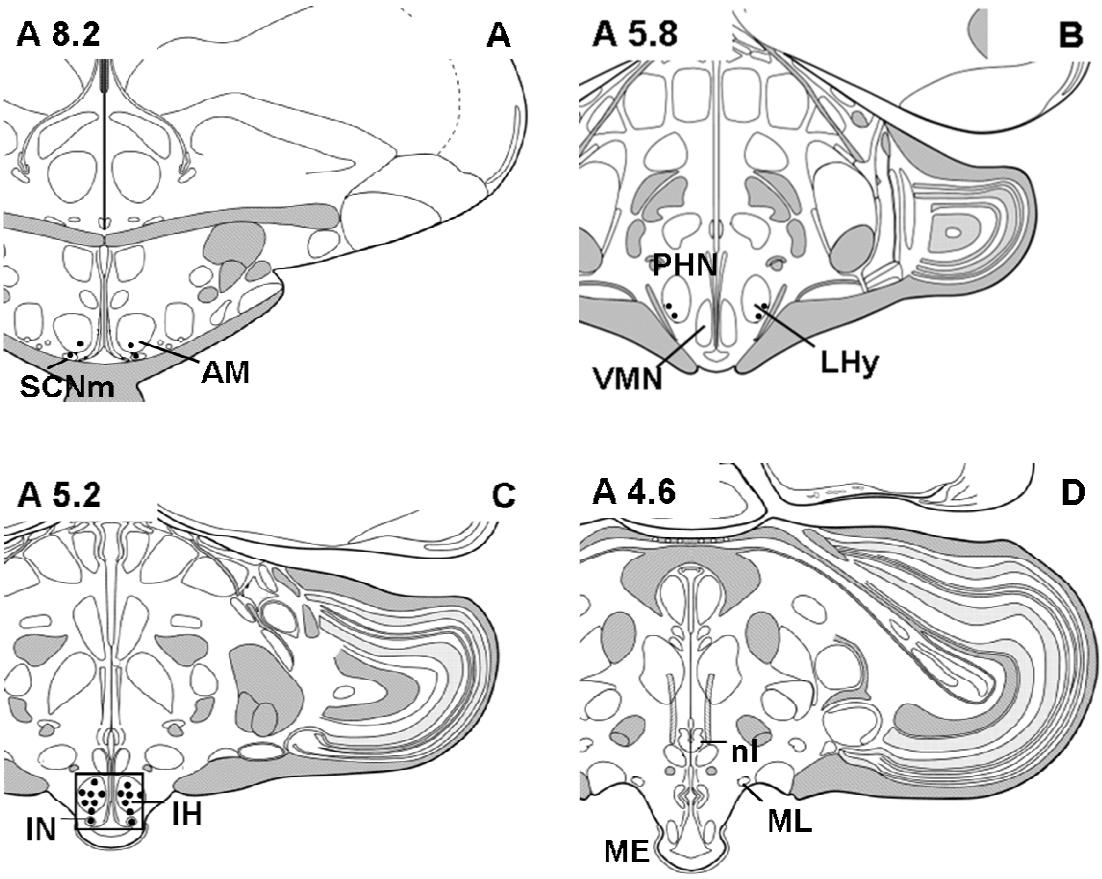

Figure 4.1 Schematic coronal brain sections showing the areas where the expression of VIP-ir (black dots) was observed (**A-D**). The sampling region for counting the number of VIP-ir neurons in the IH-IN (**C**) is represented by rectangles. Coronal illustrations were redrawn from the stereotaxic atlas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

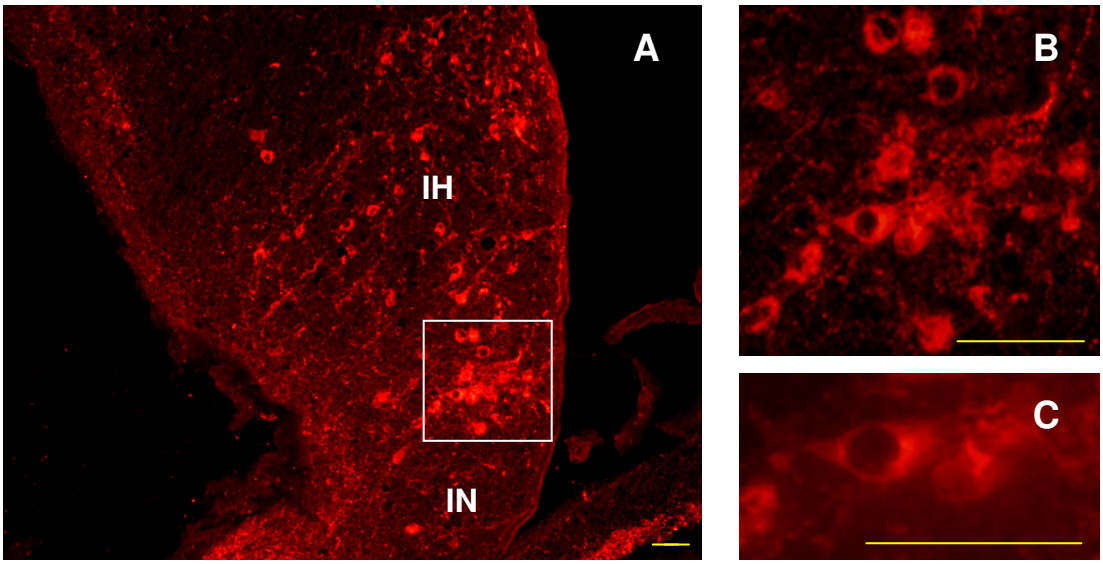

Figure 4.2 Photomicrographs illustrating the distributions of VIP-ir neurons and fibers in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) of the native Thai chicken (**A**). Rectangle indicates area from which following photomicrographs are taken. Higher magnification of the VIP-ir neurons in the IH-IN (**B** and **C**). Bar = 50 µm.

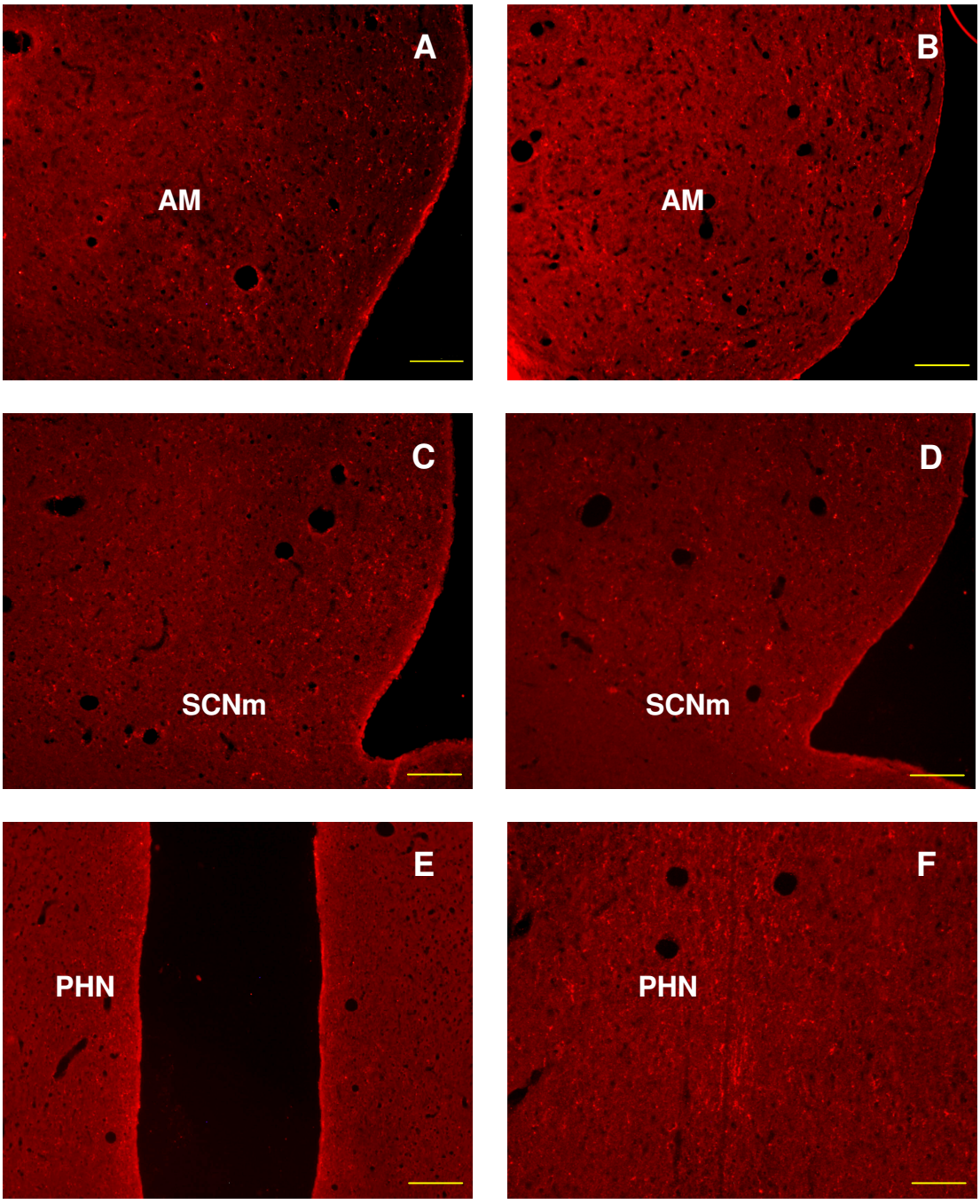

Figure 4.3 Photomicrographs illustrating the distributions of VIP-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, and **Q**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**, and **R**) native Thai hens. For abbreviations, see Table 4.1. Scale bar = 100 µm.

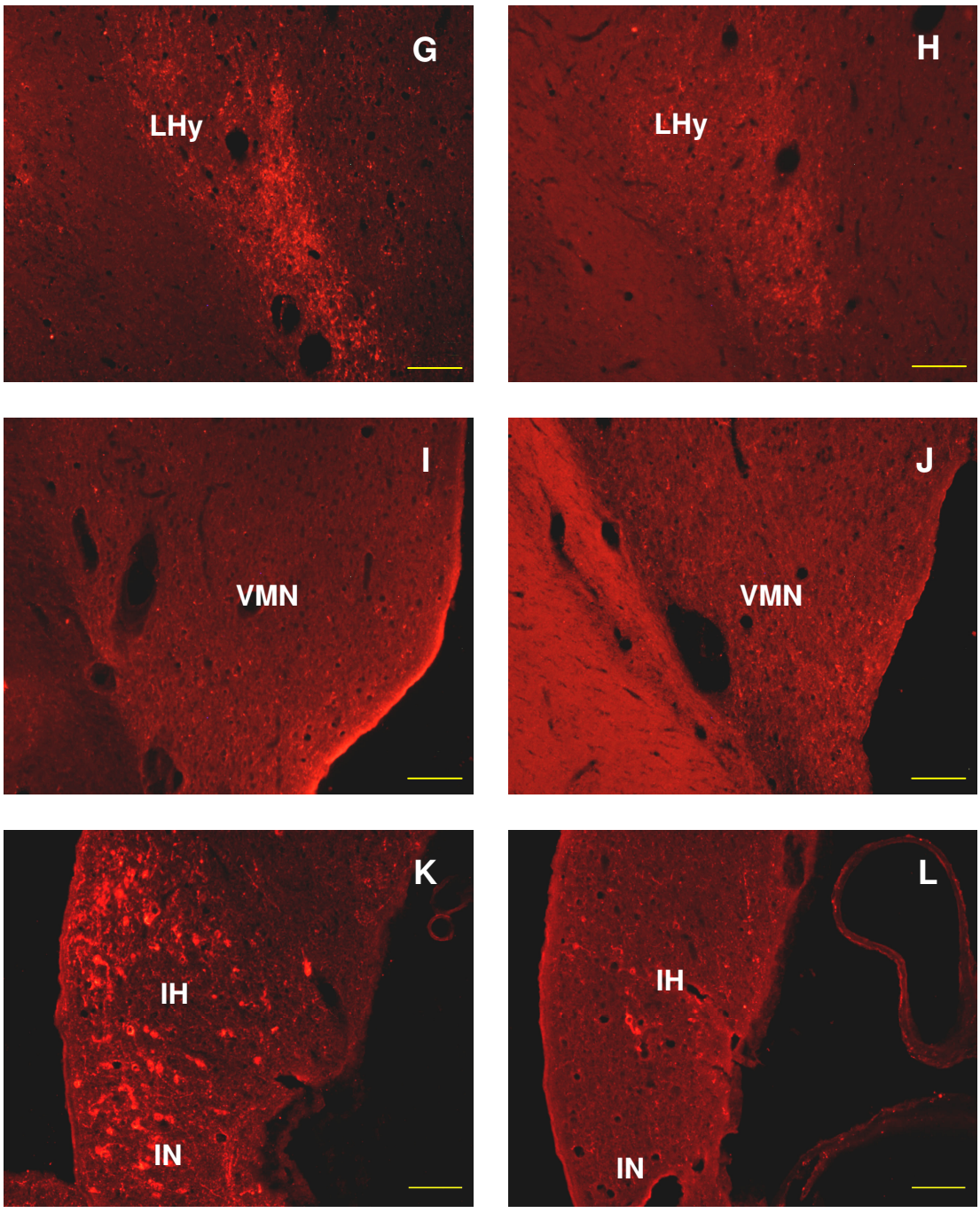

Figure 4.3 Photomicrographs illustrating the distributions of VIP-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, and **Q**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**, and **R**) native Thai hens. For abbreviations, see Table 4.1. Scale bar = 100 µm (continued).

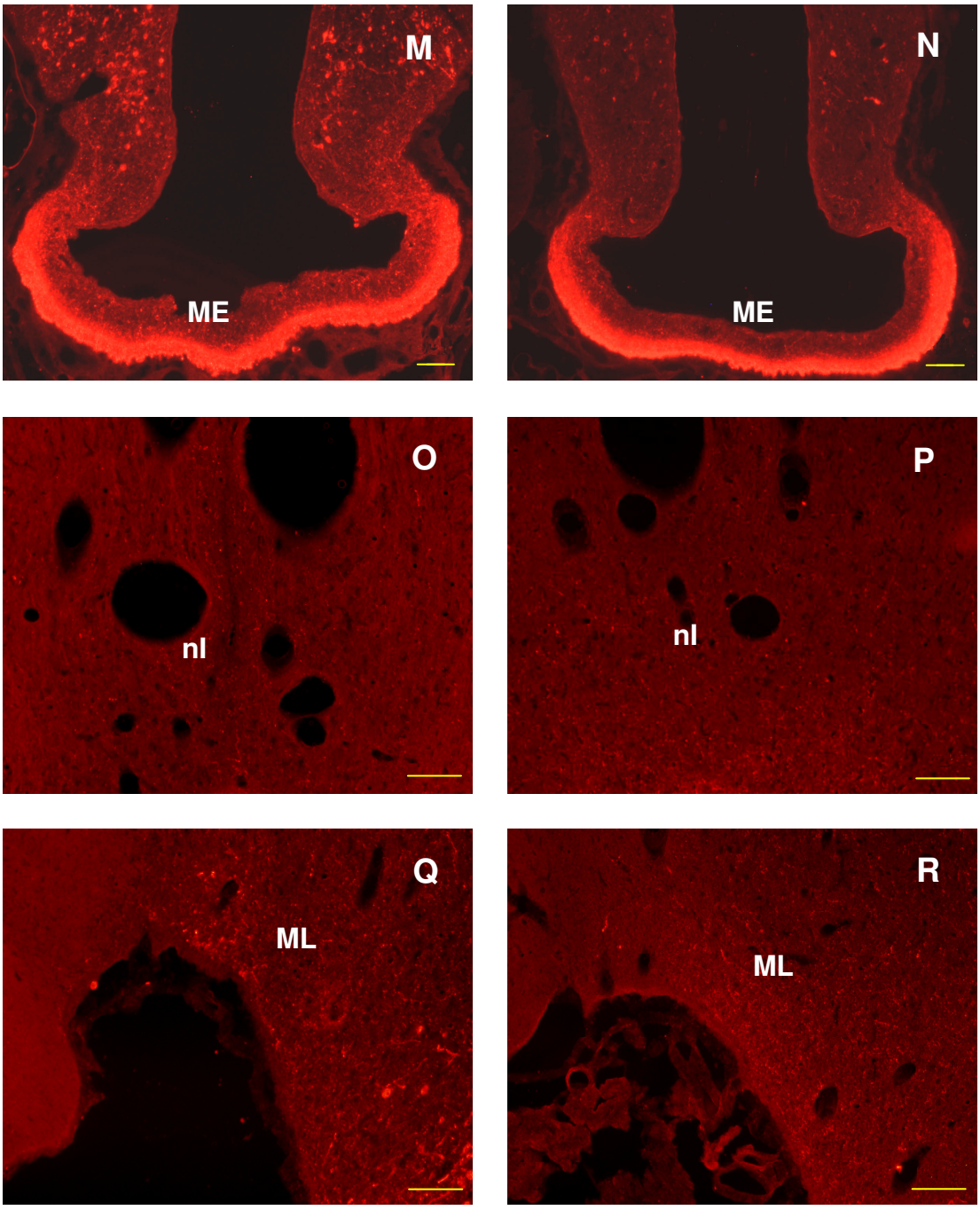

Figure 4.3 Photomicrographs illustrating the distributions of VIP-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, and **Q**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**, and **R**) native Thai hens. For abbreviations, see Table 4.1. Scale bar = 100 µm (continued).

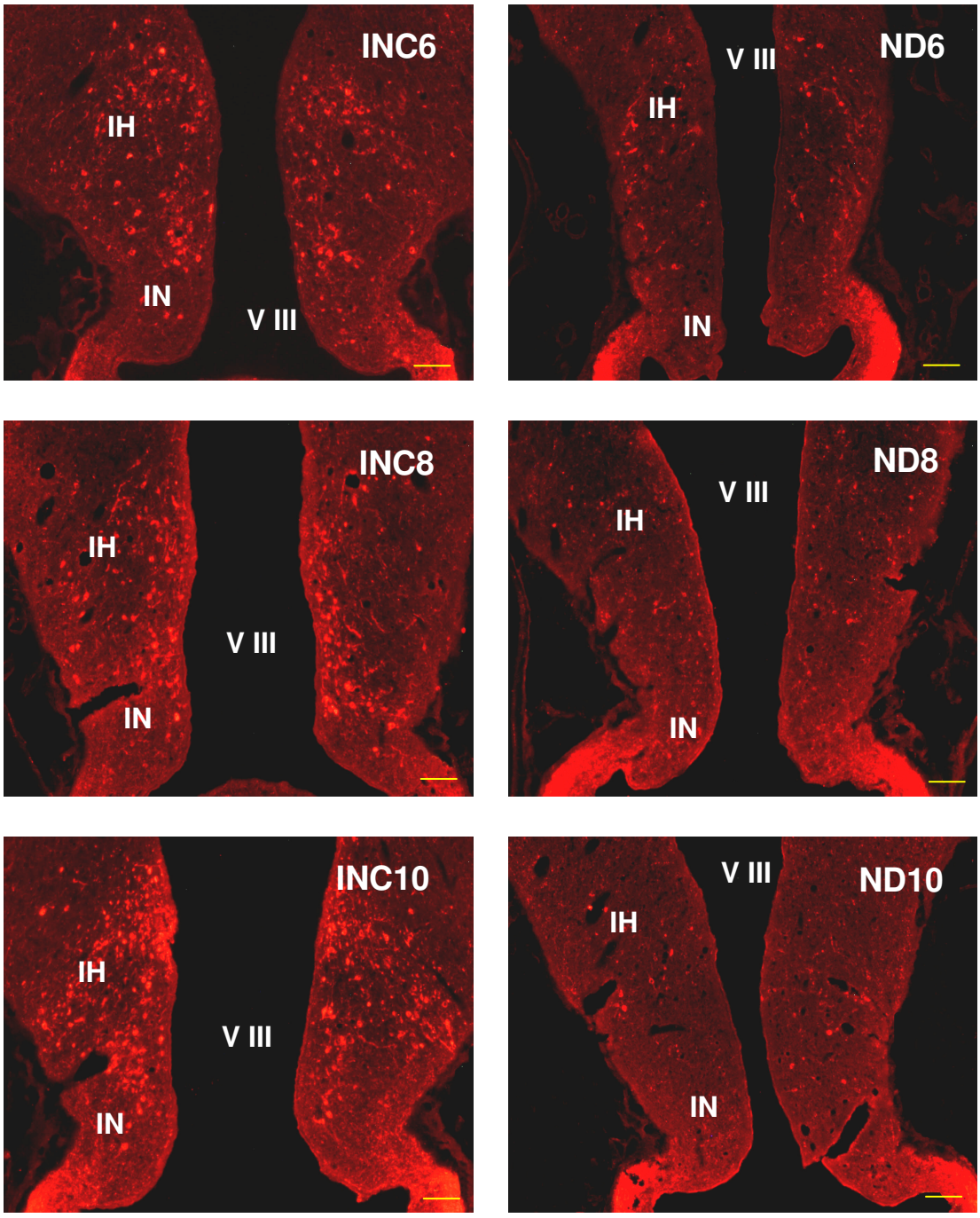

Figure 4.4 Photomicrographs showing the distributions of VIP-ir neurons and fibers in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 4.1. Scale bar = 100 µm.

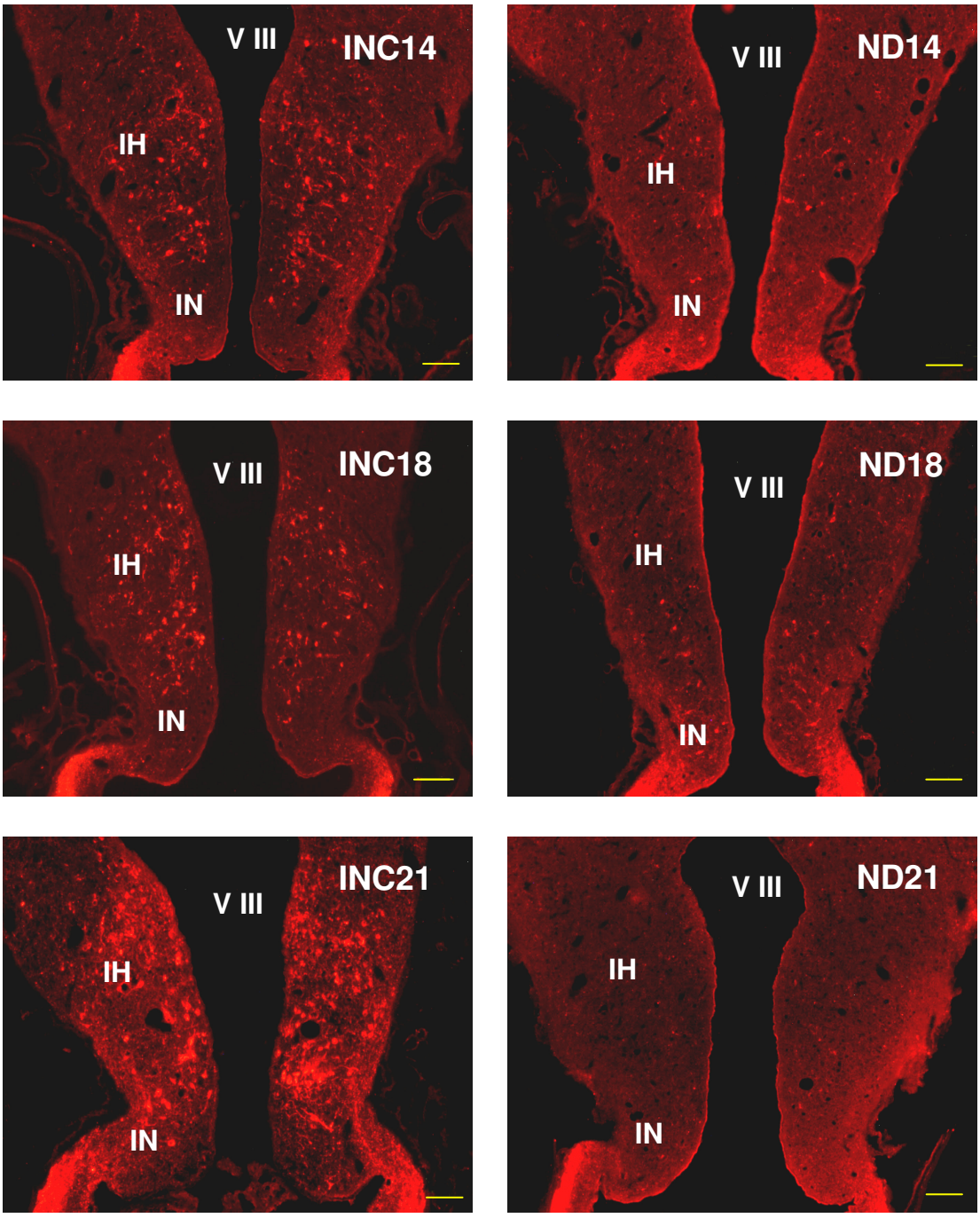

Figure 4.4 Photomicrographs showing the distributions of VIP-ir neurons and fibers in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 4.1. Scale bar = 100 µm (continued).

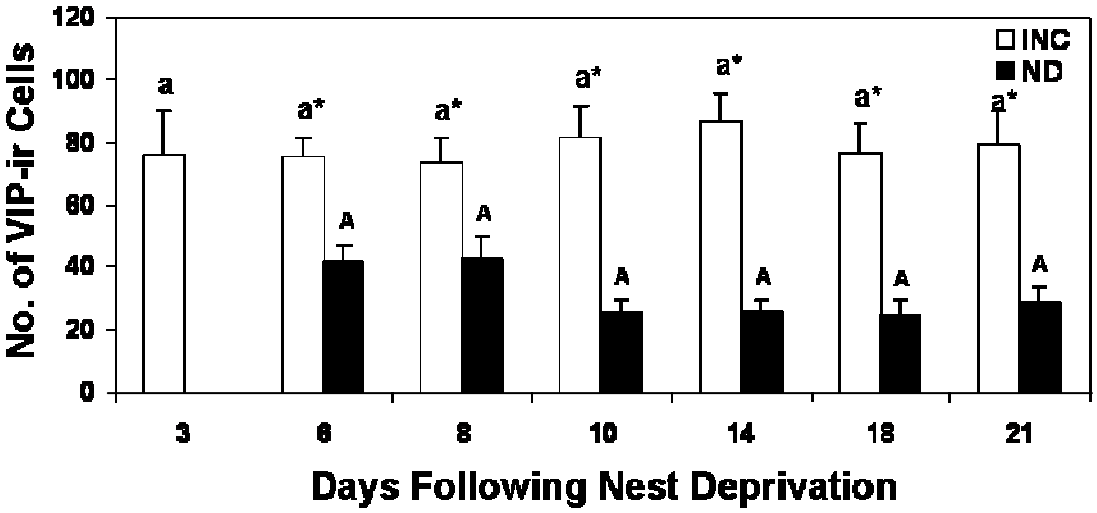

Figure 4.5 Changes in the number of VIP-ir neurons in the IH-IN of incubating (INC) and nest-deprived (ND) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ 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 incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation (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 Nest Deprivation						
	3	**6**	**8**	**10**	**14**	**18**	**21**
INC	75.75 ± 14.05 [a]	75.17 ± 6.10 [a*]	73.79 ± 7.71 [a*]	81.79 ± 9.69 [a*]	86.88 ± 8.60 [a*]	76.64 ± 9.19 [a*]	79.26 ± 10.53 [a*]
ND	N/A	42.15 ± 4.61 [a]	42.40 ± 7.58 [a]	25.38 ± 4.10 [a]	25.83 ± 3.68 [a]	25.00 ± 4.50 [a]	28.70 ± 4.87 [a]

4.5 Discussion

The results from this present study revealed that the VIP-ir neurons and fibers were extensively distributed throughout the brain of the incubating native Thai chickens and were predominantly expressed in the IH-IN area. The expression of VIP-ir neurons and fibers within the diencephalon was also observed in the AM, SCNm, PHN, LHy, VMN, and ME. No significant differences of the VIP-ir neurons and fibers were observed in each hypothalamic area except in the IH-IN area. Changes in the VIP-ir neuron populations within the IH-IN area were compared between INC and ND hens. The greatest density of VIP-ir neurons was found in INC group and the neuronal densities were decreased in ND group. The number of VIP-ir neurons in the IH-IN remained high throughout 21 days of incubation period. When the hens were nest-deprived, the number of VIP-ir neurons was declined by day 6 of nest deprivation. These findings are consistent with an investigation stating the role of VIPergic system in the regulation of PRL and incubation behavior in the other avian species (El Halawani et al., 2001). The findings also provide an additional evidence that VIP is also the PRF in this non-photoperiodic, continuously breeding avian species.

Results of the present study reveal that nest deprivation of incubating native Thai chickens suppressed hypothalamic VIPergic activity. The greatest expression of VIP-ir neurons was found in the IH-IN which located in the INF. Scattered VIP-ir neurons were observed in the AM, LHy, and VMN areas. A dense density of VIP-ir fibers were found in the external layer of the ME and also in the LHy (Kosonsiriluk et al., 2008; this study). The results from this present study are in good agreement with the previous findings that the large group of VIP-ir neurons are found in the IH-IN

area (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). 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 in the INF is correlated with increased levels of circulating PRL level and LH-β mRNA in the anterior pituitary (Bhatt et al., 2003). An elevation of PRL secretion during incubation period is associated with an increase in pituitary lactotroph abundance (Lopez et al., 1996). Chronic exposure to VIP can increase the population of lactotrophs *in vitro* (Porter et al., 2006). Nest-deprivation of incubating hens inhibits the PRL releasing mechanism(s) independently of PRL transcription, decreasing pituitary PRL mRNA and programmed cell death of lactotrophs (Talbot et al., 1991; Tong et al., 1997; Ramesh et al., 2001).

The differential expression of VIP within the IH-IN has been reported across the reproductive cycle. It has been reported that the VIP immunoreactivity is the greatest during incubating period of turkeys (Mauro et al., 1989; Chaiseha and El Halawani, 1999), bantam hens (Sharp et al., 1989) and native Thai chickens (Kosonsiriluk et al., 2008). Changes in hypothalamic VIP-ir neurons within the IH-IN, but not other areas of the hypothalamus, are observed and directly correlated with concentrations of circulating PRL throughout the reproductive cycle of the native Thai chickens (Kosonsiriluk et al., 2008). The number of VIP-ir neurons is gradually

increased in the IH-IN during the transition from non-laying to laying peroid, with the greatest number of VIP-ir neurons is observed in incubating hens. However, the number of VIP-ir neurons is decreased when birds shifted from incubating to rearing period (Kosonsiriluk et al., 2008). Nest deprivation of incubating native Thai chickens results in a decline in the number of VIP-ir neurons in the IH-IN (Prakobsaeng et al., 2009; this study) and this disruption of incubation behavior is accompanied by a precipitous decline in plasma PRL levels (Prakobsaeng et al., 2009). In addition, an increase in the number and size of VIP-ir neurons within the medio-basal hypothalamus when the concentrations of plasma PRL are high has been demonstrated in the domesticated pigeons and ring doves during the initiation of crop milk secretion and feeding of 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).

It is well established that VIP is the avian PRF (Sharp et al., 1989; El Halawani et al., 1997). In incubating hens, tactile stimuli from the nest 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; this study). The increased number of VIP-ir neurons which have been shown to be correlated with the up-regulation of VIP peptide contents and its mRNA (Mauro et al., 1989; 1992; Chaiseha and El Halawani, 1999), and the maximum plasma PRL levels reached in incubating hens could result from the presence of eggs and persistent nesting activity (El Halawani et al., 1980). 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, induce ovarian regression (Zadworny et al., 1988; Youngren et al., 1991), and commence nest protective behavior and anorexia (Zadworny and Etches, 1987). These behavioral and neuroendocrine changes have been attributed to increased PRL levels and the state of hyperprolactinemia to initiate and establish incubation behavior (El Halawani et al., 1986; Opel and Proudman, 1989; Chaiseha and El Halawani, 2005). It is possible that PRL and the state of hyperprolactinemia may also be importance in the increased the number of VIP-ir neurons in the IH-IN observed in incubating 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 incubation behavior. Indeed, in this present study, disruption of incubation behavior by nest deprivation reduces the number of VIP-ir neurons in the IH-IN and is corresponded with the studies in turkeys that indicate the number of VIP-ir neurons in the INF is increased during incubating period and decreased when hens are disrupted incubation via nest deprivation and these changes of VIP-ir neurons in the INF paralleled with the changes in plasma PRL concentrations (Mauro et al., 1989).

In conclusion, the present findings indicate an association between VIP and incubation behavior, confirming the role of VIP as the PRF in this equatorial bird. 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 the native Thai chicken, the non photoperiodic species. Nest deprivation of incubating chickens decreases the number of VIP-ir neurons in the IH-IN. Thus, the VIPergic system in

the IH-IN of the hypothalamus may involve in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of incubation behavior in the native Thai chickens.

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CHAPTER V

EFFECTS OF INCUBATION BEHAVIOR UPON THE 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 which play an important role in the avian reproductive cycle. One system involves gonadotropin releasing hormone-I (GnRH-I) and the subsequent release of luteinizing hormone (LH) and follicle stimulating hormone, named as GnRH/FSH-LH system. The other system involves the avian prolactin (PRL) releasing factor (PRF), vasoactive intestinal peptide (VIP) and the subsequent release of PRL, known as VIP/PRL system. The onset of incubation behavior is correlated with declining plasma levels of LH and gonadal steroids and increasing plasma levels of PRL. The syntheses and secretions of FSH and LH are regulated by GnRH, a decapeptide which is secreted from hypothalamic neuronal cells. There are many data supported the role of GnRH-I in the reproduction of several temperate zone species. However, there is limited study delineating the anatomical distribution and functional aspects of the GnRH system on incubation behavior in the native Thai chickens, the non-temperate zone birds. To further

understand the role of GnRH-I in the regulation of reproductive cycle, especially incubation behavior in this bird, native Thai chickens were divided into two groups; incubating (INC) and nest-deprived (ND) hens. The differential expression of GnRH-I-immunoreactive (GnRH-I-ir) neurons in the hypothalamus of INC and ND hens were compared utilizing immunohistochemical technique. The expression of hypothalamic GnRH-I-ir neurons within the nucleus anterior medialis hypothalami, nucleus suprachaiasmaticus, pars medialis, nucleus commissurae pallii (nCPa), nucleus septalis lateralis, nucleus paraventricularis magnocellularis, and regio lateralis hypothalami areas were observed. High expression of GnRH-I-ir neurons was found in the nCPa of ND hens, whereas less numbers of them were observed in the nCPa of INC hens. The number of GnRH-I-ir neurons in the nCPa was low in INC group and significantly increased by days 6 of nest deprivation. These findings implicate that the expression of incubation behavior in the native Thai chicken might be, in part, regulated by the differential expression of GnRH-I neurons in the nCPa. This study also confirms a pivotal role of GnRH-I in controlling of avian reproduction of this non-seasonally breeding, equatorial species.

5.2 Introduction

Avian reproductive system is regulated by the integration of the hypothalamus, the pituitary, and the gonads (testis and ovary). 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. The HPG axis involves two major neuroendocrine systems controlling avian reproduction. These

neuroendocrine systems include the chicken gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH), and vasoactive intestinal peptide/prolactin (VIP/PRL) neuroendocrine systems. Both systems are influenced by dopaminergic (DAergic) neurotransmission (Bhatt et al., 2003; Chaiseha et al., 2003).

GnRH is a hypothalamic neuronal secretory decapeptide being important for the control of reproduction in many vertebrates. Hypothalamic GnRH is first isolated from porcine hypothalamus and has been sequenced (Peczely, 1989; Rivier, 2001). Three types of GnRH have been found in the avian brain (Sun et al., 2001), which 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). 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 the domestic chickens (Sharp et al., 1990). There are growing evidences indicating that the three forms of GnRH influence avian gonadotropins secretion but their abilities are different. Like in mammals, GnRH is synthesized by neurosecretory cells in the hypothalamus, released from the median eminence (ME) into the hypophysial portal vessels, and transported to the pituitary gland, where it stimulates the secretions of 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). An *in vivo* study reveals that injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH concentration in hens (Guemene and Williams, 1999). Incubation of turkey anterior pituitary cells with GnRH results in an increase in LH-β-subunit mRNA 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).

In birds, the egg laying period is associated with relatively high levels of circulating FSH, LH, and gonadal steroids, and is regulated by hypothalamic GnRH (El Halawani et al., 1988). Up to date, cGnRH-I is thought to be the main hypophysiotropic factor stimulating the release of LH since immunization against cGnRH-I, but not cGnRH-II, caused 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, suggesting an involvement of cGnRH-II in the control of reproduction (Teruyama and Beck, 2000). The various distributions of cGnRH-II and GnRH-III in avian brain suggest their functional significances. It is reported that cGnRH-II may act as 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).

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 the pituitary sensitivity to GnRH in birds (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 (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). Increasing of sex steroid levels exerts a negative feedback on the GnRH system. Gonadectomy increases the synthesis of GnRH in the hypothalamus and the release of LH from the pituitary gland (Knight et al., 1983). Active VIP immunoneutralization increases pituitary content of LH-β and FSH-β mRNAs and is accompanied by a decline in PRL mRNA expression (Ahn et al., 2001). In addition, GnIH inhibited LH and FSH synthesis and release *in vitro* (Ciccone et al., 2004).

GnRH regulates LH secretion in both spontaneous and induced ovulating mammalian species (for review, see Bakker and Baum, 2000). Similarly, GnRH also plays a pivotal role in the control of avian reproduction. GnRH contents change during the avian reproductive cycle. At the peak level of reproductive activity, birds have more GnRH-immunoreactive (GnRH-ir) neurons and fibers than those of sexually inactive or photorefractory birds (Sharp et al., 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998). 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). GnRH-I concentration is significantly elevated in the POA during incubation (Millam et al., 1995). In turkeys, 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), and is greater in laying hens than those of the non-photostimulated and incubating hens. In addition, the least mRNA expression is observed in photorefractory hens (Kang et al., 2006). Measurements of hypothalamic 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) indicate that there is no change or a decrease in incubating birds. Moreover, removal of incubating hens from their nests results in an increase in LH secretion and is associated with an increase in the amount of GnRH mRNA in the hypothalamus (Dunn et al., 1996).

In mammals, GnRH perikaya axons are terminated 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 in mammals, GnRH perikarya and fibers are more widely distributed throughout the avian brain. A number of previous studies have examined the distributions of cGnRH-I throughout the avian brain including chickens (Kuenzel and Blahser, 1991), ducks (Bons et al., 1978), white-crowned sparrows (Blahser et al., 1989), Japanese quails (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; Sartsoongnoen, 2007). 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). Specific GnRH-I-ir neurons are also found in several hypothalamic regions. Several studies have reported the distributions of the cGnRH-I mRNA and its protein in the avian brains (Millam et al., 1989; Dunn and Sharp, 1999; Sun et al., 2001; Dawson et al., 2002; Kang et al., 2006). It has been indicated that the greatest cGnRH-I mRNA expressions are in the nCPa and around the organum vasculosum laminae terminalis

(OVLT). The cGnRH-I mRNAs are more abundance within the nCPa, OVLT, and SL of the laying turkey hens than those of the non-photostimulated and incubating ones (Kang et al., 2006).

As aforementioned, the differential expression of GnRH-I neurons has been reported in many temperate zone species, but little is known about the data regarding neuroendocrine regulation in the 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; Kosonsiriluk et al., 2008; Sartsoongnoen, 2007). The findings indicate that changes in the number of GnRH-I-ir neurons in the nCPa are observed across the reproductive cycle. The highest number of GnRH-I-ir neurons is observed in the nCPa of laying hens compared to other reproductive stages (Sartsoongnoen, 2007). There is limited study delineating the anatomical distribution and functional aspect of the GnRH system in controlling incubation behavior in the native Thai chickens. The aim of this study was to investigate whether the differential expression of GnRH-I-ir neurons within the hypothalamic areas were correlated with incubation behavior in the native Thai chickens. Differences in the number of GnRH-I-ir neurons within the hypothalamic areas of incubating hens with those of nest-deprived hens were compared. The findings of differential expression of GnRH-I in the hypothalamic areas with the degree of hyperprolactinemia may give an insight into the mechanism(s) underlying the regulation of incubation behavior in this equatorial species.

5.3 Materials and Methods

5.3.1 Experimental Animals

Female native Thai chickens (*Gallus domesticus*), Pradoohangdam breed, were used. They were reared and housed with mature roosters (8-9 females : 1 male) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of dark; 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; incubating eggs (INC) and non-incubating or nest deprivation (ND). Hens in the INC group had stopped laying and allowed to sit on the nests for three to four times per day showing incubating behavior. They were allowed to incubate their eggs naturally. Hens in the ND group were disrupted from incubating behavior and not allowed to incubate their eggs by removing them from their nests 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

5.3.2.1 Experiment I

Twelve female and 2 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 2 floor pens (6 hens : 1 rooster) and observed their daily behaviors. Hens were divided into two groups; INC and ND. The hens were sacrificed at day 10 after they started to incubate their eggs or after nest deprivation. The brains were pressure-perfused, sectioned with a cryostat, and

processed by immunohistochemistry (IHC) to localize and identify GnRH-I-ir neurons in the brain. The reproductive stages were identified by behavioral observation and confirmed by postmortem examination at the end of the experiment.

5.3.2.2 Experiment II

Seventy eight female and 10 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 10 floor pens (7-8 hens : 1 rooster) and observed their daily behaviors. Hens were divided into two groups; INC and ND. The hens were then sacrificed at different time periods (day 3, 6, 8, 10, 14, 18, and 21; n=6) after they started to incubate their eggs or after nest deprivation. The brain of each hen was pressure-perfused, sectioned with a cryostat, and processed by IHC to visualize and analyze the changes in the number of GnRH-I-ir neurons in the nCPa area. The reproductive stages were identified by behavioral observation and confirmed by postmortem examination at the end of the experiment.

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, 2007). 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 brain area were processed by IHC in order to visualize and analyze the changes in the number of GnRH-I-ir neurons.

5.3.4 Immunohistochemistry

Changes in the number of GnRH-I-ir neurons in the hypothalamus of INC and ND hens were conducted by IHC according to a previously described method (Sartsoongnoen, 2007). 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 INC and ND hens in the individual hypothalamic areas 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 in the nCPa. 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 per section (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. The 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

5.4.1 Experiment I

The expression of hypothalamic GnRH-I-ir neurons was observed at day 10 of incubation and nest deprivation. The expression of GnRH-I-ir neurons within the nucleus anterior medialis hypothalami (AM), nucleus suprachaiasmaticus, pars medialis (SCNm), nCPa, SL, nucleus paraventricularis magnocellularis (PVN), and regio lateralis hypothalami (LHy) areas are shown in (Figures 5.1, 5.2, and 5.3). High expression of GnRH-I-ir neurons was found in the nCPa of ND hens (Figure 5.2), whereas less expression was observed in the nCPa of INC hens. Some GnRH-I-ir fibers were observed in the AM, SCNm, SL, PVN, and LHy of both INC and ND groups but the difference were not noted.

5.4.2 Experiment II

The differential expression of GnRH-I-ir neurons in the nCPa of INC and ND hens are shown in Figure 5.4. Changes in the number of GnRH-I-ir neurons in the nCPa of INC and ND hens at different time periods are shown in Figure 5.5 and Table 5.2. In the comparison of INC and ND groups, the number of GnRH-I-ir neurons in the nCPa was low in INC group. The number of GnRH-I-ir neurons increased when the hens were deprived of the nests showing a significant difference ($P<0.05$) at day 6

(INC6 vs ND6; 0.54 ± 0.31 vs 2.00 ± 0.56 cells), day 14 (INC14 vs ND14; 0.46 ± 0.28 vs 1.70 ± 0.28 cells), and day 21 (INC21 vs ND21; 0.21 ± 0.08 vs 2.56 ± 1.03 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

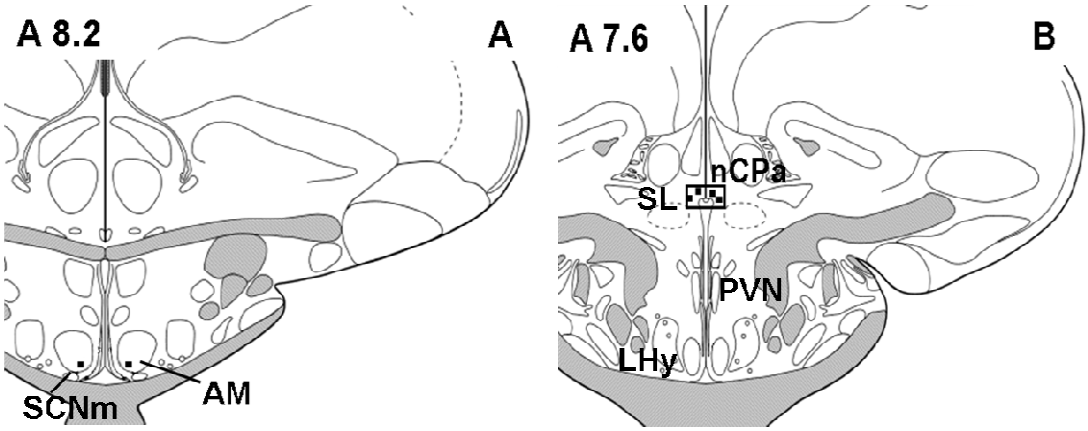

Figure 5.1 Schematic coronal brain sections showing the areas where the expression of GnRH-I-ir (black squares) was observed (**A-B**). The sampling regions for counting the number of GnRH-I-ir neurons in the nCPa (**B**) are represented by rectangles. Coronal illustrations were redrawn from the stereotaxic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

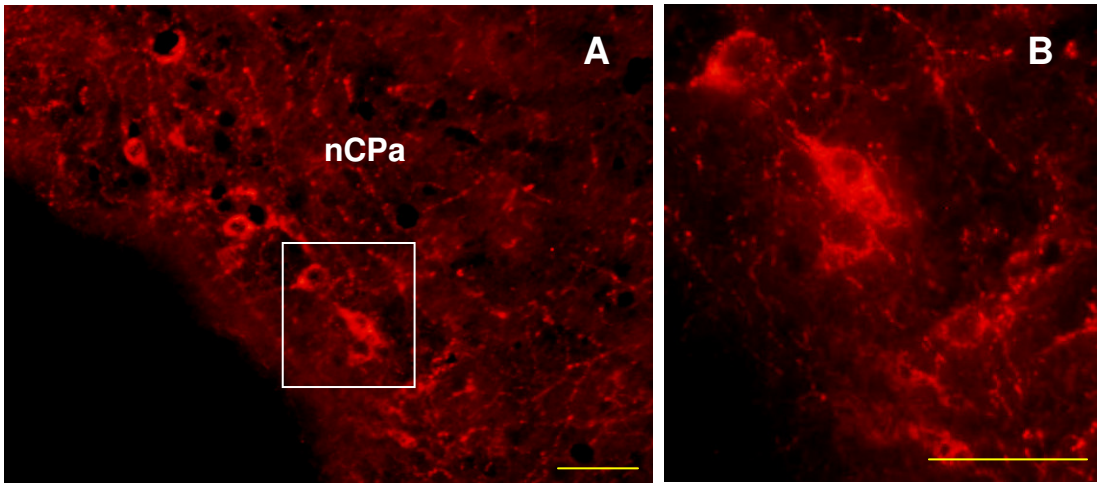

Figure 5.2 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the nucleus commissurae pallii (nCPa) of the native Thai chickens (**A**). Rectangle indicates area from which following photomicrograph is taken. Higher magnification of the GnRH-I-ir neurons in the nCPa (**B**). Bar = 50 µm.

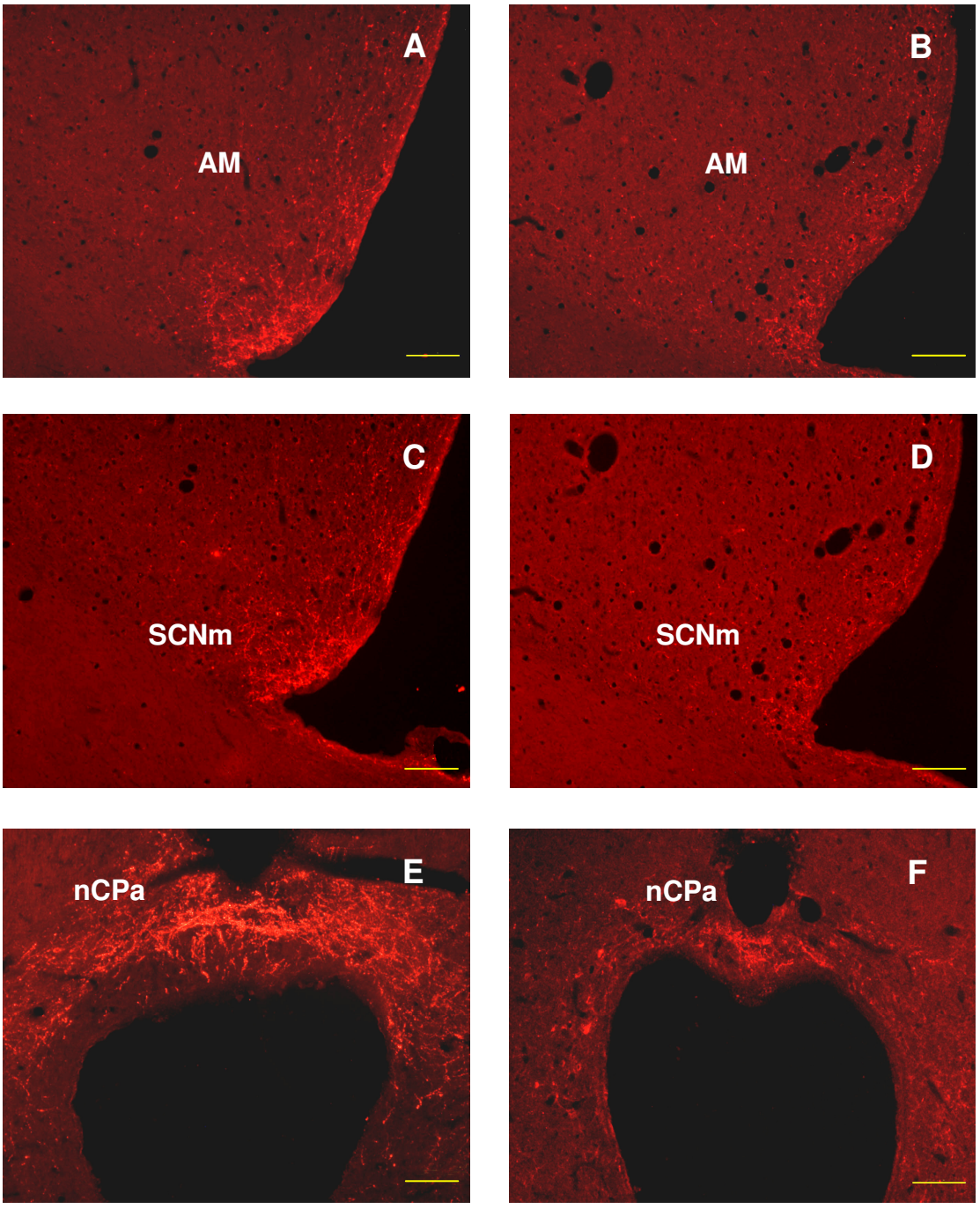

Figure 5.3 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, and **K**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, and **L**) native Thai hens. For abbreviations, see Table 5.1. Scale bar =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

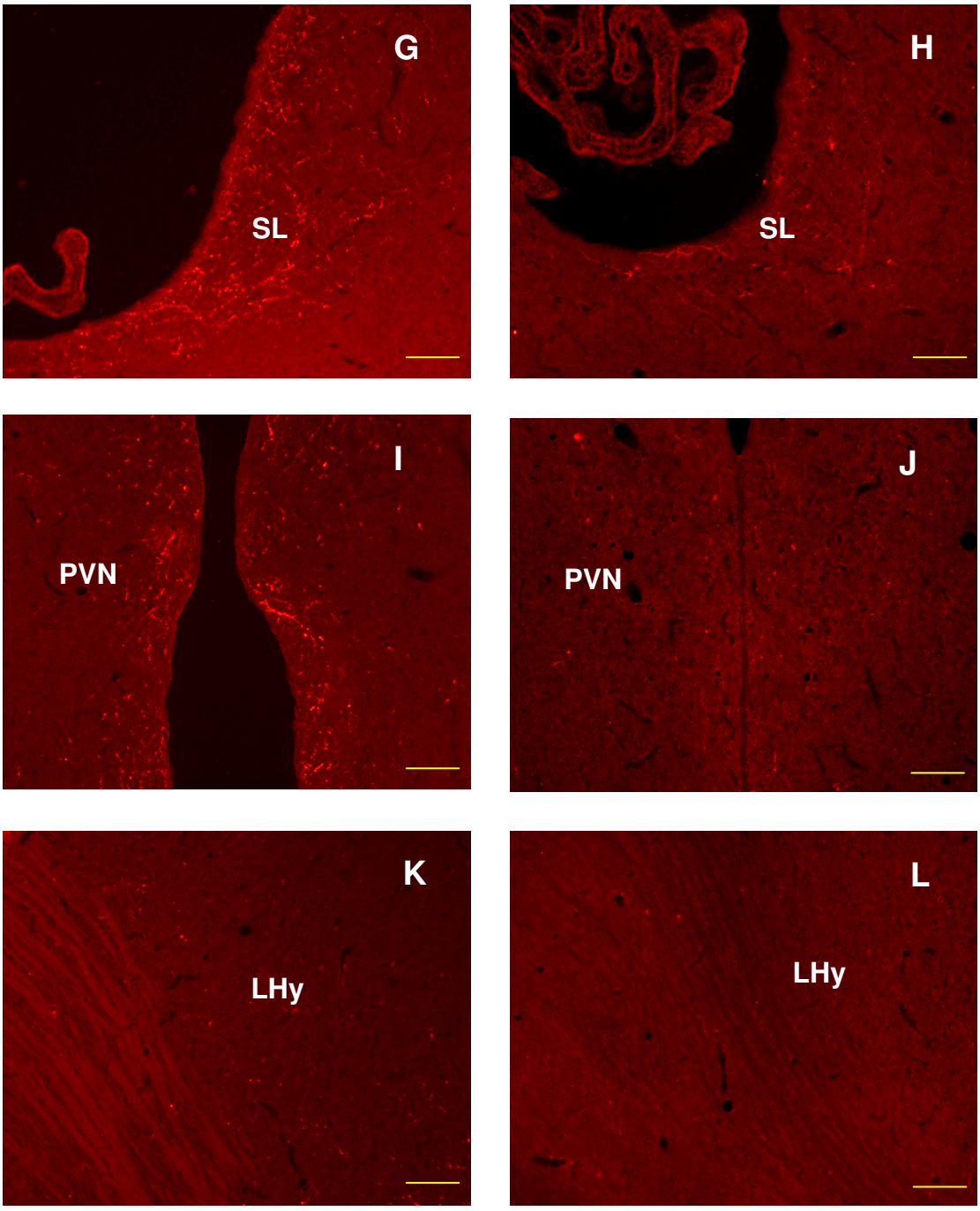

Figure 5.3 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, and **K**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, and **L**) native Thai hens. For abbreviations, see Table 5.1. Scale bar =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

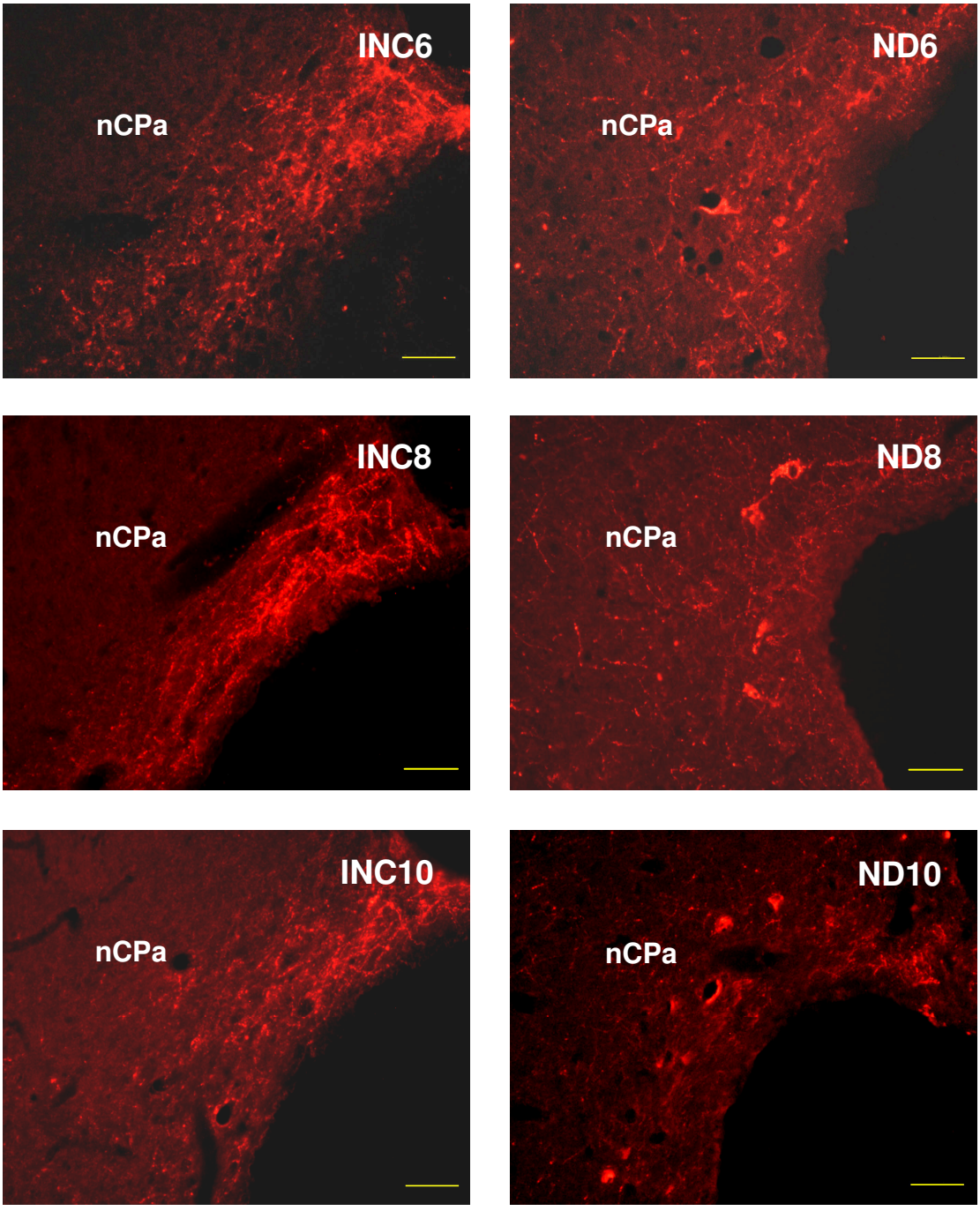

Figure 5.4 Photomicrographs showing the distributions of GnRH-I-ir neurons and fibers in the nucleus commissurae pallii (nCPa) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 5.1. Scale bar = 100 µm.

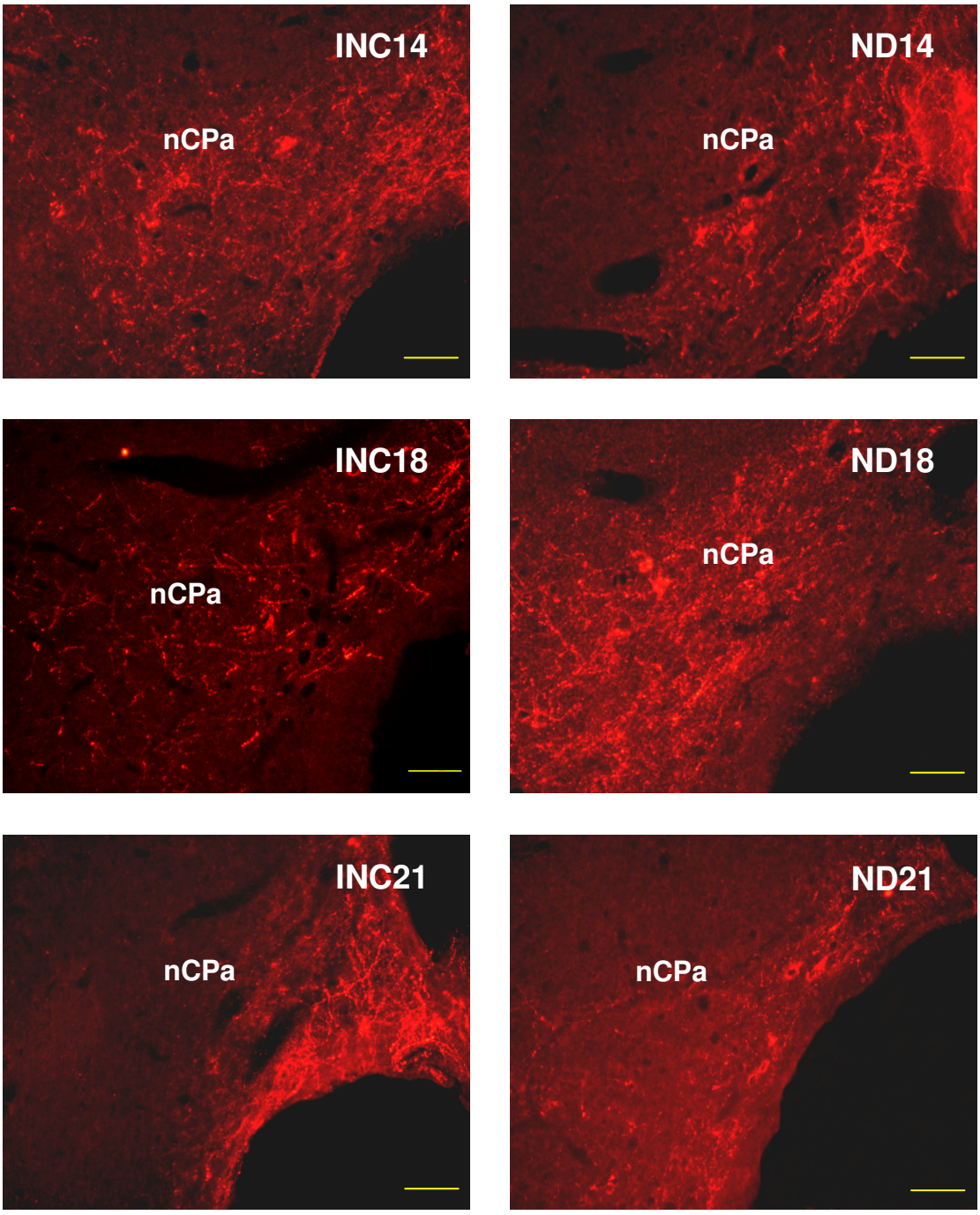

Figure 5.4 Photomicrographs showing the distributions of GnRH-I-ir neurons and fibers in the nucleus commissurae pallii (nCPa) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 5.1. Scale bar = 100 µm (continued).

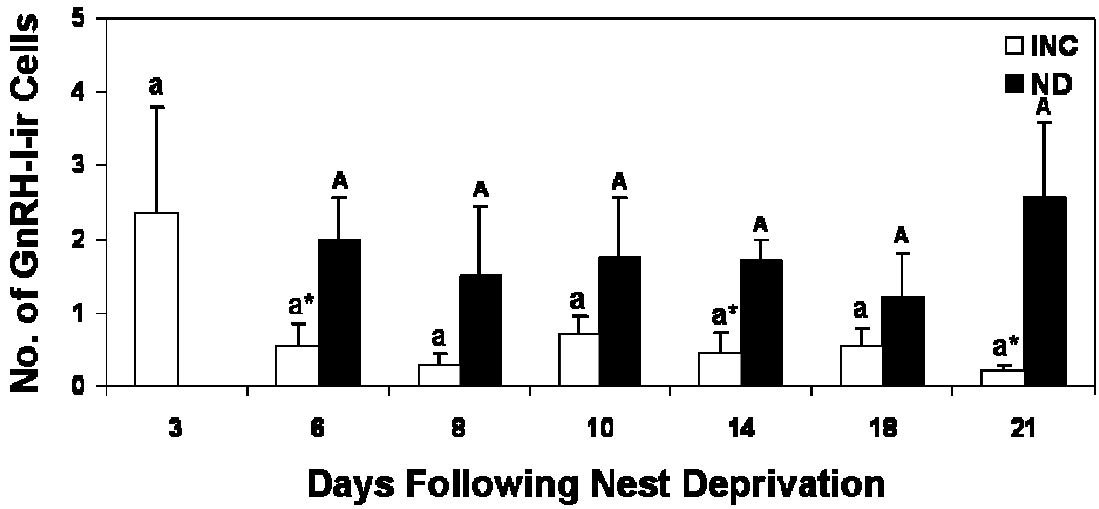

Figure 5.5 Changes in the number of GnRH-I-ir neurons in the nCPa of incubating (INC) and nest-deprived (ND) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ 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 incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation (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 Nest Deprivation**						
	3	**6**	**8**	**10**	**14**	**18**	**21**
INC	2.35 ± 1.44 [a]	0.54 ± 0.31 [a]	0.29 ± 0.15 [a]	0.71 ± 0.24 [a]	0.46 ± 0.28 [a]	0.54 ± 0.25 [a]	0.21 ± 0.08 [a]
ND	N/A	2.00 ± 0.56 [a*]	1.50 ± 0.95 [a]	1.75 ± 0.82 [a]	1.70 ± 0.28 [a*]	1.20 ± 0.60 [a]	2.56 ± 1.03 [a*]

5.5 Discussion

The results from this present study revealed that the GnRH-I-ir neurons and fibers were distributed throughout the brain of the nest-deprived native Thai chickens and were predominantly expressed in the nCPa area. GnRH-I-ir fibers were found in the AM, SCNm, SL, PVN, and LHy of both INC and ND hens. The high accumulation of GnRH-I-ir neurons was found in the nCPa of ND hens. Changes in the number of GnRH-I-ir neurons in the hypothalamus of INC and ND hens were also observed in the nCPa. The number of GnRH-I-ir neurons in the nCPa was low in INC group and significantly increased by days 6 of nest deprivation. These findings indicate the association of GnRH/FSH-LH system with the incubation behavior in this non-photoperiodic, continuously breeding avian species.

The obvious group of GnRH-I-ir neurons and fibers found in the nCPa in this present study is corresponded with the previous studies in native Thai chickens (Sartsoongnoen, 2007) and turkeys (Teruyama and Beck, 2001). Changes in the number of GnRH-I-ir neurons in the nCPa of INC and ND hens also correspond with the changes of these neurons in the nCPa across the reproductive cycle of the native Thai chickens (Sartsoongnoen, 2007). The greatest number of GnRH-I-ir neurons in the nCPa is found in laying and then decrease in incubating and non-laying, with the lowest numbers are found in rearing stages (Sartsoongnoen, 2007). It has been reported that birds at the peak level of reproductive activity have more GnRH-I-ir cells and fibers than those of sexually inactive or photorefractory birds (Sharp et al., 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998; Stevenson and MacDougall-Shackleton, 2005). The 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 the laying hens than that of the non-laying hens (Advis et al., 1985). Furthermore, GnRH-I contents is significantly elevated in the POA during incubation period (Millam et al., 1995). However, the changes in the number of GnRH-I-ir neurons within the nCPa are not observed in the turkeys, but changes in the intensity of GnRH-I-ir neurons in this area are found across the reproductive cycle (Al-Zailaie, 2003).

Disruption of incubation behavior by nest deprivation results in the increased in plasma LH and estradiol concentrations, and decreased in plasma PRL levels (El Halawani et al., 1980; Sharp et al., 1988; Dunn et al., 1996; Richard-Yris et al., 1998). The changes in plasma concentrations of LH and PRL are reversed when hens re-nested (Sharp et al., 1988). In the present study, the number of GnRH-I-ir neurons in the nCPa of ND hens was increased by day 6 of nest deprivation when compared with INC hens. These data are in good agreement with the previous study indicating that after incubating hens were deprived of their eggs for 5 days, the LH secretion was increased. This increase in LH levels is associated with a significant increased in hypothalamic contents of cGnRH-I mRNA (Dunn et al., 1996). These findings support the previous data showing that a decrease in the expression of GnRH-I influenced in maintaining the depression of LH secretion in incubating chickens.

Removal of native Thai chickens from their nests results in an increase in the number of GnRH-I-ir neurons in the nCPa (Prakobsaeng et al., 2009; this study) and VIP-ir neurons in the IH-IN, and a dramatic decline in plasma PRL levels. In addition, this disruption of incubation behavior increases the ovary and oviduct weights

(Prakobsaeng et al., 2009). Therefore, the number of GnRH-I-ir neurons in the nCPa which decrease in INC hens but increase in ND hens may involve in the regulation of PRL and LH secretion and incubation behavior.

It has been suggested that 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). Moreover, PRL acts concomitantly with VIP to inhibit LH by means of reduction of GnRH at the hypothalamic level (Rozenboim et al., 1993b). Immunoneutralization of VIP increases the pituitary content of LH-β and FSH-β mRNAs and is accompanied by a decline in PRL mRNA expression (Ahn et al., 2001). In addition, the relationship between the DAergic and the GnRH-I systems have been demonstrated by photostimulation in the turkeys. 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). Moreover, it has been reported that GnRH perikaya axons are terminated in the ME, which is closed proximity to the terminals of tuberoinfundibular DA neurons (Ajika, 1979; Merchenthaler et al., 1984; Ugrumov et al., 1989). It has 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 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). These data support an association of GnRH-I, VIP, and DA in the regulation of reproductive cycle in birds.

In summary, this present study demonstrates that nest deprivation of incubating chickens increases the number of GnRH-I-ir neurons in the nCPa, suggesting that GnRH-I neurons in this brain area may involve in the regulation of PRL and LH secretion and incubation behavior. Furthermore, the onset of incubation behavior in the native Thai chickens might be, in part, regulated by the differential expression of GnRH-I neurons in the nCPa.

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CHAPTER VI

EFFECTS OF INCUBATION BEHAVIOR UPON THE NEUROENDOCRINE REGULATION OF THE REPRODUCTIVE SYSTEM IN THE FEMALE NATIVE THAI CHICKENS: ROLE OF DOPAMINE

6.1 Abstract

Dopamine (DA) is a neurotransmitter/neuromodulator found in both central and peripheral nervous systems of many vertebrate species. DA influences gonadotropin releasing hormone/luteinizing hormone-follicle stimulating hormone and vasoactive intestinal peptide (VIP)/prolactin (PRL) systems in the regulation of avian reproductive cycle. In mammals, DA is released from the hypothalamic tuberoinfundibular DA neurons and serves as the physiological inhibitor of PRL secretion and is mediated through the D_2 DA receptors located on pituitary lactotrophs. Removal of this dopaminergic (DAergic) inhibition results in an increase in PRL secretion and hyperprolactinemia. This is not the case in birds, where removal of hypothalamic inputs results in the completed cessation of PRL secretion. In birds, it has been well established that DAergic influences are involved in stimulating and inhibiting of avian PRL secretion. It is suggested that DA stimulates PRL secretion at the hypothalamic level via D_1 DA receptors residing in the infundibular nuclear complex, whereas the VIP neurons are located and inhibits PRL at the pituitary level

via D_2 DA receptors by blocking the action of VIP. It has been suggested that the differential expression of DA neurons may play a significant role in the control of VIP secretion and subsequent PRL release. In addition, in birds, DAergic activity and DA receptor subtype mRNA expression change according to different physiological states and reproductive behaviors. The objective of this study was to investigate whether the DAergic neurons are associated in the regulation of incubation behavior in the native Thai hens using immunohistochemistry technique. Changes in DAergic neurons in the brain of incubating (INC) with those of nest-deprived (ND) native Thai hens were compared, utilizing tyrosine hydroxylase (TH, the rate-limiting enzyme for DA synthesis) as a marker for DAergic activity. The differential expression of TH-ir neurons within the hypothalamic areas correlated with incubation behavior and the degree of hyperprolactinemia in the native Thai chickens were determined. The results revealed that the expression of hypothalamic TH-ir neurons within the nucleus anterior medialis hypothalami, nucleus suprachaiasmaticus, pars medialis, organum paraventriculare, regio lateralis hypothalami, nucleus ventromedialis hypothalami, nucleus inferioris hypothalami (IH), nucleus infundibuli hypothalami (IN), nucleus intramedialis (nI), and nucleus mamillaris lateralis (ML) areas were observed in both treatment groups. The high density of TH-ir neurons was noted in the nI and ML areas. Significance changes in the number of TH-ir neurons of INC and ND hens were observed in the nI and ML areas. The number of TH-ir neurons in the nI was high during incubating period and significantly decreased by day 10 of nest deprivation. In the ML, the number of TH-ir neurons significantly decreased by day 6 of nest deprivation. This study implicates that nest deprivation of incubating chicken decreases the number of TH-ir neurons in the nI and ML. The findings from other

studies indicated that nest deprivation of incubating chicken reduces circulating PRL levels and is associated with a reduction in the number of hypothalamic VIP-immunoreactive neurons in the IH-IN and an increase in the number of hypothalamic GnRH-I-immunoreactive neurons in the nucleus commissurae pallii. The finding from this study also reveals a parallel decrease in the number of TH-ir neurons observed in the nI and ML of nest-deprived chickens which suggests that nesting activity stimulates PRL secretion by the activation of the DAergic system at the nI and ML, which in turn, stimulates VIP, the avian PRL releasing factor. These elevated PRL levels increase nesting activity and maintain incubation behavior in the native Thai chickens.

6.2 Introduction

There are two major neuroendocrine systems play a pivotal role in the reproductive cycle of avian species. One system involves gonadotropin releasing hormone-I (GnRH-I) and the subsequent secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH; Sharp et al., 1998), GnRH/FSH-LH system and the other system involves vasoactive intestinal peptide (VIP) and the subsequent secretion of prolactin (PRL; Chaiseha and El Halawani, 2005), VIP/PRL system. Both systems are influenced by dopamine (DA; Bhatt et al., 2003; Chaiseha et al., 2003).

DA is a neurotransmitter/neuromodulator found in both central and peripheral nervous systems of many vertebrate species. Limited production of DA occurs in the adrenal medulla and also non-neuronal tissues such as pancreas and anterior pituitary gland (Ben-Jonathan and Hnasko, 2001). In mammals, DA has the main function to inhibit the release of PRL from the anterior pituitary as the principle PRL-inhibiting

factor. It has been reported that the concentrations of DA in hypophysial portal blood are maintained at the physiologically active levels (Ben-Jonathan et al., 1977; Gibbs and Neill, 1978; Ben-Jonathan et al., 1980) and the pituitary lactotrophs contain DA receptors (Caron et al., 1978; Cronin et al., 1978; Goldsmith et al., 1979). It has been suggested that DA which is released from the hypothalamic tuberoinfundibular DA (TIDA) neurons serves as the physiological inhibitor of PRL secretion (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001) and is mediated through the D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). DA and its agonists attenuate PRL secretion, PRL gene expression, and lactotrophs proliferation (Shaar and Clemens, 1974; Lamberts and MacLeod, 1990). Removal of this dopaminergic (DAergic) inhibition results in an increase in PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). This is not the case in birds, where removal of hypothalamic inputs results in the completed cessation of PRL secretion. However, several studies have been reported that DA at low concentrations stimulates PRL secretion (Shin, 1978; Denef et al., 1980; Burris et al., 1991; 1992; Arey et al., 1993; Porter et al., 1994). These suggest that all lactotrophs have the potential to respond to the inhibitory and stimulatory effects of DA (Kineman et al., 1994) or that a subpopulation of lactotrophs sensitive to the stimulatory effect of DA exists (Burris et al., 1992; Burris and Freemen, 1993) and the two opposite effects of DA upon PRL secretion may be mediated by distinct guanine nucleotide-binding proteins (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994). In rats, the stimulation of PRL secretion from the pituitary may be mediated through the D_1 and/or D_5 DA receptors (Porter et al., 1994). These data support the role of DA as the PRL-releasing factor.

In birds, it has been reported and well established that DAergic influences are involved in stimulating and inhibiting of avian PRL secretion. DA inhibits pituitary PRL release *in vitro* (Harvey et al., 1982; Hall and Chadwick, 1984; Hall et al., 1986; Xu et al., 1996). Intracerebroventricular (ICV) infusion of DA can either stimulate or inhibit PRL secretion depending upon the concentrations used (Youngren et al., 1995). Both stimulatory and inhibitory effects on avian PRL secretion are depended on multiple DA receptors (Youngren et al., 1996). It is suggested that DA stimulates PRL secretion at the hypothalamic level via D_1 DA receptors residing in the infundibular nuclear complex (INF), where the VIP neurons are located. DA also inhibits PRL at the pituitary level via D_2 DA receptors by blocking the action of VIP (Youngren et al., 1995; 1996; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003).

In birds, DAergic activity and DA receptor subtype mRNA expression change according to different physiological stages and reproductive behaviors. In bantam hens, DAergic activity in the anterior hypothalamus markedly increases in incubating birds when compared with laying or nest-deprived ones (Macnamee and Sharp, 1989). Stimulatory D_1 DA receptor mRNA expression has been found to increase in the hypothalamus of incubating turkey hens with hyperprolactinemia and in the pituitary gland of laying hens, whereas inhibitory D_2 DA receptor mRNA expression increases in the pituitary gland of photorefractory hens with hypoprolactinemia (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003). In addition, changes in DAergic activity during the turkey reproductive cycle mirrored the changes in plasma PRL levels, VIP immunoreactivity, VIP peptide contents, and VIP mRNA expression within INF (El Halawani et al., 1980; 1984; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003; 2004).

In avian species, the anatomical distribution of the avian DAergic system obviously resembles to that of mammals (Moons et al., 1994; Reiner et al., 1994). DA has been measured and visualized in many avian species including domestic fowls (Knigge and Piekut, 1985), quails (Bailhache and Balthazart, 1993; Absil et al., 2001), pigeons (Berk, 1991; Divac et al., 1994; Durstewitz et al., 1998), zebra finches (Bottjer, 1993; Mello et al., 1998), chickens (Moons et al., 1994; 1995), budgerigars (Roberts et al., 2001), collared doves (den Boer-Visser and Dubbeldam, 2002), turkeys (Al-Zailaie and El Halawani, 2000), and canaries (Appeltants et al., 2001). DA neurons are found throughout the avian hypothalamus (Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been shown to be immunoreactived for VIP (Hof et al., 1991; Mauro et al., 1992) and VIP mRNA (Kuenzel et al., 1997; Chaiseha and El Halawani, 1999). Moreover, several DA neuronal groups have been observed in the preoptic hypothalamic areas of the turkeys (Al-Zailaie and El Halawani, 2000; Al-Zailaie, 2003). The distributions of tyrosine hydroxylase-immunoreactive (TH-ir) positive and DA-ß-hydroxylase (DBH) negative cells are found in the hypothalamus of turkeys and other avian species (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Reiner et al., 1994; den Boer-Visser and Dubbeldam, 2002) and TH-ir neurons are predominantly located within the diencephalon and mesencephalon.

The changes in the number of TH-ir neurons are observed in the nucleus intramedialis (nI) across the reproductive cycle of the native Thai chickens (Sartsoongnoen et al., 2008). Given their widespread distributions, the findings that DA axons and terminals are found intermingled with VIP neurons in the INF, GnRH neurons in the preoptic areas, and with both VIP and GnRH terminals in the external

layer of the median eminence (ME; Contijoch et al., 1992; Fraley and Kuenzel, 1993), it is reasonable to consider whether any regional specificity exists in those DA neurons that are neuroendocrine in nature, i.e., controlling the release and expression of VIP/PRL and GnRH/FSH-LH systems.

A study in turkeys has indicated an association between DAergic cells in the nucleus mamillaris lateralis (ML) with GnRH-I and VIP neurons. (Al-Zailaie et al., 2006). Recent findings demonstrate that the presence of DA-melatonin (MEL) neurons in the nucleus premamillaris (PMM) of the turkey hypothalamus, where DA and MEL are synthesized and co-localized. It is suggested that the pattern of serotonin/catecholamine neuronal distributions and their variable interaction with PMM DA-MEL neurons during different reproductive stages may offer a significant neuroanatomical basis for understanding the control of avian reproductive seasonality and may constitute a critical cellular process involved in the generation and expression of seasonal reproductive rhythms and suggests a previously undescribed mechanism(s) by which light signals gain access to neural targets in seasonally breeding temperate zone birds (Al-Zailaie et al., 2006; Kang et al., 2007; 2009; 2010; Thayananuphat et al., 2007a; 2007b; El Halawani et al., 2009).

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; Kosonsiriluk et al., 2008; Sartsoongnoen, 2007). In native Thai chickens, study by using TH, the rate-limiting enzyme for DA synthesis, has been reported. The changes in the number of TH-ir neurons in the nI are correlated with changes in PRL levels across the reproductive cycle of the native Thai chickens (Sartsoongnoen et al., 2008). The

population of TH-ir neurons in the nI increases significantly during the egg incubation period compared to non-laying hens, while plasma PRL levels show the same tendency of rising during egg laying and then reach the peak during incubating period (Sartsoongnoen et al., 2008). It is suggested that the differential expression of DA neurons in the nI may play a role in the control of VIP secretion and subsequent PRL release in this avian species (Sartsoongnoen et al., 2008). To date, there is limited study delineating the anatomical distribution and functional aspects of the DAergic system with incubation behavior in the native Thai chickens. The aim of this present study was to investigate whether the differential expression of TH-ir neurons (a marker for DAergic activity) within the hypothalamic areas are correlated with incubation behavior in the native Thai chickens. Changes in the number of TH-ir neurons within the hypothalamic areas of incubating hens with those of nest-deprived hens were compared. The findings of the differential expression of TH-ir neurons in the hypothalamus with the degree of hyperprolactinemia may provide an insight of the role of DA in the regulation of incubation behavior of the native Thai chickens.

6.3 Materials and Methods

6.3.1 Experimental Animals

Female native Thai chickens (*Gallus domesticus*), Pradoohangdam breed, were used. They were reared and housed with mature roosters (8-9 females : 1 male) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of dark; 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; incubating eggs (INC) and non-incubating or nest

deprivation (ND). Hens in the INC group had stopped laying and were allowed to incubate their eggs naturally by sitting on the nests for three to four times per day and showed incubating behavior. Hens in the ND group were disrupted from incubating behavior and not allowed to incubate their eggs by removing them from their nests 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.

6.3.2 Experimental Design

6.3.2.1 Experiment I

Twelve female and 2 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 2 floor pens (6 hens : 1 rooster) and observed their daily behaviors. Hens were divided into two groups; INC and ND. The hens were sacrificed at day 10 after they started to incubate their eggs or after nest deprivation. The brains were pressure-perfused, sectioned with a cryostat, and processed by immunohistochemistry (IHC) to localize and identify TH-ir neurons in the brain. The reproductive stages were identified by behavioral observation and confirmed by postmortem examination at the end of the experiment.

6.3.2.2 Experiment II

Seventy eight female and 10 male native Thai chickens at 20 weeks old were used. The chickens were randomly divided into 10 floor pens (7-8 hens : 1 rooster) and observed their daily behaviors. Hens were divided into two groups; INC and ND. The hens were then sacrificed at different time periods (day 3, 6, 8, 10, 14, 18, and

21; n=6) after they started to incubate their eggs or after nest deprivation. The brain of each hen was pressure-perfused, sectioned with a cryostat, and processed by IHC to visualize and analyze the changes in the number of TH-ir neurons in the nI and ML areas. The reproductive stages were identified by behavioral observation and confirmed by postmortem examination at the end of the experiment.

6.3.3 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 method previous described by Sartsoongnoen 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. Four adjacent sections of each individual brain area were processed by IHC to visualize and analyze the changes in the number of TH-ir neurons.

6.3.4 Immunohistochemistry

Changes in the number of TH-ir neurons in the hypothalamus of INC and ND hens by IHC were conducted according to a previously described method (Sartsoongnoen et al., 2008). The primary and secondary antibodies used for detecting TH-ir neurons were primary mouse monoclonal antibody raised directly against TH (ImmunoStar, Inc., Hudson, WI, USA) and Cy^{TM}3-conjugated AffiniPure donkey anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.), respectively. Four adjacent sections from INC and ND hens at different time periods 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 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 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 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.

6.3.5 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 TH-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 TH-ir neurons in the nI and ML. 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-TH antibody was tested by omission of the primary antibody during that step of immunohistochemistry. No immunostaining of TH was observed in control sections.

6.3.6 Statistical Analysis

Significant differences in the number of TH-ir neurons per section (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. The probability less than 0.05 ($P<0.05$) was considered statistically significant. All statistical tests were analyzed employing the SPSS for Windows software (version 13.0, SPSS Inc., Chicago, IL, USA).

6.4 Results

6.4.1 Experiment I

The expression of hypothalamic TH-ir neurons was observed at day 10 of incubating and nest-deprived native Thai hens. The expression of TH-ir neurons within the nucleus anterior medialis hypothalami (AM), nucleus suprachaiasmaticus, pars medialis (SCNm), organum paraventriculare (PVO), regio lateralis hypothalami (LHy), nucleus ventromedialis hypothalami (VMN) nucleus infundibuli hypothalami (IH), nucleus infundibuli hypothalami (IN), nI, and ML areas are shown (Figures 6.1 , 6.2, and 6.3). The high density of TH-ir neurons was observed in the nI and ML areas. The numbers of hypothalamic TH-ir neurons of INC and ND hens were compared. The results revealed that the highest accumulation of TH-ir neurons was found within the nI of INC hens (Figure 6.2; 36.58 ± 2.32 cells) and the number was decreased in ND hens ($P<0.05$; 21.38 ± 1.70 cells). TH-ir neurons abundance was also observed in the ML (Figure 6.2; INC vs ND; 16.63 ± 1.95 vs 13.14 ± 2.60 cells), but the difference between treatment groups was not statistically significant ($P>0.05$). In contrast, the number of TH-ir neurons in the AM tended to increase in the ND group. Some of the TH-ir neurons were found in the PVO in both groups (Figure 6.4 and Table 6.2). Moreover, a few number of TH-ir neurons were also found in the IH-IN of INC hens and the LHy and SCNm of both INC and ND hens. No TH-ir neurons were observed in the VMN of both groups. A dense accumulation of TH-ir fibers were found in the ME and nucleus mamillaris medialis (MM) as shown in Figure 6.5.

6.4.2 Experiment II

The differential expression of TH-ir neurons in the nI of INC and ND hens are illustrated in Figure 6.6. The number of TH-ir neurons in the nI of INC and ND hens are shown in Figure 6.7 and Table 6.3. When compared between INC and ND groups, TH-ir neurons counted significantly decreased in hens deprived of their nests for 10, 18, and 21 days ($P<0.05$; INC10 vs ND10; 36.58 ± 2.32 vs 21.38 ± 1.70, INC18 vs ND18; 40.83 ± 3.28 vs 26.21 ± 1.69, INC21 vs ND21; 33.13 ± 2.22 vs 24.50 ± 2.49 cells). The number of TH-ir neurons showed no difference across the 21 day period in both INC and ND groups.

The differential expression of TH-ir neurons in the ML of INC and ND hens are also shown in Figure 6.8. The numbers of TH-ir neurons in the ML of INC and ND hens are shown in Figure 6.9 and Table 6.3. The number of TH-ir neurons in the ML markedly declined by day 6 and day 8 of nest deprivation ($P<0.05$; INC6 vs ND6; 21.78 ± 1.32 vs 14.38 ± 1.17, INC8 vs ND8; 23.58 ± 2.35 vs 13.10 ± 1.67 cells) and then the numbers remained essentially the same in both groups. In the INC group, the number of TH-ir neurons in the ML tended to decrease by day 10 of incubation, whereas the number of TH-ir neurons in the ML of the ND group stayed in the same levels from day 6 throughout day 21. The distribution patterns of TH-ir neurons in the nI and ML areas were consistent in every INC hen. When the hens were nest-deprived, the number of TH-ir neurons decreased in the same discrete patterns.

Table 6.1 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
PVO	Organum paraventriculare
LHy	Regio lateralis hypothalami
VMN	Nucleus ventromedialis hypothalami
IH	Nucleus inferioris hypothalami
IN	Nucleus infundibuli hypothalami
ME	Eminentia mediana (Median eminence)
nI	Nucleus intramedialis
MM	Nucleus mamillaris medialis
ML	Nucleus mamillaris lateralis
V III	Ventriculus tertius (Third ventricle)

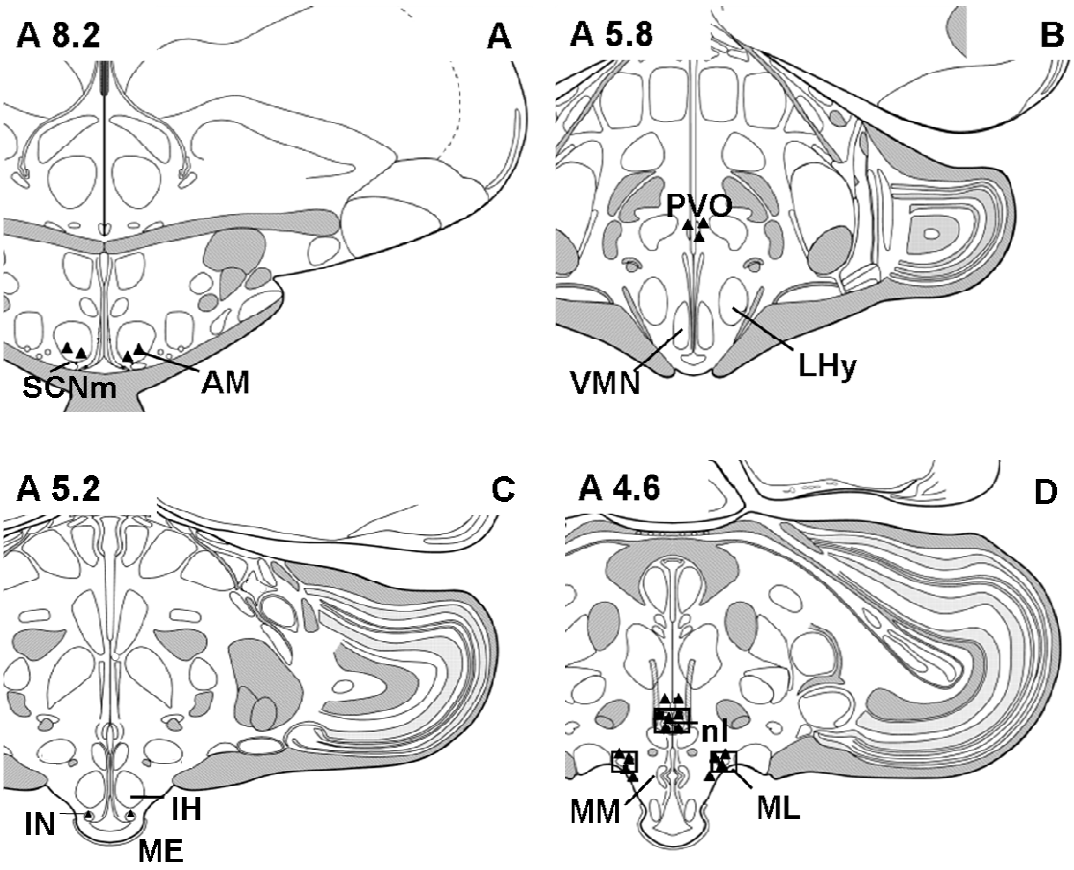

Figure 6.1 Schematic coronal brain sections showing the areas where the expression of TH-ir (black triangles) was observed (**A-D**). The sampling regions for counting the number of TH-ir neurons in the nI and ML (**D**) are represented by rectangles. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988).

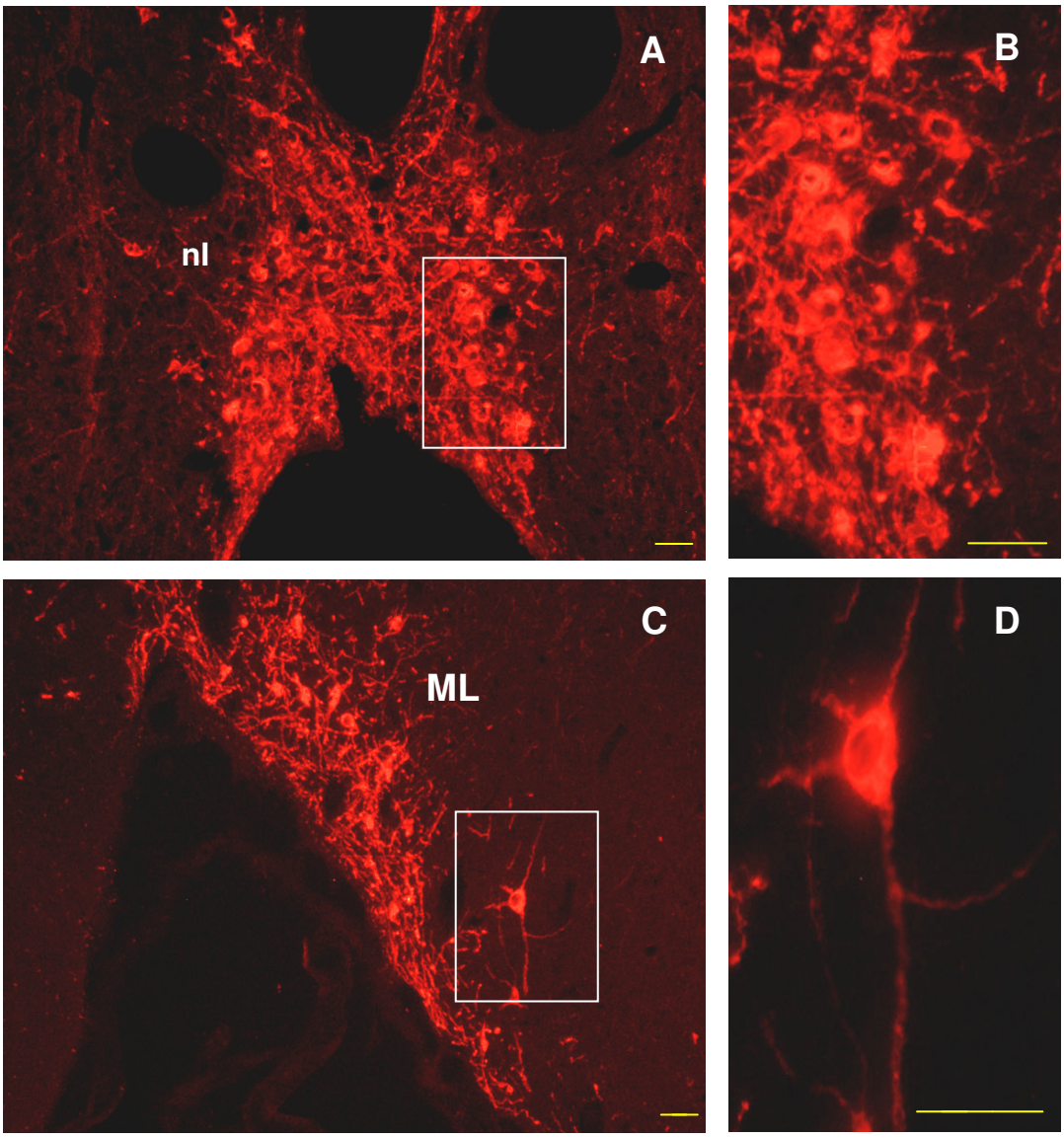

Figure 6.2 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the nucleus intramedialis (nI; **A**) and the nucleus mamillaris lateralis (ML; **B**) of the native Thai chicken. Rectangles indicate areas from which following photomicrographs are taken. Higher magnification of the TH-ir neurons in the nI (**B**) and ML (**D**). Bar = 50 µm.

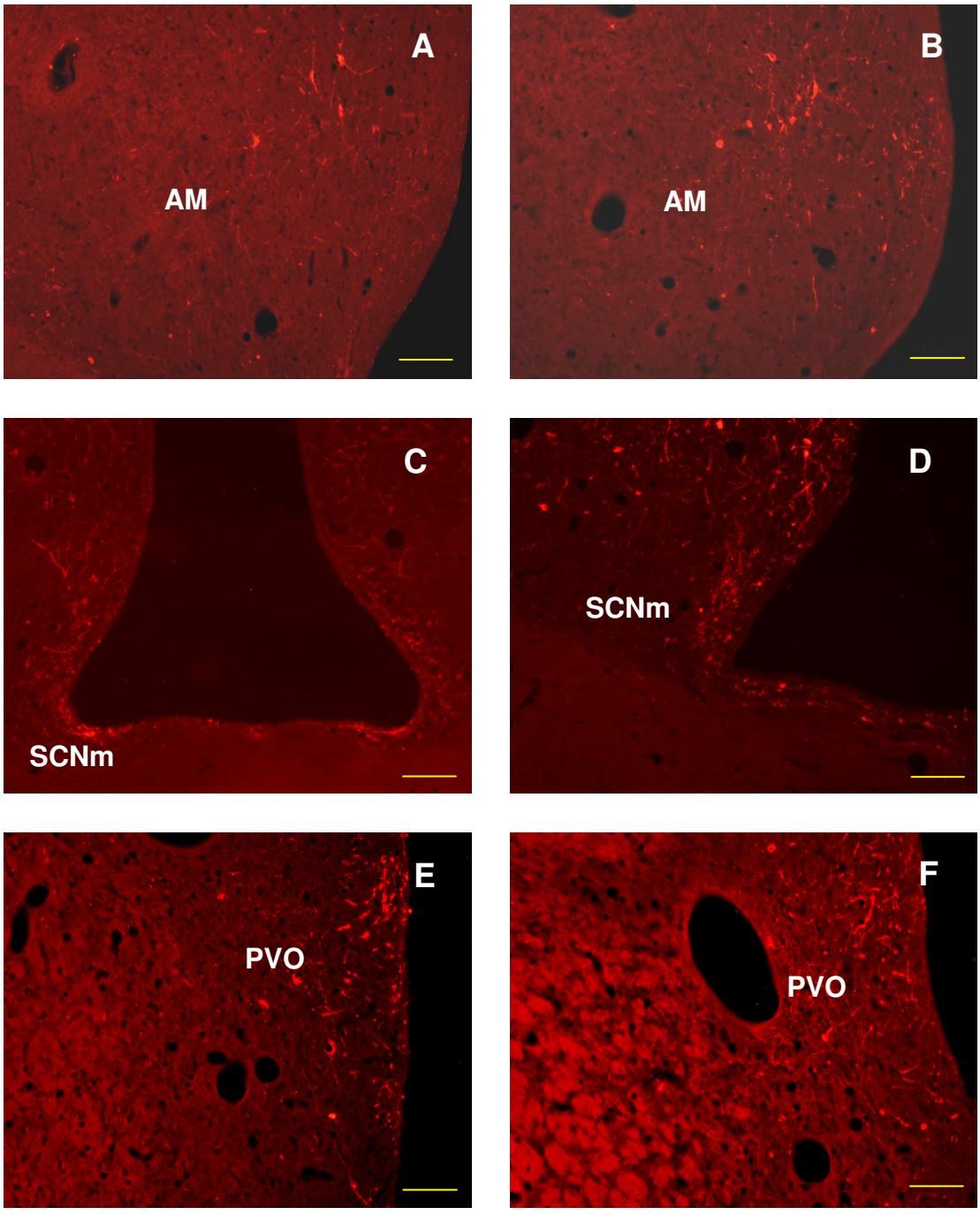

Figure 6.3 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P**) native Thai hens. For abbreviations, see Table 6.1. Scale bar = 100 µm.

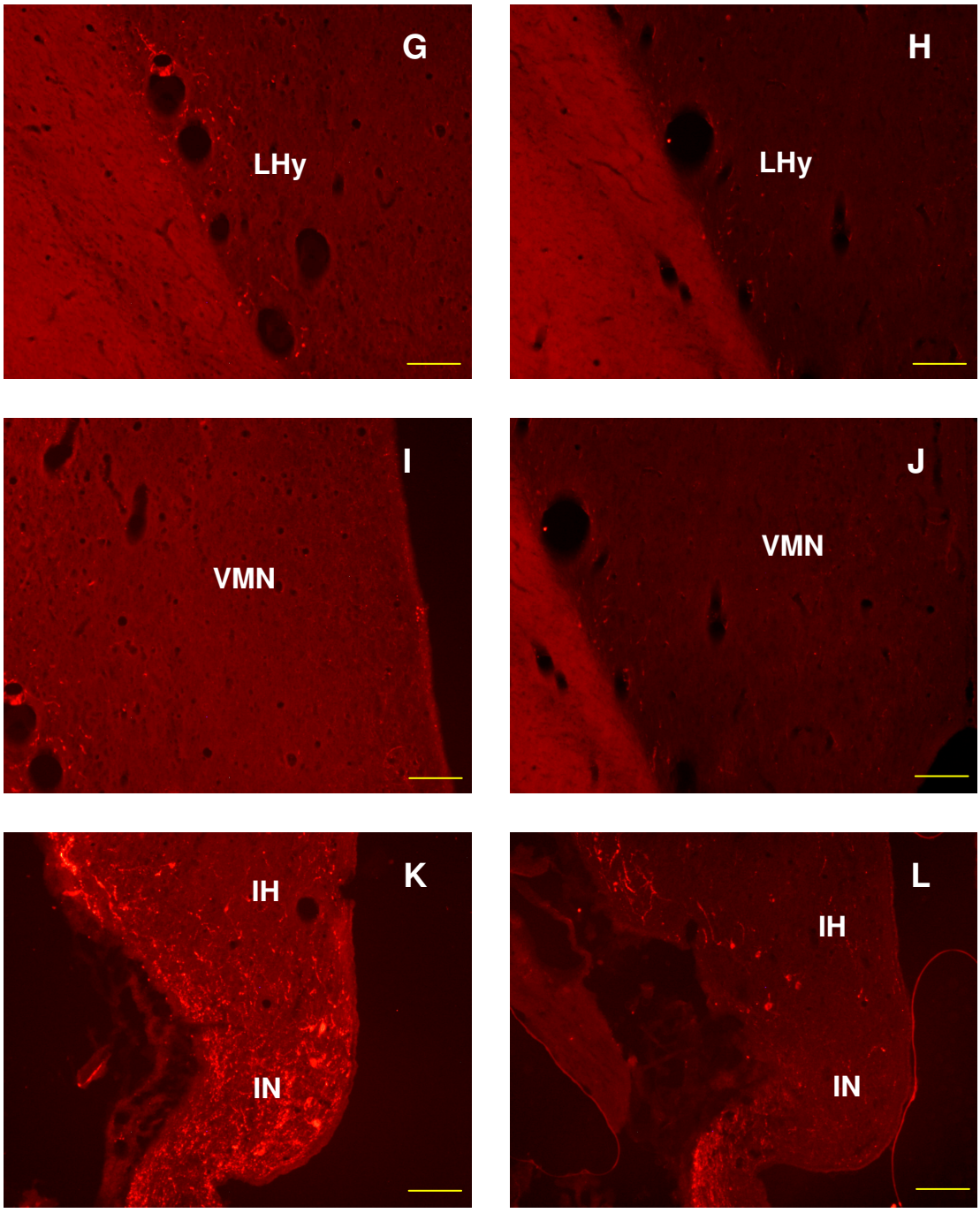

Figure 6.3 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P**) native Thai hens. For abbreviations, see Table 6.1. Scale bar = 100 µm (continued).

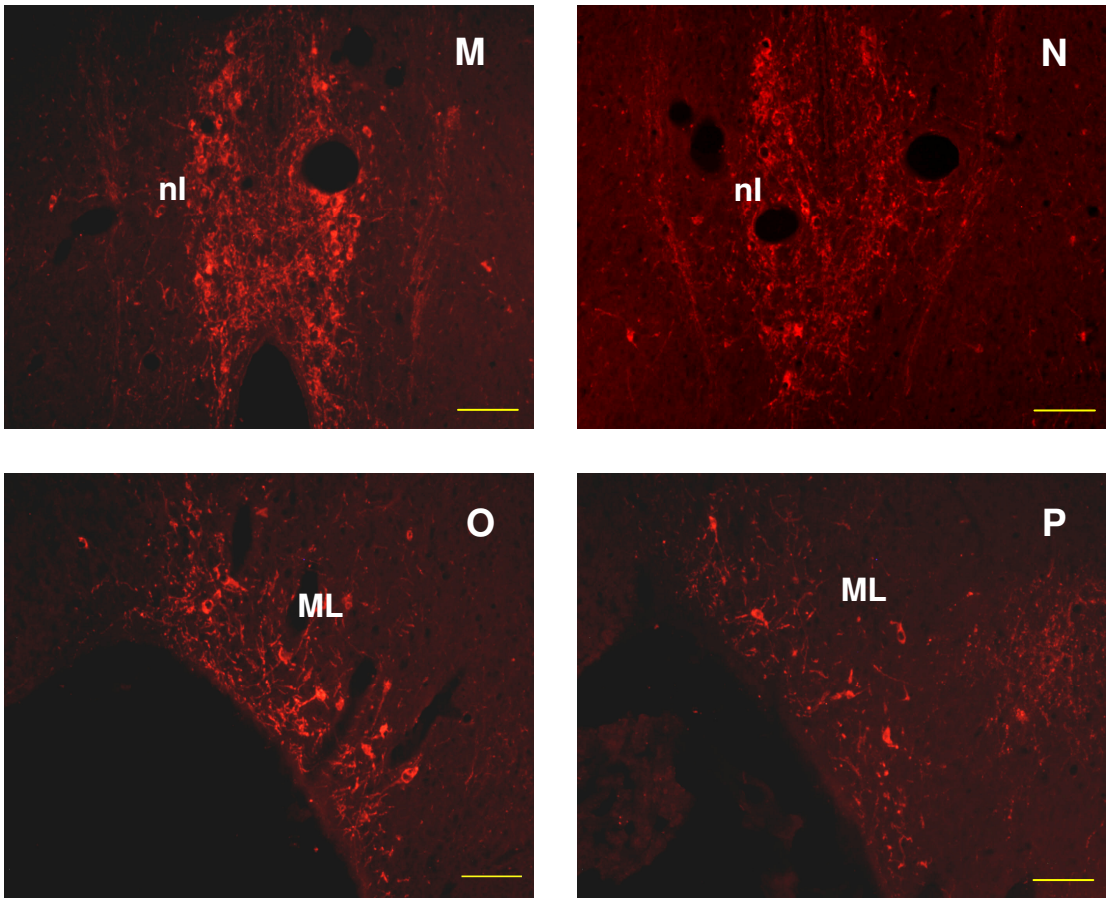

Figure 6.3 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the hypothalamus of incubating (**A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O**) and nest-deprived (**B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P**) native Thai hens. For abbreviations, see Table 6.1. Scale bar = 100 µm (continued).

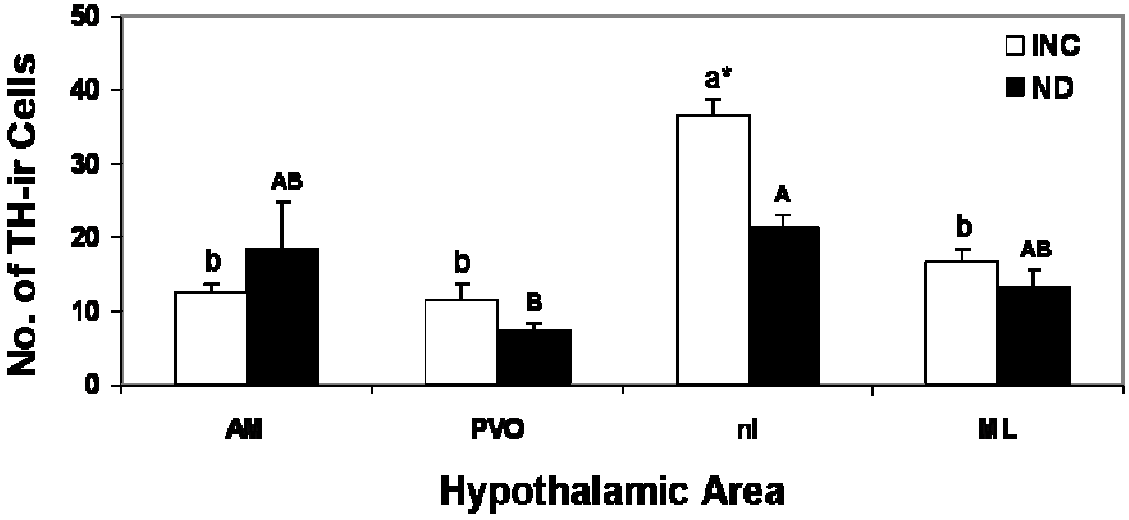

Figure 6.4 Changes in the number of TH-ir neurons in individual hypothalamic areas (AM, PVO, nI, and ML) of incubating (INC) and nest-deprived (ND) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different areas are denoted by different letters ($P<0.05$) and * $P<0.05$ for a comparison between group in each area.

Table 6.2 The number of TH-ir neurons in individual hypothalamic areas (AM, ML, nI, and PVO) of incubating (INC) and nest-deprived (ND) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different areas are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group in each area.

Group	**Hypothalamic Area**			
	AM	**PVO**	**nI**	**ML**
INC	12.33 ± 1.47^{b}	11.50 ± 2.24^{b}	36.58 ± 2.32^{a}*	16.63 ± 1.95^{b}
ND	18.47 ± 6.33^{ab}	7.29 ± 1.17^{b}	21.38 ± 1.70^{a}	13.14 ± 2.60^{ab}

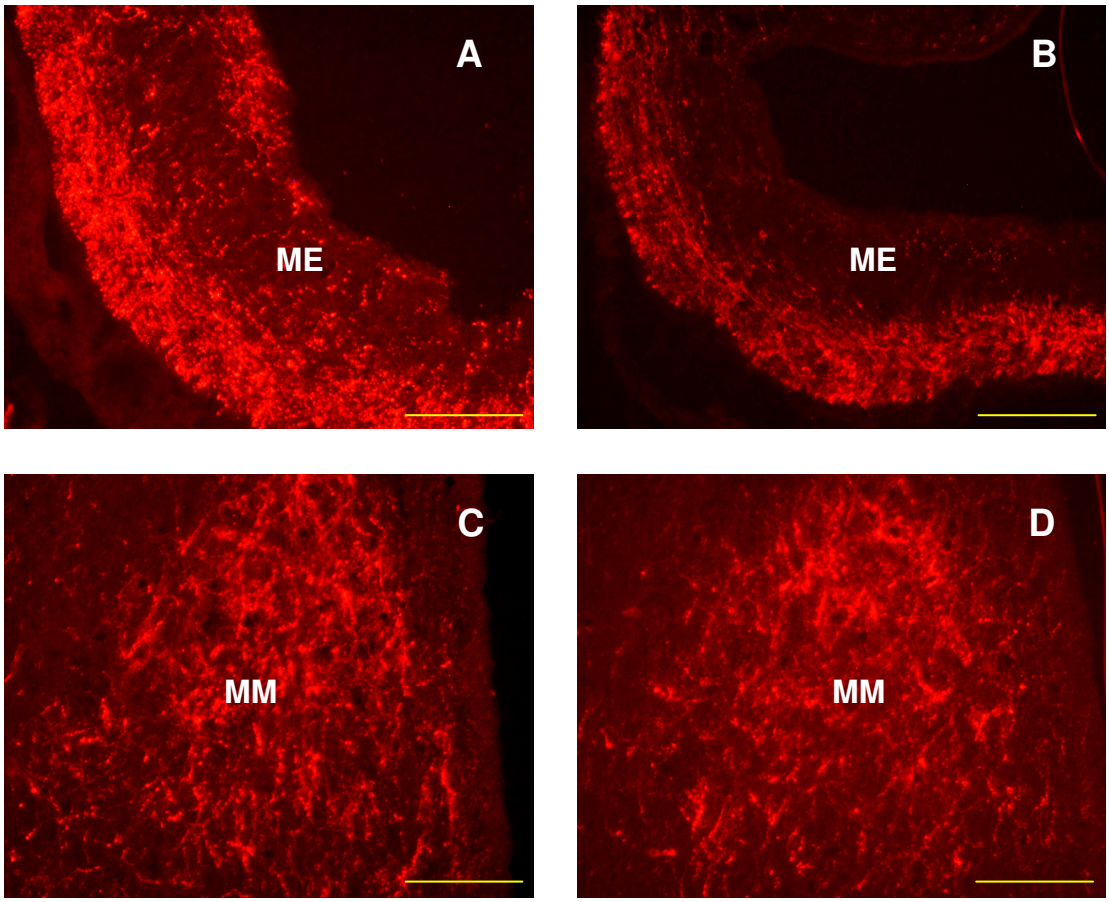

Figure 6.5 Photomicrographs showing the accumulations of TH-ir fibers in the median eminence (ME) and nucleus mamillaris medialis (MM) of incubating (**A** and **C**) and nest-deprived (**B** and **D**) native Thai hens. For abbreviations, see Table 6.1. Scale bar = 100 µm.

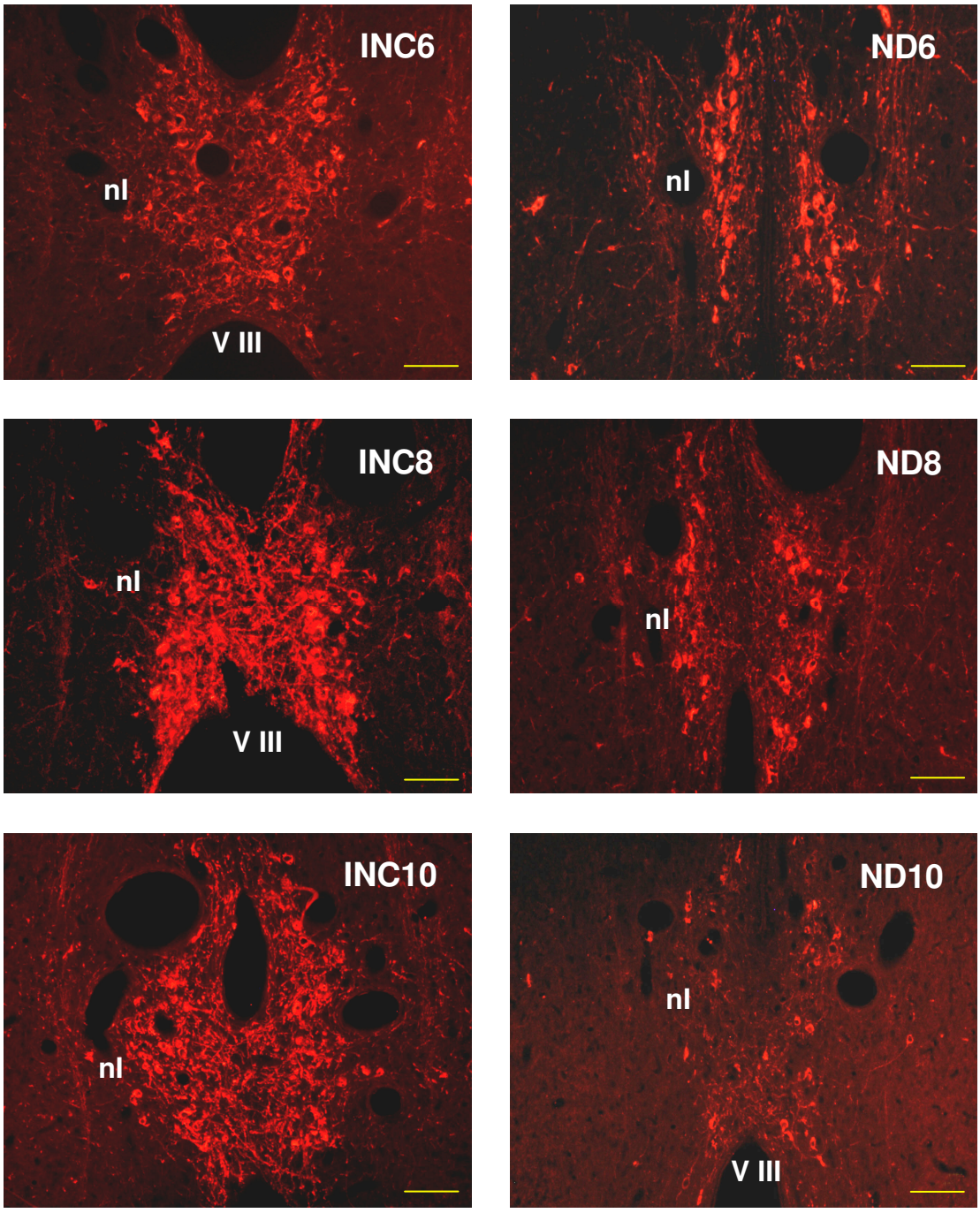

Figure 6.6 Photomicrographs showing the distributions of TH-ir neurons and fibers in the nucleus intramedialis (nI) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 6.1. Scale bar = 100 µm.

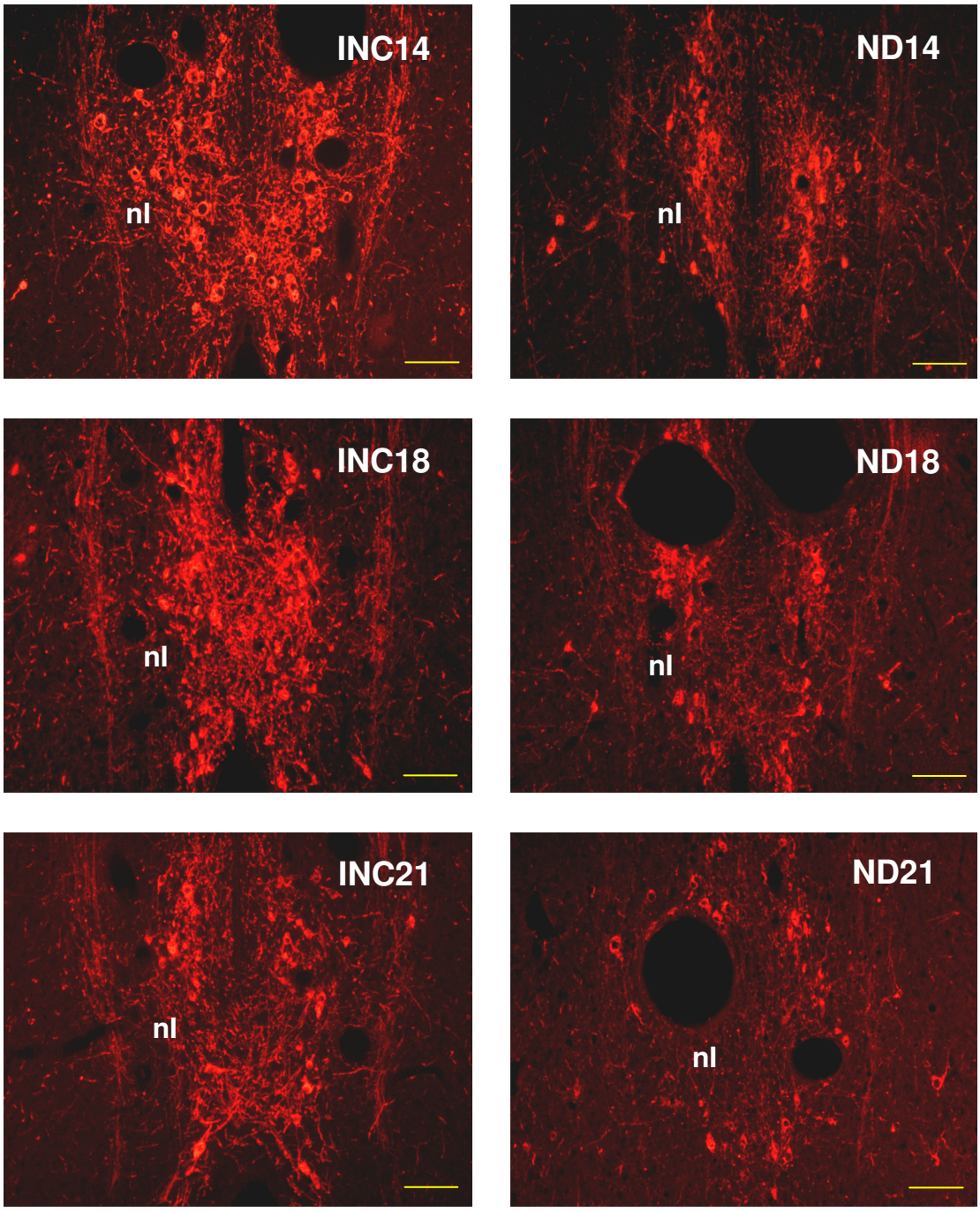

Figure 6.6 Photomicrographs showing the distributions of TH-ir neurons and fibers in the nucleus intramedialis (nI) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 6.1. Scale bar = 100 µm (continued).

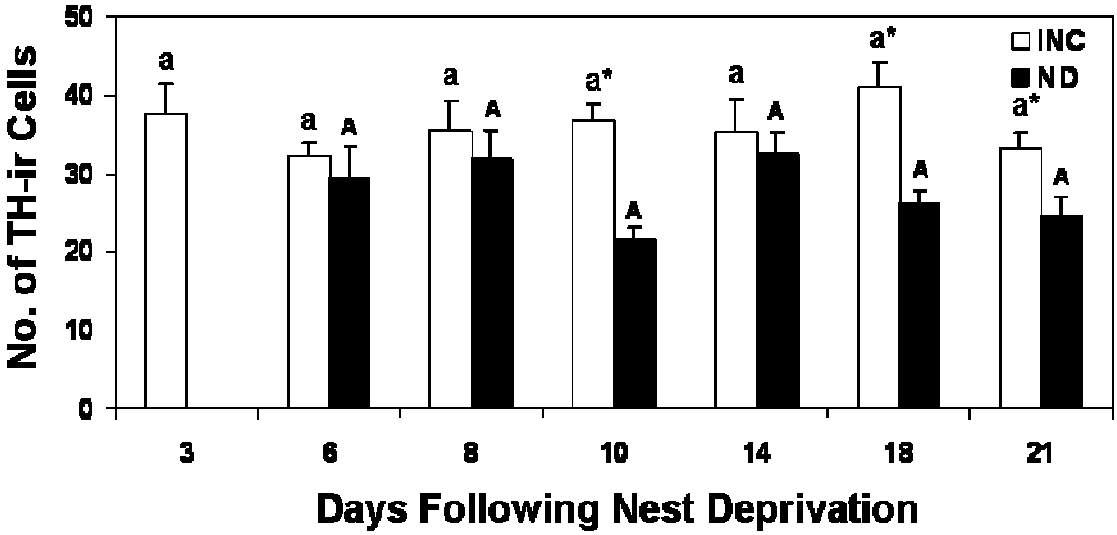

Figure 6.7 Changes in the number of TH-ir neurons in the nI of incubating (INC) and nest-deprived (ND) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

Table 6.3 The number of TH-ir neurons (Mean ± SEM) in the nI and ML of incubating (INC) and nest-deprived (ND) native Thai hens at different days of incubation or nest deprivation (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.

Area	Group	Days Following Nest Deprivation						
		3	**6**	**8**	**10**	**14**	**18**	**21**
nI	**INC**	37.70 ± 3.57 [a]	32.33 ± 1.49 [a]	35.63 ± 3.61 [a]	36.58 ± 2.32 [a]*	35.21 ± 4.27 [a]	40.83 ± 3.28 [a*]	33.13 ± 2.22 [a*]
	ND	N/A	29.54 ± 3.88 [a]	31.75 ± 3.76 [a]	21.38 ± 1.70 [a]	32.38 ± 2.82 [a]	26.21 ± 1.69 [a]	24.50 ± 2.49 [a]
ML	**INC**	16.92 ± 4.07 [a]	21.78 ± 1.32 [a*]	23.58 ± 2.35 [a]*	16.63 ± 1.95 [a]	19.63 ± 2.32 [a]	14.33 ± 2.18 [a]	14.29 ± 1.87 [a]
	ND	N/A	14.38 ± 1.17 [a]	13.10 ± 1.67 [a]	13.14 ± 2.60 [a]	13.58 ± 2.73 [a]	13.46 ± 2.12 [a]	9.40 ± 1.48 [a]

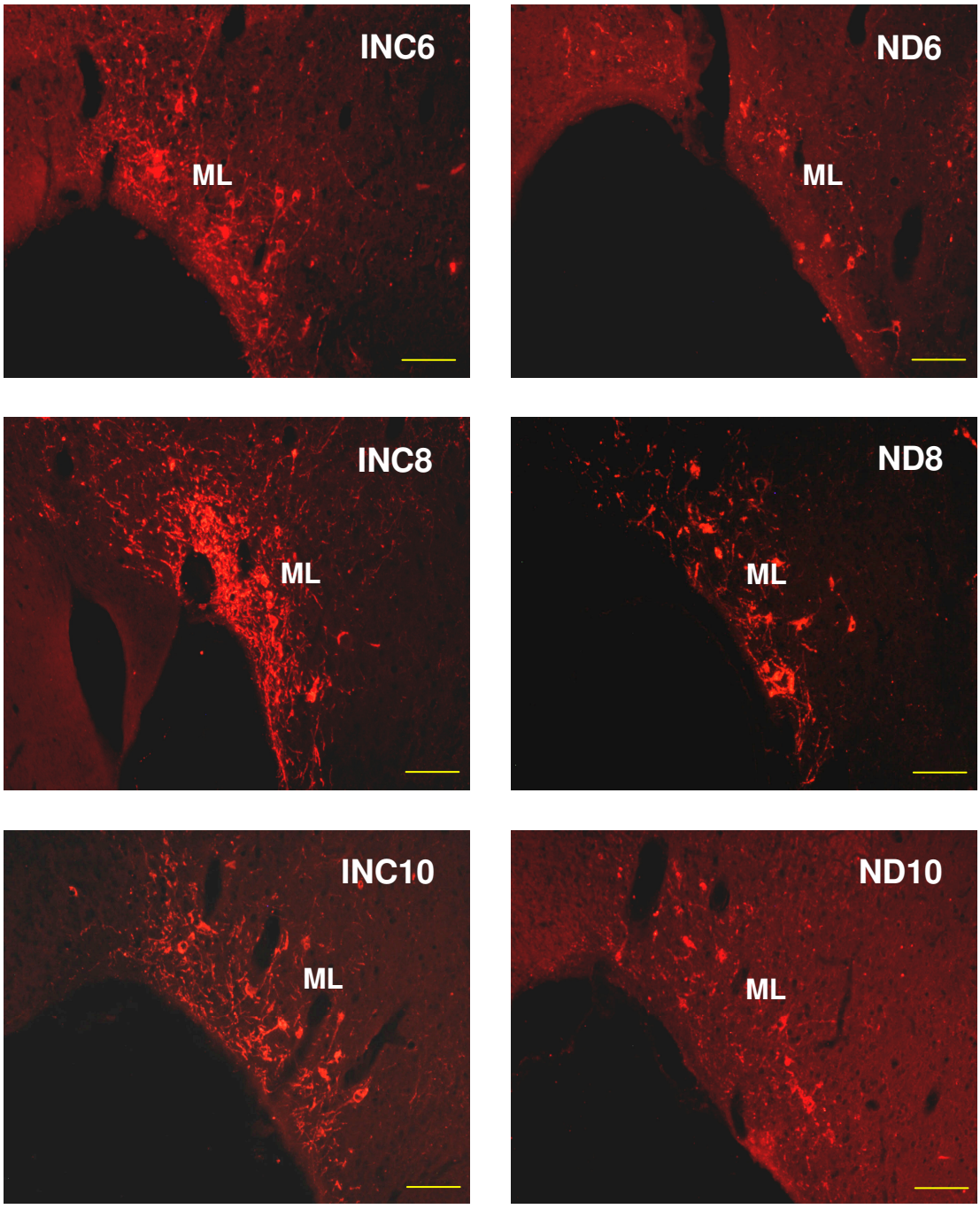

Figure 6.8 Photomicrographs showing the distributions of TH-ir neurons and fibers in the nucleus mamillaris lateralis (ML) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 6.1. Scale bar = 100 µm.

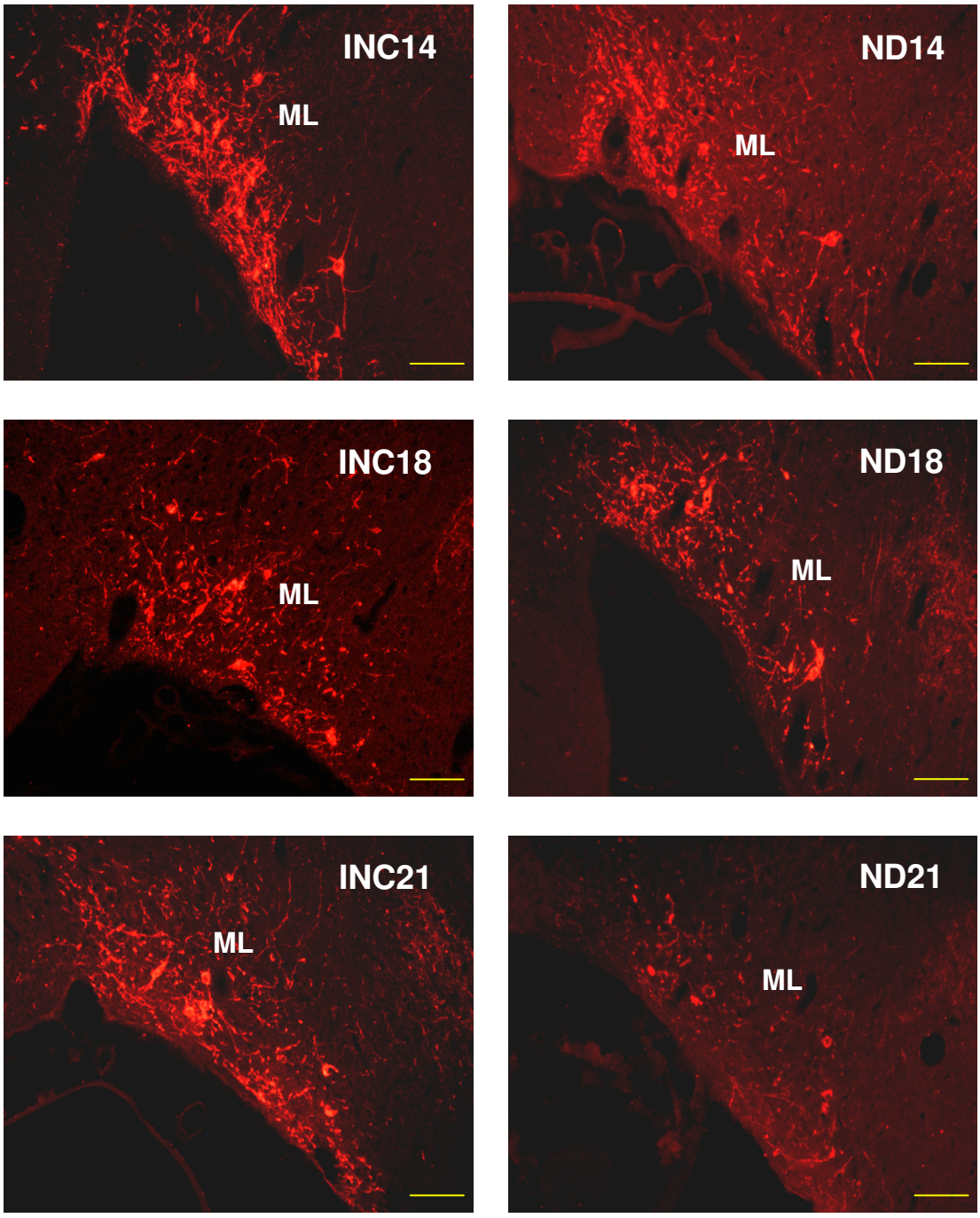

Figure 6.8 Photomicrographs showing the distributions of TH-ir neurons and fibers in the nucleus mamillaris lateralis (ML) of incubating (INC) and nest-deprived (ND) native Thai hens on different days following the initiation of incubation or nest deprivation. For abbreviations, see Table 6.1. Scale bar = 100 µm (continued).

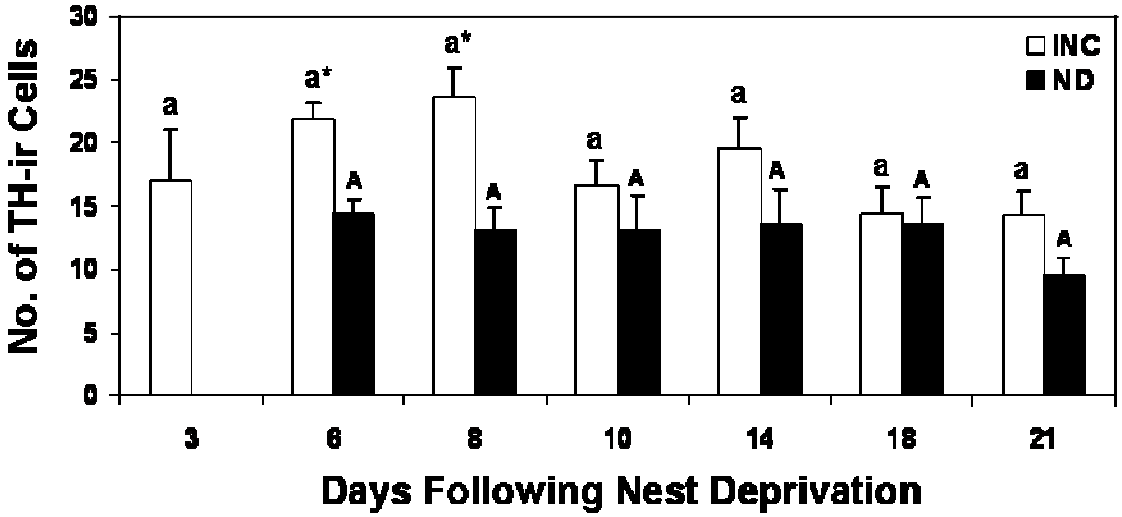

Figure 6.9 Changes in the number of TH-ir neurons in the ML of incubating (INC) and nest-deprived (ND) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

6.5 Discussion

The results from this present study revealed that the TH-ir neurons and fibers were extensively distributed throughout the brain of the incubating native Thai chickens and were predominantly expressed in the nI and ML areas. The expression of the hypothalamic TH-ir neurons in the AM, SCNm, PVO, LHy, VMN, IH-IN, nI, and ML areas were also observed. A dense accumulation of TH-ir neurons was found in the nI of INC hens and TH-ir fibers in the MM and ME of both treatment groups. TH-ir neuron abundance was found in the ML and a few number of TH-ir neurons were observed in the AM, SCNm, PVO, and LHy of both INC and ND hens. TH-ir neurons were also observed in the IH-IN of INC hens. Significance changes in the number of TH-ir neurons in the hypothalamus of INC and ND hens were observed in the nI and ML areas. The number of TH-ir neurons in the nI was high during incubating period and significantly decreased by day 10 of nest deprivation. In the ML, the number of TH-ir neurons significantly decreased by day 6 of nest deprivation. It is well known that these brain areas are involved in the regulation of PRL secretion. These findings are interpreted that nesting activity stimulates PRL secretion by the activation of the DAergic system at the nI and ML areas.

DAergic regulation of the VIP/PRL system and avian reproduction is well documented (El Halawani et al., 2001). DA can have both stimulatory and inhibitory effects on PRL secretion, depending on its site of action and DA receptor subtypes. ICV infusion of DA at low concentrations activates D_1 DA receptors in the hypothalamus and increases PRL secretion by stimulating the release of VIP from the INF (Youngren et al., 1996). The INF is rich with VIP neurons projecting to the ME (Yamada et al., 1982; Mikami and Yamada, 1984; Macnamee et al., 1986; Peczely

and Kiss, 1988; Mauro et al., 1989; Chaiseha and El Halawani, 1999; 2005). In addition, various studies have shown that these hypothalamic VIP neurons express D_1 DA receptors (Chaiseha et al., 2003). When higher concentrations of DA are infused, PRL is inhibited. This is most likely as a result of DA diffusing from the infundibular recess of the third ventricle to the pituitary, activating D_2 DA receptors on pituitary lactotrophs and inhibiting PRL secretion even in the presence of strong VIP stimulation (Youngren et al., 1996).

In the current study, utilizing TH as a marker for DAergic neurons, no significant differences in the number of hypothalamic TH-ir neurons in the AM, ML, and PVO of INC or ND hens were observed during day 10 of incubation or nest deprivation. These results are consistent with previous data reported in the native Thai chickens that changes in TH-ir neurons number in the AM, nucleus paraventricularis magnocellularis (PVN), and ML are less dramatic during the reproductive cycle and no significant differences are observed in non-laying (NL), laying (L), INC, and rearing birds (Sartsoongnoen et al., 2008). TH-ir neurons are found to be abundant in the ML and nI. The highest density of TH-ir neurons is found within the nI, where these neurons increase during incubation and decrease when the hens are deprived of their nests. These findings are also consistent with previous results showing that the number of TH-ir neurons in the nI are low in NL and significantly increase in INC birds (Sartsoongnoen et al., 2008). In deed, nest deprivation correlates with decline in both VIP-ir neurons in the IH-IN and plasma PRL levels and is accompanied with increase in the ovary and oviduct weights (Prakobsaeng et al., 2009). In addition, a marked increase in DAergic activity is observed in the anterior hypothalamus of incubating bantam hens when compared

with laying or nest-deprived hens (Macnamee and Sharp, 1989) as well as in the periventricular mid-hypothalamic regions of ring doves (Lea et al., 2001).

In this present study, the number of TH-ir neurons were found in the AM, SCNm, PVO, LHy, and VMN areas of both INC and ND birds. As mentioned above, a dense group of TH-ir neurons was observed in the nI. The result from this study corresponds with previous reports that DAergic neurons have been observed in the nI of chick (Kuenzel et al., 1992) and L-3,4-dihydroxyphenylalanine and DA-ir neurons in chicken brain (Moons et al., 1994). The greatest density of TH-ir neurons is observed in the nI of native Thai chickens (Sartsoongnoen et al., 2008). A dense accumulation of TH-ir fibers are located within the MM and the external layer of ME in the INF. Surprisingly, some of TH-ir neurons are found in the IH-IN of INC hens but not in those of the ND hens. The results of this present study consistent with the studies in mammals, which have been suggested that these areas are involved in the regulation of PRL secretion. PRL secretion is regulated by the inhibitory control of TIDA neurons (A12 DA group) residing in the INF (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001), which release DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). However, the result is not in a good agreement with the studies in birds, since it has been suggested that TIDA neurons in birds are absent (Reiner et al., 1994), and the DA in the avian hypothalamus may not be the primary PRL inhibiting factor (Kiss and Peczely, 1987). It has been reported that the tuberoinfundibular area is lack of hypothalamic TH-ir cells (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Appeltants et al., 2001). Furthermore, previous study has been reported that TH immunoreactivity found in the tuberal hypothalamus is limited to a single discrete

area of the MM and to the external layer of ME, where only TH-ir fibers are found (Sartsoongnoen et al., 2008). The results of this present study are different from the previous study, since this study observed TH-ir neurons in the brain of INC and ND hens, whereas the previous study reported the TH-ir neurons in L hens. Thus, TH-ir neurons were not observed in the INF of L hens as same as in ND hens, suggesting that TH-ir neurons found in the INF of INC hens may involve in the regulation of PRL secretion and incubation behavior and need to be further investigated.

In chickens, TH-ir neurons in the nI is corresponded to the mammalian DA A11 group (Moons et al., 1994; Lookingland and Moore, 2005), which consists of cells that may play a role in the onset of puberty (Fraley and Kuenzel, 1993). Also, the A11 DA group is shown to be involved in the regulation of reproductive seasonality in the turkeys (Thayananuphat et al., 2007a) and its activity reflects the performance of courtship singing in zebra finches (Bharati and Goodson, 2006). No double-labeled immunoreactive neurons for both TH and DBH, the enzyme for noradrenalin synthesis, are found in the hypothalamus of quails (Bailhache and Balthazart, 1993), turkeys (Al-Zailaie, 2003), and other avian species (Reiner et al., 1994). Therefore, it is suggested that TH-ir neurons found in the nI of the native Thai chicken could be DAergic neurons.

In the present study, there appears to be a differential decline in DAergic activity in the nI and ML following nest deprivation. The number of TH-ir neurons in the ML tended to decrease within six days following nest deprivation and this is associated with a decline in the number of VIP-ir neurons in the IH-IN. The changes in the number of TH-ir neurons in the nI was delayed to day 10 following nest deprivation. The functional significance of the differential DAergic neurons responses

of the ML and nI to nest deprivation and the suppression of VIPergic/DAergic systems remains to be determined. However, the changes in ML TH-ir neurons in response to nest deprivation are correlated with a decrease in VIP-ir neurons within the IH-IN; consequently, the decrease of PRL levels (Prakobsaeng et al., 2009). It is of interest to note that activation of DAergic cells in the ML is linked to the activation of GnRH-I and VIP neurons and the release of LH and PRL in turkeys (Al-Zailaie et al., 2006). Nest deprivation of native Thai chickens decreases VIP-ir neurons counted in the IH-IN, increases the number of GnRH-I-ir neurons in the nCPa, and decreases plasma PRL concentrations (Prakobsaeng et al., 2009). In addition, it has been suggested that DAergic neurons located within the PVN and ML might be possibly influencing gonadal maturation (Kuenzel, 2000). Moreover, it has been suggested that the avian ML DA neurons is corresponded to the A12 DA group in mammals (Cheung et al., 1997; Kuenzel, 2000). These neurons are involved in the regulation of PRL secretion and stress-related processes in mammals (Anderson et al., 2005; Hollis et al., 2005; Khodr et al., 2008) and in zebra finches (Bharati and Goodson, 2006).

This present study demonstrated that disruption of incubation behavior by nest deprivation decreased the number of TH-ir neurons in the nI and ML. Previous reports demonstrate that disruption of broodiness in INC hens results in decrease the number of VIP-ir neurons in the IH-IN, increase in the number of GnRH-I-ir neurons in the nCPa, and precipitous decline in plasma PRL levels (Prakobsaeng et al., 2009). In addition, it has been reported that the numbers of VIP-ir neurons in the IH-IN and plasma PRL concentrations are decreased in hens that are not allowed to rear chicks as compared to those hens that rearing chicks (Chaiyachet et al., 2010). These data suggest an association between VIP neurons in the IH-IN, GnRH-I neurons in the

nCPa, and TH-ir neurons in the nI and ML with the degree of hyperprolactinemia. Thus, it has been proposed that DA neurons in the nI and ML may influence the VIP neurons in the IH-IN and GnRH-I neurons in the nCPa in the regulation of PRL secretion and the reproduction of the native Thai chicken.

In conclusion, the findings of the present study indicate that external cues including the presence of the nest and eggs are involved in the stimulation of PRL secretion and maintenance of incubation behavior in the native Thai chickens. The findings from other studies indicated that nest deprivation of incubating chicken reduces circulating PRL levels and is associated with a reduction in the number of hypothalamic VIP-ir neurons in IH-IN and an increase in the number of hypothalamic GnRH-I-ir neurons in the nCPa, and the findings from this study, a parallel decrease in the number of TH-ir neurons observed in the nI and ML of nest-deprived chickens, are interpreted to suggest that nesting activity stimulates PRL secretion by the activation of the DAergic system at the nI and ML, which in turn, stimulates VIP, the avian PRL releasing factor. The elevated PRL levels increase nesting activity and maintain incubation behavior.

6.6 References

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CHAPTER VII

CONCLUSION

Incubation behavior is one of the maternal behaviors which highly expresses in the native Thai chickens. This behavior affects the production of the native Thai chickens by reduces egg production and terminates egg laying. In order to increase the productivity of these chickens, it is very important to understand the basic neuroendocrinology influencing its incubation behavior of native Thai chickens. The primary components of the integrated female reproductive system are the brain, especially the hypothalamus, the pituitary, and the ovary. This integrated system is referred to as the hypothalamic-pituitary-gonadal axis. The neuroendocrine systems that play a pivotal role in the avian reproductive cycle are gonadotropin releasing hormone (GnRH)/follicle stimulating hormone (FSH)-luteinizing hormone (LH) and vasoactive intestinal peptide (VIP)/prolactin (PRL) neuroendocrine systems. Both systems are influenced by dopaminergic (DAergic) neurotransmission. Thus, the aim of this study was to observe the roles of PRL, VIP, dopamine (DA), and GnRH on the neuroendocrine regulation of incubation behavior of the native Thai chickens.

The results revealed that plasma PRL levels were high in incubating hens (INC) throughout the incubation period. Nest deprivation of incubating hens reduced circulating PRL concentrations within a day of nest deprivation (Figure 7.1A). The levels of PRL remained low throughout the period of nest deprivation. The ovary and oviduct weights of INC hens were decreased during incubating period and

significantly increased by day 8 of nest deprivation (Figures 7.1B and C). In addition, disruption of incubation behavior by nest deprivation increased the presence of ovarian follicles and the number of egg laying hens. The decrease of plasma PRL concentrations in nest-deprived hens (ND) paralleled with the decline in the number of VIP-immunoreactive (VIP-ir) neurons in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN; Figure 7.2A). The number of VIP-ir neurons in the IH-IN remained high throughout incubating period. When the hens were nest-deprived, the number of VIP-ir neurons was declined by day 6 of nest deprivation. In contrast with the levels of PRL and the numbers of VIP-ir neurons, the number of GnRH-I-immunoreactive (GnRH-I-ir) neurons in the nucleus commissurae pallii (nCPa) was low in INC hens and increased in ND hens (Figure 7.2B).

In this study, tyrosine hydroxylase (TH), the rate limiting enzyme for DA synthesis was used as a marker for DAergic neurons. The number of TH-immunoreactive (TH-ir) neurons in the nucleus intramedialis (nI) was high during incubating period and significantly decreased by day 10 of nest deprivation (Figure 7.2C). In addition, the number of TH-ir neurons in the nucleus mamillaris lateralis (ML) was also significantly decreased by day 6 of nest deprivation (Figure 7.2D).

In summary, the present study indicated an association between VIP neurons in the IH-IN, GnRH-I neurons in the nCPa, and DA neurons in the nI and ML with the degree of hyperprolactinemia, suggesting that the expression of incubation behavior in the native Thai chicken might be, in part, regulated by the differential expression of VIP neurons in the IH-IN, GnRH-I neurons in the nCPa, and DA neurons in the nI and ML. DA neurons in the nI and ML may influence the VIP neurons in the IH-IN and GnRH-I neurons in the nCPa in the regulation of PRL

secretion and maintenance of incubation behavior. Nest deprivation of incubating Thai chickens suppressed hypothalamic DAergic and VIPergic activities and reduced circulating PRL levels. The increase in GnRH-I neurons may affect the changes in gonadotropin secretion. These neuroendocrine changes are associated with ovarian and oviduct recrudescence and initiation of a new laying cycle of the native Thai chickens.

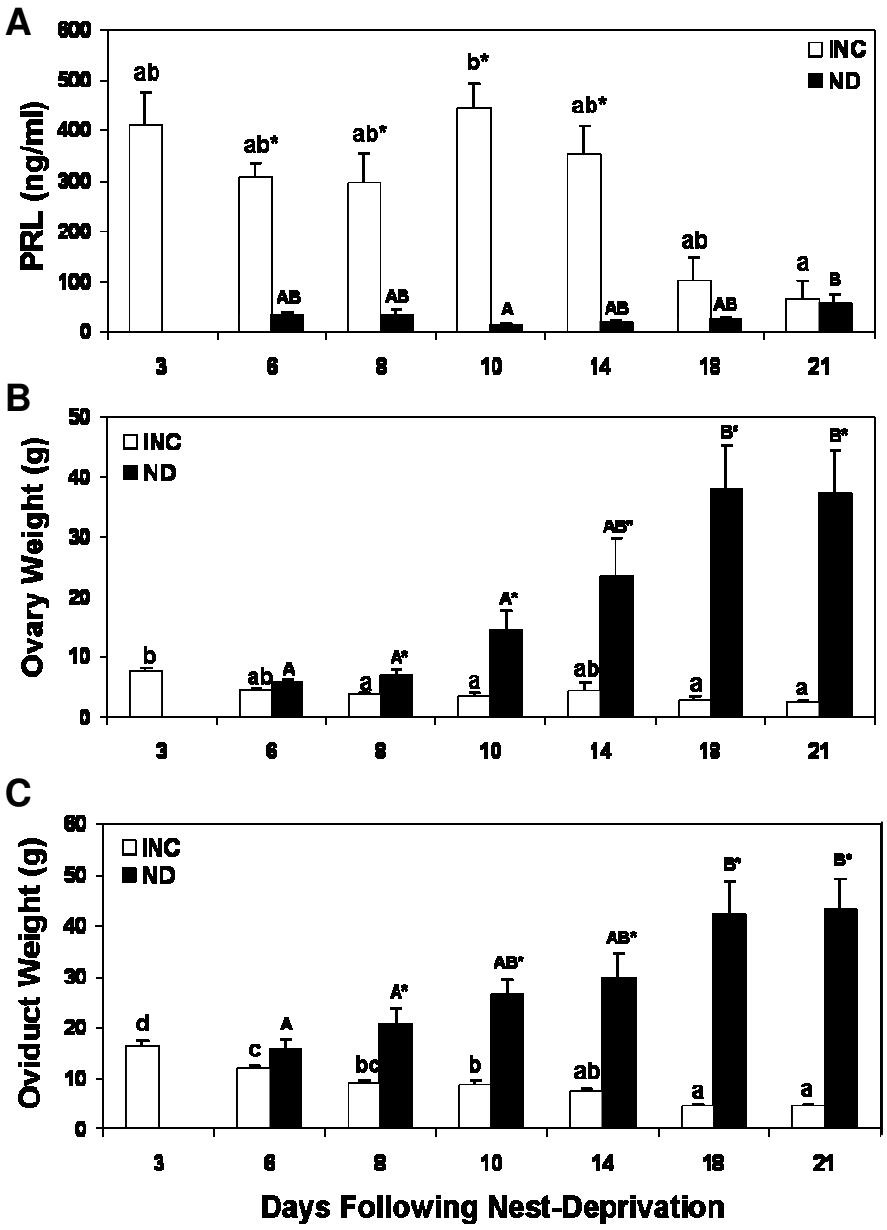

Figure 7.1 Changes in: **A**, plasma PRL concentration; **B**, ovary; **C**, oviduct weights of incubating (INC) and nest-deprived (ND) native Thai hens. Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between group at a given time point.

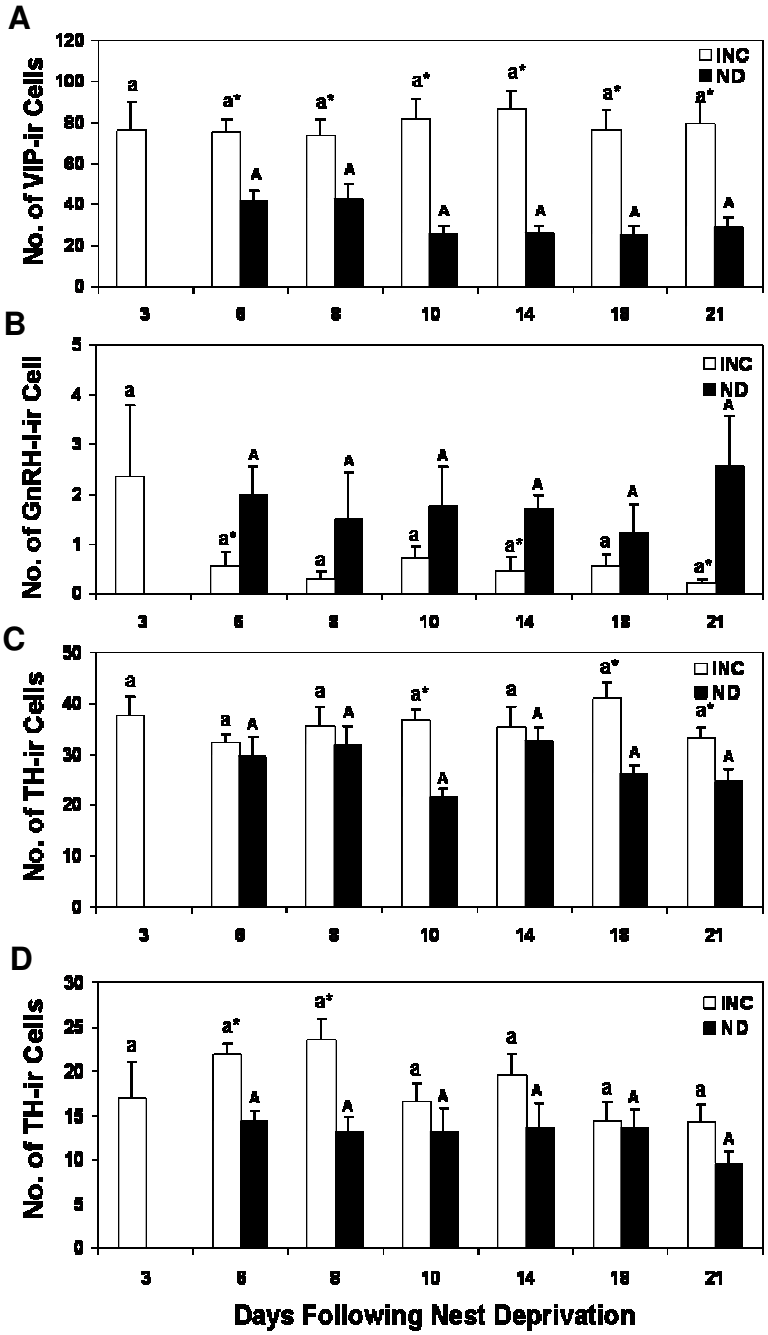

Figure 7.2 Changes in: **A**, the number of VIP-ir neurons in the IH-IN; **B**, the number of GnRH-I-ir neurons in the nCPa; **C**, the number of TH-ir neurons in the nI; **D**, the number of TH-ir neurons in the ML of incubating (INC) and nest-deprived (ND) native Thai hens. Values are presented as 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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