



## Changes of Hypothalamic GnRH-I, POMC and NPY mRNA Expression and Serum IGF-I and Leptin Concentrations during Maturation of Shaoxing Ducks (*Anas platyrhynchos*)

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**ABSTRACT** : Sexual maturity in poultry is controlled by a complex neural circuit located in the basal forebrain, which integrates the central and peripheral signals to activate hypothalamic gonadotrophin-releasing hormone (GnRH) secretion. This study demonstrated the changes of GnRH-I, POMC and NPY mRNA transcription in hypothalamus and IGF-I and leptin levels in serum of Shaoxing ducks during puberty. Body weight increased progressively from d30 to d120 and at d120 the flock reached 5% of laying rate. A significant up-regulation of hypothalamic GnRH-I mRNA expression was observed from d60, reaching the peak at d120. POMC and NPY mRNA expression in hypothalamus showed a similar pattern, which increased from d30 to d60, followed by a significant decrease towards sexual maturity. Serum IGF-I levels exhibited two peaks at d30 and d120, respectively. Serum leptin displayed a single peak at d90. The results indicate that the down-regulation of POMC and NPY genes in hypothalamus coincides with the up-regulation of GnRH-I gene to initiate sexual maturation in ducks. In addition, peripheral IGF-I and leptin may relay the peripheral metabolic status to the central system and contribute to the initiation of the reproductive function in ducks. (**Key Words** : GnRH-I, POMC, NPY, Leptin, IGF-I, Sexual Maturity, Duck)

### INTRODUCTION

The acceleration of hypothalamic GnRH neurons is regarded as the proximal signal for the activation of the pituitary-gonadal axis at the initiation of reproduction (Ebling, 2005). In birds, two forms of chicken GnRH (cGnRH), cGnRH-I and II, have been isolated but only cGnRH-I appears to be released from the median eminence to stimulate gonadotrophin secretion (Knight et al., 1985; Sharp et al., 1990). Pubertal alterations in GnRH pulsatility have been assessed in many species, including domestic chickens (Sharp et al., 1990; Stevenson and Dougall-Shackleton, 2005). In rodents, the levels of GnRH peptide content roughly parallel the changes of cellular GnRH mRNA in hypothalamus which increased progressively during pubertal development. However, in domestic chickens, no data is available, regarding the changes of GnRH caused by gene transcriptional or translational level.

Additionally, till now, studies on the profile of hypothalamic GnRH-I mRNA expression from earlier growth stage to sexual maturity have not been reported in domestic birds.

Remarkable progress has been made in understanding the molecular and cellular mechanisms underlying the pubertal activation of the GnRH generator, which involve the combined effects of intra-hypothalamic actions of inhibitory and stimulatory neuropeptides (Genazzani et al., 2000) and metabolic cues including leptin (Barash et al., 1996), GH and IGF-I (Daftary and Gore, 2003). NPY and POMC neurons are two important components located in the hypothalamus, secreting peptides to link the energy status and reproductive function (Prasad et al., 1993). NPY is one of the most highly conserved neuropeptides and the sequence of the chicken differs from that of rats and human by only one amino acid (Blomqvist et al., 1992). It's well documented that NPY exerts both stimulatory and inhibitory effects on pubertal initiation depending on the intrinsic estrogenic state (El et al., 2000). Beta-endorphin, a product derived from a pro-opiomelanocortin (POMC) precursor, is involved in regulating reproductive function as one of main inhibitory factors in mammals (Kalra and Kalra,

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1986), rodents (Gruenewald and Matsumoto, 1991) and birds (Stansfield and Cunningham, 1987). In order to better understand the effects of NPY and beta-endorphin on regulating GnRH-I synthesis during sexual maturity in birds, as the first step, it is necessary to determine the developmental profiles of hypothalamic GnRH-I and NPY and POMC gene expression.

Leptin plays an important role in regulating feed intake, energy balance, and reproduction in many species, including birds (Barb et al., 2005). Nevertheless, a body of publications showed that leptin treatment advanced sexual maturation in restricted and *ad libitum* fed rodents (Chehab et al., 1997), although there has been considerable controversy as to whether leptin serves as a permissive or a triggering role in this regard. Whether leptin serves as a permissive factor, whose presence is required or sufficient, as suggested the role in mammals is still a mystery. IGF-I is a growth-related signal that links the peripheral energy status to the hypothalamus to regulate the developmental increase of GnRH secretion. In addition to its well-documented effects on gonads, IGF-I is essential for reproductive maturation (Daftary and Gore, 2005).

In the present study, we sought to determine whether the profile of hypothalamic GnRH-I mRNA expression is changed abruptly or gradually during sexual maturity of the duck, and to examine the association with NPY and POMC genes in the hypothalamus at transcriptional level. Additionally, in order to primarily elucidate the role of circulating leptin and IGF-I on regulation of reproductive initiation in birds, radioimmunoassay (RIA) was employed to measure the developmental changes in serum leptin and IGF-I from d30 to sexual maturity in Shaoxing ducks.

## MATERIALS AND METHODS

### Animals

The Shaoxing duck is a local breed in China, characterized by small body size, early maturity (about D102) and high productivity (more than 300 eggs a year). In this study, 200 Shaoxing female ducklings were raised from hatch to sexual maturity. Feeding areas were comprised of three parts: indoor floor pens, adjacent open field with shed and a connecting water area for the ducks to paddle. Ducks were given free access to water and food under natural lighting. The first egg was seen at d102 and the laying rate of the flock reached 5, 50 and 80% at d120, d140 and d160, respectively. Fifteen ducks were sacrificed at 8:00 am at d30, d60, d90 and d120, respectively. Blood and hypothalamus were collected for analysis. Hypothalamus was immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. The sera were stored at -20°C until hormone assays.

### Materials

The avian myeloblastosis virus (AMV) reverse transcriptase, Taq DNA polymerase and other related reagents were products of Promega, USA. Agarose was a product of Roche Applied Science, Switzerland (catalog no. 1441).

### Radioimmunoassay for serum hormone levels

The serum IGF-I and leptin were measured using commercial RIA kits purchased from Shanghai Institute of Biological Products, as previously described by Hu et al. (2008). The detection range for leptin was between 0.5 and 24.0 ng/mL, and the inter- and intra-assay coefficient of variation were 5% and 10%, respectively. The ranges for IGF-I were between 0.3 and 10.0 pg/mL, and the inter- and intra-assay coefficient of variation were 10% and 15%, respectively.

### RNA extraction

Total cellular RNA was extracted by commercial kit from Takara Biocompany (Haojia, China) and was estimated by reading the absorbance at 260 nm on a spectrophotometer (Eppendorf Biophotometer). Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose-formaldehyde gel to verify their integrity.

### Reverse transcription (RT) and polymerase chain reaction (PCR)

Reverse transcription reaction mix for first-strand cDNA synthesis included 1×RT-buffer (50 mmol/L pH 8.3 Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, 10 mmol/L DTT), 10 mmol/L dNTPs, 20U RNase inhibitor, 10U AMV reverse transcriptase, 2.5 μmol/L oligo (dT15) primer, and 2 μg of total RNA in a final volume of 20 μL. RNA samples were denatured at 80°C for 5 min and placed on ice for 5 min together with Oligo (dT15) primer and dNTP before reverse transcription (RT). Tubes were incubated for 1 h at 42°C, 5 min at 95°C, and then chilled to 4°C.

Two μL RT reaction mix was used for PCR in a final volume of 50 μL containing 1 U Taq DNA polymerase, 5 mmol/L Tris-HCl (pH 9.0), 10 mmol/L NaCl, 0.1 mmol/L DDT, 0.01 mmol/L EDTA, 5% (w/v) glycerol, 0.1% (w/v) Triton X-100, 0.2 mmol/L each dNTP, 1.0-2.0 mmol/L MgCl<sub>2</sub>, 0.5 μmol/L specific primers for respective target genes. The PCR primers for GnRH-I, POMC, NPY and β-actin were designed using Premier 5.0 and synthesized by Takara Biocompany (Haojia, China). The nucleotide sequences of these primers and the PCR conditions set for target genes are shown in Table 1. In each reaction, an additional pair of primers specific to the chicken β-actin gene was included as an internal control. By adjusting the ratio of β-actin primers to the target genes primers in the

**Table 1.** Nucleotide sequences of specific primers and PCR conditions

Target genes	Reference/ GenBank accession	PCR products	Primer sequences	PCR conditions
cGnRH-I	(ref)	202 bp (171-372)	F: 5'-GCTTGGCTCAACACTGGTCT-3' R: 5'-CTGGCTTCTCCTTCGATCAG-3'	94°C 30 s, 55°C 30 s, 72°C 30 s, 30 cycles
cPOMC	AB019555	369 bp (366-734)	F: 5'-GCTTGGCTCAACACTGGTCT-3' R: 5'-CTGGCTTCTCCTTCGATCAG-3'	94°C 30 s, 55°C 40 s, 72°C 40 s, 32 cycles
cNPY	M87295	242 bp (118-360)	F: 5'-TCCGGTTTGAAGACCCT-3' R: 5'-CATCGTTTGTTCATTTAGT-3'	94°C 30 s, 55°C 30 s, 72°C 30 s, 28 cycles
cβ-actin	L08165	282 bp (721-1,002)	F: 5'-ACGTCGCACTGGATTTCGAG-3' R: 5'-TGTCAGCAATGCCAGGGTAC-3'	-

c = Chicken, ref means the cDNA sequence of cGnRH-I is cited from Dunn, I.C et al. (shown in citation).

reaction system, the overall PCR amplification efficiency of β-actin can be reduced to the level comparable to that of the target genes. Different controls were set to monitor the possible contaminations of genomic and environmental DNA both at the stage of RT and PCR. The pooled samples made by mixing equal quantity of total RNA from all samples were used for optimizing the PCR condition and normalizing the intra-assay variations. All samples were included in the same run of RT-PCR and repeated at least for 3 times. Both RT and PCR were performed in a Gene Amp PCR System 9600 (Perkin Elmer, USA).

#### Quantitation of PCR products and statistical analysis

An aliquot (10-20 μL) of PCR products was analyzed by electrophoresis on 2% agarose gels. The gel was stained with ethidium bromide and photographed with a digital camera. The net intensities of individual bands were measured using Kodak Digital Science 1D software (Eastman Kodak Company Rochester, NY, USA).

The ratios of net intensity of target genes to β-actin were used to represent the relative abundance of target gene expression. The average level of three repeats was used for statistical analysis. The results were expressed as mean±SEM and differences were considered significant when  $p < 0.05$  tested by ANOVA with SPSS 11.0 for windows.

## RESULTS

#### Serum concentrations of IGF-I and leptin

As shown in Table 2, body weight of Shaoxing ducks increased progressively from d30 to d120. Serum IGF-I

levels exhibited two peaks during this period of time, the highest level was detected on d30, followed by a decrease from d30 to d60. From d60 serum IGF-I increased progressively to reach a second and less prominent peak at d120. Serum leptin displayed a single peak on d90.

#### Hypothalamic expression of GnRH-I, POMC and NPY mRNA

Hypothalamic GnRH-I mRNA expression did not show a significant change from d30 to d60. However, a significant up-regulation of hypothalamic GnRH-I mRNA expression was observed after d60, reaching a peak at d120 when the flock had reached 5% of laying rate (Figure 1). POMC and NPY mRNA expression in the hypothalamus showed a similar pattern, which increased from d30 to d60, followed by a significant decrease towards sexual maturity, as shown in Figure 2 and Figure 3, respectively.

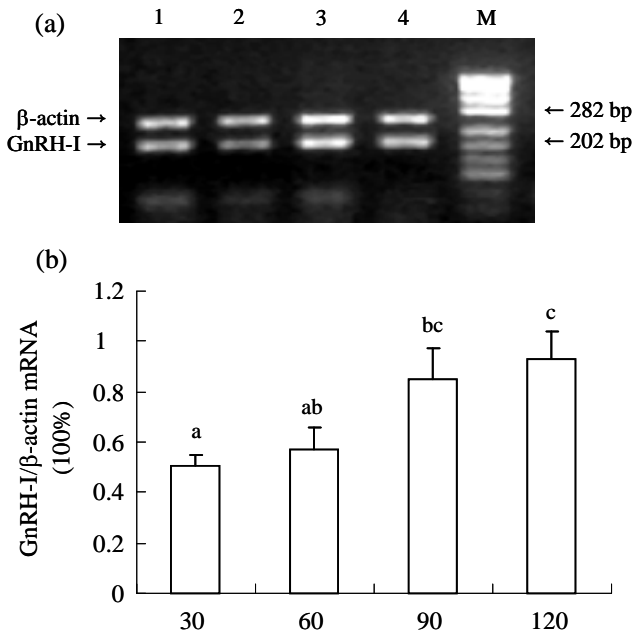
## DISCUSSION

In mammals and birds, GnRH is released from nerve terminals in the median eminence into portal capillary vessels. An increased release in pulsatile GnRH is responsible for initiation of reproduction. Compared to juvenile birds, the basal release of GnRH-I and the number of ir-cGnRH-I cell bodies in the central system is higher in mature birds (Knight, 1983). In the present study, hypothalamic GnRH-I mRNA expression increased from d30 to sexual maturity in Shaoxing laying ducks, reaching a peak at d120 when 5% ducks in the flock had commenced lay. This developmental pattern of GnRH-I mRNA

**Table 2.** Developmental changes of body weight and the levels of serum IGF-I and leptin during sexual maturity in Shaoxing female breed ducks

Parameters	d30	d60	d90	d120
Body weight (kg)	0.553±0.02 <sup>a</sup>	0.941±0.02 <sup>b</sup>	1.284±0.06 <sup>c</sup>	1.541±0.05 <sup>d</sup>
Serum IGF-I (ng/mL)	97.48±5.06 <sup>a</sup>	60.38±4.57 <sup>b</sup>	67.30±6.48 <sup>b</sup>	82.10±5.17 <sup>c</sup>
Serum leptin (ng/mL)	1.09±0.09 <sup>ab</sup>	0.88±0.09 <sup>a</sup>	1.18±0.08 <sup>b</sup>	1.03±0.11 <sup>ab</sup>

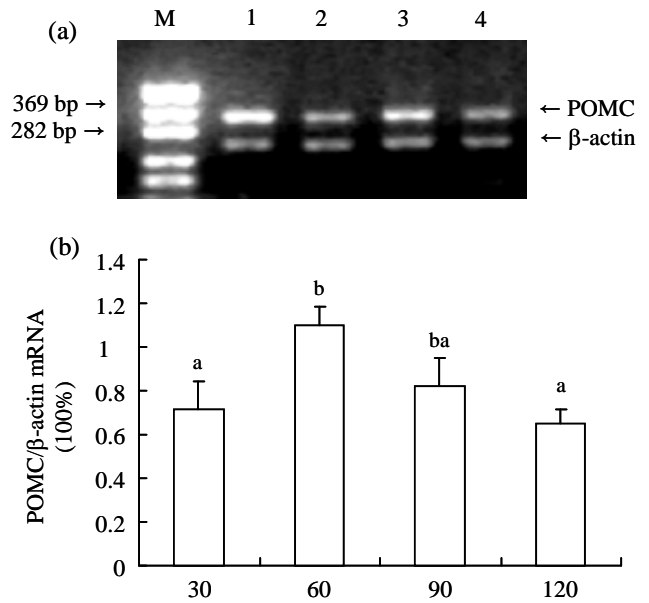
Values are means±SEM. Mean values without common superscript (a, b, c, d) differ significantly between ages ( $p < 0.05$ ,  $n = 15$ ).



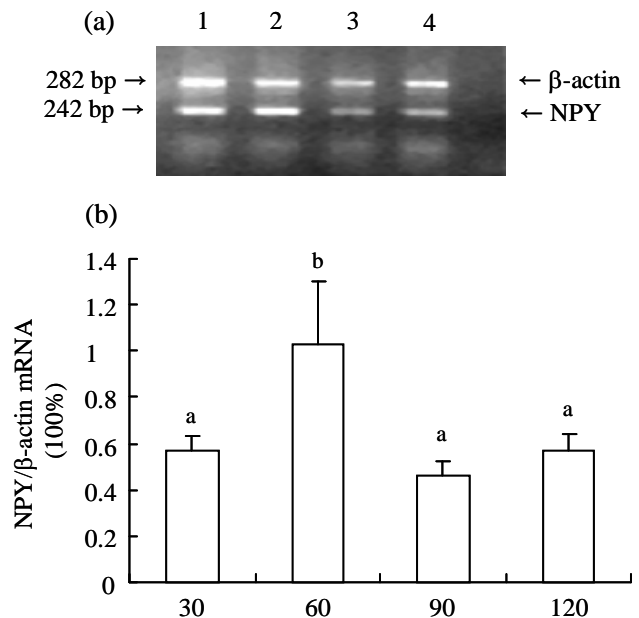
**Figure 1.** Developmental changes of the relative abundance of hypothalamic GnRH-I mRNA in the duck. (a) Representative electrophoresis photo of RT-PCR products for GnRH-I and  $\beta$ -actin mRNA, respectively. M: DNA molecular weight marker PUC19. Lanes 1-4 lanes stand for d30, d60, d90 and d120, respectively. (b) Results of RT-PCR for GnRH-I mRNA level expressed as arbitrary units relative to  $\beta$ -actin mRNA. Values are means $\pm$ SEM. ANOVA (Posthoc) was used to test the significance of difference. \*  $p < 0.05$ ,  $n = 10$ .

expression reflects a slow maturational process of the GnRH neurons during maturation in birds. However, the biosynthetic mechanism(s) responsible for developmental changes in GnRH-I mRNA levels is (are) currently unknown.

The increase of pulsatile GnRH release is mediated by interactions between neuropeptides such as NPY, POMC, orexin, and peripheral hormones (e.g., leptin and IGF-I) that act at the hypothalamus (Genazzani et al., 2000). Studies by Knight et al. (1990) also support the conclusion that the onset of sexual maturity in the cockerel is primarily determined by a central neural mechanism (Knight et al., 1990). NPY and POMC are two putative neuronal inputs to GnRH neuronal terminals at the medial eminence and are involved in the control of both reproduction and food intake. In rodents, NPY in the stalk-median eminence is highly stimulatory to GnRH release, and injection with NPY into the 3rd ventricle of peripubertal female rats induced precocious puberty accompanied with elevated LH levels (Minami et al., 1990). In this study, hypothalamic NPY mRNA expression increased greatly from d30 to d60, followed by a significant decrease approaching sexual maturation, but accompanied with the significant increase of NPY peptide content in plasma (data not shown), which



**Figure 2.** Developmental changes of the relative abundance of hypothalamic POMC mRNA in the duck. (a) Representative electrophoresis photo of RT-PCR products for POMC mRNA and  $\beta$ -actin mRNA, respectively. (b) Results of RT-PCR for POMC mRNA level expressed as arbitrary units relative to  $\beta$ -actin mRNA. Values are means $\pm$ SEM. ANOVA (Posthoc) was used to test the significance of difference. \*  $p < 0.05$ ,  $n = 10$ .



**Figure 3.** Developmental changes of the relative abundance of hypothalamic NPY mRNA in the duck. (a) Representative electrophoresis photo of RT-PCR products for ER- $\beta$  mRNA and  $\beta$ -actin mRNA, respectively. (b) Results of RT-PCR for NPY mRNA level expressed as arbitrary units relative to  $\beta$ -actin mRNA. Values are means $\pm$ SME. ANOVA (Posthoc) was used to test the significance of difference. \*  $p < 0.05$ ,  $n = 10$ .

indicated that the high translation rate of mRNA to protein may account for the lower level of NPY mRNA in the hypothalamus. Fraley et al. (1993) reported that exogenous NPY injection can advance the sexual maturity in hens (Fraley and Kuenzel, 1993). These results suggest that NPY may play a stimulatory effect on the process of GnRH neurons maturity, then to regulate the onset of reproduction under the high steroids levels when approaching to sexual maturity in ducks.

$\beta$ -endorphin is one of several peptides cleaved from the precursor molecule POMC, and has been suggested to exert a tonic inhibitory effect upon hypothalamic GnRH secretion, which is well documented in many species of mature animals, including domestic hens (Stansfield and Cunningham, 1987). In the present study hypothalamic POMC mRNA abundance decreased significantly right before or at the onset of egg lay, which coincides with up-regulation in hypothalamic GnRH-I mRNA expression, supporting the proposition that a decrease in tonic opioid inhibition of GnRH secretion is an important factor for the onset of sexual maturity in ducks.

Two growth-derived signals, leptin and IGF-I, are leading candidates for linking peripheral energy status to the hypothalamus to regulate GnRH secretion. To be an important blood-borne metabolic signal initiating the pubertal increase in GnRH secretion, the circulating substance must be quantitatively different between the sexually immature and the sexually mature individuals. Our results show that circulating leptin levels increased significantly 30 days before sexual maturity in Shaoxing female ducks and that serum IGF-I concentrations exhibited two peaks during prepubertal development, one at d30 and the other at onset of lay. It is tempting to speculate that IGF-I plays a role as important as leptin in timing the onset of reproduction in ducks and has a role in this process later than leptin.

In conclusion, we have shown in this study that hypothalamic expression of GnRH-I, POMC and NPY mRNA changed greatly at the time of sexual maturity in ducks, accompanied significant changes of circulating leptin and IGF-I levels. These results suggest that the up-regulation of the GnRH-I and the down-regulation of POMC and NPY genes in hypothalamus work in a synergic way to initiate the sexual maturity. As metabolic signals, IGF-I and leptin are involved in this process although their role is still to be determined.

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