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ORIGINAL ARTICLE

Cloning of PRL and VIP cDNAs of the Java sparrow (*Padda oryzivora*)

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ABSTRACT

Complementary DNA (cDNA) of prolactin (PRL) and vasoactive intestinal polypeptide (VIP) of the Java sparrow were cloned and sequenced. The proximal region of the PRL promoter was also identified. Java sparrow PRL was found to have 88.3, 88.3, and 89.1% sequence identity at the cDNA level to PRL of chicken, turkey, and duck, respectively. The predicted amino acid sequence had an overall similarity with a comparable region of chicken (91.4%), turkey (88.9%) and duck (92.0%) PRL. Based on the cDNA sequence and genomic structure of the chicken PRL gene, the proximal promoter was characterized. Sequence analysis of the proximal region of Java sparrow PRL promoter revealed a high degree of similarity to that of chicken, turkey and duck PRL promoters. Moreover, cDNA of prepro-VIP was also cloned and sequenced. Java sparrow prepro-VIP shows high similarity to chicken and turkey prepro-VIP. However, the region upstream of the 5′ untranslated region of Java sparrow prepro-VIP did not show similarity to that of chicken. These results suggest that the mechanisms, which regulate expression of the VIP gene, may be different between precocial and altricial birds, but expression of the PRL gene may be widely conserved in avian species.

Key words: *Java sparrow, mRNA, PRL, promoter, VIP.*

INTRODUCTION

Broody behavior occurs during the breeding season in many avian species. Broody behavior consists of two phases: the incubation of eggs and raising of young. Many studies have indicated the relation between increase of plasma prolactin (PRL) levels and expression of broodiness. In general, birds can be classified into precocial species and altricial species. The words 'precocial' and 'altricial' describe the degree of development in young birds at hatching. In the precocial species, chicks are hatched with eyes open and covered with down. These chicks leave the nest within two days. Chickens, turkeys, ducks and geese are precocial. Altricial chicks are hatched with their eyes closed, they are not feathered, they are unable to walk, and they are unable to feed themselves. Songbirds, crows, pigeons and raptors are altricial. In altricial species, the parents feed to young in the nest and remove fecal sacs containing their eliminations.

In many precocial species, anterior pituitary content and plasma levels of PRL increase as egg laying proceeds, reaches and is maintained at maximum levels during incubation and decreases after hatching of young (Sharp *et al*. 1979; Goldsmith & Williams 1980; Lea *et al*. 1981; Lea & Sharp 1982; Bluhm *et al*. 1983; Hall & Goldsmith 1983; Sharp *et al*. 1989). Levels of PRL mRNA in the anterior pituitary gland correlate with the plasma levels of PRL (Shimada *et al*. 1991; Talbot *et al*. 1991; Wong *et al*. 1991; Kansaku *et al*. 1994). On the other hand, plasma PRL levels increase at late stages of incubation and are maintained at high

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levels until the middle stage of raising of young in altricial species (Goldsmith *et al*. 1981; Ramsey *et al*. 1985; Kikuchi *et al*. 1999; Lormée *et al*. 2000).

Vasoactive intestinal polypeptide (VIP), a hypothalamic peptide, is considered to be the main physiological releasing factor of PRL in birds. VIP stimulates PRL release *in vitro* (Knapp *et al*. 1988; El Halawani *et al*. 1990a,b; Kansaku *et al*. 1995) and *in vivo* (Opel & Proudman 1988; Pitts *et al*. 1994a,b; Maney *et al*. 1999; Vleck & Patrick 1999). In addition, both passive- and active-immunization against VIP have been shown to induce various physiological responses including disruption of incubation behavior, suppression of plasma PRL and pituitary PRL mRNA levels, disruption of crop sac development, and increased egg production (Sharp *et al*. 1989; Lea *et al*. 1991; Talbot *et al*. 1991; Youngren *et al*. 1994; El Halawani *et al*. 1995; Kulick *et al*. 2005). Moreover, the number of immunoreactive VIP neurons and content and/or mRNA levels of VIP in the hypothalamus are highly correlated to changes of plasma PRL levels in both precocial and altricial birds (Macnamee *et al*. 1986; Mauro *et al*. 1989; Sharp *et al*. 1989; Cloues *et al*. 1990; Mauro *et al*. 1992; Rozenboim *et al*. 1993; Chaiseha *et al*. 1998).

Both the gene encoding PRL and its cDNA had been cloned in chickens, turkeys and domestic ducks (Hanks *et al*. 1989; Watahiki *et al*. 1989; Karatzas *et al*. 1990; Wong *et al*. 1991; Kansaku *et al*. 2005). In galliforms, the regulatory region controlling PRL gene expression is highly conserved (Kurima *et al*. 1995; Ohkubo *et al*. 2000) and a VIP response element (VRE) is located within the proximal promoter region (Kang *et al*. 2004). Interestingly, the proximal region including a VRE is also conserved in the galloanserae (Kansaku *et al*. 2005). These results may indicate the conservation of the proximal PRL promoter structure in precocial and perhaps in altricial species. However, there is little information concerning the sequence of PRL cDNA or its structure of regulatory region in the altricial birds. Moreover, a comparison of the pattern of VIP mRNA expression in the hypothalamus during reproductive cycles between precocial and altricial species has not been well documented. Thus, it is unknown whether expression of PRL mRNA in altricial species is regulated in a similar manner as in the precocial species.

In many avian species that breed at middle to high latitudes, exposure to long day photoperiods stimulate the hypothalamus-pituitary-gonadal axis, increasing the secretion of LH. Recently, the regulatory mechanism controlling photoperiodic stimulation of gonadal activity was identified. Photoperiodic type 2 deiodinase gene expression in the hypothalamus of Japanese quail regulates activity of the gonad via GnRH neurons and GnRH secretion (Yoshimura *et al*. 2003; Yamamura *et al*. 2006). Conversely, some species breed on the short day photoperiod. For example, reproductive activity of the Java sparrow is stimulated by a decrease in day length (Saito *et al*. 1992). A possible interaction between GnRH and VIP secreting neurons has been suggested (Teruyama & Beck 2001). However, to date a mechanism regulating reciprocal immunoreactivity and/or mRNA expression between GnRH and VIP has not been identified. In addition, very little is known regarding the regulation of VIP and PRL in altricial and/or short day breeding species.

Accordingly, this study was conducted to clone and ultimately, to investigate the regulatory mechanisms controlling gene expression of PRL and VIP in the Java sparrow (*Padda oryzivora*).

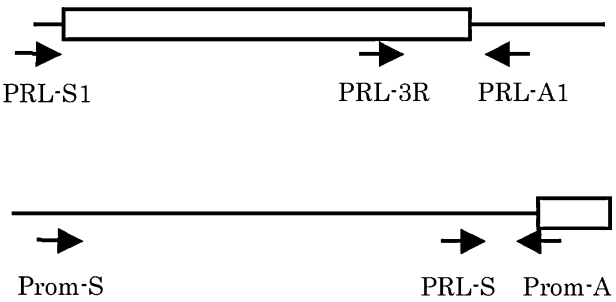
MATERIALS AND METHODS

Tissue sampling and RNA isolation

The anterior pituitary glands and hypothalamus of 2-year-old non-laying Java sparrows ($n = 5$) obtained from the local breeder were collected after decapitation. These were then snap frozen by liquid nitrogen and stored at −80°C until total RNA extraction. The total RNA was extracted using an RNA isolation reagent (TRIzol; Invitrogen, San Diego, CA, USA) according to the method described by Chomczynski (1993). The amount of total RNA was estimated by spectrophotometry (GeneQuant; GE Healthcare UK, Little Chalfont, England). Handling of Java sparrows and sampling of tissues were conducted according to the guidelines for animal experimentation at Azabu University.

PCR cloning of Java sparrow PRL cDNA

Total RNA (1 μg) isolated from the anterior pituitary gland was denatured at 70°C for 10 min with random hexamer primers and reverse-transcribed with 200 units of SuperScript II (Invitrogen) in a 20 μL mixture. Based on the sequences of chicken, turkey and duck PRL cDNA, primers (PRL-S1: TTCTGGTAGAGCAAGTCATC, PRL-A1: TGAAAAGTGGC AAAGCAACA) were designed. The location of the primers and the sequencing strategy are indicated in Figure 1. The reverse-transcribed product was subjected to 35 cycles of PCR amplification using Z-Taq polymerase (Takara, Shiga, Japan) in a total volume of 25 μL. The amplification profile consisted of 2 min of denaturation at 94°C for the first cycle and 30 s per cycle thereafter, 15 s annealing at 55°C, and 20 s extension at 72°C for the first 34 cycles and 10 min extension on the final cycle. PCR products were directly sequenced on both strands after purification (Rapid PCR Purification system; Marligen Biosciences, Ijamsville, MD, USA). Based on the sequence cloned in this study, primer (PRL-3R: GATGAG

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PRL-S1: TTCTGGTAGAGCAAGTCATC
PRL-A1: TGAAAAGTGGCAAAGCAACA
PRL-3R: GATGAGGACTCCAGGCTCTT
Prom-S: TGAATATGAATGTGGAAGAA
Prom-A: CTTGGTGCTCATGGTAGAGA
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Figure 1 Primer location and cloning strategy of Java sparrow PRL cDNA and 5′ flanking region. The open box represents open reading frame.

GACTCCAGGCTCTT) was designed for cloning of 3′ region. The 3′ region was cloned by rapid amplification cDNA end (RACE) using Takara-3′-RACE kit (Takara, Shiga, Japan) according to the manufacturer's protocol. Amplified 3′-RACE PCR products were purified and sequenced. DNA sequencing was performed using an Applied Biosystems Model 310 sequencer and the dideoxy-mediated chain-termination method (Sanger *et al*. 1977). The cloned sequence was analyzed by using FASTA and PHYLIP.

Western blot analysis

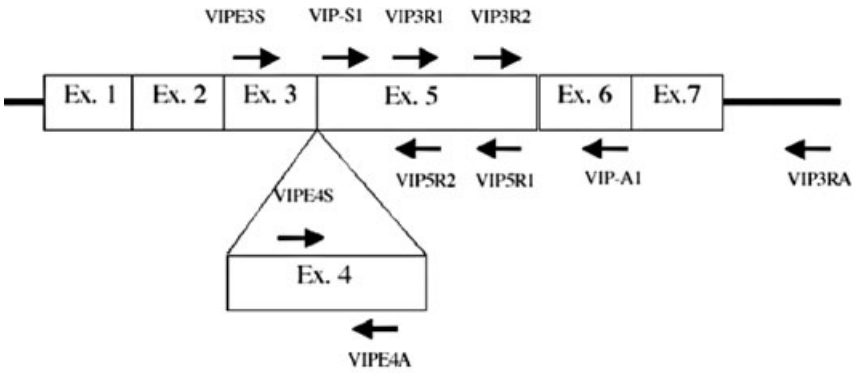
Anterior pituitary glands from male and female non-laying Java sparrows were homogenized individually in 10 μL of 10 mmpl/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, and 0.5% Tween 20 using a microhomogenizer (Wheaton Science, Millville, NJ, USA). After centrifugation, an aliquot of the extract was mixed with an equal volume of 10 mmol/L Tris–HCl, 1 mmol/L EDTA, 0.1 mmol/L PMSF, 1 μmol/L pepstatin, and 1 μmol/L leupeptin and used for immunoblotting. Pituitary homogenates were adjusted to 500 ng protein/μL and separated on a 13.5% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred onto Hybond-ECL (GE Healthcare UK) at constant voltage of 30 V for 1 h using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated for 1 h with rabbit anti chicken PRL antibody at a 1/100 000 dilution in TBS-0.5% Tween 20. The membrane was washed three times with TBS-0.5% Tween 20 and incubated with HRP-linked goat anti rabbit IgG antibody (GE Healthcare UK) at room temperature for 1 h. The membrane was washed four times (5 min each) before addition of detection reagents (GE Healthcare UK). Luminescence was assessed by exposure to instant film (FP-3000B; Fujifilm, Tokyo, Japan) using ECL mini-camera (GE Healthcare UK) at room temperature for 1 h.

PCR cloning of Java sparrow PRL regulatory region

Genomic DNA was obtained from red blood cells of Java sparrows. In short, 20 μL of blood was taken and centrifuged at 3000 rpm for 10 min. Precipitated blood cells were washed 2 times with saline (0.935% NaCl, 0.5 mmol/L EDTA). After aspiration of supernatant, cells were lyzed by 500 μL of lysis solution (0.5% TritonX-100, 50 mmol/L MgCl, 100 mmol/L Tris (pH 8.0), 5.48% sucrose). The solution was spun at 3000 rpm and the supernatant was removed. Precipitates were dispersed by 50 μL of saline. Proteinase K solution (500 μL) (0.1% Proteinase K, 10 mmol/L Tris HCl-10 mmol/L EDTA-0.5%SDS) was added into the nuclear dispersion solution and incubated for 12 h at 37°C. After proteinase K treatment, residual protein was removed by phenol-chloroform and chloroform extraction twice. After chloroform extraction, 2.5 volumes of ethanol and 1/10 volume of 5 mol/L NaCl were added. After 1 h rotation at room temperature, genomic DNA was precipitated by centrifugation (12 000 rpm for 10 min at 4°C). Genomic DNA was washed by 70% ethanol, dried and dissolved in 200 μL of TE buffer. Based on the nucleotide sequence of the chicken, turkey and duck genomic DNA, and the Java Sparrow PRL cDNA obtained in this study, primer pairs were designed to amplify the regulatory region of the Java Sparrow PRL gene. PCR was conducted using newly designed primers, Prom-S (TGAATATGAATGTGGAAGAA) designed based on a highly conserved region of the proximal promoter and Prom-A (CTTGGTGCTCATGGTAGAGA) which had complementary sequence to putative Exon1 of the Java sparrow PRL cDNA.

PCR cloning of Java sparrow VIP cDNA

Based on the sequences of chicken and turkey VIP cDNA (McFarlin *et al*. 1995; Talbot *et al*. 1995; You *et al*. 1995) and human genomic DNA structure (Linder *et al*. 1987), primers were designed (VIP-S1: AGGACAGTCCTGTCAAACG, VIP-A1: GGAAGGTTCAAGAATTTCTGC) to amplify a partial fragment of the Java sparrow VIP gene (Fig. 2). Genomic DNA was subjected to 35 cycles of PCR amplification using Z-Taq polymerase in a total volume of 25 μL. The amplification profile consisted of 2 min of denaturation at 94°C for the first cycle and 30 s per cycle thereafter, 20 s annealing at 55°C, and 45 s extension at 72°C for the first 34 cycles and 10 min extension on the final cycle. PCR products were sequenced as previously described. Based on the obtained sequence of the VIP gene, primers (VIP5R1: CATCTGCTTTCGAAAGCG GCTG, VIP5R2: GTGAAGACAGCATCAGAGTG, VIP3R1: CGCCACTCTGATGCTGTCTT, VIP3R2: CAGCCGCTTTC-GAAAGCAGATG) were designed to amplify the 5′ and 3′ regions (Fig. 2). The 5′ and 3′ regions were cloned using total RNA isolated from the hypothalamus and a 5′-RACE (Invitrogen) or 3- RACE kit (Takara) according to the manufacturer's protocol. To investigate the possibility of expression of the long form of prepro-VIP mRNA containing PHI, primers (VIPE3S: GATTGGGAAACAGAATGC, VIPE4S: CATGCT GATGGAATTTTCAC, VIPE4A: TTCTAATAAGCGAATG CAGA) were designed based on the sequence of the PHI/PHM coding region of chicken, turkey, rat, mouse, dog and



VIP-S1: AGGACAGTCCTGTCAAACG
VIP-A1: GGAAGGTTCAAGAATTTCTGC
VIP5R1: CATCTGCTTTCGAAAGCGGCTG
VIP5R2: GTGAAGACAGCATCAGAGTG
VIP3R1: CGCCACTCTGATGCTGTCTT
VIP3R2: CAGCCGCTTTCGAAAGCAGATG
VIPE3S: GATTGGGAAACAGAATGC
VIPE4A: TTCTAATAAGCGAATGCAGA
VIPE4S: CATGCTGATGGAATTTTCAC
VIP3RA: TACAGCATATGGACTCACAG

Figure 2 Primer location and cloning strategy of Java sparrow prepro-VIP. The open box represents open reading frame.

human. PCR amplification was conducted between VIPE3S and VIPE4A, and VIPE4S and VIP5R1, respectively. Since 3′-UTR amplification produces two different sizes of products due to the two alternative polyadenylation-sites, VIP3RA (TACAGCATATGGACTCACAG) was designed to sequence the 3′-UTR region. Products obtained were sequenced as previously described.

RESULTS

PCR cloning of Java sparrow PRL cDNA

Approximately 700 bp of cDNA encoding the putative PRL in Java sparrow was obtained by PCR amplification between primers PRL-S1 and PRL-A1. The up- and down-stream sequences containing untranslated regions were cloned by RACE. The sequence of 5′-RACE contained the sequence ACCATGA similar to the consensus sequence (5′-ACCATGG-3′) for the initiation site of eukaryotic ribosomes (Kozak 1986) and the translation initiating methionine codon was detected in almost the same position as in chickens and turkeys. Thus, it is likely that this ATG codon is the translation initiation site. The highly conserved AATAAA hexanucleotide polyadenylation signal (Proudfoot & Brownlee 1976) was detected 13 nucleotides from the poly dA tract. Of the 983 nucleotides that were sequenced, 16, 280 and 687 bases represented the 5′-UTR, 3′-UTR and open reading frame which predicted a peptide of 229 amino acids (Fig. 3). Comparison of the predicted amino acid sequence of Java sparrow PRL with other species and hydrophobicity analysis was done. The prepeptide consists of a 30-amino acid signal peptide and 199-amino acid mature PRL. Java sparrow PRL was found to have 88.3, 88.3, and 89.1% sequence identity at the cDNA level compared to PRL of chickens, turkeys, and ducks, respectively. The mature Java sparrow PRL had an overall similarity with a comparable region of chicken (91.0%), turkey (88.9%) and duck (92.0%) PRL (Table 1).

Western blot analysis

Two bands corresponding to approximately 23 and 25 kDa of signal were detected in the anterior pituitary gland of male and female Java sparrow by Western blotting (Fig. 4). The intensity of the signal in the male was almost equal of that observed in the female.

PCR cloning of Java sparrow PRL regulatory region

By PCR amplification using genomic DNA as a template, approximately 200 bp of regulatory region including putative 5′-UTR was obtained and

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                                        AGAGCAAGTCATCATCCAGAATCCCAACC       29

ATGAGCACCAAGGGGGCTTCACTGAAAGGTTTGTTGCTGGCAGCCCTTCTGGTGTCCCAC       89
MetSerThrLysGlyAlaSerLeuLysGlyLeuLeuLeuAlaAlaLeuLeuValSerHis       20

ATGCTTCTGACAAAGGAAGGAGTGACCTCTTTGCCAATCTGCCCCAATGGATCTGTCAAT      149
MetLeuLeuThrLysGluGlyValThrSerLeuProIleCysProAsnGlySerValAsn       40

TGCCAACTCTCCCTTGAGGAGCTTTTTGACCGAGCAGTTAAACTTTCACACTACATTCAC      209
CysGlnLeuSerLeuGluGluLeuPheAspArgAlaValLysLeuSerHisTyrIleHis       60

TTCCTCTCTTCGGAAATGTTCAATGAATTTGATGAACGCTACGCCCAGGGCCGGGGTTTC      269
PheLeuSerSerGluMetPheAsnGluPheAspGluArgTyrAlaGlnGlyArgGlyPhe       80

ATTGCAAAAGCTGTCAACAGCTGCCACACTGCGTCTTTAACCACTCCTGAAGATAAGGAG      329
IleAlaLysAlaValAsnSerCysHisThrAlaSerLeuThrThrProGluAspLysGlu      100

CAGGCTCAGCAGATTCATCACGAAGACCTACTGAATTTAATACTGGGAGTTCTGCGTTCC      389
GlnAlaGlnGlnIleHisHisGluAspLeuLeuAsnLeuIleLeuGlyValLeuArgSer      120

TGGAATGATCCCCTGATACACCTGGCCTCTGAAGTACAAAGAATCAAAGAAGCTCCAGAA      449
TrpAsnAspProLeuIleHisLeuAlaSerGluValGlnArgIleLysGluAlaProGlu      140

ACCATTCTCTGGAAGGCTGTGGAGATTGAAGAACAAAACAAGCGACTTCTAGAAGGAATG      509
ThrIleLeuTrpLysAlaValGluIleGluGluGlnAsnLysArgLeuLeuGluGlyMet      160

GAGAAAATAGTTGGGCGGGTTCACTCTGGGGAGGTCGAAAATGACATTTACACTCCTTGG      569
GluLysIleValGlyArgValHisSerGlyGluValGluAsnAspIleTyrThrProTrp      180

GATGGACTCCCATCCCTGCAGCTTGCTGATGAGGACTCCAGGCTCTTTGCCTTTTACAAC      629
AspGlyLeuProSerLeuGlnLeuAlaAspGluAspSerArgLeuPheAlaPheTyrAsn      200

CTGCTTCACTGCCTCCGCCGAGATTCCCACAAAATTGACAACTATCTCAAGGTTTTGAAG      689
LeuLeuHisCysLeuArgArgAspSerHisLysIleAspAsnTyrLeuLysValLeuLys      220

TGCCGCCTAATCCACGACAACAATTGTTGAGTAGTCATGGGCCTGATCATGTACTGAAGT      749
CysArgLeuIleHisAspAsnAsnCys***                                    229

CATTCATCATGTGTTCTTGATGCTTTCCCACTTTATGAAAATCACACTGTGCAGAAGCTG      809
TATAATCAGTAACTTTCAGGCATGTTTGTATGAATTCTGGCTGCAACAATTAGCACCACA      869
CGTGTCAGTGCTTTAAAATACTACCAACTACTTGTATGACAAGAATATACTTTTCCGTCT      929
AATCTTCTCCCCTAGTAGTATTTCAGTGACAAACAGGTAATGCATATAAAAATAAAAATT      989
CTTAAA                                                            995
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Figure 3 Nucleotide and deduced amino acid sequence of Java sparrow PRL cDNA. Nucleotides are numbered on the right side of the sequence. Untranslated regions of Java sparrow PRL cDNA are reported in lowercase letters and the open reading frame in uppercase letters. Poly A signal is underlined.

Table 1 Sequence homology and amino acid similarity (%) of mature bird PRLs

	J. Sparrow	Chicken	Turkey	J. Quail	Duck	C. Duck
J. Sparrow		88.3	88.3	88.8	89.1	88.8
Chicken	91.0		95.4	95.5	92.0	91.7
Turkey	88.9	93.0		95.1	91.7	91.6
J. Quail	91.0	96.5	92.5		91.5	91.2
Duck	92.0	96.0	92.5	95.5		99.0
C. Duck	91.0	95.0	92.5	94.5	99.0	

Sequences of Japanese quail and comb duck was deduced from DNA databank accession number AB162003 and AM180924, respectively. Values above and under represent homology of cDNA and amino acids, respectively.

sequenced. The 5′ flanking region of Java sparrow PRL promoter shows 87.7%, 85.8% and 87.1% sequence similarity to a comparable region of the chicken, turkey and duck PRL proximal promoters (Fig. 5).

PCR cloning of Java sparrow VIP cDNA

Approximately 790 bp of gene fragment of Java sparrow VIP gene representing exon 5–6 and the

intervening intron was obtained by PCR amplification between primers VIP-S1 and VIP-A1. The sequence at the exon-intron junction in the partial VIP gene is in agreement with the GT-AG rule (Breathnach & Chambon 1981). Following amplification from total hypothalamic RNA, two different sizes of 3′RACE products were detected using the antisense primer complementary to downstream of 3′-UTR (VIP3RA: TACAGCATATGGACTCACAG) to identify the poly-adenylation sites. To identify the differences, products were ligated into a TA vector (Invitrogen) and sequenced. Sequence analysis identified the 2 polyadenylation-sites. By the 5′- and 3′-RACE, 825 bp of Java sparrow VIP cDNA was characterized. PCR amplifications between primers VIPE3S and VIPE4A, and between primers VIPE4S and VIP5R1 result in 240 bp and 171 bp of product, respectively (Fig. 6).

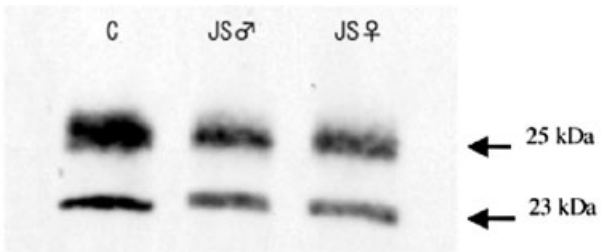

Figure 4 Example of SDS-PAGE followed by Western blot analyzes of Java sparrow pituitary PRL. Total 5 μg of protein from male and female extract of pituitary gland was electrophoresed on 13.5% SDS-PAGE gel, transferred to membrane and visualized by polyclonal antibody against chicken PRL. C, chicken (non-laying) pituitary extract; JS ♂, Java sparrow male pituitary; and JS ♀, Java sparrow female pituitary.

The sequence of these products contained PHI coding region and partial region of exon-3 or exon-5. Of the 1045 nucleotides that were sequenced, 170, 281 and 594 bases represented the 5′-UTR, 3′-UTR and open reading frame, which predicted a peptide of 198 amino acids (Fig. 7). Comparison of the predicted amino acid sequence of Java sparrow VIP with chicken and turkey and hydrophobicity analysis was done. The prepeptide

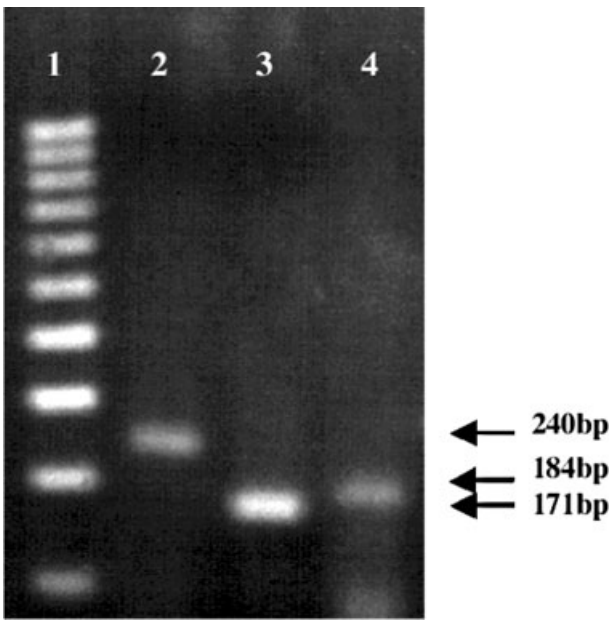

Figure 6 Detection of presence of long form of Java sparrow prepro-VIP by PCR. Lane 1: 100 bp ladder, Lane 2: primer combination VIPE3S and VIPE4A (240 bp), Lane 3: primer combination VIPE4S and VIP5R1 (171 bp), Lane 4: primer combination VIPE3S and VIP5R1 (184 bp).

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Sparrow   CTGAATATGA ATGTGGAAGA AAGGCAATTT GATGTTTGTA ATTATGGAGG   50
Duck      CTGAATATGA ATGTGGAAGA AAGGCAGTTT GATGTTTGTA ATTATCGAGG   50
Chicken   TTGAATATGA ATGTGGAAGA GAGGCAATTT GATGTTTGTA ATTATCGAGG   50
Turkey    TTGAATATGA ATGTGGAAGA GAGGCAATTT GATGTTTGTA ATTACCGAGG   50

Sparrow   CAAACTCCAC AACCTGCTGA ATGTATGCAA AA-TGGACCC TGCATGGTGT  100
Duck      TAAACTCCAC GACCTGTTGA ATATATGCAA AA-TGGACCC CGGATGGTGT  100
Chicken   TAAACTCCAC GACCTGCTGA ATGTATGCAA AAGTGGACCC CGGATGGTGT  100
Turkey    TAAACTCCAC AACCTGCTGA ATGTATGCAA A-CTGGACCC CGGATGGTGT  100

Sparrow   ATATAAGAGC AGTATGTGCA GAGAATAGCA GCAAGAATTG AGATTTCTTT  150
Duck      ATATAAATCT GGTATGTGCA GAAAATAAAA GCAAGTATTG AGACTTCTTT  150
Chicken   ATATAAATCT G--ACGTGCA GAAAGTAAGA GCAGGTATTG AGATTTCTTT  150
Turkey    ATATAAATCT G--ACATGCA GAAAGTAAGA GCAGGTATTG AGACTTCTTT  150

Sparrow   CTGGTAAAGA AAGTCATCAT CCAGAATCTC TACCATGAGC ACCAAG....  200
Duck      CTGGCAGAGC AAGTCATCCT ACAGGGTCTC TACCATGAGC ACCAAG....  200
Chicken   CTGGTAGAGC AAGTCATCAC ACAGAATCCC TACCATGAGC AACAGA....  200
Turkey    CTGGTAGAGC AAGTCATCAC AGAGAATCCC TACCATGAGC AACACA....  200
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Figure 5 Comparison of Java sparrow, duck, turkey and chicken PRL promoter. Potential TATA box was double underlined. Highly conserved sequence between birds and mammalian PRL gene is underlined. VIP response element and translation starting codon were indicated by dashed-underlined and bold letters, respectively.

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          AGAGCGTGCGGCGAGACGGAGCCCTCCTCTTCATCCTCCTCCTCCTCCTC        50
ACTCTCTGCTGCTGCCGCCCCGGCAGCCGCGCAGCGGAGCAGGGCGCACTGCCCCGGCAC      110
CGCTCCGCTCCGCGCCCGCCCAGCCAGACCCACCGACGGACTCGCGGCTCCGTGGCGGCC      170

ATGGAGCACCGCGGCGCCTCCCCGCTCCTCCTCGCCCTCGCCCTCCTCAGCGCCCTCTGC      230
MetGluHisArgGlyAlaSerProLeuLeuLeuAlaLeuAlaLeuLeuSerAlaLeuCys       20

TGGCGGGCGCGGGCGCTGCCCCCGCGGGGCGCCGCCTTCCCTCCCGTCCCGCGATTGGGA      290
TrpArgAlaArgAlaLeuProProArgGlyAlaAlaPheProProValProArgLeuGly       40

AACAGAATGCCATTTGATGGAGCCAGTGAACCTGACCATGCCCGTGGGTCATTAAAGTCA      350
AsnArgMetProPheAspGlyAlaSerGluProAspHisAlaArgGlySerLeuLysSer       60

GAATCAGATATTTTGCAGAACACACTACCTGAAAATGAGAAATTCTATTTTGATCTGTCC      410
GluSerAspIleLeuGlnAsnThrLeuProGluAsnGluLysPheTyrPheAspLeuSer       80

AGAATTATTGATAGAAATGCAAGGCATGCTGATGGAATTTTCACCAGTGTCTACAGCCAT      470
ArgIleIleAspArgAsnAlaArgHisAlaAspGlyIlePheThrSerValTyrSerHis      100

CTTTTGGCTAAACTTGCTGTGAAGAGATATCTGCATTCGCTTATTAGAAAACGAGTTAGC      530
LeuLeuAlaLysLeuAlaValLysArgTyrLeuHisSerLeuIleArgLysArgValSer      120

TCCCAGGACAGTCCTGTCAAACGCCACTCTGATGCTGTCTTCACTGACAACTACAGCCGC      590
SerGlnAspSerProValLysArgHisSerAspAlaValPheThrAspAsnTyrSerArg      140

TTTCGAAAGCAGATGGCTGTGAAGAAATACTTAAACTCAGTTTTAACCGGAAAAAGAAGC      650
PheArgLysGlnMet aIaValLysLysTyrLeuAsnSerValLeuThrGlyLysArgSer     160

CAGGAAGAGCTAAATCCTGCTAAACTTCGAGATGAAGCAGAACATCTTGAACCATCCTTT      710
GlnGluGluLeuAsnProAlaLysLeuArgAspGluAlaGluHisLeuGluProSerPhe      180

TCAGAAAACTACGATGCTGTAGATGAGCTGCTGAGCCACCTCCCACTGGACCTCTGAAGG      770
SerGluAsnTyrAspAlaValAspGluLeuLeuSerHisLeuProLeuAspLeu***         198

ACACCTGGTAAAGTCTATGACAAGAACAAGCTATTTTTGAGTTCCACATAGTATTTCAAA      830
GAGATGACTTTAGTCATCAAACCAGAACAAATATGTTGTGAAGTGAAAGTTGTGATATAT      890
TTGTTTCTTACGTAATAAAAGTTGATATTTACATTGTAAATATTACTCTAGCATTCCCTA      950
CTGAAAGCTGTACATAGGATGCCAGTTTAACTCATGAGAAGTCTGTGAGTCCATATGCTG     1010
TAAATCTTTACTTCAATAAATTCATTTGAAAATGA                              1045
```

Figure 7 Nucleotide and deduced amino acid sequence of Java sparrow VIP cDNA. Nucleotides are numbered on the right side of the sequence. Untranslated regions of Java sparrow VIP cDNA are reported in lowercase letters and the open reading frame in uppercase letters. The poly A signal is underlined.

consists of a 20-amino acid signal peptide and 178-amino acid mature prepro-VIP. Java sparrow prepro-VIP was found to have 94.2 and 92.3% sequence identity at the cDNA level compared to PHI-lacking alternative splicing form of prepro-VIP of chicken and turkey, respectively. When compared to chicken and turkey prepro-VIP, Java sparrow prepro-VIP lacked 2 amino acids. However, the VIP encoding region had the same amino acid sequence as in chickens and turkeys. Although 35 bp from the ATG codon had high similarity, more upstream regions of the 5′-UTR did not have similarity to chicken 5′-UTR. Sequence homology and amino acid similarity of VIP in birds are listed in Table 2.

Table 2 Sequence homology and amino acid similarity (%) of prepro-VIP in bird

	J. Sparrow	Chicken	Turkey
J. Sparrow		94.2	92.3
Chicken	94.5		97.5
Turkey	93.0	97.5	

Values above and under represent homology of cDNA and amino acids, respectively.

DISCUSSION

This paper describes nucleotide sequence information of Java sparrow PRL cDNA and its regulatory region, and prepro-VIP cDNA. The Java sparrow PRL cDNA encoded a predicted protein of 229 amino acids. The predicted protein includes a 30-amino acid signal peptide followed by the 199-amino acid mature PRL. Within the signal peptide (30 amino acids), 6, 4, and 5 amino acids were different when compared to chicken, turkey and duck, whereas within the mature peptide (199 amino acids), 18, 22 and 16 of amino acids differed, respectively. However, most of the amino acid

residues that participate in the formation of disulfide bonds and folding structure via characteristic secondary structure within the mature peptide were fully conserved when compared to chickens, turkeys and ducks. Thus, it is considered that structure of Java sparrow PRL is similar to those of precocial birds previously cloned.

One of the notable results in this study is the presence of consensus sequence for N-linked glycosylation (Asn-X-Ser) in Java sparrow PRL at position 36 as in pekin duck (Kansaku *et al*. 2005) and comb duck (DNA database accession number: AM180924). Since neither chicken nor turkey PRLs has this sequence, an alternative glycosylation site (Asn-X-Cys) for N-glycosylation has been proposed in galliforms (Corcoran & Proudman 1991). In chickens, this site is located at position 56, whereas turkeys have two sites at positions 56 and 197. Java sparrows have two alternative glycosylation sites at positions 56 and 197. To date, the possibility of glycosylation at position 36 or at alternative sites of Java sparrow PRL is unknown. However, the presence of a glycosylated form of PRL in Java sparrow anterior pituitary gland at least suggests that position 36 or 56 is glycosylated.

The proximal region of the Java sparrow PRL promoter cloned in this study shows high similarity to that of turkeys, chickens and ducks (Kurima *et al*. 1995; Ohkubo *et al*. 2000; Kansaku *et al*. 2005). The mechanisms regulating the expression of PRL have not been extensively studied in altricial birds. Unlike galliforms, the Java sparrow proximal region of the promoter has no consensus sequence for Pit-1 binding, but a sequence similar to the VIP response element (VRE) observed in turkeys was noted. In the turkey, position −74/−30 of PRL gene is identified as a VIP *cis*-acting element (Kang *et al*. 2004). The homologous region in the Java sparrow had only 1 base nucleotide difference to that of turkeys, and thus the PRL gene may be responsive to VIP as in chickens and turkeys. In chickens and turkeys, the transcription start site is located 53 bp upstream of the ATG codon (Kurima *et al*. 1995; Ohkubo *et al*. 2000). The proximal promoter and putative 5′-UTR of Java sparrow cloned in this study showed high similarity to those of chickens and turkeys. Although this study did not identify the transcription start site, sequence similarity may indicate that the transcription starting site and transcriptional regulation of Java sparrow PRL are similar to those in chickens, turkeys and ducks.

This study is the first to report cloning of prepro-VIP in an altricial bird. In chickens and turkeys, there are two forms of prepro-VIP (Talbot *et al*. 1995; You *et al*. 1995). The long form contains both PHI and VIP, whereas the short form contains only VIP. Moreover, the short form is more abundant than the long form. The sequence cloned in this study is identical to the short form of chicken and turkey prepro-VIP. Although PCR amplifications betweens primers VIPE3S and VIPE4A and primers VIPE4S and VIP5R1 suggest the presence of long form of prepro-VIP mRNA, a single product was obtained by PCR between VIPE3S and VIP5R1 (Fig. 6). These results are in accordance with the results of the 5′-RACE PCR and sequencing, and suggest that the short form of prepro-VIP mRNA in Java sparrows is much more abundant than the long form of prepro-VIP as in chickens and turkeys. When compared to chicken and turkey prepro-VIP, Java sparrow prepro-VIP has high similarity and the amino acid sequence of mature VIP is identical. The same amino acid sequence of mature VIP is also identified in American alligator, African clawed frog, European green frog, geese and ducks (Wang & Conlon 1993; Chartrel *et al*. 1995; BC043792; DQ023161; DQ200173). These results may indicate that the amino acid sequence of VIP is conserved and common in the birds.

Although the end part of putative exon 1 (120–160) has a similar sequence to chicken prepro-VIP, the upstream region of 5′-UTR (1–138) of Java sparrow shows no similarity to that of chickens. This result indicates the position of splicing is shared between chickens and Java sparrows. However, no similarity in more distal regions of the 5′-UTR may indicate that different regulatory mechanisms controlling expression of VIP may exist between precocial and altricial birds. Cloning and sequencing of the regulatory region of VIP may clarify this issue. On the other hand, the 3′-UTR shows very high similarity to chickens and turkeys. Interestingly, 3′-RACE PCR produced two different sized bands and sequence analysis detected two polyadenylation sites at positions 928 and 1044. Although chickens and turkeys contain polyadenylation signals at homologous positions, only the downstream signal is functional in chickens and turkeys (Talbot *et al*. 1995; You *et al*. 1995).

To investigate the relationship between the usage of polyadenylation sites and alternative splicing, two sets of PCR (VIPE3S and VIP3RA, and VIPE4S and VIP3RA) were examined. PCR between VIPE4S and VIP3RA resulted in no amplification, but PCR between VIPE3S and VIP3RA amplified a product of 625 bp

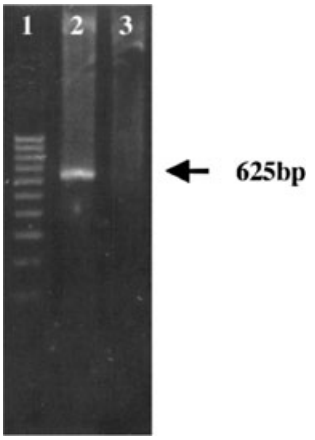

Figure 8 Relationship between alternative splicing and polyadenylation of VIP. Lane 1 : 100 bp ladder, Lane 2: primer combination VIPE3S and VIP3RA (625 bp), Lane 3: primer combination VIPE4S and VIP5R1 (no PCR product).

(Fig. 8). This result clearly indicates that polyadenylation at position 1044 is only used for the PHI lacking a short form. To date, the function of the upper polyadenylation signal of chickens and turkeys is unknown. Two different length mRNA transcripts (1700 bp and 1000 bp) originating from polyadenylation signals in different positions were identified in the rat small intestine and hypothalamus (Lamperti *et al*. 1991). Interestingly, the amount of short form of rat VIP mRNA increases after estrogen treatment in the anterior pituitary gland (Lam *et al*. 1990). Thus, different positions of polyadenylation in the Java sparrow prepro-VIP may be associated with the ratio of short and long forms produced by alternative splicing. The ratio may be altered during a reproductive cycle and/or different physiological states.

In conclusion, sequence information for Java sparrow PRL cDNA and its flanking region, and prepro-VIP cDNA will be useful to investigate the mechanisms regulating expression of the PRL and VIP genes, and also as a tool for genomic cloning. The latter may provide information on both the general and species specific mechanisms of PRL and VIP mRNA expression in birds. Moreover, structural conservation of amino acids of PRL and VIP in the various birds provides a good indication of the reliability of a previous report, which measured plasma PRL levels using heterologous radioimmunoassay systems, or immunocytochemical analysis of VIP in altricial species.

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REFERENCES

Bluhm CK, Phillips RE, Burke WH. 1983. Serum levels of Luteinizing hormone (LH), prolactin, estradiol and progesterone in laying and nonlaying canvasback ducks (*Aythya valisineria*). *General and Comparative Endocrinology* **52,** 1–16.

Breathnach R, Chambon P. 1981. Organization and expression of eucaryotic split genes coding for proteins. *Annual Review of Biochemistry* **50,** 349–383.

Chaiseha Y, Tong Z, Youngren OM, El Halawani ME. 1998. Transcriptional changes in hypothalamic vasoactive intestinal peptide during a photo-induced reproductive cycle in the turkey. *Journal of Molecular Endocrinology* **21,** 267–275.

Chartrel N, Wang Y, Fournier A, Vaudry H, Conlon JM. 1995. Frog vasoactive intestinal polypeptide and galanin: primary structures and effects on pituitary adenylate cyclase. *Endocrinology* **136,** 3079–3086.

Chomczynski PA. 1993. Reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* **15,** 532–537.

Cloues R, Ramos C, Silver R. 1990. Vasoactive intestinal polypeptide-like immunoreactivity during reproduction in doves: influence of experience and number of offspring. *Hormone and Behavior* **24,** 215–231.

Corcoran DH, Proudman JA. 1991. Isoforms of turkey prolactin: evidence for differences in glycosylation and in tryptic peptide mapping. *Comparative Biochemistry and Physiology* **99B,** 563–570.

El Halawani ME, Silsby JL, Mauro LJ. 1990a. Vasoactive intestinal peptide is a hypothalamic prolactin-releasing neuropeptide in the turkey (*Meleagris gallopavo*). *General and Comparative Endocrinology* **78,** 66–73.

El Halawani ME, Silsby JL, Mauro LJ. 1990b. Enhanced vasoactive intestinal peptide-induced prolactin secretion from the anterior pituitary cells of incubating turkeys (*Meleagris gallopavo*). *General and Comparative Endocrinology* **80,** 138–145.

El Halawani ME, Silsby JL, Rozenboim I, Pitts GR. 1995. Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (*Meleagris gallopavo*). *Biology of Reproduction* **52,** 179–183.

Goldsmith AR, Edwards C, Koprucu M, Silver R. 1981. Concentrations of prolactin and luteinizing hormone in plasma of doves in relation to incubation and development of the crop gland. *Journal of Endocrinology* **90,** 437–443.

Goldsmith AR, Williams DM. 1980. Incubation in mallard (*Anas platyrhynchos*); changes in plasma levels of prolactin

and luteinizing hormone. *Journal of Endocrinology* **86,** 371–379.

Hall MR, Goldsmith AR. 1983. Factors affecting prolactin secretion during breeding and incubation in the domestic Duck (*Anas platyrhynchos*). *General and Comparative Endocrinology* **49,** 270–276.

Hanks MC, Alonzi JA, Sharp PJ, Sang HM. 1989. Molecular cloning and sequence analysis of putative chicken prolactin cDNA. *Journal of Molecular Endocrinology* **2,** 21–30.

Kang SW, Gazzillo LC, You S, Wong EA, El Halawani ME. 2004. Turkey prolactin gene regulation by VIP through 35-bp cis-acting element in the proximal promoter. *General and Comparative Endocrinology* **138,** 157–165.

Kansaku N, Ohkubo T, Okabayashi H, Guemene D, Kuhnlein U, Zadworny D, Shimada K. 2005. Cloning of duck PRL cDNA and genomic DNA. *General and Comparative Endocrinology* **141,** 39–47.

Kansaku N, Shimada K, Saito N. 1995. Regionalized gene expression of prolactin and growth hormone in the chicken anterior pituitary gland. *General and Comparative Endocrinology* **99,** 60–68.

Kansaku N, Shimada K, Terada O, Saito N. 1994. Gene expression of prolactin, growth hormone, and luteinizing hormone-beta subunit gene expression in the cephalic and caudal lobes of the anterior pituitary gland during embryogenesis and different reproductive stages in the chicken. *General and Comparative Endocrinology* **96,** 197–205.

Karatzas CN, Zadworny D, Kuhnlein U. 1990. Nucleotide sequence of turkey prolactin. *Nucleic Acids Research* **18,** 3071.

Kikuchi M, Ishii S, Imanishi S, Kansaku N, Shimada K. 1999. Changes in plasma levels of prolactin, luteinizing hormone and progesterone in relation to breeding activities in the bull-heated shrike (*Lanius bucephalus*). In: Kwon HB, Joss JMP, Ishii S (eds), *Recent Progress in Molecular, Comparative En docrinology*, pp. 471–476. Chonnam National University, Kwangju, South Korea.

Knapp TR, Fehrer S, Silsby JL, Porter TE, Behnke EJ, El Halawani ME. 1988. Gonadal steroid modulation of basal and vasoactive intestinal polypeptide-stimulated prolactin release by turkey anterior pituitary cells. *General and Comparative Endocrinology* **72,** 226–236.

Kozak M. 1986. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proceedings of the National Academy of Sciences of the United States of America* **83,** 2850–2854.

Kulick RS, Chaiseha Y, Kang SW, Rozenboim I, El Halawani ME. 2005. The relative importance of vasoactive intestinal peptide and peptide histidine isoleucine as physiological regulators of prolactin in the domestic turkey. *General and Comparative Endocrinology* **142,** 267–273.

Kurima K, Proudman JA, El Halawani ME, Wong EA. 1995. The turkey prolactin-encoding gene and its regulatory region. *Gene* **156,** 309–310.

Lam KS, Srivastava G, Lechan RM, Lee T, Reichlin S. 1990. Estrogen regulates the gene expression of vasoactive intestinal peptide in the anterior pituitary. *Neuroendocrinology* **52,** 417–421.

Lamperti ED, Rosen KM, Villa-Komaroff L. 1991. Characterization of the gene and messages for vasoactive intestinal polypeptide (VIP) in rat and mouse. *Brain Research Molecular Brain Research* **9,** 217–231.

Lea RW, Dods ASM, Sharp PJ, Chadwick A. 1981. The possible role of prolactin in the regulation of nesting behavior and the secretion of luteinizing hormone in broody bantams. *Journal of Endorinology* **91,** 89–97.

Lea RW, Sharp PJ. 1982. Plasma prolactin concentrations in the broody turkey; lack of agreement between homologous chicken and turkey prolactin radioimmunoassays. *British Poultry Science* **23,** 451–459.

Lea RW, Talbot RT, Sharp PJ. 1991. Passive immunization against chicken vasoactive intestinal polypeptide suppresses plasma prolactin and crop sac development in incubating ring doves. *Hormone and Behavior* **25,** 283–294.

Linder S, Barkhem T, Norberg A, Persson H, Schalling M, Hökfelt T, Magnusson G. 1987. Structure and expression of the gene encoding the vasoactive intestinal peptide precursor. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 605–609.

Lormée H, Jouventin P, Lacroix A, Lallemand J, Chastel O. 2000. Reproductive endocrinology of tropical seabirds: sex-specific patterns in LH, steroids, and prolactin secretion in relation to parental care. *General and Comparative Endocrinology* **117,** 413–426.

Macnamee MC, Sharp PJ, Lea RW, Sterling RJ, Harvey S. 1986. Evidence that vasoactive intestinal polypeptide is a physiological prolactin-releasing factor in the bantam hen. *General and Comparative Endocrinology* **62,** 470–478.

Maney DL, Schoech SJ, Sharp PJ, Wingfield JC. 1999. Effects of vasoactive intestinal peptide on plasma prolactin in passerines. *General and Comparative Endocrinology* **113,** 323–330.

Mauro LJ, Elde RP, Youngren OM, Phillips RE, El Halawani ME. 1989. Alterations in hypothalamic vasoactive intestinal peptide-like immunoreactivity are associated with reproduction and prolactin-release in the female turkey. *Endocrinology* **125,** 1795–1804.

Mauro LJ, Youngren OM, Proudman JA, Phillips RE, El Halawani ME. 1992. Effects of reproductive status, ovariectomy, and photoperiod on vasoactive intestinal peptide in the female turkey hypothalamus. *General and Comparative Endocrinology* **87,** 481–493.

McFarlin DR, Lehn DA, Moran SM, MacDonald MJ, Epstein ML. 1995. Sequence of a cDNA encoding chicken vasoactive intestinal peptide (VIP). *Gene* **154,** 211–213.

Ohkubo T, Tanaka M, Nakashima K. 2000. Molecular cloning of the chicken prolactin gene and activation by Pit-1 and cAMP-induced factor in GH3 cells. *General and Comparative Endocrinology* **119,** 208–216.

Opel H, Proudman JA. 1988. Stimulation of prolactin release in turkeys by vasoactive intestinal peptide. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine* **187,** 455–460.

Pitts GR, Youngren OM, Silsby JL, Rozenboim I, Chaiseha Y, Phillips RE, Foster DN, El Halawani ME. 1994a. Role of vasoactive intestinal peptide in the control of prolactin-induced turkey incubation behavior. I. Acute infusion of vasoactive intestinal peptide. *Biology of Reproduction* **50,** 1344–1349.

Pitts GR, Youngren OM, Silsby JL, Rozenboim I, Chaiseha Y, Phillips RE, Foster DN, El Halawani ME. 1994b. Role of

vasoactive intestinal peptide in the control of prolactin-induced turkey incubation behavior. II. Chronic infusion of vasoactive intestinal peptide. *Biology of Reproduction* **50,** 1350–1356.

Proudfoot NJ, Brownlee GG. 1976. 3′ non-coding region sequences in eukaryotic messenger RNA. *Nature* **263,** 211–214.

Ramsey SM, Goldsmith AR, Silver R. 1985. Stimulus requirements for prolactin and LH secretion in incubating ring doves. *General and Comparative Endocrinology* **59,** 246–256.

Rozenboim I, Sislby JL, Tabibzadeh C, Pitts GR, Youngren OM, El Halawani ME. 1993. Hypothalamic and posterior pituitary content of vasoactive intestinal peptide and gonadotropin-releasing hormones I and II in the turkey hen. *Biology of Reproduction* **46,** 622–626.

Saito N, Shimada K, Nomura N, Oguri K, Sato K, Wada M. 1992. Seasonal changes in the reproductive functions of Java sparrow (*Padda oryzivora*). *Comparative Biochemistry and Physiology* **101A,** 459–463.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74,** 5463–5467.

Sharp PJ, Scanes CG, Williams JB, Harvey S, Chadwick A. 1979. Variations in concentrations of prolactin, luteinizing hormone, growth hormone and progesterone in the plasma of broody bantams (*Gallus domesticus*). *Journal of Endocrinology* **80,** 51–57.

Sharp PJ, Sterling RJ, Talbot RT, Huskisson NS. 1989. The role of hypothalamic vasoactive intestinal polypeptide in the maintenance of prolactin secretion in incubating bantam hens, observations using passive immunization, radioimmunoassay and immunohistochemistry. *Journal of Endocrinology* **122,** 5–13.

Shimada K, Ishida H, Sato K, Seo H, Matsui N. 1991. Expression of prolactin gene in incubating hens. *Journal of Reproduction and Fertility* **91,** 147–154.

Talbot RT, Dunn IC, Wilson PW, Sang HW, Sharp PJ. 1995. Evidence for alternative splicing of the chicken vasoactive intestinal polypeptide gene transcript. *Journal of Molecular Endocrinology* **15,** 81–91.

Talbot RT, Hanks MC, Sterling RJ, Sang HM, Sharp PJ. 1991. Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: effects of manipulating plasma levels of vasoactive intestinal polypeptide. *Endocrinology* **129,** 496–502.

Teruyama R, Beck MM. 2001. Double immunocytochemistry of vasoactive intestinal peptide and cGnRH-I in male quail: photoperiodic effects. *Cell and Tissue Research* **303,** 403–414.

Vleck CM, Patrick DJ. 1999. Effects of vasoactive intestinal peptide on prolactin secretion in three species of passerine birds. *General and Comparative Endocrinology* **113,** 146–154.

Wang Y, Conlon JM. 1993. Neuroendocrine peptides (NPY, GRP, VIP, somatostatin) from the brain and stomach of the alligator. *Peptides* **14,** 573–579.

Watahiki M, Tanaka M, Masuda N, Sugisaki K, Yamamoto M, Yamakawa M, Nagai J, Nakashima K. 1989. Primary structure of chicken pituitary prolactin deduced from the cDNA sequence. Conserved and specific amino acid residues in the domains of the prolactins. *Journal of Biological Chemistry* **264,** 5535–5539.

Wong EA, Ferrin NH, Silsby JL, El Halawani ME. 1991. Cloning of a turkey prolactin cDNA, expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (*Meleagris gallopavo*). *General and Comparative Endocrinology* **83,** 18–26.

Yamamura T, Yasuo S, Hirunagi K, Ebihara S, Yoshimura T. 2006. T (3) implantation mimics photoperiodically reduced encasement of nerve terminals by glial processes in the median eminence of Japanese quail. *Cell and Tissue Research* **324,** 175–179.

Yoshimura T, Yasuo S, Watanabe M, Iigo M, Yamamura T, Hirunagi K, Ebihara S. 2003. Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. *Nature* **426,** 178–181.

You S, Silsby JL, Farris J, Foster DN, El Halawani ME. 1995. Tissue-specific alternative splicing of turkey preprovasoactive intestinal peptide messenger ribonucleic acid, its regulation, and correlation with prolactin secretion. *Endocrinology* **136,** 2602–2610.

Youngren OM, Sislby JL, Rozenboim I, Phillips RE, El Halawani ME. 1994. Active immunization with vasoactive intestinal peptide prevents the secretion of prolactin induced electrical stimulation of the turkey hypothalamus. *General and Comparative Endocrinology* **95,** 330–336.