Studies on Prolactin and its Receptor During Late Embryogenesis in Turkeys and Chickens

by

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February 2006

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-27810-9
Our file Notre référence
ISBN: 978-0-494-27810-9

ABSTRACT

Ph.D. Degree Benoît Leclerc Animal Science

Changes in the levels of expression of the prolactin receptor (PRLR) mRNA in the pituitary gland, hypothalamus, liver, pancreas, kidney and gonad from embryonic day (ED) 15 and ED21 to 1 day post-hatch, respectively, in chickens and turkeys were measured by real-time PCR. In both species, PRLR mRNA increased from low levels during the last week of ED to reach maxima at the peri-hatch period. Similarly, circulating levels of prolactin (PRL) also increased during this interval and were correlated with the observed increases in tissue content of PRLR mRNA. This suggested that PRL was up-regulating its own receptor during late embryogenesis. In support of this, *in vitro* stimulation of the pituitary gland of turkeys with VIP on ED24 resulted in a 4 fold and 3 fold increase in PRL and PRLR, respectively. Stimulation with VIP of either the hypothalamus or gonad had no effect on either levels of the PRLR transcript. This suggests that VIP acts indirectly through increased PRL to up-regulate the number of receptors. In order to investigate the transcription of genes that may be induced/suppressed by PRL, suppressive subtractive hybridization (SSH) libraries from control or VIP stimulated ED24 turkey pituitary glands were constructed. Stimulation with VIP resulted in a 5.7 and 2.8 fold increase in media and pituitary content of PRL, respectively. The changes in PRL were consistent with endogenous levels of PRL observed just prior to hatch. Following sequence analysis of random clones (n=96) from each library, a total of 145 non-redundant putative genes were obtained. About 51 % of the putative genes have as yet no assigned function, whereas, 15 % were housekeeping genes and 34 % had known functions within various

pathways. Real-time PCR was used to confirm the differential expression of 21 of these genes in VIP treated and control pituitaries. Since the majority of these genes were expressed at levels consistent with the direction of subtraction, these data suggest that these libraries may be useful to study the direct and indirect effects of increasing levels of PRL on anterior pituitary function at about the time of hatch.

RÉSUMÉ

Ph.D. Benoît Leclerc Science animale

Les changements dans les niveaux d'expression de l'ARNm du récepteur de la prolactine (PRLR) dans la glande pituitaire, l'hypothalamus, le foie, le pancréas, le rein et la gonade ont été mesuré par PCR en temps réel à partir du jour embryonnaire 15 et 21 jusqu'au jour 1 suivant l'éclosion chez les poulets et les dindes, respectivement. Chez les deux espèces, les faibles niveaux de l'ARNm du PRLR augmentait durant la dernière semaine du développement embryonnaire (ED) pour atteindre un niveau maximal durant la période d'éclosion. Pareillement, les niveaux circulant de la prolactine (PRL) ont aussi augmenté durant cet interval et ont été corrélés avec l'augmentation observée du contenu tissulaire en ARNm du PRLR. Ceci suggère que la PRL hausse son propre récepteur durant l'embryogénese tartif. Pour prouver cette affirmation, des expériences de stimulation *in vitro* avec le VIP sur la glande pituitare de dindes en ED24 résultaient d'une augmentation de 4 et 3 fois en PRL et en PRLR, respectivement. La stimulation avec le VIP sur l'hypothalamus ou la gonade n'avait eu aucun effet sur les niveaux de transcription du PRLR. Ceci suggère que le VIP indirectement agit via l'augmentation de la PRL afin de hausser le nombre de récepteur. Afin d'examiner la transcription des gènes qui ont pu être induits/supprimés par la PRL, des libraries d'hybridization subtractive à suppression (SSH) ont été construites à partir de glandes pituitaires de dinde en ED24 servant de contrôle ou stimulées par le VIP. La stimulation par le VIP résultait d'une augmentation de 5.7 et 2.8 fois en PRL en milieu de culture et en contenu pituitaire, respectivement. Les changements en PRL étaient conformes avec les niveaux endogènes de PRL observés juste avant l'éclosion. Suivant

l'analyse de séquence de clones sélectionnés au hasard (n=96) dans chaque librarie, un total de 145 gènes putatif non redondant ont été obtenu. Environ 51% des gènes putatif n'ont pas encore de fonction attribuée, alors que 15% étaient des gènes de maintien et 34% avaient des fonctions connues impliquées dans des signalisations diverses. La PCR en temps réel était utilisée pour confirmer l'expression différentielle de 21 de ces gènes dans des glandes pituitaires de contrôle et traitées au VIP. Puisque la majorité de ces gènes-ci ont été exprimés à des niveaux conforment suivant la direction de la soustraction, ces données-ci suggèrent que ces libraries peuvent être utilisées pour étudier les effets directes et indirectes des niveaux augmentant de la PRL sur la function de la glande pituitaire antérieure au alentour de la période d'éclosion.

ACKNOWLEDGEMENTS

The author wishes to express his best gratitude towards his supervisor, Dr. D. Zadworny for his scientific guidance, enthusiastic encouragement, intellectual help, and academic and finance supports through years.

In addition, I would like to address my gratitude to other members of my advisory committee, Dr. U. Kühnlein for his invaluable scientific advices and suggestions during my studies and his generous help for the academic support, and Dr. J.F. Zhou for his indispensable help and advice.

A special thank is also addressed to Dr. G. Bédécarrats for his precious contribution to the prolactin project. Moreover, I thank him for his cordial invitation at the University of Guelph.

I wish to thank the staff of the Department of Animal Science for their academic support. Also, I thank Dana Praslickova and all my fellow students for their technical help and encouragement. Moreover, I wish to thank Ming-Kai Ho, my fellow student, for his technical help on the SSH project.

To extend, I appreciate Vicki Muise, the technician, of the ABI Laboratory in Plant Science for her technical support.

Finally, my deepest gratitude goes to my best friend, Haifeng Wu, for her strong desire I succeed my Ph.D. degree.

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AP	adenohypophysis
Asn-X-Cys	asparagine-X-cysteine
A.U.	arbitrary units
AVT	arginine vasotocin
BLAST	basic local alignment search tool
bp	base pair
cDNA	complementary deoxyribonucleic acid
CIS	cytokine-inducible SH2-containing protein
cpm	counts per minute
DEPC	diethyl pyrocarbonate
dNTP	deoxynucleotide triphosphate
ED	embryonic day
EPOR	erythropoietin receptor
EST	espressed sequence tag
EtBr	ethidium bromide
FS	forward subtracted
FSH	follicle-stimulating hormone
G-PRL	glycosylated prolactin
GAS	gamma-interferon activated sequence

GH	growth hormone
GHF-1	growth hormone factor 1
hVIP	human vasoactive intestinal peptide
IL2	interleukin2
IL2R	interleukin2 receptor
IPTG	isopropyl thiogalactoside
JAKs	janus kinases
kb	kilobase
kDa	kilo Dalton
LB	Luria Broth
LH	luteinizing-hormone
LHRH	luteinizing hormone releasing hormone
MAPK	mitogen-activated protein kinase
MHCII	major histocompatibility complex class II
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NG-PRL	nonglycosylated prolactin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pit-1	pituitary-specific POU (Pit-1, Oct-1 and Oct-2, unc-86)-domain desoxyribonucleic acid binding factor
Pit+Hyp	pituitary glands cultured with hypothalami
PL	placental lactogen

PMSF	phenylmethanesulfonylfluoride
PRF	prolactin-releasing factor
PRL	prolactin
PRLR	prolactin receptor
PVDF	polyvinylidine difluoride
Q-PCR	quantitative polymerase chain reaction
rctPRL	recombinant turkey prolactin
rRNA	ribosomal ribonucleic acid
RPA	ribonuclease protection assay
RS	reverse subtracted
RT	reverse transcription
rTaq	recombinant *Thermus aquaticus*
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SE	standard error
SH2	src homology domain 2
SMART	switching mechanism at the 5' end of the RNA transcript
SOCS	suppressors of cytokine signaling
SSC	sodium chloride-sodium citrate buffer
SSH	suppressive subtractive hybridization
STAT	signal transducer and activator of transcription
T3/T4	triiodothyronine/thyroxine
TBS	tris buffered saline

TNE	tris base, NaCl, EDTA
tPRLR	turkey prolactin receptor
TRH	thyrotropin releasing hormone
Tris	tris(hydroxymethyl)aminomethane
TSH	thyroid-stimulating hormone
VIP	vasoactive intestinal peptide
VIPR	vasoactive intestinal peptide receptor
WSXWS	tryptophan serine X tryptophan serine
X-gal	5-bromo-4-chloro-3-idoly-β-D-galactopyranoside

CONTRIBUTIONS TO KNOWLEDGE

1) In Chapter 3, we have developed for the first time a semi-quantitative real-time PCR (Q-PCR) test to assess levels of PRLR mRNA transcript in chickens and turkeys and applied this technique to study the hypothalamo-hypophyseal axis during ontogeny. During the last week of embryogenesis in both species, we observed that the expression of PRLR mRNA increased in both the hypothalamus and pituitary gland to reach a maxima in the peri-hatch interval. Since PRLR mRNA and the concentration of circulating PRL were highly correlated, this suggested that PRL was up-regulating its own receptor.

2) The latter was confirmed by *in vitro* analysis of embryonic day 24 turkey pituitary glands where VIP stimulation resulted in a concomitant increase in both PRL and its receptor. An autocrine and/or paracrine role of PRL in controlling PRLR number was proposed, whereas, analysis of hypothalami suggested a short-loop feedback regulatory mechanism.

3) In Chapter 4, using Q-PCR, the content of PRLR mRNA in both species was measured in some putative target organs during ontogeny. Levels of transcript were highest in kidney followed by gonad, liver and pancreas during the last week of embryogenesis and correlated with circulating levels of PRL. This suggested the possible involvement of these organs and increased levels of PRL with the adaptation of the embryo to *ex ovo* life post-hatch.

4) In Chapter 5, the role of PRL on pituitary function during late embryogenesis was investigated by stimulating embryonic day 24 turkey pituitary glands with VIP. At this stage of ontogeny, the expression of PRL was minimal. However, following stimulation, levels of PRL were induced to levels consistent with the time of hatch. Post-stimulation, suppressive subtraction hybridization (SSH) libraries were constructed for the first time in the embryonic pituitary gland. Sequencing of clones from libraries derived from VIP stimulated or control RNA (n=96 each) revealed 145 non-redundant sequences. About half of these putative genes have no assigned function as yet, whereas, the other half consisted of cell proliferation genes, ubiquitin-proteasome pathway genes, apoptotic pathway genes, protein trafficking genes, cytoskeletal and extracellular matrix genes and house keeping genes. Clones derived from these libraries may be useful to study the direct and indirect effects of increasing levels of PRL on anterior pituitary function at about the time of hatch.

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CONTRIBUTIONS OF CO-AUTHORS

TO MANUSCRIPTS FOR PUBLICATION

The main body of this thesis consists of three manuscripts. Manuscript 1 (Chapter 3) has been submitted in the *General and Comparative Endocrinology*. Manuscript 2 (Chapter 4) has been submitted in the *Poultry Science*. Manuscript 3 (Chapter 5) is in preparation. Drs. D. Zadworny and U. Kühnlein are co-authors on all manuscripts. Dr. Zadworny, my supervisor, was responsible for the overall design of this research project and for correcting the manuscripts. Dr. Kühnlein, co-investigator of the project, provided scientific guidance in this research project. Dr. G. Bédécarrats is co-author on manuscripts 1 and 2. Dr. Bédécarrats helped measuring plasma and medium PRL levels during the study. Ming-Kai Ho is co-author on manuscript 3. Mr. Ho participated to the construction of the SSH libraries.

CHAPTER 1. INTRODUCTION

The hormone prolactin (PRL) has been recognized to possess a wide range of biological functions (Bern and Nicoll, 1968). Since its discovery by Riddle and colleagues in the early 1930's as causative hormonal factor inducing incubation behaviour in "broody strains" of actively laying chicken, more than 300 distinct functions of PRL have been recorded in various vertebrates (Bole-Feysot *et al.*, 1998). These different biological functions have been broadly categorized into those which affect water and electrolyte balance, growth and development, metabolism and endocrine regulation, brain and behaviour, reproduction, and immune regulation and function (Forsyth and Wallis, 2002).

In mammals, many of the pleiomorphic effects of PRL in target tissues are modulated by its structural polymorphism, by changes in its sites of local expression and processing, by changes in sites and developmental pattern of receptor expression, by its intracellular signaling pathways, and by its internalization and nuclear actions via regulatory mechanisms on target genes (Ben-Jonathan *et al.*, 1996; Freeman *et al.*, 2000; Goffin *et al.*, 2002; Forsyth and Wallis, 2002). In avian species, the actions of PRL have been shown to be modified by the combination of changes in circulating concentration of PRL and its site of synthesis (Ishida *et al.*, 1991; Kansaku *et al.*, 1994; Karatzas *et al.*, 1997; Bédécarrats *et al.*, 1999a), changes in ratio of post-translationally modified PRL (Bédécarrats *et al.*, 1999a,b,c), variation in levels of its receptor (Zhou *et al.*, 1996; Ohkubo *et al.*, 1998b), and the presence of cell specific variants of its receptor (Mao *et al.*, 1999, Pitts *et al.*, 2000; Tanaka *et al.*, 2000).

The wide distribution of the prolactin receptor (PRLR) in vertebrates is consistent with the multiplicity of physiological processes that PRL is known to be involved in (Bole-Feysot *et al.*, 1998; Zadworny *et al.*, 2002). However, there is scanty information about the expression of PRLR mRNA during avian embryogenesis. At the prehatch period, Yamamoto *et al.* (2003) observed an independent relationship between plasma levels of PRL and expression levels of PRLR in kidney, intestine and allantoic membrane. In the adult pituitary gland and hypothalamus, expression of PRLR mRNA seems to be up- and down-regulated by PRL, respectively (Zhou *et al.*, 1996). Conversely, in rodents, levels of PRLR mRNA increase between days 17.5 and 20.5 of gestation in several fetal tissues, including the pituitary. This suggests that PRL is among several endocrine factors, such as growth hormone (GH), luteinizing-hormone (LH) and prolactin releasing factor (PRF), necessary to coordinate developmental activities (Royster *et al.*, 1995; Brown-Borg *et al.*, 1996).

Vasoactive intestinal peptide (VIP) has been shown to be the major hypophysiotropic PRL releasing factor (PRF) (Sharp *et al.*, 1989) regulating PRL gene expression by acting both at the transcriptional and at the messenger RNA (mRNA) stability levels (Tong *et al.*, 1998). Recently, it has been observed that VIP has a stimulatory effect on PRL gene expression and its release by modulating the Ca^{2+} signaling activity (Al Kahtane *et al.*, 2005). Although the intracellular mechanism that regulates PRL transcription by VIP are still unknown, it is most likely that VIP up-regulates PRL transcription by modulating the activity of transcription factors through second messenger systems (Tong *et al.*, 1998, Kansaku *et al.*, 1998; Kang *et al.*, 2002),

possibly via the presence of a VIP response element in the proximal PRL promoter (Kang *et al.*, 2004).

Since PRL is involved in the early steps of lactotrope differentiation during late embryogenesis its expression will be expected to increase in parallel with its PRLR gene transcript and both will be modulated following VIP stimulation. Therefore, measurement of PRLR mRNA transcript provides an indirect way to evaluate the expression of the PRLR protein. In this thesis, our objective is to investigate the expression of PRL and its receptor gene transcript in pituitary and extra-pituitary tissues during avian embryogenesis. A real-time polymerase chain reaction (PCR) assay was developed to assess levels in different tissues during prenatal and postnatal development of both turkeys and chickens. Levels of plasma and medium PRL, measured by radioimmunoassay, were correlated with levels of PRLR mRNA at several developmental time points in each species. Furthermore, the effects of hypothalamic hypophyseal co-culture and/or VIP stimulated release on induction of PRLR transcription were examined in turkeys. Finally, a suppressive subtractive hybridization (SSH) was designed to study the genomic effect of VIP on turkey pituitary gland. This may provide insights into the roles of PRL on adenohypophyseal gland function and to which extent this hormone can coordinate development activities during the pre-hatch period.

CHAPTER 2. LITERATURE REVIEW

2.1. Prolactin

Early studies indicated that PRL affects the reproduction cycle of avian species. Prolactin was shown to stimulate the growth of the pigeon crop-sac, induce broodiness in chickens (Riddle *et al.*, 1933; 1935; Saeki and Tanabe, 1955), cause gonadal regression in both sexes (Bates *et al.*, 1935; Nalbandov, 1945), have a role in molting (Juhn and Harris, 1958), stimulate brood patch formation (Hohn and Cheng, 1965), and inhibit gonadotropin-stimulated ovulation (Tanaka *et al.*, 1971). With the advent of avian radioimmunoassays (Scanes *et al.*, 1976), the role of PRL has been broadened and its circulating levels were associated with photoperiodism (Dawson, 1997; Proudman, 1998), reproduction (Zadworny *et al.*, 1989, Tabibzadeh *et al.*, 1995; You *et al.*, 2000), metabolism (Harvey *et al.*, 1978a; Barron *et al.*, 1999), osmoregulation and stress (Arad *et al.*, 1986; Rozenboim *et al.*, 2004), and development (Ishida *et al.*, 1991; Kansaku *et al.*, 1994; Bédécarrats *et al.*, 1999c).

In avian species, PRL was mainly studied in the adult for its role on reproduction and more extensively during the expression of incubation behaviour. Many studies have indicated that hyperprolactinemia is associated with the onset and maintenance of incubation behaviour in chickens and turkeys, but has also been associated with ovarian regression and the relatively poor egg production in turkey hens that result in a huge economic loss to the turkey industry (El Halawani and Rozenboim, 1993; Ramesh *et al.*, 1998; El Halawani, 1998). The role(s) of PRL during incubation behaviour are not known, but experiments using active immunization against PRL clearly suggest that high levels of PRL are a prerequisite aspect for this behaviour

(Crisostomo *et al.*, 1998; El Halawani *et al.*, 2000a). Furthermore, immuno-neutralization of the hypothalamic VIP neuronal system has been reported to be intimately linked to photoperiodic mechanism-induced PRL secretion during reproduction (El Halawani *et al.*, 1996).

Incubation behaviour is characterized by large changes in physiology such as high circulating PRL levels, reduced gonadotropin and ovarian steroid levels, cessation of lay and ovarian regression, nesting activity, aggressive nest protection, anorexia and altered brain neurotransmitter activity (El Halawani *et al.*, 1988a; Karatzas *et al.*, 1997). Prolactin inhibits reproductive function and probably acts at all levels of the hypothalamic-pituitary-gonadal axis (Sharp *et al.*, 1998). For example, PRL decreases reproductive activity by acting on the hypothalamus to inhibit gonadotropin releasing hormone release (Rozenboim *et al.*, 1993), on the pituitary to reduce LH-β subunit mRNA expression and LH release (You *et al.*, 1995), and directly down-regulates the ovarian intact form of LH receptor by increasing truncated LH receptor isoforms (You *et al.*, 2000) through inhibition of steroidogenic enzymes mRNA expression from the theca cells and/or small white follicles (Lien *et al.*, 1989; Morrison *et al.*, 1990; Tabibzadeh *et al.*, 1994, 1995; You *et al.*, 2000). During incubation behaviour, nesting stimulus and/or PRL act in concert with ovarian steroids to negatively feedback on hypothalamic luteinizing hormone releasing hormone (LHRH) release, resulting in low concentrations of circulating LH, and consequently inducing gonadal regression (El Halawani and Rozenboim, 1993). Since high PRL levels have been shown to reduce photo-induced LH release and delay the onset of sexual maturity (El Halawani *et al.*, 1991), indirect evidence supports a possible correlation between pituitary LH content

and LH gene expression with LH receptor isoforms during reproductive cycle (Wong *et al.*, 1992; You *et al.*, 2000).

2.1.1. Prolactin Gene

Genes encoding PRL, GH, and placental lactogen (PL) have evolved from a common ancestral protein by gene duplication at least 500 million years ago (Nicoll and Baldocchi, 1988). Based on its similar genetic, structural, immunologic, binding and functional properties, PRL belongs to the PRL/GH/PL family referred to as hematopoietic cytokines (Miller and Eberhardt, 1983; Horseman and Yu-Lee, 1994; Goffin *et al.*, 2002). The gene encoding the human and chicken PRL gene has been located on chromosome 6 and 2, respectively (Owerbach *et al.*, 1981; Miao *et al.*, 1999). The gene has an overall length of 10 kb, 6.7 kb and 6.16 kb in humans, turkeys and chickens, respectively (Truong *et al.*, 1984; Kurima *et al.*, 1995; Au and Leung, 2002). Similar to mammals, the avian PRL gene exists as a single copy per haploid genome (Truong *et al.*, 1984; Hanks *et al.*, 1989; Shimada *et al.*, 1993) and is composed of five exons and four introns (Shimada *et al.*, 1993; Kansaku *et al.*, 2005). Sequence of turkey PRL gene indicates one putative TATA box located 24 nucleotides upstream from the transcription start point found 51-53 nucleotides upstream from the methionine start codon (Kurima *et al.*, 1995). Two putative polyadenylation signals (AATAAA) are identified 209 and 276 nucleotides downstream from the stop codon (Kurima *et al.*, 1995). They predicted the final PRL gene transcript to have a length around 1.1 kb. Two regions similar to mammalian Pit-1/GHF-1 (pituitary-specific POU-domain DNA binding factor/growth hormone factor) binding sites were identified by computer

analysis at -127 and -61 sequences of the 5'-flanking region of the turkey PRL gene Kurima *et al.* (1995). This suggested that Pit-1/GHF-1 may modulate the transcription of the turkey PRL gene. Since in mammals the distal enhancer element of the PRL gene contained four Pit-1 binding sites located adjacent to an estrogen response element (Nelson *et al.*, 1988), activation of the PRL gene most likely requires both Pit-1 (Mangalam *et al.*, 1989; Cohen *et al.*, 1996) and the estrogen receptor (Weatherly *et al.*, 2001). However, in avian species, neither estrogen nor its receptor is involved in transcription (Knapp *et al.*, 1988). Furthermore, the significance of the Pit-1 element remains controversial. Some studies indicate that Pit-1 is involved in the activation of PRL gene expression in chicken lactotrophs and during early embryogenesis (Kurima *et al.*, 1998; Van As *et al.*, 2000; Ohkubo *et al.*, 2000; Fu and Porter, 2004). However, other studies suggest that Pit-1 binding to the promoter is not required for PRL activation (Weatherly *et al.*, 2001; Kang *et al.*, 2004).

2.1.2. Prolactin Protein, Structure and its Variants

The PRL cDNA contains a 687-nucleotide open reading frame encoding a pre-PRL of 229 amino acids. The protein was predicted to have a 30 amino acid signal sequence which would be cleaved to give a mature PRL protein of 199 amino acids with a molecular weight around 23 kDa. The deduced amino acid sequence of turkey PRL has a high degree of identity to chicken (> 90%) and both have about 70% identity to human (Hanks *et al.*, 1989; Watahiki *et al.*, 1989; Wong *et al.*, 1991; Shimada *et al.*, 1993; Sinha, 1995). The amino acid sequences of chicken PRL deduced from cDNAs derived from broiler chicken (Watahiki *et al.*, 1989) or broody bantam hen pituitary

gland (Hanks *et al.*, 1989) differ at positions 141, 150, 175; thus possibly reflecting their different ancestries (Shimada *et al.*, 1993).

Similar to mammalian PRL, avian PRLs have three intramolecular disulfide bridges between cysteine residues 4-11 (N-terminal), 58-174, and 191-199 (C-terminal). The final molecule gives rise to three disulfide loops that provide stability to the structural conformation (Sinha, 1995). A comparative analysis of the amino acid structures of PRLs from a range of vertebrate species showed that conserved residues cluster in five highly conserved domains of the PRL molecules (11-51, 58-71, 80-98, 108-133, and 160-192) and form determinants for PRL specific binding to receptors which are likely essential for biological activity (Kurima *et al.*, 1995; Zadworny *et al.*, 2002). To date, the three-dimensional structure of PRL via x-ray crystallography or nuclear magnetic resonance has not been reported. However, based on the structure/function similarities between PRL and GH, the three dimensional structure of human PRL corresponded to the better characterized porcine GH (Abdel-Meguid *et al.*, 1987). The partial three dimension of the human PRL molecule is composed of four alpha helices organized in an anti-parallel fashion, with the binding sites of the receptor located on the same side of the folded protein (Sinha, 1995). Comparison of avian GH which was assumed to have a three dimensional structure resembling recombinant methionine-porcine GH with avian PRL indicated that the conserved domains overlap within four helices (Zadworny *et al.*, 2002) at residues 18-32, 58-72, 83-98, and 160-199 (Watahiki *et al.*, 1989, Shimada *et al.*, 1993, Sinha, 1995). Likely, avian PRLs may

have similar receptor binding domains to the better known mammalian PRLs, therefore may act in a similar fashion in signal transduction pathways (Zadworny *et al.*, 2002).

The majority of PRL variants can be the result of posttranslational modifications of the amino acid chain (Freeman *et al.*, 2000) which include proteolytic cleavage, glycosylation, phosphorylation, deamidation, sulphation, dimerization and polymerization of PRL or PRL bound to binding proteins (reviewed by Sinha, 1995). In avian species, variants of normal PRL (23 kDa, the major form), produced by alternative splicing, may be smaller (21 kDa) because of the removal of an exon or larger (25 kDa) because an intron is not removed (Shimada *et al.*, 1993). Aramburo *et al.* (1992) demonstrated that PRL can be phosphorylated by incubating PRL *in vitro* with the catalytic subunit of protein kinase A. In avian species, further investigation is required to determine the biological significance of phosphorylated PRL in cells and tissues. Nevertheless, the phosphorylated PRL form has been shown to change its structure and, consequently, the binding to its receptor (Wicks and Brooks, 1995). Recently, the non phosphorylated PRL form has been suggested to participate through the Jak2-Stat5 pathway, whereas, the phosphorylated PRL form signaled primarily through the MAPK pathway after prolonged exposure (Wu *et al.*, 2003). In addition, phosphorylated PRL form decreases tumor growth (Xu *et al.*, 2001; Schroeder et al., 2003) and has a role in normal development of the immune system of offspring (Yang *et al.*, 2001; Guzman *et al.*, 2005). For example, the administration of phosphorylated PRL form in pregnant rats has resulted in increasing the apoptosis level in the thymus of new borns through the decrease of epidermal gamma delta T cells (Yang *et al.*, 2002). A

body of evidence has focused on the glycosylation of PRL for its roles in regulating its binding activity to its receptor and its underlying biological significance or its clearance (Corcoran and Proudman, 1991; Sinha, 1995; Freeman *et al.*, 2000) because the sites of glycosylation have been noted to affect the secondary structure associated with disulfide bond formation (Bédécarrats *et al.*, 1999b). In the turkey, both a non-glycosylated prolactin (NG-PRL) isoform (24 kDa) and a glycosylated prolactin (G-PRL) isoform (27 kDa) are observed (Corcoran and Proudman, 1991; Bédécarrats *et al.*, 1999a, b). Bédécarrats *et al.* (1999a, b) reported that N-linked G-PRL containing sialic acid is exclusively present in the pituitary gland of hens, and N-linked consensus sequences (Asn-X-Cys) are located at position 56 and 197. Furthermore, they showed that the ratio between G- and NG-PRL isoforms in the pituitary gland varied during the reproductive cycle.

2.1.3. Pituitary Cell Differentiation and Prolactin Expression

Previous immunocytochemistry studies have shown that PRL immunoreactive cells were seen predominantly in the cephalic lobe of the adenohypophysis (Barabanov *et al.*, 1985; Berghman *et al.*, 1992; Ramesh *et al.*, 1995). In turkeys, during the transition from laying to incubating stage, a proportion of lactotrophs replaced somatotrophs in the caudal lobe (Ramesh *et al.*, 1996), whereas, upon nest-deprivation of incubating hens, somatotrophs progressed from the junction of the regenerated cephalic and caudal lobes concomitant with a decline in PRL-immunoreactive and an increase in GH-immunoreactive cells (Ramesh *et al.*, 2001). Associated with these changes were transitional mammosomatotrophs expressing both GH and PRL (Ramesh

et al., 1998), as observed in mammals (Frawley and Boockfor, 1991). Interestingly, during *in ovo* development (Fu *et al.*, 2004) very few cells containing both hormones were detected, suggesting somatotrophs and lactotrophs arise from separate precursor cell populations, residing in the caudal lobe and cephalic lobe, respectively.

Indirect evidence indicates that during the hen's reproductive cycle, cellular events in the pituitary gland are associated with changes in the levels of plasma PRL, pituitary PRL and PRL mRNA, and ultimately control, in part, physiological responses such as the onset of incubation behaviour, termination of egg laying and ovarian regression (Lopez *et al.*, 1996). Indeed, steady-state amounts of pituitary PRL mRNA have been shown to be directly related to changes in concentration of pituitary and plasma PRL (Wong *et al.*, 1991; Karatzas *et al.*, 1997). A similar observation has been made in embryos (Ishida *et al.*. 1991; Bédécarrats *et al.*, 1999c). However, during the reproductive cycle, pituitary PRL mRNA, plasma and pituitary PRL levels did not increase to the same extent (Wong *et al.*, 1991). This suggested that the PRL gene is regulated at both transcriptional and post-transcriptional levels and that different cellular regulatory mechanisms such as mRNA stability, nuclear mRNA processing, transport of transcripts and PRL transcription may be involved in the individual reproductive stages (Wong *et al.*, 1991; Tong *et al.*, 1997). Thus, the enhanced PRL transcription may be due to an increase in the rate of transcription associated with the recruitment of lactotrophs in the pituitary gland of incubating hens (Tong *et al.*, 1997). Increased serum levels of PRL are accompanied with an increased proportion of PRL-secreting cells and a greater capacity of each lactotroph to secrete PRL (Lopez *et al.*,

1996; Woods and Porter, 1998), an effect only observed following chronic VIP stimulation for 6 days in the pituitary gland of adult laying hens (Porter *et al.*, 2006).

2.1.4. Extrapituitary Prolactin

In addition to being synthesized and secreted by lactotrophs, PRL is also produced in various regions of the brain (Ramesh *et al.*, 2000). In Japanese quail, PRL immunoreactive neurons were detected in the mediobasal hypothalamus (Berghman *et al.*, 1992). The presence of immunoreactive PRL was also found in the portal blood vessels that surround the external zone of the median eminence in the turkey. Most immunoreactive PRL perikarya and fibers were found in the medial and lateral septal area, the hippocampus and bed nucleus of the stria terminalis pars magnocellularis in both turkeys and doves (Ramesh *et al.*, 2000). The presence of PRL immunoreactive perikarya and fibers in several hypothalamic nuclei suggests that PRL may act centrally to influence parental behaviour, food intake, autonomic nervous system function and reproduction (Buntin *et al.*, 1999; Ramesh *et al.*, 2000). Recently, in support of this, it was reported that PRL stimulates the production of neuronal progenitors in the forebrain of female mice during pregnancy followed by enhanced olfactory capability (Shingo *et al.*, 2003; Lennington *et al.*, 2003); thus contributing to adaptive parental behaviour in both mammals and birds (Bridges and Mann, 1994; Buntin, 1996). Up to now, PRL has not been described to be synthesized and produced in other avian extrapituitary tissues, but, in mammals, PRL has been shown to be produced by immune cells (Gala and Shevach, 1994; Kooijman *et al.*, 2000; Mendez *et al.*, 2004). However, in birds, PRL hormone has been mentioned to increase the proliferation of lymphocytes and

phagocytic function of heterophils during the incubation period (Rodriguez *et al.*, 1996; Ibars *et al.*, 1997).

2.1.5. Control of Prolactin Secretion

So far, a number of biogenic amines, amino acids, and neurotransmitters have been implicated in the regulation of PRL secretion in avian species (Hall *et al.*, 1986), as well as in mammals (reviewed by Freeman *et al.*, 2000). It is generally accepted that the hypothalamic control of PRL secretion is mediated by the hypophysial portal vascular system which transports regulatory neuropeptides and neurotransmitters released from the median eminence to the anterior pituitary gland (Follett, 1984). In addition, these neurochemicals have been shown to have direct effects on PRL release in the pituitary gland via specific receptors in pituitary and hypothalamus (Hall *et al.*, 1986; El Halawani *et al.*, 1997).

2.1.5.1. Regulation by Hypothalamic Neuropeptides

2.1.5.1.1. Vasoactive Intestinal Peptide

It has long been established that hypothalamic VIP is a physiologically important PRL-releasing hormone in galliformes, columbiformes and passeriformes (Macnamee *et al.*, 1986; Lea and Vowles, 1986; Vleck and Patrick, 1999). Several criteria defined VIP as the major hypophysiotropic PRF in avian species (El Halawani *et al.*, 1997). Firstly, fluctuations in the number of VIP immunoreactive neurons in the infundibular nuclear complex were correlated with plasma PRL during the reproductive cycle of the turkey hen (Mauro *et al.*, 1989; Kuenzel *et al.*, 1997). Secondly, VIP

concentration and pulsatility in turkey hypophysial portal blood plasma were correlated to levels of PRL in peripheral circulation (Lopez *et al.*, 1989; El Halawani *et al.*, 1997; Chaiseha *et al.*, 1998). Thirdly, levels of VIP receptor mRNA in the cephalic lobe of the anterior pituitary increase in concert with the capacity to secrete PRL (Rozenboim and El Halawani, 1993; Gonzales *et al.*, 1995; You *et al.*, 2001). Fourthly, a 5'-flanking regulatory element of the turkey PRL promoter, named the VIP response element, is an essential *cis*-acting element for VIP-stimulated PRL gene expression in the adenohypophysis and represents a site where specific DNA-protein complexes occur at the transcriptional level (Kang *et al.*, 2004). During the turkey reproductive cycle, VIP is regulated in large part at the transcriptional level coupled to the photoperiodic receptor mechanisms to induce PRL secretion (Chaiseha *et al.*, 1998). For instance, Silver *et al.* (1988) observed that VIP is colocalized with an opsin-like pigment in the infundibular region, thought to contain extra-retinal hypothalamic photoreceptors important for the induction of seasonal reproductive function in avian species. Lastly, immunoneutralization of VIP in the hypothalamus (median eminence) reduced PRL gene expression, PRL secretion induced by electrical stimulation and PRL secretagogues (serotonin, dopamine, dynorphin) which are mediated via the hypothalamic terminal VIP neuronal system (Tong *et al.*, 1998; Youngren *et al.*, 1994, 1999). These findings implied that among PRL-releasing hormones, only VIP directly stimulates PRL secretion involved in the induction and maintenance of incubation behaviour in galliformes and in the proliferation of crop-sac during incubation in columbiformes (Sharp *et al.*, 1989; Lea *et al.*, 1991).

2.1.5.1.2. Thyrotropin Releasing Hormone

In birds, thyrotropin releasing hormone (TRH) was shown to stimulate PRL release from the pituitary gland via cyclic adenosine 3',5'-monophosphate, but its role in the physiological release of PRL remains uncertain since treatment with TRH *in vivo* and *in vitro* gave inconsistent effects on PRL release (Harvey *et al.*, 1978b; Hall *et al.*, 1985a, 1986). Shimada *et al.* (1991) suggested that TRH at high dose may contribute, at least in part, to the increase in transcription of PRL mRNA for PRL biosynthesis. This may be due to an indirect effect since Van As *et al.* (2004) observed that Pit-1 mRNA expression was significantly increased following TRH injection in prenatal chicks. Nevertheless, it is unlikely that TRH is the major PRF in avian hypothalami (Hall *et al.*, 1986) since PRL releasing activity of hypothalamic extracts cannot be identified only by the content of TRH (Hall and Chadwick, 1983). It remains to be established whether TRH may act inside the brain by modulating the release of other secretagogues which can transmit a hypothalamic message directly to the lactotrophs (El Halawani *et al.*, 1997).

2.1.5.1.3. Arginine Vasotocin

A number of other neuropeptides which have been considered as possible PRF like-activity (Hall *et al.*, 1985b; Proudman and Opel, 1988) were not thought to be physiologically significant (Sharp *et al.*, 1989). Nevertheless, it was observed that arginine vasotocin (AVT) was a potent releaser of PRL in cultured turkey anterior pituitary cells, but not *in vivo* (Proudman and Opel, 1988; Opel and Proudman, 1988). This suggested that AVT may have a minimal PRF activity in the posterior pituitary (El

Halawani *et al.*, 1992). The possibility that this hormone may influence the release of PRL needs further investigation since large amount of bioactive and immunoreactive AVT were found in the anterior pituitary (Jackson and Nalbandov, 1969; Robinzon *et al.*, 1988). There is increasing evidence that PRL and AVT are active in osmoregulation and electrolyte balance in adult birds as well as during *in ovo* development. Likely, AVT and PRL may enhance electrolyte transport to favour the movement of water from the allantois to the embryonic circulation by acting on the chorioallantoic membrane directly, on the kidneys, or both (Murphy, 1997).

2.1.5.2. Regulation by Hypothalamic Neurotransmitters

2.1.5.2.1. Dopamine

It was shown that dopamine plays an intermediary role in PRL secretion, requiring an intact vasoactive intestinal peptidergic system in order to release PRL since active immunization against VIP prevented this effect (Youngren *et al.*, 1996a). *In vitro* studies demonstrated the inhibitory effect of dopamine on PRL release (Hall and Chadwick, 1984; Xu *et al.*, 1996). However, *in vivo* studies demonstrated biphasic actions of dopamine, which can either stimulate or inhibit PRL secretion, depending upon the dose infused (Youngren *et al.*, 1995). Subsequent infusion experiments within the turkey brain indicated that dopamine indirectly stimulates the secretion of PRL via activation of D_1 dopamine receptors mainly expressed in the infundibular region of the hypothalamus which cause the release of VIP (Youngren *et al.*, 1996b; Chaiseha *et al.*, 1997). Conversely, dopamine directly inhibits PRL secretion via activation of D_2 dopamine receptors mainly expressed in the anterior pituitary (Youngren *et al.*, 1996b;

Xu *et al.*, 1996). Schnell *et al.* (1999) observe that only pituitary D_{1D}, D_{2L} and D_{2S} dopamine receptors mRNA are differentially expressed during the turkey hen's reproductive cycle which may affect the degree of prolactinemia by modulating VIP and/or PRL secretion. For instance, major expression of $D1_D$ dopamine receptor mRNA located within the same infundibular region as the VIP neurons indicates that this dopamine subtype is involved in stimulating PRL secretion by modulating the activity of VIP neurons in incubating hens (Youngren *et al.*, 2002; Chaiseha *et al.*, 2003). In contrast, major expression of D2 dopamine receptor mRNA located within the pituitary gland is involved in inhibiting VIP-inducing PRL secretion in photorefractory hens (Chaiseha *et al.*, 2003). Moreover, co-localization of both dopamine transcripts with neurons expressing VIP mRNA confirms the dual role played by dopamine to regulate PRL at the pituitary level (Chaiseha *et al.*, 2003). Both VIP and dopamine play a major role in the regulation of PRL gene expression at both the transcriptional and post-transcriptional levels since Al Kahtane *et al.* (2003) observed that a D_2 dopamine agonist reduces VIP-stimulated PRL mRNA level, PRL mRNA half-life, and PRL at the pituitary level. However, the molecular mechanism(s) by which activation of D_2 dopamine receptor destabilizes PRL mRNA half-life and served as an inhibitor of PRL secretion is not known.

2.1.5.2.2. Serotonin

Evidence indicates that serotonin stimulates PRL release in the chicken *in vivo* and *in vitro* (Hall *et al.*, 1983a, b, 1984a, b). Prolactin release required the functional integrity of serotonergic neurons within the ventromedial nucleus (El Halawani *et al.*,

1988b) since it had no effect on PRL secretion when added to pituitary cells *in vitro* (Fehrer *et al.*, 1985). Furtheremore, the integrity of both dopaminergic and vasoactive intestinal peptidergic systems is required (El Halawani *et al.*, 1995; Youngren *et al.*, 1998). Dynorphin, serotonin, dopamine and VIP, all appear to stimulate avian PRL secretion via a common pathway expressing κ opioid, serotonergic, dopaminergic and vasoactive intestinal peptidergic receptors at synapses arranged serially in that functional order, with the vasoactive intestinal peptidergic system as the final mediator (Youngren *et al.*, 1999, El Halawani *et al.*, 2000b). The ability of serotonin to induce PRL secretion is modulated by reproductive states associated with photoperiodism (Pitts *et al.*, 1996; Youngren *et al.*, 1998), and heat stress in turkey hens (Rozenboim *et al.*, 2004), but its role in the regulation of PRL secretion requires further study (Youngren *et al.*, 1998).

2.2. Prolactin Receptor

The physiological effects of PRL on target tissues are mediated through its specific transmembrane receptor, the PRLR, localized on plasma membrane of numerous peripheral and neural tissues (Posner *et al.*, 1974; Dube *et al.*, 1980; Muccioli *et al.*, 1988; Tanaka *et al.*, 1992). Initially, PRL binding assays provided an indirect way to study PRLR function, thus associating a PRL biological response to its target tissue (Kelly *et al.*, 1991). Subsequently, PRLR was cloned in rat, human, chicken, tilapia, and brushtail possum (Boutin *et al.*, 1988; Kelly *et al.*, 1989; Tanaka *et al.*, 1992; Sandra *et al.*, 1995; Demmer, 1999) allowing the study of its developmental expression and biological function.

2.2.1. Prolactin Receptor Gene

The gene encoding human, rat, mouse and chicken PRLR are located on chromosome 5, 2, 15 and Z, respectively (Arden *et al.*, 1990; Barker *et al.*, 1992; Suzuki *et al.*, 1999). The single gene of human and mouse PRLR contain 11 and 13 exons respectively, (Ormandy *et al.*, 1998; Hu *et al.*, 2001) and has an overall length exceeding 100 kb (Arden *et al.*, 1990; Ormandy *et al.*, 1998). The gene spans about 70 kb and 40 kb in the rat and chicken, respectively (Banville *et al.*, 1992; Mao *et al.*, 1999). However, very little is known about the organization of the avian gene.

In rat and human, several PRLR transcripts resulting from alternative transcription initiation sites were reported suggesting different PRLR gene promoters (Hu *et al.*, 1996, 1999). In addition, alternative splicing of noncoding and coding exon transcripts (Hu *et al.*, 2001; Leondires *et al.*, 2002), alternative polyadenylation sites, or any combination of these events (Mao *et al.*, 1999) have also been reported. In avian species, Chen and Horseman (1994) initially observed the presence of multiple PRLR mRNA transcripts in several tissues, this was further confirmed by Ohkubo *et al.* (1998a) and Pitts *et al.* (2000) on Northern blot. Among the transcripts, the 3.1 kb and 3.3 kb were similar in size to those previously reported (Tanaka *et al.*, 1992; Chen and Horseman, 1994; Zhou *et al.*, 1996). The significance of the 7.3 kb and 7.5 kb PRLR transcripts are uncertain, but one possibility is that the two PRLR mRNA transcripts used different transcription start sites explaining the size differences (Ohkubo *et al.*, 1998a). Ohkubo *et al.* (1998a) and Pitts *et al.* (2000) might have identified different turkey PRLR transcript isoforms after amplification of the intracellular domain,

whereas, Zhou *et al.* (1996) observed only one PRLR transcript isoform after amplification of the extracellular domain of the receptor. The evidence of multiple PRLR transcripts size translated as distinct protein isoforms remains uncertain in avian species (Pitts *et al.*, 2000). In addition, it was reported that the truncated chicken PRLR forms contain two exons (Mao *et al.*, 1999; Tanaka *et al.*, 2000). Two testis-specific first exons, named TSE-1 and TSE-2 encode multiple 5'-truncated PRLR transcripts containing only the cytoplasmic domain in the testis, termed (+) Box 1-A, 1-B, 1-C, 1-D, and (-) Box 1, whereas, exon 1 encodes for the full-length transcript (Tanaka *et al.*, 2000). During maturation of the chicken testis, a shift in the transcription initiation sites of the PRLR gene occurs from exon 1 to TSE-1 and TSE-2 encoding (+) Box 1 and (-) Box 1 transcripts, respectively, which are predicted to parallel the increase of testosterone levels (Mao *et al.*, 1999; Tanaka *et al.*, 2000). However, it remains to be established which regulatory mechanisms are involved in the expression of 5'-truncated PRLR transcripts, and whether the sequence of TSE-1 and TSE-2 exon are used as initiation sites for the translation of the truncated PRLR proteins (Tanaka *et al.*, 2000).

2.2.2. Prolactin Receptor Structure

In galliformes, the open reading frame of the PRLR predicted a peptide of 831 amino acid residues composed of a leader peptide, an extracellular domain, a single transmembrane domain, and an intracellular domain (Tanaka *et al.*, 1992; Zhou *et al.*, 1996). The mature PRLR is composed of 807 amino acid residues after excluding the leader peptide and has a molecular mass of 92 kDa (Zhou *et al.*, 1996). Unlike mammalian PRLRs, the avian PRLRs possessed two highly homologous units in their

extracellular domain (Tanaka *et al.*, 1992; Chen and Horseman, 1994; Zhou *et al.*, 1996). The membrane distal and proximal units consisted of 201 and 204 amino acids, respectively (Zhou *et al.*, 1996). Each unit has similar identity to the singular extracellular domain of the mammalian PRLRs (Tanaka *et al.*, 1992; Chen and Horseman, 1994; Zhou *et al.*, 1996). The extracellular cysteines located at positions 36, 46, 75, 86, 239, 249, 278, and 289 and the WSXWS motif located at positions 213-217 and 419-423 are conserved in both repeat units (Chen and Horseman, 1994; Zhou *et al.*, 1996). The transmembrane and intracellular domains consisted of 24 and 369 amino acids, respectively (Tanaka *et al.*, 1992; Zhou *et al.*, 1996). The intracellular domain of the characterized avian PRLR was equivalent to the long form of mammalian PRLRs (Zhou *et al.*, 1996). The expression of multiple 5'-truncated PRLR transcripts encoding only the intracellular domain in the testis prior to sexual maturity in male chicken suggested a functional role in testicular activity (Mao *et al.*, 1999; Tanaka *et al.*, 2000). However, it is not known if these transcripts were translated.

2.2.3. Prolactin Receptor Family, Domains and Binding

The PRLRs are structurally and functionally related to other members of the class I superfamily of cytokine/hematopoietin receptors which includes more than 30 members such as the receptors for GH, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, leukemia inhibitory factor, oncostatin M, erythropoietin, thrombopoietin, glycoprotein130, obesity factor leptin and several interleukins (Cosman, 1993; Bole-Feysot *et al.*, 1998; Goffin *et al.*, 2002). These transmembrane receptor proteins share highly conserved sequences in their

extracellular and intracellular domains (Bole-Feysot *et al.*, 1998; Corbacho *et al.*, 2002). However, although they show a relatively low degree of overall sequence identity, they have very similar tertiary structures (Somers *et al.*, 1994; Forsyth and Wallis, 2002).

The avian PRLR is structurally different from the mammalian PRLR due to the presence of two conserved potential ligand-binding regions in the extracellular domain (Tanaka *et al.*, 1992). Chen and Horseman (1994) showed that the mutated PRLR containing only the proximal domain had equal specificity and binding affinity to rat PRL when compared to the full-length PRLR. The biological significance of the second repeat unit is not clear. However, the use of heterologous ligand (rat PRL to pigeon PRLR expressed in a mammalian cell line) may not be appropriate since both the affinity and stoichiometry of the PRLR to its ligand may be changed. Conversely, it is possible that the proximal domain of the PRLR has the only ligand binding region since deletion of N-terminal module indicated that the duplication does not play a fundamental role in ligand-binding affinity, ligand specificity or signal transduction (Chen and Horseman, 1994; Horseman and Buntin, 1995; Gao *et al.*, 1996). The disulfide bonds (subdomain D1) and the WSXWS motif (subdomain D2) are essential for the proper folding and trafficking of the receptor, although they are not responsible for binding the ligand itself (Wells and De Vos, 1996; Goffin *et al.*, 1998). It appears that the avian PRL has a similar receptor binding domain to the better characterized mammalian PRLR, and thus PRL is likely to induce signal transduction in a similar fashion in birds (Zadworny *et al.*, 2002).

In mammal and avian species, the transmembrane domain of the PRLR is characterized as a single-pass transmembrane chain (Boutin *et al.*, 1988; Zhou *et al.*, 1996). The functional activity of this receptor region is currently unknown (Clevenger *et al.*, 2003; Li *et al.*, 2005). However, Constantinescu *et al.* (2001) demonstrated that the erythropoietic receptor (EPOR) transmembrane domain mediates ligand-independent oligomerization and allows the PRLR to associate with the EPOR following the use of chimeric constructs where the transmembrane domain of the EPOR was swapped with those of the PRLR.

In most mammalian species, the intracellular domains of PRLR differ in length and composition. However, in the cytoplasmic domain two conserved regions have been identified, termed box 1 and box 2 (Murakami *et al.*, 1991). Box 1 is a membrane-proximal region composed of 8 amino acids rich in prolines and hydrophobic residues (Bole-Feysot *et al.*, 1998). The last proline residue is particularly crucial for the consensus folding of the PRLR recognized by Jak2 whose tyrosine kinase activity is then implicated in the downstream protein phosphorylation such as Stat molecules (Pezet *et al.*, 1997a; Bole-Feysot *et al.*, 1998). Although less conserved than Box 1, Box 2 is located downstream of the cytoplasmic domain of the PRLR and consists of hydrophobic, negatively and positively charged residues in that sequence order (Bole-Feysot *et al.*, 1998). Both sequences are important in signal transduction even though the box 2 signaling function is largely uncharacterized (Clevenger and Kline, 2001). Currently, the 3 dimension structure of the intracellular domain of the PRLR has not been determined.

Although very few studies in avian ligand-PRLR binding have been conducted, information from mammalian model has accumulated. Briefly, activation of the PRLR involves ligand-induced sequential receptor dimerization (Bole-Feysot *et al.*, 1998). In a first step, interaction of helices 1 and 4 of PRL (binding site 1) to PRLR leads to the formation of an inactive H1:R1 complex. Formation of this complex appears to be essential for PRL binding site 2. Binding site 2 involves helices 1 and 3 of PRL and these interact with a second receptor, which leads to PRLR dimerization and formation of an active H1:R2 complex. Mutation of PRL binding site 2 blocks PRLR activation. Prolactin that binds to the receptor only through site 1 behaves as antagonist of the wild-type hormone. Detailed analyses of individual residues required for tight receptor binding and its antagonistic properties were reported by Goffin *et al.* (2005).

2.2.4. Signal Transduction Pathways and PRLR Processing

Several ligands can bind to human PRLR including GH (Kelly *et al.*, 1991; Kato *et al.*, 1996); however there is no evidence that the type of ligand can affect the nature of the signal transmitted into the cell (Goffin *et al.*, 2002). The best-known signaling cascades involve the Jak/Stat (Janus kinases/signal transducer and activator of transcription) pathway, the Ras-Raf-MAPK pathway, and the Src tyrosine kinases (Freeman *et al.*, 2000; Goffin *et al.*, 2002). Dimerization of the PRLR induces tyrosine phosphorylation of numerous cellular proteins including the receptor itself (Bole-Feysot *et al.*, 1998; Freeman *et al.*, 2000). Both the presence of the proline-rich box 1, variable box and box 2 motifs (Clevenger and Kline, 2001) and strict homodimeric stoichiometry of PRLR dimers are essential to activate Jak2, the major PRLR-

associated Janus kinase (Han *et al.*, 1997). This kinase is constitutively associated with the intracellular domain of the receptor (Lebrun *et al.*, 1994). After receptor dimerization, Jak2 kinases transphosphorylate each other and this leads to phosphorylation of other receptor tyrosine residues (Pezet *et al.*, 1997b; Yu-Lee, 2002). The phosphorylated receptor tyrosine residues are potential binding/docking sites for the binding of src homology domain 2 (SH2)-containing proteins, which include Stat1, Stat3, Stat5; phosphatases; and other adaptor molecules (Shuai, 2000; Freeman *et al.*, 2000).

The Stat1, Stat3, and especially Stat5a and Stat5b are the central transducer molecules of the signal transduction pathways initiated by PRLR (Bole-Feysot *et al.*, 1998; Freeman *et al.*, 2000). Stat1 and Stat3 are likely to interact with membrane-proximal regions of the receptor complex, whereas, the major binding site of Stat5a and Stat5b are found in the C-terminal region (Bole-Feysot *et al.*, 1998). Stat molecules are composed of a SH2 domain, which allows these proteins to recognize a phosphorylated tyrosine residue at the C-terminal region. The Stat molecules will bind to the receptor to form a complex which is then phosphorylated by the Janus kinases belonging to the complex. Then, the phosphorylated Stat dissociates from the receptor and dimerizes in a heterologous or homologous fashion through its phosphotyrosine residues with the SH2 domain of another phosphorylated Stat molecule. Finally, the Stat dimer translocates to the nucleus, activates a Stat DNA-binding motif (GAS: gamma-interferon activated sequence) in the promoter of target genes to regulate gene transcription (Luo and Yu-Lee, 1997; Bole-Feysot *et al.*, 1998; Freeman *et al.*, 2000; Yu-Lee, 2002). Numerous promoters contain the GAS consensus motif (Gouilleux *et al.*, 1995; Ferrag *et al.*,

1996), and within 10 minutes following the ligand-receptor binding, PRL-inducible transcription of target genes is detected in the nucleus (Yu-Lee, 2002). Besides tyrosine phosphorylation, activation of Stat also involves serine/threonine phosphorylation (Freeman *et al.*, 2000). Protein kinase C alpha and casein kinase II have been proposed as the phosphorylases activating Stat5 (Beadling *et al.*, 1996).

Although Jak/Stat are the most important pathways initiated by activation of PRLR, the mitogen-activated protein kinase (MAPK) cascade is involved in the activation of a wide range of transcription factors/immediate early genes by phosphorylation (Freeman *et al.*, 2000). Phosphotyrosine residues of the PRLR can also serve as docking sites for adapter protein Shc which binds to the Grb2 (growth factor receptor-binding protein 2) and the latter to the SOS connecting the receptor to the Ras/Raf/MAPK cascade (Das and Vonderhaar, 1995). Additional data suggest interconnection between Jak/Stat and MAPK pathways (Guerra *et al.*, 1998). Furthermore, PRL can induce the activation of members of the Src kinase family, c-src and Fyn, which are involved in the tyrosine phosphorylation of phosphatidylinositol 3-kinase (Berlanga *et al.*, 1995; Al-Sakkaf *et al.*, 1997). Moreover, box 1 is involved in the activation of tyrosine kinase-dependent K^+ channels by Jak2, whereas the C-terminal of the PRLR is involved in the production of inositol 1,3,4,5-tetrakisphosphate (IP_4) and inositol hexakisphosphate (IP_6) that open voltage-independent Ca^{2+} channels (Sorin *et al.*, 1998; Ratovondrahona *et al.*, 1998). Other proteins are activated by PRL stimulation such as Tec family of tyrosine kinases, Vav family of guanine nucleotide exchange factors, ZAP-70 (70 kDa tyrosine kinase ζ-chain associated protein), focal adhesion kinase, Jun kinase, and insulin receptor substrate-1 (Clevenger and Kline,

2001). Thus, activation of different signaling cascades with the Jak/Stat signaling pathway determines the expression of specific genes induced upon ligand-receptor binding in diverse cells or tissues. Taken together, the actions of PRL are exerted on cell proliferation, differentiation, or apoptosis are based on differential activation of these coordinated kinase cascades (Yu-Lee, 2002).

Since most signaling molecules are activated by tyrosine phosphorylation (Jaks, Stats, Src, Tec), the involvement of tyrosine phosphatases to regulate the PRLR signaling cascades has been described (Bole-Feysot *et al.*, 1998; Goffin *et al.*, 1999). For instance, Jak2 has been demonstrated to activate the SH2 domain of protein tyrosine phosphatase (SHP-2) which then acts as a positive regulator of PRLR-dependent induction of β-casein gene transcription (Ali *et al.*, 1996). More recently, Ali *et al.* (2003) indicated that SHP-2 plays a positive role in signaling the kinase Jak2 and PRLR by dephosphorylating SOCS-1 recruitment site within Jak2, consequently allowing signal propagation maintenance. By using a mutant form of the PRLR or a phosphatase inactive mutant of SHP-2, these authors were able to block the interaction of Jak2/SOCS-1 which is a major mechanism by which SHP-2 regulates PRLR signaling. Besides phosphatases, a family of cytokine inducible inhibitors of signaling has been identified that includes: CIS (cytokine-inducible SH2-containing protein) and members of SOCS (suppressors of cytokine signaling) family. The SOCS interacts with Jaks or CIS competes with Stats for binding to the PRLR which provides a way to down-regulate the Jak/Stat signaling pathways (Bole-Feysot *et al.*, 1998; Goffin *et al.*, 1999; Clevenger and Kline, 2001). Accordingly, the PRL-inducible SOCS proteins (SOCS-1 and SOCS-3) can bind to the PRLR and turn off signaling at the receptor level in a

negative-feedback loop (Naka *et al.*, 1999; Tam *et al.*, 2001) by inhibiting the catalytic activity of Jak2 and activation of Stat proteins. In contrast, the CIS and SOCS-2 were still elevated 24 hours after PRL treatment (Goffin *et al.*, 1999), and SOCS-2 seems to restore the sensitivity of the cell to PRLR stimulation probably by suppressing the inhibitory effect of SOCS-1 via Jak2 kinase activity (Pezet *et al.*, 1999).

Generally, upon any ligand-receptor binding, the internalization process is subsequently initiated and results in multiple possible outcomes. These include recycling of receptors back to the plasma membrane, degradation of ligand and/or its receptor by lysosomes or proteasomes, or translocation of ligand and/or its receptor to the nucleus to induce genomic effects (Freeman *et al.*, 2000; Schuler *et al.*, 2001). Although very little is known about the PRLR and its related cytokine receptors superfamily endocytic pathways (Lu *et al.*, 2002; Clevenger *et al.*, 2003), the internalization fate processes may be differently regulated among species and depend on the concentration of the receptor expressed on the cellular surface (Schuler *et al.*, 2001). For example, Lu *et al.* (2002) identified two regions of the long bovine PRLR isoform which seem to differentially regulate internalization among isoforms. These motifs required for endocytosis correspond to a phenylalanine (amino acid 290) in close proximity to a di-leucine (amino acids 286-287), and three di-leucines (amino acids 243-244, 259-260, 268-269) motifs close to the transmembrane domain. They suggested that the similarity between the tyrosine and phenylalanine residues and di-leucine motifs could bind to the adaptor protein 2 which allow internalization through clathrin-coated pits. Alternatively, the phenylalanine residue of the long PRLR and GHR may be

part of an ubiquitin-dependent endocytosis motif which is involved in internalization. However, Lu *et al.* (2002) noted that the internalization of the PRLR was twice rapid as the GHR. Prolactin receptor uses perhaps different motifs linked to different endocytic pathways or the presence of multiple motifs compared to the single dominant motif of the GHR may explain this difference (Lu *et al.*, 2002). In bovine, the rate of internalization differs between the long and short PRLR isoforms, with a faster rate observed for the long form. Interestingly, Vincent *et al.* (1997) reported the opposite in rodents. Different rates of internalization are the result of different PRLR cytoplasmic domains between species (Clevenger *et al.*, 2003). Since internalization of the long form appears to be faster than the short, an increase in ligand stimulation of cells expressing both receptor isoforms would result in a relative predominance of short PRLR over the long isoform on the cell surface (Schuler *et al.*, 2001; Lu *et al.*, 2002). Consequently, this would lead to an overall attenuation of PRL-induced signals, by decreasing the dominant-positive signal of the remaining long isoform and increasing the dominant-negative action of the short PRLR isoform (Schuler *et al.*, 2001). Indirect evidence predicts that ligand-receptor internalization process is interconnected to downstream signaling effectors via caveolae, microdomains in the plasma membrane, glycosphingolipids and lipid-anchored membrane proteins (Clevenger *et al.*, 2003). For instance, studies conducted on GHR have demonstrated that it is internalized both by clathrin-coated pits and caveolar pathways (Govers *et al.*, 1997; Lobie *et al.*, 1999); a similar demonstration has been made in bovine PRLR using a dominant-negative mutant of dynamin-1 (Lu *et al.*, 2002). Vincent et al. (1997) suggested that di-leucine peptides are bound directly to alpha-adaptin, an adaptor protein-2, which serves as a

mediator connecting cargo to clathrin-coated pits to facilitate endocytosis. However, other pathways by which the internalization processes occur exist. For example, internalization processes occur throughout Rho family members such as the GTPase RhoG involving in caveolar trafficking (Prieto-Sanchez *et al.*, 2006) and throughout Src family members such as the CIS depending on GHR internalization via signaling to Stat5b demonstrated by using a dominant-negative CIS mutant (Landsman and Waxman, 2005). Following the ligand-receptor binding steps, Lu *et al.* (2005) used proteasome inhibitors to measure the degradation process and observed the appearance of extracellular domain of PRLR fragment which was dependent on proteasomal activity. They hypothesized that proteasomes have a limited proteolytic role to generate a stable long PRLR isoform cleavage product. It has been clearly demonstrated that internalization of PRL-receptor complex occur, but whether it is translocated to the nucleus after internalization is still not known (Goffin *et al.*, 2002). Rycyzyn *et al.* (2000) noted significant nuclear translocation of PRL which required the presence of co-stimulators such as epithelium growth factor and interleukins, although the molecular mechanisms underlying this observation remain unknown. Nevertheless, using a yeast two-hybrid system, they observed that the enhanced action of PRL by cyclophilin B was accompanied by a dramatic increase in the nuclear translocation of PRL and by an increase of PRL mitogenic activity. Indeed, Rycyzyn and Clevenger (2002) demonstrated that the intra-nuclear PRL/cyclophilin B complex acts as an inducer of transcription by interacting directly with Stat5. This is caused by the removal of the Stat repressor protein inhibitor of activated Stat3 and, consequently, enhances Stat5 DNA-binding activity and PRL-induced Stat5-mediated gene expression.

2.2.5. Prolactin Receptor Distribution

In mammals, the PRLR was detected in a wide range of tissues including several area of the central nervous system, the pituitary, the reproductive system of the male and female, the gastrointestinal tract, lymphoid tissue, bone tissue, and skin. The receptor was also expressed in ganglia, adrenal cortex, gill (fish), lung, heart, skeletal muscle, adipocytes and brown adipose tissue, liver, submandibular and submaxillary glands, pancreas, kidney, bladder, and area postrema which is one of the main chemosensitive areas of the brain lacking the blood-brain barrier (Nagano and Kelly, 1994; Bole-Feysot *et al.*, 1998; Mangurian *et al.*, 1999). In avian species, PRLR is also expressed ubiquitously. In addition to the tissues mentioned above, PRLR mRNA was also detected in crop-sac, thyroid gland, blood, gizzard, brood patch, pineal gland, leukocyte, heterophil, shell gland, caeca, bursa of Fabricius, choroid plexus and a variety of forebrain and midbrain sites (Buntin *et al.*, 1993; Chen and Horseman, 1994; Rodriguez *et al.*, 1996; Di Carlo *et al.*, 1996; Zhou *et al.*, 1996; Ohkubo *et al.*, 1998a; Pitts *et al.*, 2000). Recently, PRLR mRNA has been detected in the allantoic membrane during late stage embryogenesis of the chick (Yamamoto *et al.*, 2003). The widespread distribution of PRLR mRNA in brain and peripheral tissues indicates that PRL acts to exert multiple effects in target tissues (Ohkubo *et al.*, 1998a).

2.2.6. Prolactin Receptor Expression

The pattern of PRLR expression changes within tissues during development. Using *in situ* hybridization and immunohistochemistry, Royster *et al.* (1995) and

Freemark *et al.* (1997) noted a similar tissue distribution between PRLR immunoreactivity to that of PRLR mRNA, indicating that PRLR mRNA is translated to receptor protein in the human fetus and fetal rat *in vivo*. Levels of expression of PRLR mRNA and protein increased during gestation in a number of fetal tissues. However, steady-state PRLR mRNA and protein levels were not always correlated. Nevertheless, changes in levels of transcript have been measured and these are likely to correlate with protein levels. Also, striking changes in the cellular distribution and level of expression of PRLR were noted in many tissues during development. These authors demonstrated that the expression of PRLR during ontogeny provided evidence for changing roles of PRL in organ development and function (Freemark *et al.*, 1997). For example, the PRLR was expressed at very low levels in the olfactory bulb of the adult rat but is detectable in abundance in olfactory epithelium and bulb of the fetal rat in late gestation (Freemark *et al.*, 1996). Thus, during ontogenesis, expression of PRLRs in the olfactory system adopted a different cellular distribution and level of expression, and discontinuous, of receptors within a single tissue (Freemark, 2001). During postnatal period, pituitary PRL may modulate olfactory function, by acting on the neonatal feeding response and on the interactions between the mother and its offspring (Freemark *et al.*, 1996). In avian species, protein levels of PRLR have not been assessed. However, in adult turkeys, Zhou *et al.* (1996) observed amongst 17 tissues that increasing levels of plasma PRL up-regulated the level of PRLR mRNA only in the shell gland and pituitary, whereas, PRLR transcript was significantly down-regulated in the hypothalamus. The presence of PRLR in the basal hypothalamus in the same region as VIP neurons suggested 1) that these neurons may be a target for circulating PRL

which exerts a negative feedback control over its secretion and its receptor transcript, and 2) the positive feedback effect of VIP on PRL secretion in the adenohypophysis via paracrine and/or autocrine effects caused increasing levels of PRLR transcript during hyperprolactinemia (Zhou *et al.*, 1996; Ohkubo *et al.*, 1998a). Furthermore, higher levels of PRLR mRNA in the small white follicle compared to the large follicle correlated with the action of PRL in suppressing estradiol production by small follicles (Zadworny *et al.*, 1989; Zhou *et al.*, 1996). Thus, levels of transcript are likely to correlate to protein levels of the receptor.

2.3. Pituitary Cell Differentiation during Embryogenesis

Allaerts *et al.* (1999) illustrated three distinct developmental periods relevant to the maturation of the adenohypophysis: 1) segregation of Rathke's pouch, 2) differentiation of hormone producing cells simultaneously with influx of hematopoietic cells and 3) differentiation of non-hormone-producing folliculo-stellate cells. It is generally admitted that the pituitary gland begins with an up-growth from the primitive mouth which differentiates into the adenohypophysis and with a down-growth from the diencephalon which differentiates into the neurohypophysis (Dubois *et al.*, 1997; Sasaki *et al.*, 2003). Although the origin of the andenohypophysis is still controversial, it seems to originate from each of the embryonic germ layers (reviewed by Dubois *et al.*, 1997; Allaerts *et al.*, 1999). Within 3 days of incubation, the cephalic lobe and caudal lobe of the pars distalis and the pars tuberalis of the adenohypophysis are formed from the stomodeal ectoderm (Dubois *et al.*, 1997; Sasaki *et al.*, 2003). In the chick embryo, the adenohypophysis, derived from the Rathke's pouch becomes evident around 6 days of

incubation (Dubois *et al.*, 1997; Sasaki *et al.*, 2003). Soon after, hormone-immunoreactive cells appeared. Although the time of first appearance of the various immunoreactive cells was not found to be uniform due to the antisera and methods of fixation used, Sasaki *et al.* (2003) identified in the pituitary gland the first appearance of LH-, follicle-stimulating hormone (FSH)-, adrenocorticotropic hormone (ACTH)-, thyroid-stimulating hormone (TSH)-, GH-, and PRL-immunoreactive cells appearance at 6.5, 7.5, 7.5, 9, 16, and 20 days of incubation, respectively. However, Mikami and Takahashi (1987) identified the first PRL-immunoreactive cells at 18 days of incubation in accord with the observation made by Woods and Porter (1998). In addition, Tennyson *et al.* (1985) detected the first mesotocin and arginine vasotocin-immunoreactive cells at 8.5 days of incubation. In the hypothalamus, TRH-, LHRH and VIP-immunoreactive neurons were identified at 4.5, 5.5 and 5 days of incubation, respectively (Thommes *et al.*, 1985; Woods *et al.*, 1985; Du *et al.*, 1988). Macrophages originating from the yolk sac invade the endocrine cell cords when cell differentiation is proceeding. CD45-immunopositive leukocytes and macrophages may infiltrate the adenohypophysis at embryonic day 10 and 12, respectively (Allaerts *et al.*, 1999). Final differentiation of folliculo-stellate cells, resulting in the expression of S100 protein, occurs after hatching in chickens (Van Nassauw *et al.*, 1987; Allaerts *et al.*, 1999). It was observed that major histocompatibility complex (MHC)-class II antigens from the dendritic cells were completely absent in the chicken adenohypophysis during prenatal development (Allaerts *et al.*, 1999). Nevertheless, Kaur *et al.* (2002) noted that the alteration of macrophage function could be due to altered levels of ACTH production in the adenohypophysis since this hormone is involved in immune responses by

suppressing the phagocytic activity and expression of MHC class II antigens of macrophages. In mammals, the folliculo-stellate cells, usually located among secretory cells of the anterior pituitary gland, produce many peptides such as endothelial growth factor, fibroblast growth factor, interleukin-6, follistatin, leukemia inhibitory factor, nitric oxide synthase and leptin that exert a paracrine effect on hormone-producing pituitary cells (Jin *et al.*, 2001).

It has been demonstrated that the transcripts of hormone-immunoreactive cells, such as LH, GH and PRL, are expressed earlier than their peptides during embryogenesis. In chicks, the differentiation of somatotroph and lactotroph cells in the pituitary gland occurs by 16 and 17 days of incubation, respectively (Porter *et al.*, 1995; Woods and Porter, 1998), and about this time levels of GH and PRL mRNA begin to increase, as well as circulating levels of GH and PRL (Harvey *et al.*, 1979; Ishida *et al.*, 1991; McCann-Levorse *et al.*, 1993; Kansaku *et al.*, 1994; Gregory *et al.*, 1998). After hatching, levels of GH mRNA, GH content and plasma GH increased throughout the first 4 weeks of age in chickens with a temporary decrease at day 6 posthatch (McCann-Levorse *et al.*, 1993; Gregory *et al.*, 1998). Evidence points out that the co-distribution of growth hormone receptor immunoreactive cells and its transcript in early embryonic chicken indicates that GH action is mediated in an autocrine or paracrine mechanisms (Harvey *et al.*, 2001; Harvey and Hull, 2003). Conversely, the day after hatch, levels of PRL mRNA decrease and then stay stable, whereas the plasma levels of PRL decrease the first day and then increase during the first week (Ishida *et al.*, 1991). A similar pattern was observed in turkeys (Bédécarrats *et al.*, 1999c). These authors observe a two-day delay between the increase in pituitary content and plasma levels of PRL,

suggesting PRL is probably accumulated and/or processed in the pituitary gland before it is released into the circulation. Although in avian species no information showing a relationship between the expression of PRLR transcripts and its peptide during embryogenesis has been reported, the expression of fetal rat PRLR mRNA was shown to precede the appearance of immunoreactive PRLR protein in a number of tissues (Royster *et al.*, 1995; Freemark *et al.*, 1997). This may reflect in part the normal delay between the onset of transcription and translation of protein; alternatively, the rate of turnover of PRLR protein may exceed that of PRLR mRNA, or translation of the fetal PRLR *in vivo* may itself be subject to developmental control (Royster *et al.*, 1995).

2.3.1. Prolactin Biological Actions in the Embryo

Changes in the expression of the PRL gene in the pituitary gland with changes in the synthesis and release of the different PRL isoforms before and after hatching could be correlated with its actions on the development of extrapituitary organs (Bole-Feysot *et al.*, 1998; Bédécarrats *et al.*, 1999c). Data concerning the roles of PRL in physiological processes in avian embryo are scanty. However, it has been reported that PRL when injected to less than 14-day old embryos, significantly lowered allantoic NaCl concentration. This suggested that NaCl in the urine was reabsorbed from the metanephric kidney following NA^+-K^+-ATPase stimulation (Doneen and Smith, 1982; Murphy *et al.*, 1986). The ability of PRL to stimulate NA^+-K^+-ATPase in metanephros may be related to high levels of PRLR in kidney (White and Nicoll, 1980; Krishnan *et al.*, 1991). Indeed, only a cursory research note on PRLR mRNA expression in various tissues has been done in avian embryogenesis (Yamamoto *et al.*, 2003). PRLR mRNA

is expressed abundantly in the kidney, intestine and allantoic membrane in 19 days-old chicks, suggesting a role of PRL in osmoregulation. In addition, PRL may have a role together with GH in controlling peripheral thyroid hormone metabolism during the process of hatching. Both hormones have different hepatic receptors and opposite effect on hepatic deiodinating enzyme activity (Kuhn *et al.*, 1996). Variations of PRL may therefore act on thyroid hormones (T_3 and T_4) by modulating their circulating concentrations and/or activation in the thyroid gland during hatching (Scanes *et al.*, 1987; Bédécarrats *et al.*, 1999c). It has been shown that injection of recombinant chicken PRL elevated plasma concentration of corticosterone prior to day 16 (Kuhn *et al.*, 1996). Of the specific hormones examined, corticosterone has the most prominent effects, both on pulmonary growth and on surfactant synthesis during ontogeny (Hylka and Doneen, 1983). Furthermore, evidence suggests that PRL may affect the development of the immune system in chickens (Bédécarrats *et al.*, 1999c), since PRL and growth hormone influenced the development of the immune-neuroendocrine network via receptors during embryogenesis (Herradon *et al.*, 1991; Moreno *et al.*, 1994; 1998; Harvey *et al.*, 2003; Luna *et al.*, 2005).

CONNECTIVE STATEMENT 1

It has been previously shown that the concentration of PRL during embryonic development rapidly increases during the latter stages of embryogenesis in turkeys. However, the function of PRL in the hatching process is not clear. In this chapter, levels of PRLR mRNA in the pituitary gland and hypothalamus were semi-quantified by real-time PCR in turkeys and chickens during the perihatch stage of development. Levels of transcript were correlated to blood levels of PRL measured by radioimmunoassay. In addition, VIP was used in a tissue culture system to stimulate expression of PRL and compare levels of PRLR in pituitary glands from each species.

CHAPTER 3

DEVELOPMENT OF A REAL-TIME (Q) PCR ASSAY TO ASSESS VARIATION IN THE EXPRESSION OF PROLACTIN RECEPTOR MRNA IN THE HYPOTHALAMUS AND PITUITARY GLAND DURING LATE EMBRYOGENESIS IN TURKEYS AND CHICKENS

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Submitted to General and Comparative Endocrinology

3.1. ABSTRACT

Changes in levels of prolactin receptor (PRLR) mRNA in the pituitary gland and hypothalamus of chickens and turkeys from embryonic day (ED) 15 and ED21 to 1 day post-hatch, respectively, were measured by real-time PCR. In both species, PRLR mRNA progressively increased during the last week of ED and reached maxima at the peri-hatch period. Similarly, circulating levels of PRL also increased during this interval and were highly correlated with levels of the PRLR mRNA in both the pituitary gland and hypothalamus. This suggests that PRL was up-regulating its receptor. In support of this, stimulation of the turkey pituitary gland with VIP on ED24 resulted in a 4 and 3 fold increase in PRL and PRLR, respectively. Since VIP had no direct effect on the levels of PRLR transcript in the hypothalamus it is likely that VIP is acting indirectly through increased PRL to up-regulate the number of receptors.

3.2. INTRODUCTION

In the chicken and turkey embryogenesis requires 21 and 28 days, respectively. Although the pituitary gland has differentiated by an earlier stage, lactotroph cells are detectable by staining to immunoreactive prolactin (PRL) by about embryonic day (ED) 15 to 17 in chicks (Kansaku et al., 1994; Sasaki et al., 2003; Fu et al., 2004; Zheng et al., 2005) and ED23 in turkeys (Bédécarrats et al., 1999). However, PRL transcripts are detectable at earlier stages (Kansaku et al., 1994; Bédécarrats et al., 1999). During the last few days of ED, PRL is released into the circulation and increases in concentration

(Ishida et al., 1991; Bédécarrats et al., 1999). This increased level of PRL is highly correlated to pituitary content of PRL and PRL mRNA and furthermore is associated with a shift in post-translationally modified PRL. Prior to ED23 in the turkey, PRL is predominantly non-glycosylated (NG), whereas, by ED27 about 50 % of the total PRL is glycosylated (G) (Bédécarrats et al., 1999). Since PRL is known to be involved in a large number of physiological functions in vertebrates (Bole-Feysot et al., 1998), it is likely that increases in pituitary derived PRL around the time of hatch may modulate these processes.

In galliforms, the secretion of PRL is mainly regulated by vasoactive intestinal peptide (VIP) of hypothalamic origin and numerous studies have demonstrated the stimulatory effects of VIP on the secretion of PRL (Proudman and Opel, 1988; El Halawani et al., 1990b; Talbot et al., 1991; Pitts et al., 1994a,b; Kansaku et al., 1995; Xu et al., 1996). The abundance of PRL mRNA (Talbot et al., 1991; Tong et al., 1998; Chaiseha et al., 1998) and plasma levels of PRL (Youngren et al., 1996) vary in concert with the levels of VIP in the hypothalamus during the various stages in turkey reproductive cycle. Moreover, the release patterns of VIP into hypophyseal portal blood (Youngren et al., 1996) and the number of VIP immunoreactive cells in the hypothalamus (Mauro et al., 1989) are correlated with the levels of plasma PRL (Kang et al., 2004). Tong et al. (1998) reported that VIP induced PRL gene expression by increasing transcription rate and enhancing mRNA stability. VIP enhances PRL gene transcription via a 35-bp VIP response element located in the turkey PRL promoter (Kang et al., 2004). In the adult turkey, levels of PRL are positively correlated to levels of pituitary VIP receptor mRNA (Chaiseha et al., 2004). In the chicken, the VIP

receptor mRNA is detectable by ED14 but levels substantially increase by ED18 (Kansaku et al., 2001). The latter increase is consistent with the initiation of secretion of PRL by lactotrophs.

Prolactin acts by binding to a specific receptor which is a member of the Class I cytokine receptor superfamily that includes growth hormone, erythropoietin, thrombopoietin granulocyte colony-stimulating factor, granulocyte/macrophage stimulating factor, leptin and several interleukin receptors (Bazan, 1990; Bole-Feysot et al., 1998). The receptor has been cloned in both chickens and turkeys (Tanaka et al., 1992; Zhou et al., 1996) and in adult hens, levels of the PRLR are highest in the anterior pituitary gland and hypothalamus (Zhou et al., 1996; Ohkubo et al., 1998a,b) and vary with the reproductive state of the hen. The single study measuring levels of PRLR during ED reported that the PRLR was barely detectable in chick brain tissue at ED19 and furthermore that the levels of the PRLR did not vary between ED17 and 28 days post-hatch in the kidney or intestine (Yamamoto et al., 2003). Since circulating levels of PRL significantly increase during late ED and in post-hatch development and would be expected to correlate with PRLR number, a hypothetical result which may stem from the insensitivity of the nuclease protection assay to detect changes in transcript level. As a consequence, we developed a real-time (Q) PCR assay to assess changes in the PRLR in the hypothalamus and pituitary gland in both chickens and turkeys during the last week of ED.

3.3. MATERIALS AND METHODS

3.3.1. Tissue Sampling

Eggs from two commercial strains of white Medium Hybrid turkey hens (Hybrid Turkeys Inc, Kitchener, Ontario, Canada) and white Hyline W98 Leghorn chicken hens (HyLine International, West Des Moines, Iowa, USA) were incubated under standard commercial conditions at 37.5°C, 85-86% humidity for 25 and 18 days, respectively. They were then transferred to a hatcher at 36.9°C, 90-92% humidity. In addition to the day of hatch and 1 day after hatch, turkey and chicken embryos were collected at day 21, 23, 25 and 27 or day 15, 17 and 19 of incubation, respectively. Ten embryos and poults were killed by cervical dislocation for each developmental stage in both species. Tissues were immediately collected after decapitation, snap-frozen in liquid nitrogen, and stored at –80°C until required for assay.

3.3.2. Tissue Culture

Turkey embryos were sacrificed at day 24 of incubation and the hypothalamus and anterior pituitary gland were rapidly collected and placed into 6-well culture plates. Hypothalami or pituitary glands were cultured separately (n=12), whereas, for co-culture experiments (Pit+Hyp), pituitary glands were cultured with an equal number of hypothalami (n=14). Tissues were pre-incubated at 39°C for 1 h in a 5% CO_2/95% air incubator in 10 ml M199 (Gibco BRL Products, Life Technologies, Burlington, Ontario, Canada) containing Earle salts supplemented with 25 mM Hepes, 0.1% (w/v) bovine serum albumin, 100 mg/l L-glutamine, 100 U/ml penicillin G and 100 µg/ml

streptomycin sulfate in 0.85% saline (sodium salt), and 2200 mg/l sodium bicarbonate. After 1 h, the medium was removed and replaced by the same volume of medium plus or minus 10^{-7} M hVIP (Sigma-Aldrich, St-Louis, USA). The tissues were incubated at 39°C for 4 h, individually collected, snap-frozen in liquid nitrogen, and then stored at -80°C until assayed. An aliquot of medium was collected at the start and end of VIP stimulation and stored at -80°C until assayed for the concentration of PRL.

3.3.3. Extraction of Total RNA

Total RNA from tissues was extracted using TRIzol reagent (Gibco BRL Products), according to the manufacturer's protocol. Briefly, tissues were homogenized individually in 800 µl of a monophasic solution of phenol and guanidine isothiocyanate by sonicating on ice for approximately 30 sec (2 bursts). A volume of 200 µl of chloroform was added to the homogenate, and the different phases were separated by centrifugation at 12 000 rpm for 15 minutes. The aqueous phase containing RNA was precipitated with an equal volume of isopropanol. The RNA was recovered at 12 000 rpm for 15 min and washed with 1 ml of 75% ethanol. The recovered RNA was dissolved in DEPC-treated water to a final concentration of 0.5 µg/µl (Bédécarrats et al., 1999). Integrity of the RNA was electrophoretically verified in 1.2 % formaldehyde agarose gel stained with ethidium bromide (EtBr).

3.3.4. Reverse Transcription (RT) and Real-Time (Q-PCR) Quantification of tPRLR mRNA

The RT, modified from Bédécarrats et al. (1999), was carried out in a final volume of 10 µl containing 2.0 µg of total RNA. The RNA was heat-denatured at 65°C for 10 min, then mixed with 2.5 U of Avian Myeloblastosis Virus reverse transcriptase (Amersham Biosciences, Baie-Urfe, Quebec, Canada), 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 0.25 mM each dNTPs, and 40 pmol of antisense PRLR primer (5'-CCCAAGACGATCCACACAATC-3'). This sequence binds at positions 353-1373 (L76587) and 1539-1559 (NM_204854) in the turkey and chicken, respectively. The RT was conducted at 42°C for 1 h; then the reaction mix was heated at 90°C for 5 min to inactivate the reverse transcriptase.

An external standard of 332 bp (Zhou et al., 1996) was used in Q-PCR to semi-quantify levels of PRLR cDNA. This sequence contained a 39 bp deletion of the amplicon of 371 bp amplified from tPRLR mRNA to allow for determination of co-amplification efficiency by melting point analysis and/or gel electrophoresis. The external standard was amplified by PCR at 93°C for 45 sec, 56°C for 2 min, and 72°C for 90 sec for 35 cycles (Zhou et al., 1996), purified from 1% agarose gel before use and stepwise diluted tenfold to cover the range of turkey and chicken PRLR mRNA levels in tissues amplified by Q-PCR.

Conditions for Q-PCR using the LightCycler (Roche Applied Science, Laval, Quebec, Canada) were optimized for 1) $MgCl^{2+}$ concentration, 2) primer concentration, 3) annealing temperature, and 4) concentration of the cDNA. The optimized protocol for Q-PCR was as follows. An aliquot of 2 µl of a single-strand cDNA mixture was amplified by LC PCR in a final volume of 20 µl containing 1x LC-FastStart DNA Master SYBR Green I (Roche Applied Science, Laval, Quebec, Canada), 4 mM $MgCl_2$,

and 10 pmol of both forward and reverse primers (Invitrogen Canada Inc). For the Q-PCR, a sense and an antisense tPRLR-specific primers were as designed by Zhou et al. (1996: 5'AGGAAACATTTACCTGTTGGT and 5'AAGCCATCCAGATCTGACATC). The cDNA was diluted according to the stages of the embryo development. Samples for Q-PCR were denatured at 95°C for 10 min followed by an amplification program (95°C for 10 sec, 55°C for 5 sec, 72°C for 16 sec) for 40 cycles using the second derivative maximum option of the LC software (Version 3.01). Specificity of the amplifications was determined by melting curve analysis (63°C for 15 sec to 95°C in 0.1°C/sec increments) and by gel electrophoresis.

To estimate the concentration (Co) of PRLR mRNA in the turkey and chicken cDNA samples, the following formula was used: CP = S Log (Co) + I, where CP is the crossing point value from the turkey and chicken samples i.e. the point at which the fluorescence rises appreciably above the background fluorescence. The standard slope (S) represents the overall reaction efficiency and the intercept (I) of the standard represents the crossing point value on the y-axis for which the concentration equals to zero. The linear regression line was obtained by plotting the crossing cycle number versus the logarithm of the concentration for each unknown sample. The PCR efficiency was calculated from the standard slope provided by the LC software.

3.3.5. PRL Radioimmunoassay and Western

Blood samples were collected into heparinized tubes from the chorio-allantoic blood in turkey and chicken embryos from day 21 until 25 and from day 15 until 19 of incubation, respectively, and thereafter by intra-cardiac puncture. After centrifugation,

plasma samples were stored at –80°C until assayed. For tissue culture experiments, an aliquot of medium was collected at time 0 h and 4 h post VIP stimulation and stored at -80°C until assayed.

Levels of PRL were measured by radioimmunoassay as described by Guémené et al. (1994). Recombinant turkey PRL (rctPRL) used for iodination and as standard was obtained from Dr. A.F. Parlow (National Hormone and Peptide Program, Harbor-ULCA Medical Center, Torrance, CA) and was purified from the expression construct of Karatzas et al. (1993). Aliquots of plasma (100 µl: turkey or 200 µl: chicken), media (50 to 200 µl) or in 100 µl of a 1/50 dilution of pituitary extracts were assayed in triplicate for PRL content. In brief, samples were incubated with rabbit anti-rctPRL antibody (1/1000 final dilution) for 24 h at 4°C prior to the addition of 15 000 cpm of [^{125}I] rctPRL. Following incubation for 24 h at 4°C, goat anti-rabbit second antibody (1/100 final dilution) was added and tubes were incubated for 48 h at 4°C. After addition of 1 ml phosphate buffered saline (PBS), immuno-complexes were precipitated by centrifugation at 3000 *g* for 30 minutes. Pellets were washed with 1 ml PBS and radioactivity was counted using a gamma counter. Concentration of PRL in the samples was estimated using GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA). The intra- and inter-assay coefficients of variation were 5.8 % and 0.5 %, respectively.

The protocol for preparation of pituitary extracts was adapted from Bédécarrats et al. (1999). In brief, a pool of six pituitaries from VIP stimulated or the control was homogenized in 45 µl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5% Tween 20 by sonication 5 times for 10 sec on ice. The pituitary homogenate was adjusted to 1 µg

protein / µl with 10 mM Tris-HCl (pH 8.0) containing PMSF (0.1 mM) and an aliquot of 2.5 µg protein from each pool was separated on 12% SDS polyacrylamide gel (0.75 mm, 8 cm). After electrophoresis, the proteins were electrotransferred to a PVFD membrane (Immobilon-P, 0.45 µm, Millipore) at constant voltage of 100 V for 1 h at 4 °C with constant stirring in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. The membranes were blocked with Tris buffered saline pH 7.5 (TBS: Tris base 50 mM, NaCl 150 mM) with 5% non fat dry milk for 1 hour with shaking and then incubated with rabbit anti-rctPRL antibody (Guémené et al., 1994) at a 1/2000 dilution in TBS with 0.5% non fat dry milk over night with shaking at 2 °C to 8 °C. The membranes were washed 6 times for 10 minutes with TBS 0.05% with Tween 20 with shaking prior to incubation for 1 h with anti-rabbit IgG peroxidase at 1:20000 dilution in TBS containing 0.5% non fat dry milk at room temperature. Subsequently, the membranes were washed 4 times for 5 minutes with TBS containing 0.05 % Tween 20 and the immunoreactive bands were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer's protocol. After exposure to Kodak X-ray film, the image was digitalized and the proportion of each band was measured by Quantity One Software (Bio-Rad Laboratories, Inc.).

3.3.6. Statistical Analysis

Following log transformation, all statistical tests were analyzed using NCSS 2004 software (Kaysville, Utah) and ANOVA ($P < 0.05$). Where appropriate, differences in mean levels of PRLR mRNA and PRL were determined by Tukey-

Kramer tests. Each data point represents the average ± standard error of the mean (SEM).

3.4. RESULTS

3.4.1. Levels of Pituitary and Hypothalamic PRLR mRNA and Plasma PRL in Turkey Embryos Before and After Hatching

The concentration of PRLR mRNA in the pituitary gland remained at low levels (about 80 A.U./pituitary) until 1 day before hatching (Figure 1). The levels of mRNA then increased consistently until the end of the experiment. At 1 day before hatch, at hatch and 1 day after hatch, levels of PRLR mRNA were 147 $\pm$ 34, 187 $\pm$ 38 and 381 $\pm$ 175 A.U./pituitary, respectively, compared to 60 $\pm$ 15 between 7 to 3 days before hatch. A similar increase in hypothalamic content of PRLR mRNA was observed during the same interval. Levels of PRLR mRNA in the hypothalamus increased from 81 $\pm$ 24 during 7 to 3 days before hatch to 290 $\pm$ 100, 619 $\pm$ 144 and 528 $\pm$ 182 A.U./hypothalamus 1 day before, at hatch and 1 day after hatch, respectively. Plasma levels of PRL did not vary significantly up to 24 h prior to hatch (33 $\pm$ 3 ng/ml) then increased to 72 $\pm$ 14 ng/ml by day one after hatching. The correlation between PRL and pituitary PRLR and hypothalamic PRLR was 0.75 and 0.61, respectively.

3.4.2. Levels of Pituitary and Hypothalamic PRLR mRNA and Plasma PRL in Chicken Embryos Before and After Hatching

The concentration of PRLR mRNA in the pituitary gland and hypothalamus of chickens increased in a similar fashion during embryogenesis (Figure 2). In both tissues, levels of the receptor mRNA increased consistently from low levels at day 6 before hatch (33 $\pm$ 17 and 47 $\pm$ 18) to 1 day after hatch (1190 $\pm$ 318 and 718 $\pm$ 118) in the pituitary gland and hypothalamus, respectively. Plasma levels of PRL increased in concert with the increase in levels of PRLR mRNA. The correlation between levels of PRL and hypothalamic or pituitary PRLR were 0.98 and 0.90, respectively. In addition, the correlation between levels of PRLR in the tissues was 0.65. In general, the pattern of changes in PRL and PRLR during embryogenesis between chickens and turkeys were very similar although the magnitude of the changes was greater in chickens.

3.4.3. Influence of VIP on Level of PRLR mRNA in Pituitary Glands, Hypothalami or Gonads of Embryonic Day 24 Turkeys

Pituitary glands from turkey embryos 4 days before hatch were stimulated with VIP. Levels of PRL in the incubation medium significantly increased 4.2 fold following treatment with VIP over levels in the control. In pituitary extracts, two immunoreactive bands were detected with relative molecular weights of 24 kDa and 27 kDa which represented NG-PRL and G-PRL respectively. There was about an approximate 2- fold increase in the expression of total PRL in the extract following stimulation with VIP and the relative proportion of G-PRL increased from 13 to 44 % (Figure 3). Concomitantly, levels of PRLR mRNA increased 2.8 fold (Figure 3, Table 2) following VIP stimulation and co-incubation of the hypothalamus with the pituitary gland had no

further effect. Culture of either the hypothalamus or the gonad with VIP had no effect on levels of PRL or PRLR.

3.5. DISCUSSION

Around the time of hatch, blood levels of PRL and pituitary levels of PRL mRNA have been clearly established to increase in concentration in chickens and turkeys (Ishida et al., 1991; Bédécarrats et al., 1999). However, few studies have examined changes in the concentration of the PRLR during late embryogenesis. In part, the lack of information is due to the sensitivity of the assays employed since the receptor is expressed at relatively low levels in embryonic tissues. For example, using a ribonuclease protection assay Yamamoto et al. (2003) reported that PRLR mRNA was barely detectable in the brain of chick embryos at embryonic day (ED) 19, and furthermore, there were no detectable differences in levels of PRLR transcript in somatic tissues between ED17 and day 28 post-hatch. As a result, they concluded that increased levels of PRL during the peri-hatch period had no effect on PRLR expression. In contrast, the development and use of a semi-quantitative real-time PCR (Q-PCR) in our study indicated that the peri-hatch period is associated with major increases in PRLR which occur in concert with the increase in circulating levels of PRL.

In the current study, blood levels of PRL increased progressively during the latter stages of embryogenesis in both turkeys and chickens (Figures 1 and 2) in agreement with previous studies (Ishida et al., 1991; Woods and Porter, 1998; Bédécarrats et al., 1999; Kansaku et al., 2001). These increases are consistent with the

differentiation of lactotrophes in the chicken where PRL immunopositive cells first appear about ED 16-17 in the cephalic lobe of the pituitary gland (Woods and Porter, 1998; Zheng et al., 2005) although PRL mRNA can be detected at earlier stages of development (Kansaku et al., 1994). Presumably, lactotroph differentiation occurs at a comparable embryonic stage in the turkey.

In concert with the increase in PRL about the time of hatch, levels of PRLR were shown to increase in both the hypothalamus and pituitary glands of chickens and turkeys (Figures 1 and 2). There were no qualitative differences between the patterns, although the magnitude of the increases was greater in the chicken. The reason for this quantitative difference between the species is unknown but may be related to differences in the patterns of PRL release. In the chicken, blood levels of PRL increased 3.3 fold from ED17 to 1 day after hatch, whereas, in the turkey this increase was 2.6 fold during the comparable period. The higher levels of PRL in the chicken may have up-regulated the PRLR to a greater extent as has been reported in adult hens (Zhou et al., 1996; Ohkubo et al., 1998a,b). In adult chickens and turkeys, the highest levels of PRLR are observed in the pituitary gland and hypothalamus (Zhou et al., 1996; Ohkubo et al., 1998a,b). Furthermore, levels of PRLR vary with the reproductive state of the hen and are correlated with circulating levels of PRL. This correlation is also apparent during embryogenesis and was 0.75 and 0.60 and 0.90 and 0.98 in the turkey and chicken pituitary gland and hypothalamus, respectively. In addition, stimulation of the ED24 turkey pituitary gland with VIP resulted in a greater than 4 fold increase in PRL and a 2.8 fold increase in PRLR (Figure 3). Thus, it is likely that PRL is acting to up-regulate the PRLR during the late stages of embryonic development. The presence of

receptors in the hypothalamus suggests that a short loop feedback mechanism via either retrograde transport in the hypophyseal portal system or active transport across the blood brain barrier via the choroid plexus may operate in the brain. In the pituitary gland, the PRLRs are likely to be regulated in an autocrine and/or paracrine fashion by PRL.

The major releasing factor for PRL in galliforms is VIP (Sharp et al., 1989; El Halawani et al., 1990a; You et al., 2001) and numerous studies have demonstrated that VIP stimulates PRL secretion and transcription of the PRL gene both *in vivo* and *in vitro* (Proudman and Opel, 1988; Opel and Proudman, 1988; El Halawani et al., 1990b; Talbot et al., 1991; Pitts et al., 1994a,b; Kansaku et al., 1995; Xu et al., 1996). Moreover, the pulsatile release patterns of VIP into hypophyseal portal blood (Chaiseha et al., 1998) and the number of VIP immunoreactive cells in the hypothalamus (Mauro et al., 1989) are correlated with the levels of plasma PRL (Kang et al., 2004). In the chicken, mRNA encoding the VIP receptor (VIPR) are detectable by ED14 in the pituitary gland but increase substantially by ED18 (Kansaku et al., 2001) at which time, levels of circulating PRL increase significantly (Figure 2; Ishida et al., 1991). Pituitary cells as early as ED13 are responsive to VIP which appears to induce differentiation of lactotrophs in both embryonic and adult cell cultures (Wood and Porter, 1998; Porter et al., 2006). In the turkey embryo, the ontogeny of expression of the VIPR are not known but it is likely that they are present by ED24 since pituitary secretion of PRL exceeded a 4 fold increase following stimulation with VIP (Figure 3). This increase in PRL was consistent with a later stage of ED (Figure 1) and was also associated with a shift from non-glycosylated to glycosylated PRL. Within 6 h of stimulation, the percentage of total

PRL represented by glycosylated PRL increased from 13 to 44 %, a level similar to that observed on ED27 in turkey embryos (Bédécarrats et al., 1999). The increased levels of PRL induced by VIP also increased the levels of PRLR about 3 fold which is also consistent with the pattern observed between ED24 and ED27 (Figure 1). Since VIP had no effect on either PRL or PRLR in hypothalamic tissues this suggests that PRL was acting in an autocrine and/or paracrine fashion in up-regulating its receptor and that VIP had no direct effect on transcription of the PRLR encoding gene. In support of the latter, evidence for direct up-regulation of its receptor by PRL in both normo- and pathological states has been shown in a number of mammalian species (Wu et al., 2005).

The current study presents no qualitative differences in expression of PRL or PRLR in the pituitary or hypothalamus between the White Leghorn chicken and turkey. In galliforms, many studies have indicated that hyperprolactinemia is a requisite for the egg incubation phase of broody behaviour. Mediterranean breeds of chickens such as Leghorns rarely exhibit this behaviour, whereas, in turkeys a high proportion of individuals will cease egg production and incubate eggs during a reproductive cycle. Thus, differences in the developmental patterns of expression of PRL and PRLR during ED have no obvious relationship to the demonstration of broodiness during a reproductive cycle. This is in accord with the observations of Ohkubo et al. (1998a) who compared a broody (bantam) and non-broody (Leghorn) breed of adult chicken hens. In their study, there were no apparent differences between the breeds with the expression of PRLR transcripts or the structure of the gene as analysed by Southern blotting.

The physiological roles of PRL during the late stages of ED at this time are unknown but are more than likely to involve functional maturation of the embryo and the transition to *ex ovo* life. In adult chickens and turkeys, the PRLR is expressed ubiquitously as has been observed in both adult and fetal mammals (Zhou et al., 1996; Horseman et al., 1997; Moreno et al., 1998; Ohkubo et al., 1998a,b; Bole-Feysot et al., 1998; Bouchard et al., 1999; Bédécarrats et al., 2000; Freemark, 2001). Thus, the increased levels of PRL during the last week of hatch may be associated with the final maturation of the immune system prior to exposure to new antigens.

In conclusion, changes in the expression of PRLR mRNA in the pituitary gland and hypothalamus were detected by real-time (Q) PCR assays through the embryonic development in both species. This assay is superior over northern blot, ribonuclease protection assay or semi-quantitative PCR in term of sensitivity and easiness to detect low and little variation in expression of PRLR mRNA during the development.

Figure 3.1. Changes in the hypothalamic and pituitary content of PRLR mRNA (A.U.) and levels of plasma PRL (ng/ml) before and after hatch (average ± SE) in turkey embryos. Developmental stages with common letters do not significantly differ in the amount of PRLR mRNA or PRL.

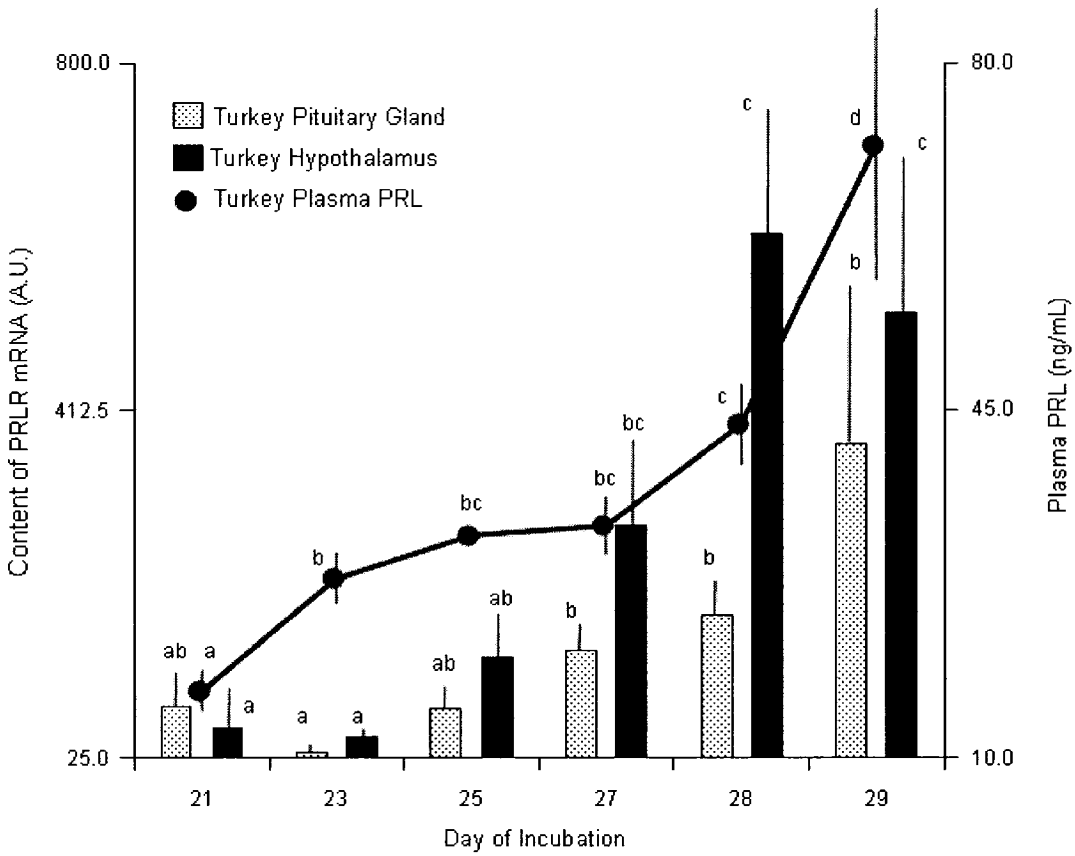

Figure 3.2. Changes in the hypothalamic and pituitary content of PRLR mRNA (A.U.) and levels of plasma PRL (ng/ml) before and after hatch (average ± SE) in chicken embryos. Developmental stages with common letters do not significantly differ in the amount of PRLR mRNA or PRL.

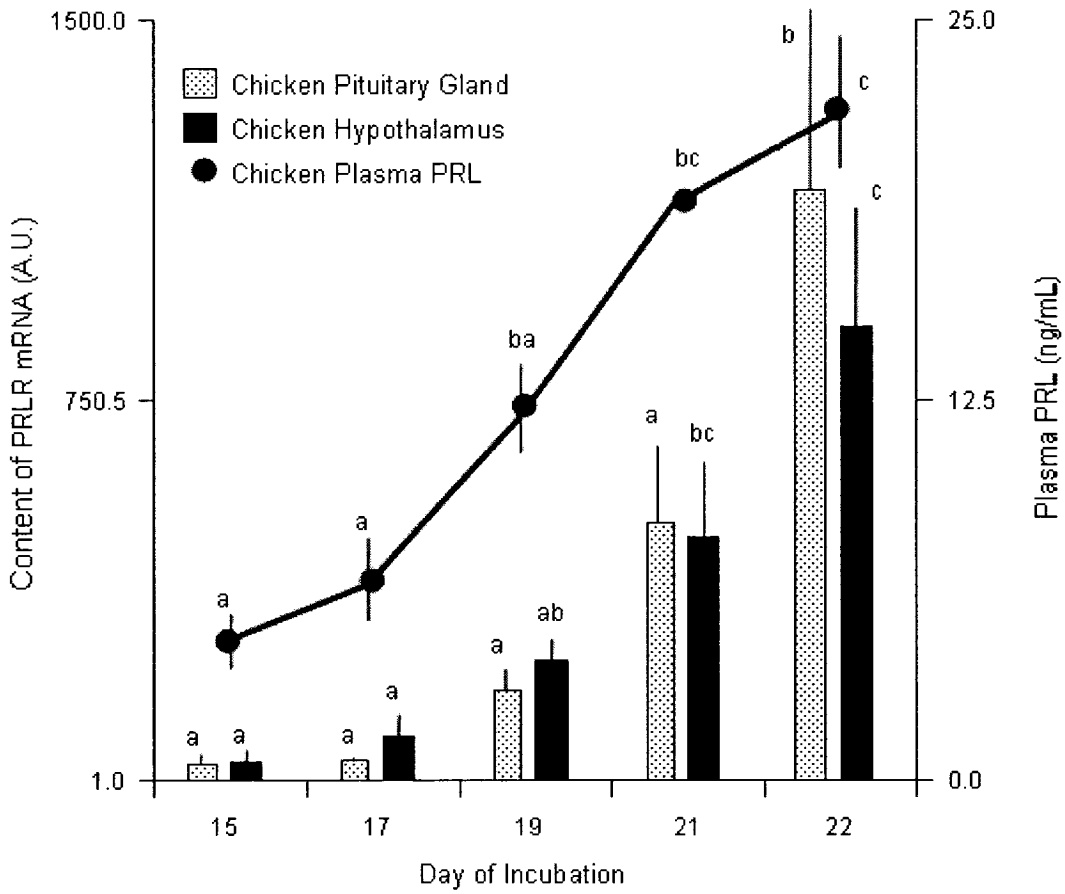

Figure 3.3. Influence of VIP (10^{-7} M) on level of PRLR mRNA (A.U.) in pituitary glands, hypothalami or gonads of embryonic day 24 in turkeys. Data are expressed as average (± SE) and significant differences between VIP treated and control are indicated by an *. The inset (western blot) represents the effect of VIP on pituitary content of PRL isoforms. The upper band represents the glycosylated (27 kDa) isoform, whereas, the lower band (25 kDa) is the non-glycosylated isoform of PRL.

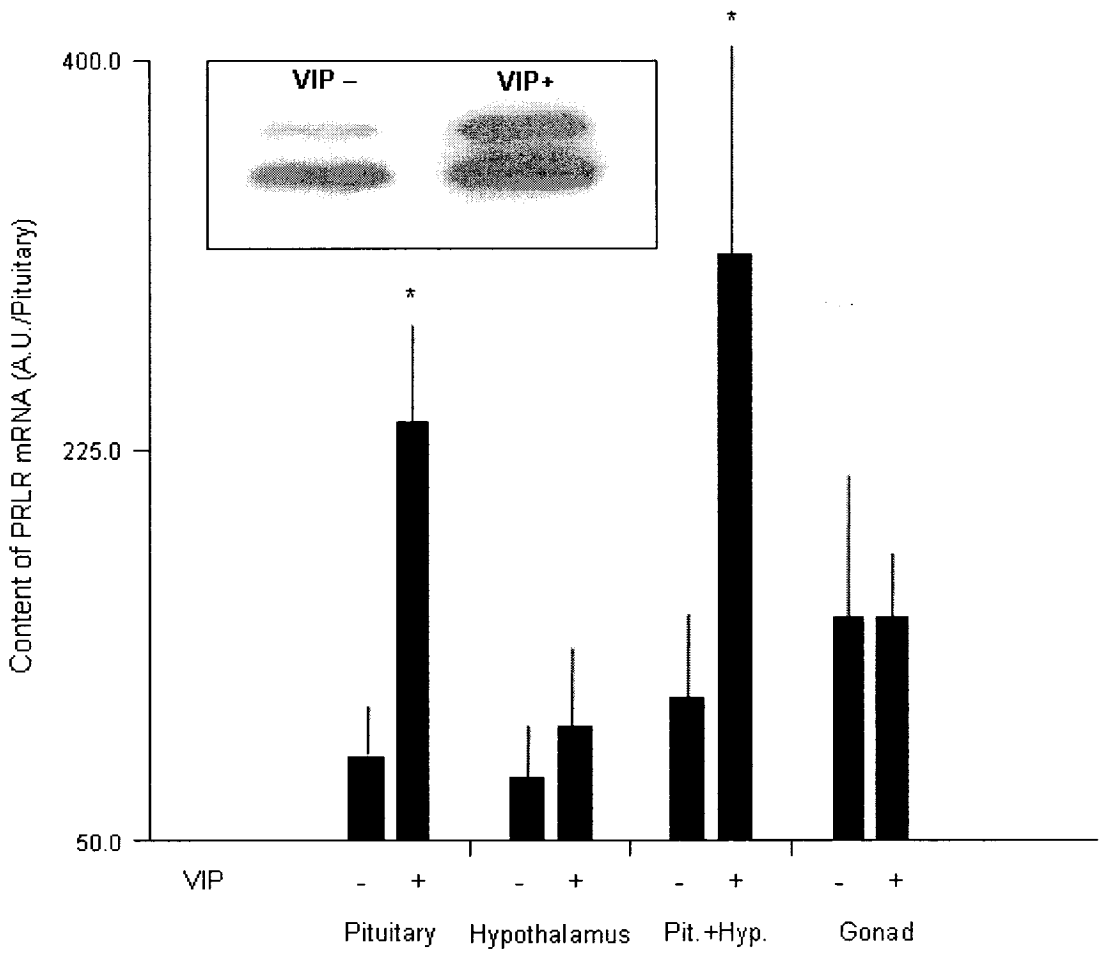

Table 3.1. Effect of VIP (10^{-7}M) on tissue levels of PRLR mRNA (A.U./tissue) and medium content of PRL (ng/ml). Tissues were isolated from turkey embryos on day 24 of embryogenesis and incubated with VIP for 4 h. Samples with common letters do not significantly differ (average ± SE). Numbers in parentheses indicate the sample size.

Tissue	VIP+	VIP–
Pituitary PRLR (A.U./Pituitary)	238 ± 43^{A} (12)	88 ± 21^{B} (14)
Medium PRL (ng/mL)	441 ± 135^{A} (5)	104 ± 11^{B} (4)
Hypothalamus PRLR (A.U./Hypothalamus)	100 ± 34^{A} (14)	78 ± 19^{A} (15)
Medium PRL (ng/mL)	ND	ND
Pituitary+Hypothalamus (A.U./Pit+Hyp)	308 ± 94^{A} (5)	113 ± 36^{B} (9)
Medium PRL (ng/mL)	181 ± 51^{A} (4)	85 ± 27^{B} (3)
Gonad (A.U./Gonad)	148 ± 28^{A} (9)	148 ± 65^{A} (6)
Medium PRL (ng/mL)	ND	ND

ND: not detectable

CONNECTIVE STATEMENT 2

In the previous chapter, we have shown that PRLR mRNA in the pituitary gland and hypothalamus is closely correlated with levels of plasma PRL during late embryonic development in chickens and turkeys. Since the actions of PRL are pleiomorphic and major changes in physiology occur at about the time of hatch, levels of PRLR mRNA were measured in organs which may be responsive to the actions of PRL. The correlation between circulating levels of PRL and the ontogeny of PRLR mRNA expression in the liver, kidney, pancreas and gonad may provide insight into the effects of PRL on the adaptations of the embryo to *ex ovo* life.

CHAPTER 4

ONTOGENESIS OF THE EXPRESSION OF PRLR mRNA DURING LATE EMBRYOGENESIS IN TURKEYS AND CHICKENS

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Submitted to Poultry Science

4.1. ABSTRACT

Changes in circulating levels of PRL and tissue content of PRLR mRNA in the liver, pancreas, kidney and gonads (testis/ovary) were measured in turkey and chicken embryos from embryonic day (ED) 21 or ED15, respectively, to 1 day after hatch by real-time PCR. There were no differences between the sexes in either chickens or turkeys. Both species had very similar patterns of PRL release and expression of PRLR and no major differences were observed between turkey or chicken embryos. Plasma levels of PRL progressively increased from low levels during the last week of embryonic development and were at significantly higher levels (about 3 fold) by hatch. Similarly, in all tissues the content of PRLR mRNA was minimal at the outset and progressively increased to reach maxima about the time of hatch. In both species, the highest levels of transcript were observed in the kidney followed by the gonad, liver and pancreas. The tissue content of PRLR was correlated (0.7 to 0.9 dependent on the tissue) to circulating levels of PRL which suggested that PRL up-regulates its receptor around the time of hatch. Since both levels of PRL and tissue content of PRLR mRNA increased around the time of hatch, this suggests that these tissues may be targets for PRL and be involved in the physiologic changes occurring in embryos around the time of hatching.

4.2. INTRODUCTION

Prolactin (PRL) is a polypeptide hormone that is produced and secreted mainly by the lactotroph cells of the adenohypophysis. In vertebrates, PRL is thought to be involved in modulating an extraordinary diversity of biological processes, and greater than 300 separate actions have been assigned to it (Bole-Feysot et al., 1998). These roles have been broadly categorized as those that affect: 1) water and electrolyte metabolism 2) growth and development 3) endocrine systems and metabolism 4) brain and behaviour 5) reproduction and 6) immunoregulation and protection. The most studied role in avian species has involved the actions of PRL during the egg incubation phase of broody behaviour (Sharp et al., 1998). However, many other functions of PRL have been studied including osmoregulation (Doneen and Smith, 1982; Murphy et al., 1986; Roberts, 1998), crop milk production (Dumont, 1965; Goldsmith et al., 1981; Anderson et al., 1984; Horseman and Buntin, 1995), liver lipid metabolism (Goodridge and Ball, 1967a, b; Simpkins and Smith, 1976; Meier, 1977; Sotowska-Brochocka et al., 1986), ovarian function (Camper and Burke, 1977; Zadworny et al., 1989; Porter et al., 1989; Tabibzadeh et al., 1995) and lymphocyte development, maturation and activation (Skwarlo-Sonta, 1990,1992; Soares and Proudman, 1991; Moreno et al., 1994, 1998; Ibars et al., 1997).

The biological actions of PRL are mediated by binding to its receptor (PRLR) and receptors have been detected in the digestive, osmoregulatory, immune, reproductive and neural system in both adult chickens and turkeys (Tanaka et al., 1992; Zhou et al., 1996; Ohkubo et al., 1998a; Mao et al., 1999). The broad distribution of the receptor is consistent with the multiplicity of the physiological processes that PRL is known to be involved in. Moreover, levels of PRLR have been observed to vary

according to the physiological status of the hen at least in some tissues such as the adenohypophysis and hypothalamus (Zhou et al., 1996; Ohkubo et al., 1998b).

During embryogenesis, the physiological roles of PRL have not been extensively studied. Lactotroph cells differentiate late in embryonic development and immunoreactive PRL is detectable by embryonic day (ED) 15 to 17 in chicks (Kansaku et al., 1994; Sasaki et al., 2003; Fu et al., 2004; Zheng et al., 2005) and ED23 in turkeys (Bédécarrats et al., 1999c) although PRL transcripts are detectable at earlier stages (Kansaku et al., 1994; Bédécarrats et al., 1999c). Subsequent to lactotroph differentiation, PRL is released into the circulation and during the last week of embryogenesis levels of PRL progressively increase and reach maxima at about the time of hatch (Ishida et al., 1991; Bédécarrats et al., 1999c). Moreover, the time of hatch is associated with a significant shift in the circulating form of PRL isoform (Bédécarrats et al., 1999c). The temporal relationship between increased levels of PRL and the hatch of the embryo suggests that PRL may participate in various physiological systems to adapt the embryo to ex ovo life. However, the biological roles of PRL during chicken or turkey ontogeny have not been extensively studied. Furthermore, the tissue distribution of PRLR during embryogenesis has not been well characterized. The single study measuring PRLR mRNA during embryogenesis compared levels of receptor in intestinal and kidney tissue between ED17 and 19 and days 2 and 28 post-hatch (Yamamoto et al., 2003). They reported that there was no change in levels of PRLR mRNA during that interval. However, since the levels of PRLR are expected to be quite low, the ribonuclease protection assay may have had insufficient sensitivity to detect changes in tissue content. Accordingly, in this study a real-time (Q) PCR assay was

designed and validated in order to assess levels of PRLR mRNA in individual tissues from both turkeys and chickens during the last week of embryogenesis.

4.3. MATERIALS AND METHODS

4.3.1. Tissue Sampling

Eggs from commercial strains of white Medium Hybrid turkeys (Hybrid Turkeys Inc, Kitchener, Ontario, Canada) and white Hyline W98 Leghorn chickens (HyLine International, West Des Moines, Iowa, USA) were incubated under standard commercial conditions at 37.5°C, 85-86% humidity for 25 and 18 days, respectively. They were then transferred to a hatcher at 36.9°C, 90-92% humidity. In addition to the day of hatch and 1 day post-hatch, turkey and chicken embryos were collected at day 21, 23, 25 and 27 or day 15, 17, and 19 of incubation, respectively, and sacrificed by cervical dislocation. Tissue samples from the liver, kidney, pancreas and left gonad were collected immediately after sacrifice, snap-frozen in liquid nitrogen, and stored at –80°C until assayed.

4.3.2. Extraction of Total RNA

Total RNA from tissues was extracted using TRIzol reagent (Gibco BRL Products, Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's protocol. Briefly, tissues were homogenized individually in 800 µl of a monophasic solution of phenol and guanidine isothiocyanate by sonicating on ice for approximately 30 sec (2 bursts). A volume of 200 µl of chloroform was added to the

homogenate, and the different phases were separated by centrifugation at 12 000 rpm for 15 minutes. The aqueous phase containing RNA was precipitated with an equal volume of isopropanol. The RNA was recovered at 12 000 rpm for 15 min and washed with 1 ml of 75% ethanol. The recovered RNA was dissolved in DEPC-treated water to a final concentration of 1 μg/μl. Integrity of the RNA was electrophoretically verified in 1.2 % formaldehyde agarose gels stained with ethidium bromide (EtBr).

4.3.3. Reverse Transcription (RT) and Real-Time (Q-PCR) Quantification of tPRLR mRNA

The RT, modified from Bédécarrats et al. (1999c), was carried out in a final volume of 10 μl containing 2.0 μg of total RNA. The RNA was heat-denatured at 65°C for 10 min, then mixed with 2.5 U of Avian Myeloblastosis Virus reverse transcriptase (Amersham Biosciences, Baie-Urfe, Quebec, Canada), 1mM dithiothreitol, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 0.25 mM each dNTPs, and 40 pmol of antisense turkey (t)PRLR-specific primer (Invitrogen Canada Inc, Burlington, Canada). The RT was conducted at 42°C for 1 h; then the reaction mix was heated at 90°C for 5 min to inactivate the reverse transcriptase.

An external standard of 332 bp (Zhou et al., 1996) was used in Q-PCR to semi-quantify levels of PRLR cDNA. This sequence contained a 39 bp deletion of the amplicon of 371 bp amplified from tPRLR mRNA to allow for determination of co-amplification efficiency by melting point analysis and/or gel electrophoresis. The external standard was amplified by PCR at 93°C for 45 sec, 56°C for 2 min, and 72°C for 90 sec for 35 cycles (Zhou et al., 1996), purified from 1% agarose gel before use and

stepwise diluted tenfold to cover the range of turkey and chicken PRLR mRNA levels in tissues amplified by Q-PCR.

Conditions for Q-PCR using the LightCycler (Roche Applied Science, Laval, Quebec, Canada) were optimized for 1) $MgCl^{2+}$ concentration, 2) primer concentration, 3) annealing temperature, and 4) concentration of the cDNA. The optimized protocol for Q-PCR was as follows. An aliquot of 2 µl of a single-strand cDNA mixture was amplified by LightCycler PCR in a final volume of 20 µl containing 1X LightCycler-FastStart DNA Master SYBR Green I (Roche Applied Science, Laval, Quebec, Canada), 4 mM $MgCl_2$, and 10 pmol of both forward and reverse primers (Invitrogen Canada Inc). For the Q-PCR, a sense and an antisense tPRLR-specific primer were as designed by Zhou et al. (1996: 5'AGGAAACATTTACCTGTTGGT and 5'AAGCCATCCAGATCTGACATC). The cDNA was diluted according to the stages of the embryo development. Samples for Q-PCR were denatured at 95°C for 10 min followed by an amplification program (95°C for 10 sec, 55°C for 5 sec, 72°C for 16 sec) for 40 cycles. Specificity of the amplifications was determined by melting curve analysis (63°C for 15 sec to 95°C in 0.1°C/sec increments) and by gel electrophoresis.

To estimate the concentration (Co) of PRLR mRNA in the turkey and chicken cDNA samples, the following formula was used: CP = S Log (Co) + I, where CP is the crossing point value from the turkey and chicken samples i.e. the point at which the fluorescence rises appreciably above the background fluorescence. The standard slope (S) represents the overall reaction efficiency and the intercept (I) of the standard represents the crossing point value on the y-axis for which the concentration equals to

zero. The linear regression line was obtained by plotting the crossing cycle number versus the logarithm of the concentration for each unknown sample.

4.3.4. PRL Radioimmunoassay

Blood samples were collected into heparinized tubes from the chorio-allantoic blood in turkey and chicken embryos from day 21 until 25 and from day 15 until 19 of incubation, respectively, and thereafter by intra-cardiac puncture. After centrifugation, plasma samples were stored at –80°C until assayed. Levels of PRL were measured in triplicate in aliquots of plasma (100 µl: turkey or 200 µl: chicken) by radioimmunoassay as described by Guémené et al. (1994). In brief, samples were incubated with rabbit anti-recombinant turkey PRL antibody (1/1000 final dilution) for 24 h at 4°C prior to the addition of 15 000 cpm of [^{125}I] recombinant turkey PRL. Following incubation for 24 h at 4°C, goat anti-rabbit second antibody (1/100 final dilution) was added and tubes were incubated for 48 h at 4°C. After addition of 1 ml phosphate buffered saline (PBS), immuno-complexes were precipitated by centrifugation at 3000 *g* for 30 minutes. Pellets were washed with 1 ml PBS and radioactivity was counted using a gamma counter. Concentration of PRL in the samples was estimated using GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA). The intra- and inter-assay coefficients of variation were 5.8 % and 0.5 %, respectively.

4.3.5. Statistical Analysis

Transcript levels of PRLR mRNA in tissues, defined as arbitrary units (A.U.), were analyzed following log transformation using one-way ANOVA ($P < 0.05$) and

where appropriate followed by the Duncan multiple range test ($P < 0.05$). Each data point represents the average ± standard error of the mean (SEM). To associate levels of PRLR mRNA with plasma PRL through the ontogenesis, the Pearson correlation test was performed.

4.4. RESULTS

4.4.1. Levels of PRLR mRNA and Plasma PRL in Turkeys

Circulating levels of PRL did not change markedly before ED27 in turkey embryos then increased significantly and reached maxima by 1 day after hatch (Table 1). Similarly, steady state levels of PRLR mRNA transcript in the tissues were maintained at low levels before hatch then subsequently increased significantly (Figures 1a-d). By 1 day after hatch, tissue content of PRLR mRNA was as follows: kidney > gonad > liver > pancreas. The correlation between the plasma concentration of PRL and PRLR mRNA content in liver, kidney, pancreas or gonad was 0.70, 0.83, 0.87 and 0.80, respectively.

4.4.2. Levels of PRLR mRNA and Plasma PRL in Chickens

The content of PRLR mRNA in the liver, pancreas, kidney and gonad was maintained at low levels until ED19 (Figures 1a-d). Subsequently, it increased significantly between ED19 and hatch in all tissues. By 1 day after hatch, the relative tissue content of PRLR mRNA was as follows: kidney > gonad > liver > pancreas. Similarly, plasma levels of PRL were at low levels on ED15 then progressively increased during the late stages of

embryogenesis and by the day of hatch were > 3 fold higher (Table 1). The correlation between the plasma content of PRL and the relative tissue content of PRLR mRNA in the liver, kidney, pancreas and gonad was 0.74, 0.74, 0.67 and 0.81, respectively. In all tissues, the levels of PRLR mRNA were greater in the chicken than in the turkey on the day of hatch but this difference was not significant.

4.5. DISCUSSION

Real-time (Q) PCR is a sensitive method used by many laboratories to quantify levels of specific transcripts in tissues. In particular, Q-PCR is advantageous over northern blotting, ribonuclease protection assay (RPA) or semi-quantitative PCR for detecting and quantifying low abundance transcripts such as PRLR or mRNAs from tissues that are available in limited amounts. For example, using RPA, Yamamoto et al. (2003) were unable to detect the PRLR in liver of ED19 chick embryos although signals were detected in the kidney and intestine. However, the amount of PRLR in the latter tissues did not vary between ED17 and 19 or between 2 and 28 days post-hatch. This is a surprising result since both circulating levels of PRL and pituitary content of PRL mRNA significantly increase during the peri-hatch and post-hatch period in chickens (Ishida et al, 1991, Kansaku et al., 1994) and turkeys (Bédécarrats et al., 1999c) and increased levels of PRL have been shown to up-regulate the number of PRLRs in some reproductive tissues (Zhou et al., 1996; Ohkubo et al., 1998b). In addition, PRL has effects on a wide variety of physiological functions (Bole-Feysot et al., 1998) that may be involved in the transition to ex ovo life of the avian embryo. Accordingly in this

study, a Q-PCR assay was developed in order to assess the ontogeny of PRLR transcript expression during the peri-hatch period in chickens and turkeys. Using this assay and in contrast to the study of Yamamoto et al. (2003), significant increases in concentration of PRLR mRNA were observed at the time of hatch in chicken and turkey embryos (Figures 1a-d). Moreover, increases in tissue content of PRLR mRNA were correlated with circulating levels of PRL.

In the liver, kidney, pancreas or gonads, there was no quantitative difference in levels of PRLR mRNA between the sexes in either chicken or turkey embryos and as a result data was pooled (data not shown). The transcript was maintained at low levels in both chicken and turkey embryos but increased during the late stages of embryogenesis and the highest levels were observed at hatch or 1 day after hatch (Figures 1a-d). In both species, the highest levels of PRLR were observed in the kidney, followed by the gonad, liver, and pancreas in descending order. Neither the pattern of change of tissue content of PRLR nor the absolute level of PRLR mRNA was different between the chicken and turkey. Thus, although the turkey is considered to be a broody breed and the white Leghorn, a non-broody breed, differences in the pattern of expression of the PRLR in these tissues during late embryogenesis had no apparent relationship to the incidence of broodiness in these species post sexual maturity. Conversely, the similar patterns of expression of PRLR suggests that PRL acting through its receptor is required to modulate the physiology of the embryo to adapt to ex ovo life in both species.

The increases in circulating level of PRL about the time of hatch (Table 1) are consistent with the differentiation of lactotrophs in the pituitary. In the chicken, immunoreactive PRL can be detected in the cephalic lobe of the adenhypophysis by

about ED 15-17 (Kansaku et al., 1994; Sasaki et al., 2003; Zheng et al., 2005) and ED23 in turkeys (Bédécarrats et al., 1999c) although PRL mRNA can be detected earlier (Kansaku et al. 1994; Bédécarrats et al., 1999c). Consistent with lactotroph differentiation, circulating levels of PRL progressively increased by 2.6 and 3.3 fold from ED23 to 1 day post-hatch or ED17 to 1 day post-hatch in turkeys and chickens, respectively. During the same interval, tissue content of PRLR mRNA progressively increased in the liver, pancreas, kidney and gonad and was correlated (0.7 to 0.9 dependent on the tissue) to the plasma concentration of PRL. This suggested that increases in PRL during embryonic development up-regulated its receptor in target tissues as has been observed in certain reproductive states in the hypothalamus and adenohypophysis of adult chickens and turkeys (Zhou et al., 1996; Ohkubo et al., 1998b). In support of this, in vitro stimulation of turkey pituitary glands from ED24 embryos with VIP for 4 h resulted in about a 4 fold increase in PRL and a 3 fold increase in PRLR mRNA (Leclerc et al., submitted). Induction of expression of PRLR was likely due to a direct effect of PRL since culture of the hypothalamus with VIP had no effect, whereas, co-culture of the hypothalamus and pituitary gland with VIP also resulted in a 3 fold increase in hypothalamic content of PRLR mRNA. Conversely, Yamamoto et al. (2003) noted no change in chicken kidney or intestinal content of PRLR on ED17 and 19 or 2 and 28 days post-hatch and they suggested that PRLR mRNA was regulated independently of the circulating levels of PRL. However, their assay system (RPA) may have not had sufficient sensitivity to detect variation in PRLR content.

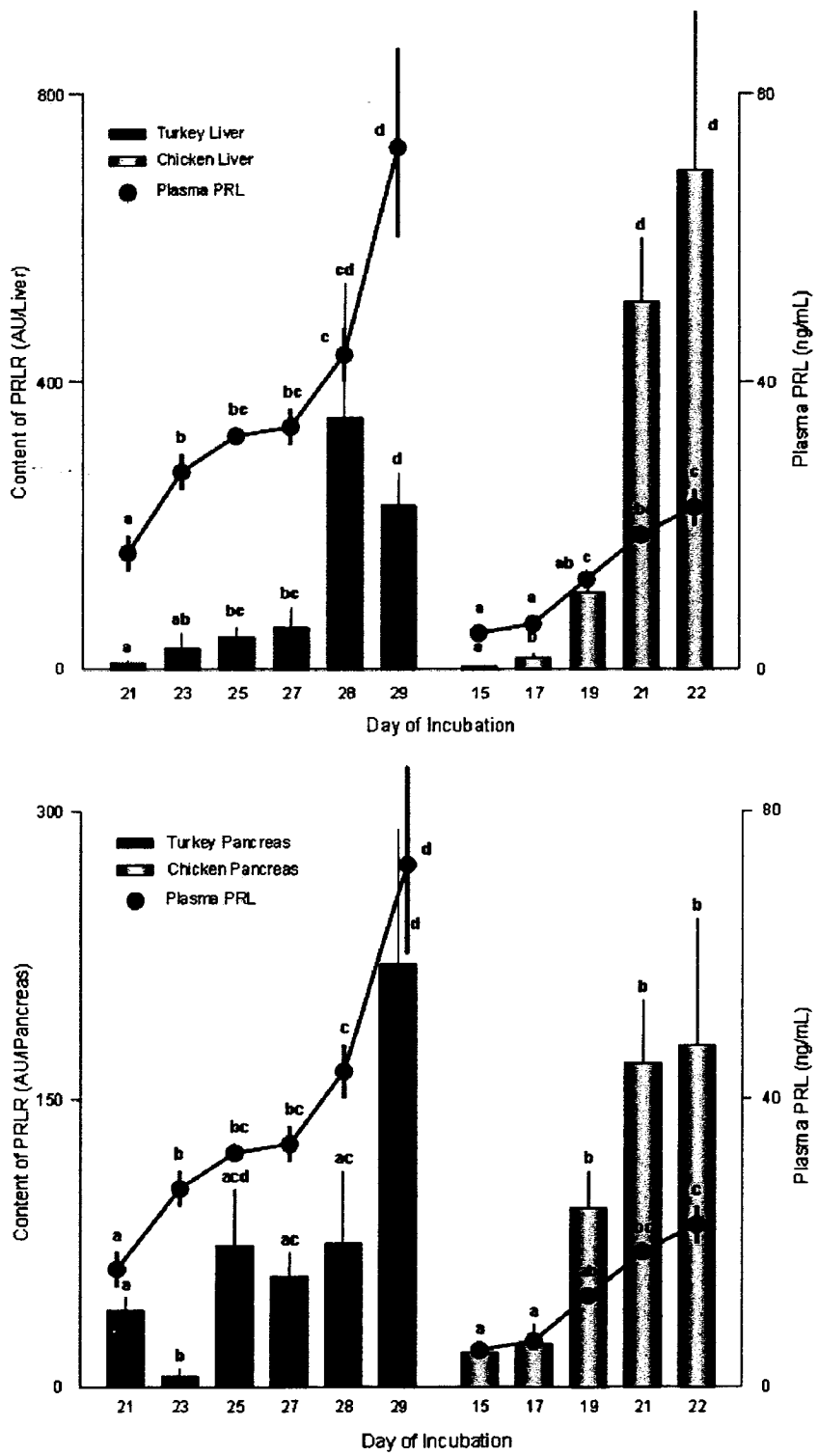
Prolactin interacts with its receptors in a wide variety of target tissues to affect physiological processes which have been broadly grouped into actions that affect reproduction, growth and development, osmoregulation, behaviour and immunoregulation (Bole-Feysot et al., 1998). The large increases in levels of receptors in the kidney, gonad, liver and pancreas about the time of hatch suggest that PRL may be acting in these target tissues. Indeed, at the time of hatch, large changes in physiology such as maturation of the immune system, change from chorio-allantoic to lung gas exchange, a large increase in metabolic rate to provide energy to pip the shell etc. must occur in preparation for ex ovo life. For example, the increase in PRLR mRNA at hatch in pancreatic tissue may indicate that PRL is acting to stimulate the endocrine pancreas. In rats, PRLR is located in the fetal and neonatal pancreatic islets and PRL stimulates β-cell hyperplasia and insulin secretion; an effect which is modulated by PRL induced expression of preadipocyte factor 1 (Carlsson et al., 1997). Conversely, in PRLR knockout mice, β-cell hypoplasia occurs (Freemark et al., 2002). In addition, macroarray analysis of pancreatic islets stimulated with PRL indicated up-regulation of genes involved with cell proliferation and differentiation and down-regulation of pro-apoptotic genes (Bordin et al., 2004). Thus, it is likely in the embryonic turkey and chicken increased levels of PRL and PRLR at hatch are associated with stimulation of the endocrine pancreas. However, very few studies have examined the effects of PRL on avian pancreatic development although PRL has been shown to be a mitogen in chick islet cells (Maiti et al., 1982). Similarly, liver metabolism may be stimulated at the time of hatch to meet the increased energy demands associated with the hatching process. The concurrent increase in circulating

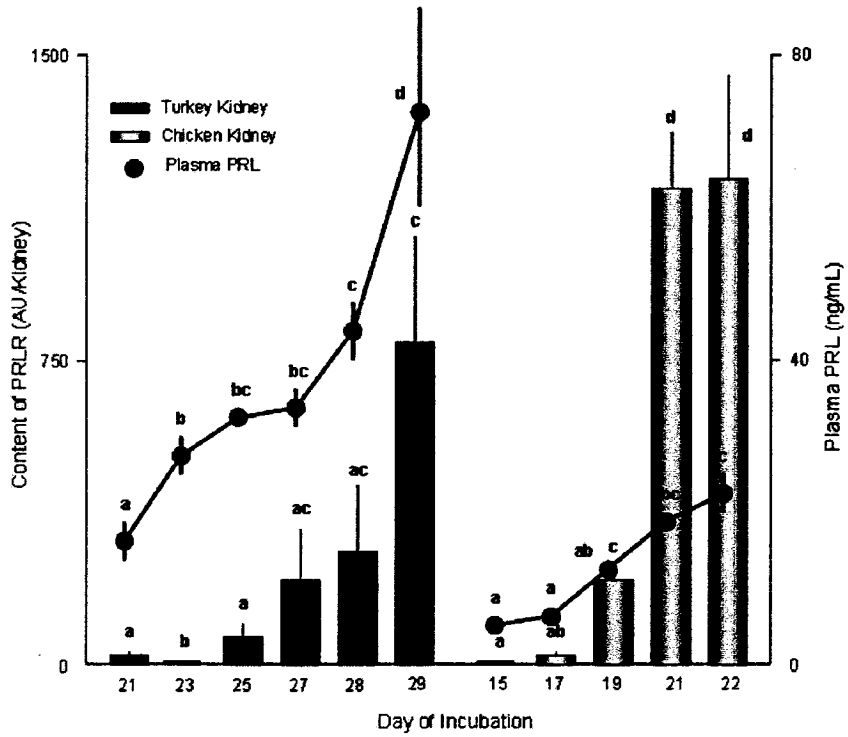
PRL and hepatic PRLR mRNA suggest that PRL may be active in stimulating this organ. In part, this effect may be moderated through thyroid hormones since injection of recombinant chicken PRL into ED19 chickens modulates hepatic metabolism of T3 and T4 (Kühn et al., 1996). The osmoregulatory ability of the embryonic kidney would also be expected to change around the time of hatch and a balance between the concentration of ions and water is essential for successful hatch (Davis et al., 1988). Around the time of hatch, glomerular filtration rate significantly increases and resorption of allantoic water reserves occurs in preparation for ex ovo life (Murphy et al., 1991). The injection of PRL into the chorio-allantoic membrane of chicken embryos has been shown to increase the Na^+-K^+-ATPase activity and reduce the concentration of Na^+ and Cl^- in allantoic fluid (Doneen et al., 1982) without altering the allantoic fluid volume (Murphy et al., 1986). Thus, the increase in circulating levels of PRL and kidney content of PRLR mRNA may be associated with the stimulation of NaCl resorption during the peri-hatch period. Evidence also suggests that PRL may affect steroid production by the gonads. During late embryogenesis, plasma levels of the sex steroids increase which coincides with the observed increases in circulating PRL and PRLR mRNA in the gonad. In the chick embryo, gonadotrophin immunoreactive cells are the first to appear in the adenohypophysis at about ED7 (Sasaki et al., 2003) and steroid biosynthesis occurs at about the same time. At about the time of hatch, however, there is increased release of LH and FSH and the gonads significantly increase the secretion of steroids (Gonzalez et al., 1987; Mendez-Herrera et al., 1998; Peralta et al., 2004). In many species, PRL is involved in the up-regulation of gonadotrophin receptors (Bjurulf et al., 1994; Porter et al., 2000; You et al., 2000) hence; increased levels of circulating PRL

and gonadal PRLR around the time of hatch suggest that PRL is involved in maintaining steroid production. The increased gonadal steroids are in turn involved in many aspects of pre- and post-hatch physiology.

Many direct and indirect effects of PRL on target tissues are likely to occur during late embryogenesis in chickens and turkeys. The identification of large increases in PRLR mRNA in the kidney, gonad, liver and pancreas at the time of hatch suggests that these tissues may be targets for PRL. To date, few physiological studies have investigated the direct effects of PRL on these tissues during embryogenesis. However, the availability of recombinant chicken (Hanks et al., 1989; Ohkubo et al., 1993) and turkey (Karatzas et al., 1993) PRL may allow for assessment of the response of these tissues to stimulation.

Figure 4.1. Changes in the expression of the tPRLR mRNA (A.U.: average ± SE) in the liver (a) pancreas (b) kidney (c) and gonad (d) during the last week of embryogenesis in turkeys and chickens. Developmental stages with common letters do not significantly differ in the amount of PRLR mRNA within each species.




Turkey Kidney
Chicken Kidney
Plasma PRL
Content of PRLR (AU/Kidney)
Plasma PRL (ng/mL)
Day of Incubation
1500
750
0
80
40
0
21 23 25 27 28 29 15 17 19 21 22

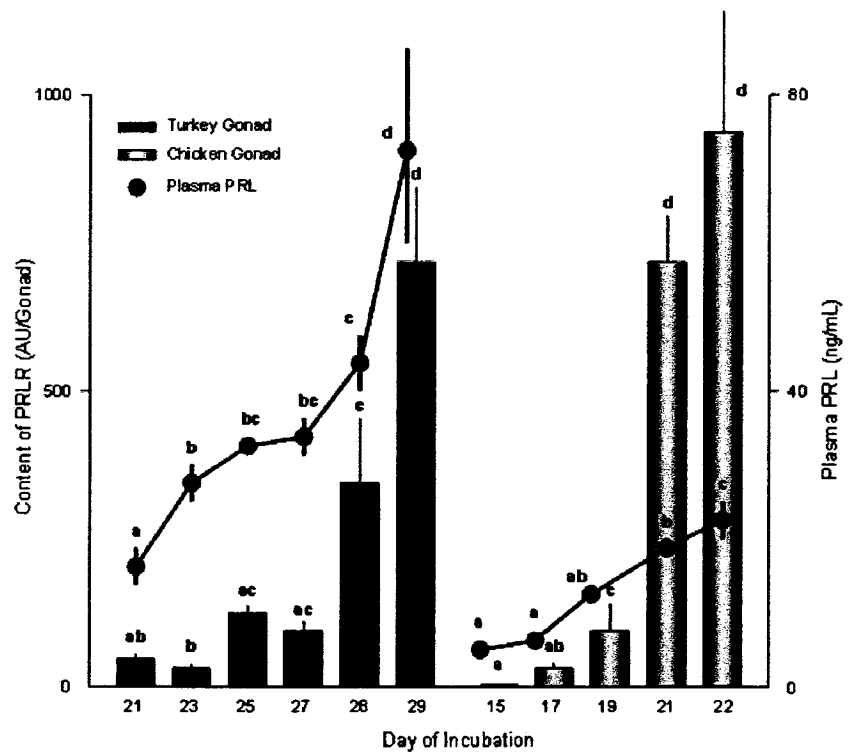

Turkey Gonad
Chicken Gonad
Plasma PRL
Content of PRLR (AU/Gonad)
Plasma PRL (ng/mL)
Day of Incubation
1000
500
0
80
40
0
21 23 25 27 28 29 15 17 19 21 22

Table 4.1. Changes in the circulating levels of PRL (ng/ml) before and after hatch (average ± SE). Samples with common letters do not significantly differ. Numbers in parentheses indicate the sample size.

Species	**-7/-6**	**-5/-4**	**-3/-2**	**-1**	**H**	**H1**
Turkey	16.4 ± 2.1^{A} (9)	28.0 ± 2.6^{B} (9)	32.30 ± 0.9^{BC} (10)	33.4 ± 3.0^{BC} (8)	43.7 ± 4.1^{C} (12)	72.2 ± 13.8^{D} (7)
Chicken	5.2 ± 0.8^{A} (8)	6.8 ± 1.2^{A} (9)	12.4 ± 1.2^{AB} (13)	NA	19.75^{BC} (1)	19.66 ± 7.7^{C} (7)

NA: not available

CONNECTIVE STATEMENT 3

Levels of turkey PRLR mRNA in the pituitary gland, hypothalamus, liver, kidney, pancreas and gonad increased during late embryogenesis in association with the observed increase in circulating levels of PRL. This suggested that PRL was up-regulating its receptor and hence tissue responsiveness to the effects of PRL was enhanced during the transition to *ex ovo* life. Moreover, stimulation of embryonic day (ED) 24 turkey pituitary glands with VIP resulted in a significant increase in levels of both PRL and PRLR mRNA. The latter increases were consistent with levels observed at about the time of hatch. In order to investigate the effects of PRL on the induction of other genes in the pituitary gland, RNA from control and VIP stimulated ED24 turkey embryos was used to construct suppression subtractive hybridization (SSH) libraries. Sequencing of clones associated with VIP stimulated or control libraries (as the source of subtractive cDNAs) may provide insights into the types of genes that may be up- or down-regulated in association with the physiological changes that occur around the time of hatch.

CHAPTER 5

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN PITUITARY GLANDS OF DAY 24 TURKEY EMBRYOS

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Manuscript in preparation for submission

5.1. ABSTRACT

Around the time of hatch, circulating levels of PRL increase in turkey embryos as lactotrophe cells differentiate. Although the increased levels of PRL are likely to modulate many physiological processes associated with final maturation and the adaptation to *ex ovo* life via the circulation, PRL may also act in an autocrine and/or paracrine fashion to affect adenohypophyseal function. In order to investigate the transcription of genes that may be induced/suppressed by PRL in the adenohypophysis (AP), libraries were constructed using suppressive subtractive hybridization (SSH). Stimulation with VIP (10^{-7} M) of AP isolated from day 24 turkey embryos for 4 h resulted in a 2.8 and 5.7 fold increase in AP and media content, respectively, of PRL. Total RNA from VIP stimulated and control AP was used to construct both forward (control cDNA as driver) and reverse (VIP stimulated cDNA as driver) SSH libraries. Random clones (n=96) from each library were sequenced and compared to data banks. A total of 66 and 79 different putative genes were identified in the forward and reverse libraries, respectively, of which about 50 % had unknown function. Real-time PCR was used to confirm the differential expression of a selected number (n=21) of these genes in VIP treated and control AP. A total of 66 distinct gene functions could be considered as a source of inducible or repressive genes associated with the direct effect of VIP on PRL biosynthesis during the developing adenohypophyseal gland. Among them, several have been shown to play a role in cell proliferation, protein trafficking, the ubiquitin-protease pathway, apoptosis and cytoskeletal and extracellular matrix.

5.2. INTRODUCTION

Many of the actions of prolactin (PRL) are associated with its functional activity in cell proliferation, differentiation, cell survival and various developmental processes (reviewed by Bole-Feysot *et al.*, 1998). During embryogenesis, PRL has many modulatory roles in the developmental and maturational process. In contrast to mammals where embryonic development is influenced by the fetal placental unit, avian development occurs independently of maternal influence although maternal hormones are transferred to the ovum during yolk accretion and prior to shell deposition. Thus, the avian embryo is useful for studying the ontogeny of PRL secretion. In chicken and turkey embryos, lactotrophe cells in the pituitary gland differentiate by embryonic day (ED) 17 and 23, respectively (Kansaku *et al.*, 1994; Zheng *et al.*, 2005; Bédécarrats *et al.*, 1999c). Subsequently, PRL is released into the circulation and levels increase to reach maxima at about the time of hatch (Ishida *et al.*, 1991; Bédécarrats *et al.*, 1999c). Presumably, this increase in PRL is associated with the final stages of maturation and the transitition to *ex ovo* life. Although the biological roles have not been extensively studied in birds, the correlated increase in prolactin receptor (PRLR) in various target tissues during embryogenesis is suggestive of major roles (Leclerc *et al.*, in press).

In turkeys, the secretion of PRL is mainly regulated by vasoactive intestinal peptide (VIP) of hypothalamic origin and many studies have indicated the stimulatory effect of exogenous VIP on circulating levels of PRL (Proudman and Opel, 1988; El Halawani *et al.*, 1990b; Pitts *et al.*, 1994a,b; Xu *et al.*, 1996). In addition, a positive correlation among the number of VIP containing neurons, hypothalamic content of VIP,

levels of VIP measured in the hypothalamo-hypophyseal portal system and circulating levels of PRL has been observed (Mauro *et al.*, 1989; Youngren *et al.*, 1996; Chaiseha *et al.*, 1998). Furthermore, active immunization against VIP has been demonstrated to inhibit PRL release (El Halawani *et al.*, 2000a). The actions of VIP are initiated by binding to its receptor (VIPR) in pituitary glands and levels of VIPR transcripts are correlated to blood levels of PRL (Chaiseha *et al.*, 2004). The transcription of the PRL gene has been shown to be activated by the presence of a VIP response element located in the PRL promoter (Kang *et al.*, 2004).

In avian embryos, the role of VIP has not been extensively studied. VIP receptor mRNA is detectable by ED14 in the cephalic lobe of the pituitary gland (Kansaku *et al.*, 2001). At this time, levels of immunoreactive PRL are very low; however, PRL mRNA is detectable by Northern blot (Kansaku *et al.*, 1994). By ED18, levels of VIPR are significantly increased consistent with the differentiation of lactotrophe cells and the increased secretion of PRL in the peri-hatch period. This suggests that maturation of the hypothalamo-hypophyseal axis to regulate the secretion of PRL is occurring during the latter stages of embryogenesis. In order to investigate, the changes in pituitary function associated with the secretion of PRL, suppressive subtraction hybridization (SSH) libraries were constructed from ED24 turkey pituitary glands stimulated with VIP. At this stage of development, synthesis and secretion of PRL were at low levels. Hence, the SSH libraries should reflect the differential expression of genes associated with direct effect of VIP and indirect effects of PRL (and other genes) on pituitary function. Analysis of such genes may provide insights into the autocrine and paracrine roles of PRL on pituitary function during the peri-hatch period.

5.3. MATERIALS AND METHODS

5.3.1. Tissue Culture and Extraction of Total RNA

Eggs from a commercial strain of white Medium Hybrid turkey hens (Hybrid Turkeys Inc, Kitchener, Ontario, Canada) were collected at day 24 of incubation. The embryos were killed by cervical dislocation and anterior pituitary glands (n=60) were rapidly collected and placed into 6-well culture plates. and equilibrated at 39°C for 1 h in a 5% CO_2/95% air incubator in 10 ml M199 (Gibco BRL Products, Life Technologies, Burlington, Ontario, Canada) containing Earle salts supplemented with 25 mM Hepes, 0.1% (w/v) bovine serum albumin, 100 mg/l L-glutamine, 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate in 0.85% saline (sodium salt), and 2200 mg/l sodium bicarbonate. After 1 h, the medium was removed and replaced by the same volume (1 ml/pituitary gland) of medium plus or minus 10^{-7} M synthetic VIP (Sigma Inc.). The 2 groups (n=30) of pituitary glands were incubated at 39°C for 4 h, snap-frozen in liquid nitrogen in three aliquots of 10 for each group, and stored at -80°C until required for assay. Aliquots of media (500 µl) were collected at the time of VIP treatment and 4 h later for radioimmunoassay (RIA) of PRL content.

Total RNA was extracted using a NucleoSpin® RNA II Kit (BD Biosciences Clontech, Inc.) according to the manufacturer's protocol. In brief, a pool of 10 pituitary glands from each group (control and VIP stimulated) was lysed using 350 µl of the supplied RA1 buffer and 3.5 µl of β-mercaptoethanol. Following centrifugation, the supernatant was mixed with 350 µl of 70% ethanol and applied to the spin column and centrifuged at 8000 x g for 30 s. After treating the RNA with 350 µl Buffer MDB and

DNase I treatment at room temperature for 15 min, the column was washed twice with 200 µl of Buffer RA2 and 600 µl of Buffer RA3. Total RNA was eluted by stepwise addition of 50 µl of nuclease-free water twice. The concentration of total RNA was estimated by UV spectroscopy at a wavelength of 260 nm.

5.3.2. Super Smart PCR cDNA Synthesis

5.3.2.1. First-Strand cDNA Synthesis and Purification

The Super Smart™ PCR cDNA Synthesis Kit (BD Biosciences Clontech, Inc.) was used to synthesize cDNA for SSH. Five hundred ng of total RNA from the pool of pituitary glands from each group was annealed with 12 µM of 3' SMART CDS Primer II A and 12 µM of SMART A oligonucleotide for 2 minutes at 65 °C, followed by 90 minutes of incubation at 42 °C for first strand cDNA synthesis. The single stranded cDNA was subsequently purified with NucleoSpin® Extraction Kit (BD Biosciences Clontech, Inc) using the protocol recommended by the manufacturer and eluted with 85 µl of RNAse free water.

5.3.2.2. Long Distance (LD) PCR Amplification

For LD PCR, a reaction mix containing 80 µl of single-strand cDNA, 172 µl of distilled water, 30 µl of 10X Advantage 2 PCR Buffer, 6 µl 50X dNTP (10 mM), 6 µl 5' PCR Primer II A (12 µM) and 6 µl 50X Advantage 2 Polymerase Mix was aliquoted into 3 tubes and placed into a MJ Research, Inc. Model PTC-100 Programmable Thermal Cycler. Samples were initially denatured at 94 °C for 1 minute followed by 36 cycles of 95 °C for 15 seconds, 65 °C for 30 seconds and 68 °C for 6 minutes. After 21 cycles, 2 aliquots were removed and refrigerated at 4 °C. From the last sample, aliquots

(5 µl) were collected every 3 cycles from cycle 21 to 36 and analyzed on a 1% agarose gel to optimize the number of cycles. After the number of cycles was optimized, the 2 tubes stored at 4 °C were subjected to additional cycles.

The amplicons were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), concentrated to 70 µl using n-butanol and purified using CHROMA SPIN™-1000 columns (BD Biosciences Clontech, Inc.) using the protocol recommended by the manufacturer.

5.3.2.3. Digestion with Rsa I

Purified PCR products were digested with *Rsa* I (10U) restriction endonuclease at 37 °C for 3 h. Completion of digestion was assessed on a 1% agarose gel prior to purification of the fragments using the NucleoSpin® Extraction Kit (BD Biosciences Clontech, Inc) according to the recommended protocol. The DNA was precipitated with ethanol and dissolved in 6.7 µl of TNE (Tris base, NaCl, EDTA) buffer.

5.3.3. Suppressive Subtractive Hybridization (SSH)

The Clontech PCR-Select™ cDNA Subtraction Kit (BD Biosciences Clontech, Inc) was used to construct two directions libraries (forward and reverse) according to the manufacturer's recommendation. In brief, 1 µl of purified *Rsa* I digested cDNA was diluted with 5 µl of sterile water and the appropriate adaptors were ligated using 400 units of T4 DNA ligase at 16 °C overnight. In the first hybridization, a reaction mix (4 µl) containing 15 ng of tester (differentially expressed transcripts) cDNA, 450 ng of driver (reference cDNA i.e. cDNA derived from either VIP stimulated or control

pituitary glands without the ligation of adaptors) and 1 µl of 4X hybridization buffer was incubated at 98 °C for 1.5 min followed by incubation at 68 °C for 8 hours. In the second hybridization, 1 µl of driver cDNA (350 ng) was overlaid with 1 drop of mineral oil and denatured at 98 °C for 1.5 min prior to the addition of the first hybridization products. Following incubation at 68 °C overnight, 200 µl of dilution buffer pH 8.3 was added (20 mM HEPES pH 6.6, 20 mM NaCl, 0.2 mM EDTA pH 8.0).

5.3.3.1. PCR Amplification

Following the second hybridization, a reaction mix contained 1 µl of diluted cDNA, 19.5 µl of deionized H_2O, 2.5 µl of 10 X Advantage 2 PCR Buffer, 0.5 µl of dNTP (10 mM), 1 µl of PCR Primer 1 (10 µM) and 0.5 µl of 50X Advantage 2 Polymerase Mix was amplified for 27 cycles (94 °C for 10 seconds, 66 °C for 30 seconds and 72 °C for 1.5 minutes). Subsequently, the PCR mixture was diluted 1:10 with sterile water and used in nested PCR. The reaction mix contained 1 µl of diluted cDNA from primary PCR, 18.5 µl of deionized H_2O, 2.5 µl of 10 X PCR Buffer, 0.5 µl of dNTP (10 mM), 1 µl of nested PCR Primer 1 (10 µM), 1 µl of nested PCR Primer 2R (10 µM) and 0.5 µl of r*Taq* DNA polymerase (5000 units/ml) (Amersham Biosciences, Inc). Following denaturation at 94 °C for 1 minute, PCR amplification consisted of 15 cycles of 94 °C for 10 seconds, 66 °C for 30 seconds and 72 °C for 1.5 minutes. An extension step of 72 °C for 10 minutes was added to the final cycle.

5.3.4. Cloning of Genes

Prior to ligation into the pDrive Cloning Vector (Qiagen Inc.), PCR products were purified with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Inc) according to the recommended protocol. Following ligation into the pDrive vector, Qiagen EZ Competent Cells were transformed using the heat shock procedure, plated onto LB agar plates containing ampicillin (100 μg/ml), IPTG (50 μM) and X-gal (80 μg/ml) and incubated at 37 °C overnight. About 100 positive clones from each library were randomly picked and inoculated into 3 ml of liquid LB medium containing ampicillin (100 μg/ml) and grown at 37 °C overnight. Plasmids from these bacteria were purified using established methods and inserts were confirmed by restriction analysis prior to DNA sequencing at the Genome Québec Innovation Centre. Clones were sequenced on both strands using SP6 and M13 reverse primers. The DNA sequences obtained were analyzed using the search engines provided by the National Center for Biotechnology Information (NCBI).

5.3.5. Dot Blot Assay

For dot blotting, 400 ng of plasmid DNA was denatured in 800 μl of 6 X SSC containing 0.1 vol of 1 M NaOH at 37 °C for 5 minutes and transferred to charged nylon membrane (Hybond N+, Amersham Biosciences, Inc) using a 96 well Minifold® System I Dot Blot apparatus (Schleicher and Schuell BioScience, Inc.). Four replicates were made for each library. Subsequently, the membranes were denatured using 1.5M NaCl and 0.5M NaOH for 5 minutes and neutralized in a solution containing 1.5M NaCl, 0.5M Tris-HCl (pH 7.2) and 1mM EDTA for 1 minute prior to baking the membrane at 80 °C for 2 hours. Probes were prepared from reverse transcribed RNA

obtained from VIP stimulated or control samples using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences, Inc). The concentration of the cDNAs was adjusted to 10 ng/µl and 100 ng of cDNA was denatured in a boiling water bath for 5 minutes and cooled on ice for 5 minutes prior to the addition of 10 µl of horseradish peroxidase reagent and 10 µl of glutaraldehyde solution. Probes were then incubated at 37 °C for 10 minutes and maintained on ice until required. Membranes were pre-hybridized with 10 ml ECL Gold Hybridization Buffer (Amersham Biosciences Inc.) at 42 °C for 15 minutes in a rotisserie hybridization incubator. Subsequently, 10 µl of the appropriately labelled probe per blot was added to the hybridization buffer and the membranes were incubated overnight at 42 °C. Following hybridization, the membranes were washed using 25 ml of a solution containing 6 M urea, 0.4 % SDS and 0.5 X SSC at 42 °C for 20 minutes. This washing step was repeated twice for 10 minutes before 2 additional washes with 10 ml of 2X SSC at room temperature for 5 minutes. Following exposure of the membrane to hydrogen peroxide and luminol for 1 minute, chemiluminescence was detected on Kodak X-ray film. The resultant images were digitalized using Quantity One Software (Bio-Rad Laboratories, Inc.).

5.3.6. Radioimmunoassay (RIA) and Western Blotting of PRL

The concentration of PRL in the media was measured in triplicate 100 µl aliquots by radioimmunoassay (RIA) as described by Guémené *et al.* (1994). The intra-assay coefficient of variation was 5.8 %. For western blotting of PRL content of pituitary glands, extracts from a pool of 6 glands from each group were prepared and

blotted as reported by Bédécarrats *et al.* (1999a). Immunoreactive bands were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer's protocol. After exposure to Kodak X-ray film, the image was digitalized and the proportion of each band was measured by Quantity One Software (Bio-Rad Laboratories, Inc.).

5.3.7. Quantitative PCR (Q-PCR) of Selected Genes

Total RNA was isolated from 10 pituitary glands of either VIP stimulated or control group and reverse transcribed using random hexamers and the SuperScript™ II Reverse Transcriptase Kit (Invitrogen Canada Inc, Burlington, Canada) according to the manufacturer's protocol. Primers were designed using DNAMAN (Lynnon Corporation) to amplify a total of 21 genes arbitrary chosen from both libraries by Q-PCR in order to confirm the efficacy of the SSH procedure. In addition, PRL was assessed by Q-PCR. The primer sequences and genes are shown in Table 5.1a and 5.1b. The gene products were short in length (approximately 100-200 bp), except the one for PRLR which was 371 bp. The Q-PCR assays were performed using Brilliant® SYBR® Green QPCR Master Mix kit (Stratagene, Inc.) according to the manufacturer's protocol and were optimized for each of 21 genes. In brief, a reaction mix containing 12.5 µl of 2X master mix, 0.5 µl of sense and anti-sense primers (10 µM), 0.375 µl of reference dye (final =30 nM), 9.125 µl of distilled water and 2 µl of diluted single strand cDNA (diluted according to the transcript abundance) was placed into the MX3000P™ real-time PCR system (Stratagene, Inc.). The PCR conditions were an initial denaturation at 95 °C for 10 minutes followed by a 2 step amplification program (95 °C for 30 seconds

and 60 °C or 55 °C for 1 minute) for 40 cycles. Following completion of the cycles, specificity of the amplification was assessed by melting curve analysis (60 °C for 30 seconds to 95 °C at 0.5 °C/second) and by electrophoretic separation of the amplicons on agarose gels to check for correct size. Fluorescence data were collected twice at the end point of the annealing step and continuously during dissociation curve. Levels of 18S rRNA (Ambion, Inc.) in the samples were analyzed by Q-PCR to standardize for RNA content. The relative changes of gene expression between the 2 samples were analyzed by using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$) described by Livak and Schmittgen (2001). In brief, Ct is the cycle at which the increase in fluorescence becomes logarithmic. The change in fold expression is calculated based on the difference of Ct of the samples, which is normalized to the Ct of the reference gene (18S rRNA). It was assumed that the amplification efficiency of the sample and the reference gene are approximately equal.

5.4. RESULTS

5.4.1. Content of PRL in Pituitary Extracts and Media

Stimulation of pituitary glands collected from ED 24 turkeys with VIP resulted in a significant increase in both pituitary content (2.8 fold increase) and levels of PRL in the medium (5.7 fold increase), whereas, levels of PRL in non-stimulated glands did not change. In addition, western blotting indicated that the proportion of PRL isoforms (24 and 27 kDa) in pituitary extracts was significantly altered by VIP treatment. In the control pituitaries, the glycosylated (G) variant represented about 13 % of total PRL,

whereas, following stimulation with VIP, G-PRL increased to 44 % during the 4 h incubation (Figure 5.1).

5.4.2. Identification of Clones from Both Libraries

Following plating of the libraries, 96 clones from each library were randomly picked and their plasmids were analyzed by restriction endonuclease digestion. A representative blot is shown in Figure 5.2 which indicated that the clones contained inserts of variable size. After the confirmation of the presence and size of DNA insert, the plasmids were submitted to the Genome Québec Innovation Centre for DNA sequencing on both strands. Five and eight clones, respectively, from the forward and reverse libraries did not produce readable sequence. The remaining 179 sequences were used to search the NCBI data banks using nucleotide and protein BLAST. Tables 5.2 and 5.3 present a summary of the blast search results for the forward and reverse libraries, respectively. A more detailed table of the blast search results is presented in appendix A and B.

In the forward library, 66 different putative genes were identified. Most of these (56) were represented once in the sequencing results, whereas, 7 genes were represented by multiple clones (2 to 9). Most of latter sequences were housekeeping genes with high abundance transcripts such as myelin basic protein (5 clones) and cytochrome I (7 clones). However, ariadne I was the most abundant clone (9 independent clones). Ariadne I is a component of the ubiquitination complex regulating cell protein levels and would normally be expressed at low levels in non-stimulated conditions (i.e. serum free medium without VIP). Of the 66 different putative genes identified, 34 (51%) have

not been assigned a function whereas 19 (29%) and 13 (20%) were identified as unique and housekeeping genes, respectively. In the reverse library, 79 different putative genes were identified. Most of these (73) were represented once in the sequencing results, whereas, 3 genes were represented by multiple clones (2 to 6). Some of latter sequences were housekeeping genes with high abundance transcripts such as cytochrome I (4 clones), whereas, ariadne 1 was the most abundant clone (6 clones). Of the 79 different putative genes identified, 40 (51%) have not been assigned a function whereas 30 (38%) and 9 (11%) were identified as putative and housekeeping genes, respectively. A total of 74 clones represented as ESTs and hypothetical genes have been identified in both libraries.

5.4.3. Differential Screening of the Subtracted Libraries by Dot Blot and Real-Time PCR

Dot blots were made in quadruplicate from the 96 clones (400 ng of plasmid) from each library and hybridized in duplicate to the appropriate labeled probe derived from the VIP treated or control group cDNA. Following densitometric analysis, the ratio of intensities was calculated to determine if genes were differentially expressed. In general, higher levels of expression were observed in the forward library hybridized to the probe derived from VIP stimulated total RNA. However, the results were difficult to interpret due to intra-assay variation between the duplicates (data not shown) and the choice of probe source. Similar inconsistencies occurred in the screening of the reverse library. As a result, about 10 clones from each library were chosen and analyzed by Q-PCR.

Ten genes from the forward library were analyzed by Q-PCR (Figure 5.3). Levels of transcript in total RNA were measured in both control and VIP stimulated pituitary glands and normalized to 18S rRNA content. The relative fold changes in expression between the groups were presented in Figure 5.3. Two genes failed to amplify (PFN and UBI) but in the other 8 cases, levels of transcript were higher in the RNA from pituitary glands stimulated with VIP. Most of the genes were expressed about 2 to 3 fold greater following VIP stimulation than in the control, whereas, levels of PRL transcript were increased about 5.5 fold. The latter increase was consistent with the observed increase in PRL content in both the pituitary gland and the medium following VIP stimulation.

Similarly, 11 genes were analyzed by Q-PCR from the reverse library and the relative fold changes in levels of transcript between the control and VIP treated groups were presented in Figure 5.4. Seven of the 11 genes were expressed at much lower levels in the pituitary gland following VIP stimulation than in the control, whereas, 4 genes were up-regulated. Most of the genes up-regulated were expressed about 3 fold greater following VIP stimulation than in the control. However, level of ariadne 1 was increased about 11 fold, suggesting that the corresponding transcript was ubiquitously expressed in pituitary gland and might have multiple roles in cell maintenance. Most of the genes down-regulated were repressed between 18.0 and 33.1 fold following VIP stimulation than in the control, whereas, the PRLR and EST6 genes were repressed about 5 and 7 fold, respectively.

5.5. DISCUSSION

On ED24, circulating levels of PRL are relatively low but gradually increase during late embryogenesis to reach a maxima during the peri-hatch period as lactotroph cells differentiate and mature (Leclerc *et al.*, in press). Stimulation of the ED24 pituitary gland with VIP resulted in a 2.8 fold and 5.7 fold increase in pituitary content and medium content of PRL, respectively (Fig. 5.1). These levels are consistent with endogenous levels of PRL observed at about the time of hatch (Leclerc *et al.*, in press; Bédécarrats *et al.*, 1999c). Moreover, stimulation with VIP induced a similar increase in proportion of glycosylated isoforms as is observed at hatch and also significantly (5.8 fold) increased the pituitary content of PRL mRNA. Hence, stimulation of the pituitary gland with VIP mimics, in part, changes in PRL which occur during a later stage of development. As a result, suppressive subtractive hybridization libraries were constructed from ED24 pituitary glands to study the influence of VIP and increased levels of PRL on gene transcription in the pituitary gland.

Preliminary analysis of clones obtained from both libraries indicated that most of the plasmids contained inserts of variable size (Fig. 5.2). Hence, 96 clones from each library were randomly chosen and sequenced. Sequence analysis revealed that about 50 % of the putative genes (74 of 145 non-redundant sequences) identified by comparison to data banks have as yet no assigned function (Tables 5.2 and 5.3). Of the remaining 71 genes with assigned function, 22 were identified as housekeeping genes. Many of the latter were represented by multiple clones indicating that not all of the commonly expressed genes in the stimulated and non-stimulated libraries were efficiently

subtracted. Although adriane1 is not usually considered a housekeeping gene, a total of 15 independent clones were sequenced (9 and 6 clones, respectively, in the forward and reverse library). Thus, this transcript was very abundantly expressed in both libraries. The ariadne1 protein homolog is involved in the ubiquitin/proteasome pathway and is capable of binding to the E2 conjugating enzyme as well as to the translation initiation factor E4. Thus, adriane1 participates in both ubiquitin mediated protein degradation as well as inhibition of translation (Tan *et al.*, 2003). The significance of the abundance of this transcript in both libraries is currently unknown. It may be related to culture of the pituitary glands in serum free medium which may inhibit transcription and translation. However, the large increase in PRL protein and PRL mRNA following VIP stimulation argues against a role in a general inhibition pathway.

In order to determine if the transcripts identified in the libraries did indeed respond to VIP stimulation, 9 and 11 clones were chosen from the forward and reverse library, respectively, and assessed using Q-PCR. In the forward library, all 9 clones had significantly higher levels of transcript in the VIP stimulated pituitary glands as expected from the cloning strategy (Fig. 5.3). Similarly analysis of transcripts in the reverse library, indicated that 7 of the 11 transcripts were expressed at lower levels in the VIP stimulated pituitary glands as would be expected from the cloning strategy (Fig. 5.4). However, 4 of the identified clones were expressed at higher levels. Three of these genes (SNX6 associated with the TGFβ receptor; Ariane1 associated with the ubiquitin pathway; and UNR associated with the removal of poly(A) tails from mRNA) are involved with cell senescence and/or the apoptotic pathway. This suggests that in the absence of growth factors, the pituitary cells may have been undergoing apoptosis or in

a pre-apoptotic state as indicated, for example, by the identification of caspase 3 in the reverse library. However, following VIP stimulation cells may have been reactivated. In support of this, genes such as thioredoxin (TRX, Fig. 5.3) were up-regulated about 2 fold suggesting a more active metabolism since TRX controls the cell redox state. Furthermore, superoxide dismutase (SOD3) was identified from the forward library although not assessed by Q-PCR. The latter protein is an important anti-oxidant involved in the detoxification of superoxide anion radicals. Whether the TRX or SOD3 genes were induced as a direct consequence of VIP or an indirect effect of biosynthesis of PRL or other gene products is not known. However, PRL is known to be a mitogen and the large increase in PRL (Fig. 5.1) following VIP stimulation suggests that PRL may be a major stimulatory factor. Moreover, both the presence of PRLR and the positive correlation between levels of PRL and the levels of PRLR in the pituitary glands of chickens and turkeys suggests that PRL may act in an autocrine/paracine fashion during the late stages of embryogenesis (Leclerc *et al.,* in press). In addition, VIP is known in mammals to have a variety of neutrotrophic and mitogenic effects (Moody *et al.,* 2003; Ravni *et al.*, 2006). Hence, it is possible that the induction of genes favouring cell survival and proliferation may be the result of the combinatorial action of both PRL and VIP.

Of the 145 putative genes identified in both libraries (Tables 5.2, 5.3), 21 were assessed by Q-PCR to determine if the transcript levels were indeed affected by VIP treatment. These measurements were only made at a single time point. Thus, further studies are required to determine how levels of these and other transcripts may vary during the late stages of ontogenesis (i.e. between ED24 and hatch) and their

physiological relevance. Notably, many of the identified sequences were related to cell proliferation genes, ubiquitin-proteasome pathway genes, apoptotic pathway genes, protein trafficking genes and cytoskeletal and extracellular matrix genes. It is beyond the scope of this thesis to further characterize these genes. However, the libraries should prove to be a valuable resource for studying the activation of the hypothalamo-lactotroph axis during late embryogenesis.

Table 5.1a. List of primers used to amplify selected genes using real-time PCR from the forward library.

Gene	Orientation	Primer	Temperature (°C)
FKBP12	Sense	5'-CGAAGATTAGAGTGGCGTTTGG-3'	60
	Antisense	5'-TCAGAGGGCGAAGATGACCA-3'	60
Hsp70	Sense	5'-CTGCTTGTCCTGGTCGCTTA-3'	60
	Antisense	5'-AACAGAGATAGGGTGGGAGC-3'	60
NAPOR	Sense	5'-CGCAGATGGTCTTACAGTTT-3'	60
	Antisense	5'-GGGTTACAGTTCCTTGGCT-3'	60
NPM	Sense	5'-GCTGGTCTCTTTGTTGAAGCA-3'	60
	Antisense	5'-GCCTGTTTATGTCAGTGGTCA-3'	60
PFN	Sense	5'-TGGCTGTCTTGTGATGTGG-3'	60
	Antisense	5'-GCAGGGAAAGAAATGGACAA-3'	60
CYP17	Sense	5'-TCTAAAGGTCACCGCATTGA-3'	60
	Antisense	5'-GCGATTCCTCATCTTACCCT-3'	60
sFRP1	Sense	5'-ATTGTTCTCTGGCTCGGGA-3'	60
	Antisense	5'-CATCCTAAATCCATCTCTCTGC-3'	60
TiPARP	Sense	5'-TCAGGGTTGCAGTAGAGTC-3'	60
	Antisense	5'-CAGGGCGCTTGTGTTTATG-3'	60
TRX	Sense	5'-GGCTGGAGATTAGACAAGACT-3'	60
	Antisense	5'-TGGTGATGTGGTGTTCATTG-3'	60
UBI	Sense	5'-GTCAAGCAAGATGCACA-3'	60
	Antisense	5'-CACTTAAACCACCGTGGAA-3'	60
PRL	Sense	5'-CTCCATTCCTTCTAAAAGC-3'	60
	Antisense	5'-GCAAACTCAGCAGATTCAT-3'	60

Abbreviations: FK506 bing protein 12 (FKBP12), Hsp70 heat shock protein (Hsp70), Neuroblastoma apoptosis-related RNA-binding protein (NAPOR), Nucleophosmin (NPM), Profilin (PFN), Steroidogenic enzyme cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17), Secreted frizzled related protein 1 (sFRP1), TCDD inducible polyADP-ribose polymerase (TiPARP), Thioredoxin (TRX), Ubiquitin I (UBI), Prolactin (PRL).

Table 5.1b. List of primers used to amplify selected genes using real-time PCR from the reverse library.

Gene	Orientation	Primer	Temperature (°C)
SNX6	Sense	5'-TCAGCAGATACCCGAG-3'	55
	Antisense	5'-GCATTAGGAACACAGGA-3'	55
ARI14/27	Sense	5'-TCAGCCGAAGAGAGAAGCG-3'	60
	Antisense	5'-CGCACTGCCACCCATTTA-3'	60
UNR	Sense	5'-CATCATCTCCTACTTTGAGC-3'	60
	Antisense	5'-AAACCTTCTCCACAACAATG-3'	60
PANK4	Sense	5'-TCACCTACAGCGAATCCC-3'	60
	Antisense	5'-GCACAGCCAGACCTTTAT-3'	60
TOMMA	Sense	5'-GTCAGCCCTAAAGACGCT-3'	60
	Antisense	5'-AACAACCACTCCCACAAC-3'	60
eIF4G2	Sense	5'-TTGGCTGGTTCTTTAGTC-3'	60
	Antisense	5'-AGTGTTCTGCTGATGTGT-3'	60
APG12	Sense	5'-GCTTCTTCATTACTGTCTTG-3'	60
	Antisense	5'-GAATCCCAGGAGGTGTG-3'	60
EST6	Sense	5'-GCATAAACCTCTCCATCT-3'	55
	Antisense	5'-TCTCTTCTTCTGACCCTT-3'	55
UNKc	Sense	5'-GAGAAATAAAGCAAGACAGC-3'	55
	Antisense	5'-AACCCAATGAAGAAGGAC-3'	55
EST45	Sense	5'-CCAAGAAAGAAATGACCTG-3'	55
	Antisense	5'-CTCAGAAACCCAAGAATGT-3'	55
PRLR	Sense	5'-AGGAAACATTTACCTGTTGGT-3'	60
	Antisense	5'-AAGCCATCCAGATCTGACATC-3'	60

Abbreviations: Sorting nexin 6 (SNX6), Ariadne homolog, ubiquitin-conjugating enzyme E2 (ARI14/27: clones 14 and 27), Upstream of NRAS (UNR), Pantothenate kinase 4 (PANK4), Translocase of outer mitochondrial membrane 70 homolog A (TOMMA), Eukaryotic translation initiation factor 4 γ 2 (eIF4G2), Autophagy protein 12-like (APG12), Clone 6 EST (EST6), Chromosome UNK clone (UNKc), Clone 45 EST (EST45), Prolactin receptor (PRLR).

Table 5.2. Brief summary of blast search results analyzing the clones (n=92) from the forward library.

Accession Number	Homologous Gene Identity to *Gallus gallus*	Brief Description of Gene Product	Clone ID
Putative genes			
M33698.1	*Meleagris gallopavo* glycoprotein hormone alpha-subunit precursor	alpha subunit of LH, FSH and TSH	FS01
NM_204371.1	Gap junction protein beta 1, 32kDa (connexin 32)	Cell to cell communication	FS09
NM_001031489.1	Hemoglobin beta chain (LOC428114)	Subunit of hemoglobin	FS28
AB055761.1	FK506 bing protein 12	Mediates immunosuppression by acting as the intracellular receptor for FK506	FS37
AY313844.1	*Taenopygia guttata* Cytochrome P450 17α-hydroxylase/17,20 lyase	Steroidogenic enzyme, partial codons	FS90
NM_205453.1	Thioredoxin	Cellular redox state	FS39
NM_205267.1	Nucleophosmin	Maturation of rRNA	FS48
XM_418622.1	Similar to vimentin (LOC420519)	Structural protein	FS69
NM_204553.1	Secreted frizzled-related protein 1	Antagonist of Wnt signaling pathway	FS83
M15861.1	Neural cell-adhesion molecule gene, exon 19	Structural protein in neural cells	FS04
NM_001006199.1	Far upstream element (FUSE) binding protein 3	Transcription factor that binds to the far upstream element of c-myc	FS40
NM_001029981.1	Oxidative stress responsive 1 (LOC419203)	Oxidative stress	FS70
XM_422828.1	Similar to TCDD-inducible poly(ADP-ribose) polymerase (LOC425026)	Adaptive response to TCDD	FS96
NM_053024.3, NM_002628.4	*Homo sapiens* Profilin 2, transcript variant 1, 2	Regulates actin polymerization	FS14
NM_001039313.1	Clathrin light polypeptide chain a (Lca)	Cellular transport	FS11
NM_001030549.1	Similar to mitogen-activated protein kinase 6 (LOC415419)	Signal transduction pathway	FS26

	Mus musculus	Regulation of RNA	
NM_010160.1	CUG triplet repeat, RNA binding protein 2 (NAPOR)	processing events such as alternative splicing and RNA editing	FS57
XM_415051.1	Similar to Rnps1 protein (LOC416756)	Pre-mRNA splicing activator	FS29
XM_420760.1	Similar to extracellular superoxide dismutase [Cu-Zn] precursor (LOC422810)	Removes superoxide radicals	FS53
Housekeeping genes			
Hsp70 AY143693.1, Hsc70 NM_205003.1, Hspa9b NM_001006147.1	Heat shock family	Multiple roles e.g. environmental stress, steroid receptor binding protein etc.	FS06, FS55, FS79, FS84.
NM_205280.1	Myelin basic protein	Binds to multiple target proteins such as calmodulin, actin, and tubulin	FS07, FS43, FS60, FS74, FS93.
Cyto. I/III AP003322.1 Cyto. VIIb AF255353.1 Cyto. b L08381.1	*Meleagris gallopavo* Cytochrome family	Oxidation-reduction reactions	Cytochrome I FS12, FS18, FS38, FS62, FS72, FS82, FS87. Cytochrome III: FS13 Cytochrome VIIb: FS58
NM_001030387.1	Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (*Drosophila*) (ariadne 1 mRNA)	Ubiquitination pathway	FS23, FS25, FS41, FS42, FS47, FS56, FS59, FS73, FS75.
r15S XM_425249.1 r60S XM_414453.1, NM_001030929.1, NM_001006241.1 r18S DQ018752.1	Ribosomal protein	Protein translation	Ribosomal 15S: FS32 Ribosomal 60S: FS03, FS81. Ribosomal 18S: FS52, FS64, FS80.
XM_415847.1	Similar to polyubiquitin (LOC417602); (Ubiquitin 1)	Post-translational modification of proteins - protein half-life	FS35, FS50, FS94.

NM_204305.1	Glyceraldehyde-3-phosphate dehydrogenase	Gene with multiple functions	FS76
NM_001030620.1	Calnexin mRNA	Folding of glycoproteins	FS88
X15841.1	Mitochondrial ATPase 8 and ATPase 6 genes	ATP synthesis	FS92
Unknown genes			
		NA	FS02, FS05, FS08, FS10, FS15, FS17, FS19, FS21, FS22, FS24, FS27, FS30, FS31, FS33, FS34, FS36, FS44, FS45, FS46, FS49, FS51, FS54, FS61, FS63, FS65, FS66, FS67, FS71, FS77, FS78, FS85, FS86, FS91, FS95.
		NA	

NA: not available

Table 5.3. Brief summary of blast search results analyzing the clones (n=88) from the reverse library.

Accession Number	Homologous Gene Identity	Brief Description of Gene Product	Clone ID
Putative genes			
XM_129836.7	*Mus musculus* PHD finger protein 3, transcript variant 1	Chromatin-mediated transcriptional regulation	RS03
XM_421235.1	Similar to sorting nexin 6 (TRAF4-associated factor 2) (LOC423319)	Association with receptor serine-threonine kinases	RS05
NM_058172.3	*Homo sapiens* Anthrax toxin receptor 2	Involved in pathogenesis	RS12
AY675346.1	Polymyositis/scleroderma autoantigen 1	Role in endosome fusion and negative regulator of cell growth	RS15
NM_205104.1	Bleomycin hydrolase (Aminopeptidase H)	Aminopeptidase and endopeptidase activities	RS17
M33698.1	*Meleagris gallopavo* Pituitary glycoprotein hormone alpha-subunit precursor	alpha subunit of LH, FSH and TSH	RS18, RS20.
NM_001006468.1	Nucleobindin 2	Calcium binding protein, modulator of matrix maturation	RS36
XM_414943.1	Similar to transcription factor III C alpha chain (LOC416646), partial mRNA	Cellular transcription factor	RS52
XM_420794.1	Similar to syntaxin 18 (LOC422848)	Involved in transport between the endoplasmic reticulum and Golgi	RS57
NM_001429.2	*Homo sapiens* E1A binding protein p300	Involved in the development of carcinomas, have multiple functions	RS58
NM_001030918.1	Cold shock domain containing E1, RNA-binding, mRNA (UNR)	Regulation of mRNA stability and internal ribosome entry site (IRES)-dependent mRNA translation	RS62
XM_417556.1	Similar to pantothenate kinase 4; hypothetical protein Fang1 (LOC419394)	Catalyzes the rate-controlling step in the coenzyme A (CoA)	RS63

		biosynthetic pathway	
XM_416605.1	Similar to translocase of outer mitochondrial membrane 70 homolog A (LOC418387)	Composed of receptors, a channel protein, and its modulators that function together to import proteins into mitochondrial compartments	RS65
AB096098.1	eIF4G-related protein NAT1	Shutoff of protein translation in programmed cell death	RS69
NM_205391.1	P311 POU mRNA	Regulated at multiple levels by pathways that control cellular growth, transformation, motility, protein half- life	RS73
XM_416595.1	Similar to transcription termination factor, RNA polymerase II; lodestar protein; human factor 2 (LOC418376)	Transcription termination, pausing, processing, interacts with transcriptional factors	RS86
NM_204725.1	Caspase-3, apoptosis-related cysteine peptidase	Cysteine protease family, Apoptotic process and proteolytic activation, cleavage of eIF4GII appears to correlate with the inhibition of translation	RS88
NM_00100847 9.1	SUB1 homolog (*S. cerevisiae*), mRNA (Activated RNA polymerase II transcription cofactor 4)	Modulated by phosphorylation, interact with transcriptional activators and the basal transcription machinery	RS90
AL122015.17	*Homo sapiens* DNA sequence from clone RP1-8L15 on chromosome 11p13 (Homeodomain-interacting protein kinase 3)	Involved in growth control pathways such as development and physiological functions of their target tissues (male steroidogenesis), apoptosis, phosphorylation, etc.	RS92
XM_424458.1	Similar to microsomal signal peptidase 21 kDa subunit (SPase 21 kDa subunit) (LOC426850)	Signal peptide processing, process of protein translocation, integration into endoplasmic reticulum	RS100
NM_00100618	Transmembrane emp24	Cellular protein transport	RS102

6.1	domain trafficking protein 2		
NM_001004411.1	Serpin peptidase inhibitor, clade I (neuroserpin), member 1	Regulators of intracellular and extracellular proteolytic pathways expressed in brain, linked to neuroendocrine cell activation	RS103
XM_414624.1	Similar to F-box and leucine-rich repeat protein 3B) (LOC416306)	Cell cycle, protein-protein interactions, ubiquitin-dependent protein degradation	RS104
AF502149.1	Regulator of G-protein signaling protein 2	Regulate membrane signaling pathways of numerous cell types	RS108
XM_419213.1	Similar to proenkephalin A precursor (LOC421131)	Activate sensory neuron--specific G protein--coupled receptors; post-translational processing	RS110
XM_424963.1	Similar to autophagy protein 12-like (LOC427390)	Multiple biological roles: protein and organelle degradation with the lysosome/vacuole, type II programmed cell death, etc.	RS112
XM_420710.1	Similar to RAS-like family 11 member B (LOC422756)	Signal transduction, pleiotropic effect on cellular growth and differentiation	RS113
NM_001031266.1	SEC22 vesicle trafficking protein homolog B (*S. cerevisiae*)	Mediators of endoplasmic reticulum-to-Golgi protein transport	RS51
NM_001031483.1	Solute carrier family 41, member 2	Novel eukaryotic gene family, homology with Mg^{2+} transporters	RS66
NM_001031142.1	Heterogeneous nuclear ribonucleoprotein D-like	mRNA biogenesis and mRNA metabolism	RS96
Housekeeping genes			
L00101.1	CaM gene encoding calmodulin, exon 6	Calcium metabolism	RS01
Cyto. I/III AP003322.1 Cyto. b L08381.1 NADH4 AP003195.2	*Meleagris gallopavo, Coturnix japonica* Cytochrome family	Oxidation-reduction reactions	Cytochrome I: RS04, RS26, RS59, RS94. Cytochrome

			III: RS55 Cytochrome b: RS81 NADH4: RS114
NM_001030387.1	Ariadne homolog, ubiquitin-conjugating enzyme E2 binding prot. 1	Development; ubiquitin/proteasome pathway	RS14, RS24, RS27, RS44, RS56, RS75.
AF069771.1	Lactate dehydrogenase H subunit mRNA	Gene with several actions : Environmental adaptation, T cell function, etc.	RS77
NM_204305.1	Glyceraldehyde-3-phosphate dehydrogenase	Gene with multiple functions	RS80
AP003322.1	16S ribosomal RNA	Protein translation	RS83
Unknown genes			
		NA	RS02, RS06, RS07, RS08, RS10, RS11, RS16, RS19, RS28, RS29, RS32, RS35, RS38, RS41, RS42, RS45, RS46, RS61, RS64, RS67, RS68, RS70, RS71, RS74, RS76, RS79, RS82, RS84, RS87, RS89, RS91, RS93, RS97, RS106, RS107, RS109, RS111, RS115, RS116, RS118.

NA: not available

Figure 5.1. The effect of VIP on pituitary content of PRL isoforms. The upper band represents the glycosylated (27 kDa) isoform, whereas, the lower band (25 kDa) is the non-glycosylated isoform of PRL.

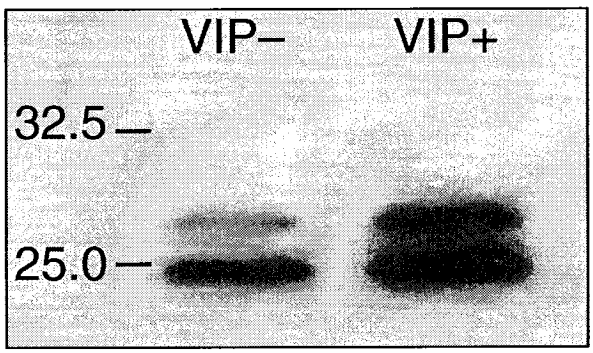


Figure 5.2. Clones were randomly chosen and their plasmids were analyzed by restriction endonuclease digestion (0.75U/μl *Eco*RI). A representative blot indicated that clones contained inserts of variable size from the forward and reverse libraries.

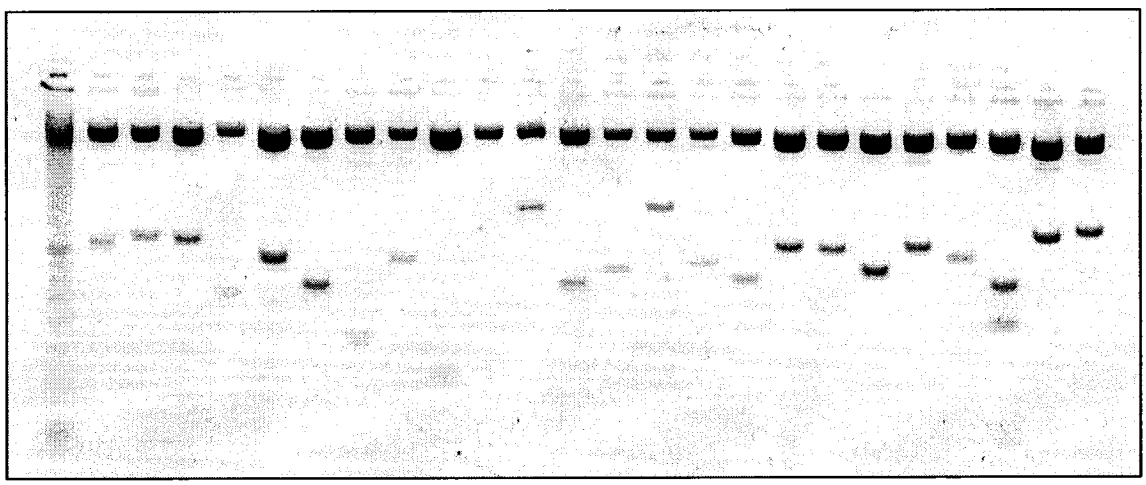


Figure 5.3. The relative fold changes in expression among ten genes from the forward library.

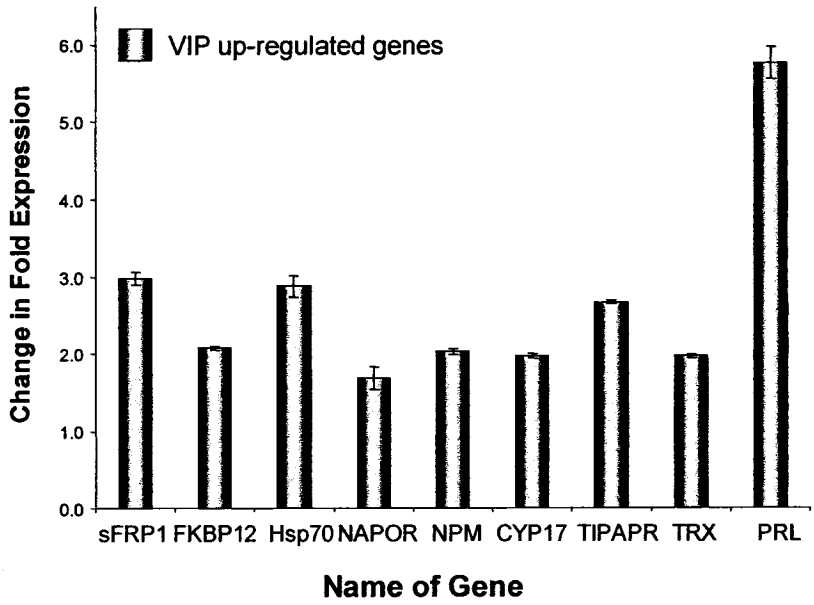
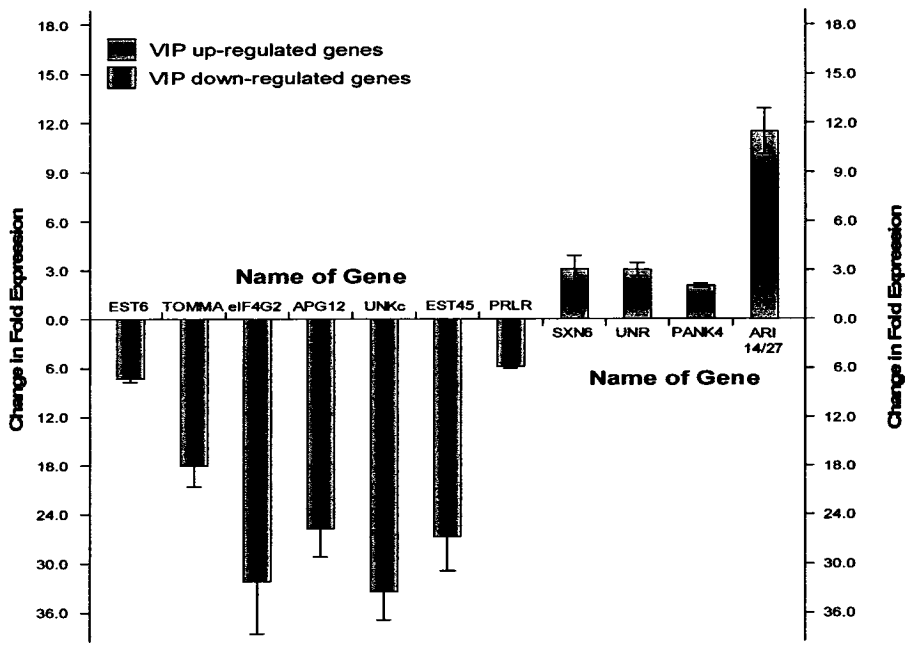


Figure 5.4. The relative fold changes in expression among eleven genes from the reverse library.



CHAPTER 6. GENERAL CONCLUSIONS

In oviparous animals, embryogenesis proceeds largely independently of maternal influences transferred across the fetal placental unit. Hence, avian species provide a unique system for studying the ontogeny of the endocrine system and the physiological adaptions that the animal must make in preparation for *ex ovo* life. The latter is especially true for precocial species such as chickens and turkeys. Associated with the hatching process is a change from chorio-allantoic to pulmonary gas exchange, the development of the complexus musculus to pip the shell, a large increase in metabolism to support the hatching process, maturation of the immune system to prepare for new antigen exposure and functional maturation of the endocrine system to adapt *ex ovo* life. During this time a large increase in circulating levels of PRL occurs and PRL may be expected to interact in a modulatory fashion in a number of these physiological adaptions.

In the current studies, pituitary content of PRL mRNA, PRL and circulating levels of PRL were shown to increase during the last week of embryogenesis in turkeys and chickens to reach maxima during the peri-hatch period. Associated with this increase was a significant increase in levels of PRLR mRNA in various putative target tissues. This suggested that PRL was up-regulating its own receptor at about the time of hatch. Increased cellular receptor number would in turn increase the responsiveness of various organs. For example, at the time of hatch the embryo adapts to *ex ovo* life and osmoregulation is an important factor that is affected by PRL. Thus, the large increase in levels of PRLR in renal tissue is likely to sensitise the fetal kidney in order to resorb

NaCl from the urine. Similarly, increases in PRLR in the pancreas, liver and gonad could be an adaption to increased responsiveness to PRL to meet the metabolic demands imposed by the hatching process.

The increased levels of PRL at hatch were also associated with an increase in receptor mRNA in both the pituitary gland and the hypothalamus. Since the hypothalamus is the source of PRL releasing factor(s), this suggested that PRL may have been modulating its own secretory control through hypothalamic receptors on neurons. Such a short loop feedback mechanism implies retrograde transport of PRL in the hypophyseal portal system or active transport across the blood brain barrier via the choroid plexus. The presence of PRLR in pituitary tissue suggests an autocrine and or paracrine effect of PRL to modulate its own secretion. However, the cell types which have these receptors were not assessed. Culture of embryonic day 24 pituitary glands with VIP (the main PRL stimulatory factor) resulted in a large increase in the release of PRL that mimicked endogenous levels observed at hatch. Again there was an associative rise in levels of PRLR mRNA. This effect seemed to be specific to PRL since non-PRL producing tissues such as the hypothalamus or the gonad had no observed increase in levels PRLR although VIP receptors are present. This provides further evidence that PRL, itself, via various feedback loops may be regulating to some extent its own secretion.

Since levels of PRL increase significantly at the time of hatch, a model system was used to investigate the roles that PRL may have on the pituitary gland. Embryonic day 24 (low levels of endogeneous PRL) pituitary glands were stimulated with VIP to achieve PRL levels consistent with endogenous levels of PRL at the time of hatch and

suppressive subtractive hybridization libraries were constructed. Clones sequenced from these libraries would be expected to represent genes associated with the direct effects of VIP but moreover, the direct effects of PRL induced by VIP. Of the non-redundant clones (n=145) sequenced about one half have as yet not been assigned a biological function. The majority of the rest were associated with functions related to cell proliferation, protein trafficking, the ubiquitin-protease pathway, apoptosis and cytoskeletal and extracellular matrix. It is beyond the scope of this thesis to further characterize the various genes and their involvement in the hatching process. However, these libraries should provide a wealth of information for future studies.

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APPENDIX A. Identification of Nucleic Acid Sequences of Clones Belonging to the Reverse cDNA Library from Turkey Pituitary Gland Embryos (ED24) after Using BLASTn (Basic Local Alignment Search Tool for nucleotide) Search Programs.

Clones	Length (bp)	Accession Number	Species	Gene	Submission	Description of the Gene Product	Bit (Score)
RS01	504	DQ217268.1	*Taeniopygia guttata*	Clone 0061P0019C01 calmodulin variant 1-like	Oct. 2005	Whole brain	137
		L00101.1/ CHKCAM6	*Gallus gallus*	CaM gene exon 6	Apr. 1993	Brain gDNA; calcium ion binding; G-prot. coupled receptor signaling protein pathway	119
RS02	378	CR406571.1	*Gallus gallus*	Clone EST	May 2004	Limbs; unknown biological function	347
RS03	414	XM_601217.2	*Bos Taurus*	similar to PHD finger protein 3	Sep. 2005	GNOMON; Domain in the central regions of transcription elongation factor S-II	161
		XM_343548.2	*Rattus norvegicus*	PHD finger protein 3	Apr. 2005	GNOMOM; DNA binding, translation elongation factor act.; development, regulation of transcription DNA-dependent	153
RS04 RS26 RS59 RS94	1084 1116 1235 962	AP003322.1	*Gallus gallus*	Mitochondrial DNA, COI	Jul. 2005	Whole Blood; Cytochrome C and Quinol oxidase polypeptide I; Oxidoreductase act.	886 854 609 850
RS05	734	CR353054.1	*Gallus gallus*	Clone EST	Mar. 2004	Whole embryo	1185
		XM_421235.1	*Gallus gallus*	Sorting nexin 6 (TRAF4-associated	Jul. 2004	GNOMOM; catalytic act., phosphoinositide binding;	636

				factor 2)		intracellular signaling cascade	
RS06	471	CR354246.1	*Gallus gallus*	Clone EST	Mar. 2004	Limbs	161
RS07	1100	AADN01060104.1	*Gallus gallus*	Chromosome 5 genomic contig44.127, whole genome shotgun sequence	Feb. 2004	Blast chicken sequences (WGS contigs); same program, but different database	652
		CF132784.1	*Gallus gallus*	Ventral neuroepithelium cDNA library cDNA similar to sulfatase	Dec. 2003	Blast chicken sequences (ESTs); different database	≤ 50
		AC145963.5	*Gallus gallus*	BAC clone CH261-20A24 from chromosome unknown	Dec. 2005	Genomic DNA, complete sequence	≤ 40
RS08	1062	AADN01060104.1	*Gallus gallus*	Chrom. 5 genomic contig44.127, whole genome shotgun sequence	Jul. 2004	Blast chicken sequences (WGS contigs)	159
		CF132784.1	*Gallus gallus*	Ventral neuroepithelium cDNA library cDNA similar to sulfatase	Dec. 2003	Blast chicken sequences (ESTs)	≤ 50
		AJ627213.1	*Gallus gallus*	Primary C-C chemokine receptor cluster	Feb. 2004	Genomic DNA	≤ 40
RS10	1121	AADN01027748.1	*Gallus gallus*	Chromosome 12 genomic contig16.28	Feb. 2004	Whole genome shotgun sequence	686
RS32	763	AADN01004890.1	*Gallus gallus*	Chromosome 2 genomic contig2.736	Feb. 2004	WGS	519
RS35	665	AADN01	*Gallus*	Chromosome 5	Feb. 2004	WGS	515

		060930.1	*gallus*	genomic contig45.56			
RS38	766	AADN01 015439.1	*Gallus gallus*	Chromosome 3 genomic contig8.305	Feb. 2004	WGS	234
RS46	595	AADN01 045697.1	*Gallus gallus*	Chromosome W genomic contig130.176	Feb. 2004	WGS	242
RS64	488	AADN01 042018.1	*Gallus gallus*	Chromosome 1 genomic contig127.137	Feb. 2004	WGS	145
RS82	321	AADN01 071566.1	*Gallus gallus*	Chromosome 1 genomic contig56.140	Feb. 2004	WGS	295
RS89	699	AADN01 100359.1	*Gallus gallus*	Chromosome 9 genomic contig86.43	Feb. 2004	WGS	309
		AC17230 4.2	*Gallus gallus*	BAC clone CH261-75C12 from chromosome ul	Jan. 2006	Genomic DNA	≤ 50
RS93	632	AADN01 002803.1	*Gallus gallus*	Chromosome 5 genomic contig1.667	Feb. 2004	WGS	307
RS109	625	AADN01 103621.1	*Gallus gallus*	Chromosome 11 genomic contig90.49	Feb. 2004	WGS	644
RS115	635	AADN01 079692.1	*Gallus gallus*	Chromosome 26 genomic contig65.139	Feb. 2004	WGS	329
RS116	500	AADN01 034372.1	*Gallus gallus*	Chromosome 2 genomic contig21.283	Feb. 2004	WGS	353
All these clones		AJ009799 .1	*Gallus gallus*	mRNA for ABC transporter protein	Apr. 2005	P-glycoprotein; gene: cmdr1; Intestine	≤ 50
All these clones		AB09599 6.1	*Gallus gallus*	Ghsr1aV mRNA for growth hormone secretagogue receptor type 1a variant	Oct. 2003	Pituitary	≤ 50
RS11	1081	BU25428 7.1	*Gallus gallus*	cDNA clone ChEST33212 5'	Nov. 2002	Limbs; blast chicken sequences (ESTs)	165

		BX927385.15	*Danio rerio*	Zebrafish DNA sequence from clone DKEY-76P7 in linkage group 10	Jan. 2006	Genomic DNA; complete sequence	84
RS12	534	CR353495.1	*Gallus gallus*	Clone EST	Mar. 2004	Liver	835
		NM_058172.3	*Homo sapiens*	Anthrax toxin receptor 2	Oct. 2005	ANTXR2; metal ion binding, cytoskeletal protein binding, receptor act.; cell growth and/or maintenance	307
RS14 RS24 RS27 RS44 RS56 RS75	1022 826 824 1220 744 1196	NM_001030387.1	*Gallus gallus*	Ariadne homolog, ubiquitin conjugating-enzyme E2 binding protein, 1 (Drosophila) (ARIH1)	Oct. 2005	Bursal lymphocytes	256 232 234 274 246 238
RS15	830	CR385575.1	*Gallus gallus*	Clone EST	Apr. 2004	Whole Embryo	347
		NM_001034828.1	*Gallus gallus*	Exosome component 9 (EXOSC9)	Oct. 2005	Polymyositis/scleroderma autoantigen 1; 3'-5'-exoribonuclease act., RNA bnding, hydrolase act., protein binding; immune response, rRNA processing	143
RS16	1088	AADN01042017.1	*Gallus gallus*	Chrom. 1 genomic contig1.127	Feb. 2004	WGS	555
RS76	1084	AADN01020126.1	*Gallus gallus*	Chrom. 4 genomic contig11.332	Feb. 2004	WGS	864
RS79	1125	AADN01018612.1	*Gallus gallus*	Chrom. 8 genomic contig10.586	Feb. 2004	WGS	551

RS84	1172	AADN01 072398.1	*Gallus gallus*	Chrom. 1 genomic contig57.41	Feb. 2004	WGS	559
RS17	1108	NM_2051 04.1	*Gallus gallus*	Bleomycin hydrolase (BLMH)	Jul. 2005	Aminopeptidase activity, bleomycin hydrolase; proteolysis	1160
RS18 RS20	814 817	TKYPGH X/M3369 8.1	*Meleagris gallopavo*	Pituitary glycoprotein hormone alpha-subunit precursor	Oct. 2001	Pituitary; hormone act.; cell communication, signal transduction	1396
RS19	1122	AC09071 1.3	*Homo sapiens*	Clone RP11-89H1 map 8q21-q23	Oct. 2002	Genomic DNA	174
RS28	786	AC14595 9.3	*Gallus gallus*	Chrom. UNK clone CH261-17M10	Mar. 2004	Genomic DNA	700
RS29	1155	AADN01 035438.1	*Gallus gallus*	Chrom. 6 genomic contig22.148	Feb. 2004	WGS	773
		Y14971.1	*Gallus gallus*	mRNA for K60 protein	Apr. 2005	Macrophage like; CXC chemokine K60; gene: K60; chemokine act.; immune response	≤ 50
RS36	531	NM_0010 06468.1	*Gallus gallus*	Nucleobindin 2 precursor (LOC423071)	Jul. 2005	NUCB2; DNA binding, calcium ion binding	706
RS41 RS42	626 1235	AJ009799 .1	*Gallus gallus*	mRNA for ABC transporter protein	Apr. 2005	P-glycoprotein; gene: cmdr1; Intestine	≤ 50
		AB09599 6.1	*Gallus gallus*	Ghsr1aV mRNA for growth hormone secretagogue receptor type 1a variant	Oct. 2003	Pituitary	≤ 50
RS45	982	AADN01 044007.1	*Gallus gallus*	Chrom. 1 genomic contig29.217	Feb. 2004	WGS	741
		XM_4168 06.1	*Gallus gallus*	Chrom X (ORF23) LOC418607	Jul. 2004	GNOMOM; catalytic act.	672
		BX64040	*Gallus*	DNA sequence from	Sep. 2003	Genomic DNA	≤ 50

		2.2	*gallus*	clone WAG-10A12			
RS51	584	NM_0010 31266.1	*Gallus gallus*	SEC22 vesicle trafficking prot. like 1	Oct. 2005	SEC22L1, bursal lymphocytes; vesicle-mediated transport	343
RS52	493	XM_4149 43.1	*Gallus gallus*	Transcription factor III C alpha chain	Jul. 2004	Partial mRNA, GNOMOM; DNA binding, oxidoreductase act.; metabolism	367
RS55	677	AB07330 1.1	*Cortunix chinensis*	Mitochondrial DNA COIII	Dec. 2003	Liver; Cytochrome-c oxidase activity, oxidoreductase activity; electron transport	494
RS57	642	XM_4207 94.1	*Gallus gallus*	Syntaxin 18	Jul. 2004	GNOMOM; catalytic act., protein transporter act.; intracellular protein transport	484
RS58	648	NM_0014 29.2	*Homo sapiens*	E1A binding protein p300	Feb. 2006	Adenovirus-associated; many functions and processes	200
RS61	260	CR390435 .1	*Gallus gallus*	Clone EST	Apr. 2004	Ovary	161
		CR353961 .1		Clone EST	Mar. 2004	Heart	159
		CR389862 .1		Clone EST	Apr. 2004	Ovary	155
		CR354235 .1		Clone EST	Mar. 2004	Limbs	155
		CR388444 .1		Clone EST	Apr. 2004	Kidney + Adrenal gland	145
RS62	1117	NM_0010 30918.1	*Gallus gallus*	Cold shock domain containing E1, RNA-binding (CSDE1)	Oct. 2005	Upstream of NRAS; Bursal lymphocytes; DNA & RNA binding; regulation of transcription DNA-dependent	1306
RS63	1116	XM_4175 56.1	*Gallus gallus*	Pantothenate kinase 4; hypothetical protein	Jul. 2004	GNOMOM; protein of unknown function	1011

				Fang1 (LOC419394)			
RS65	687	XM_416605.1	*Gallus gallus*	Translocase of outer mitochondrial membrane 70 homolog A (LOC418387)	Jul. 2004	GNOMOM; catalytic act.	876
		NM_014820.3	*Homo sapiens*	Translocase of outer mitochondrial membrane 70 homolog A (yeast) (TOMM70A)	Jan. 2006	Nuclear gene encoding mitochondrial protein; auxiliary transport protein act.; transport	147
RS66	754	NM_001031483.1	*Gallus gallus*	Solute carrier family 41, member 2	Oct. 2005	SLC41A2, bursal lymphocytes; cation transporter act.; cation transport	613
RS67	1152	NP_001026665.1	*Gallus gallus*	Serine/arginine repetitive matrix 1	Oct. 2005	Bursal lymphocyte; PWI, domain in splicing factors; nuclear mRNA splicing via spliceosome	≤ 50
		XP_426527.1	*Gallus gallus*	Chromosome 10 open reading frame 12	Jul. 2004	GNOMOM; ATP binding, catalytic act.; ATP synthesis coupled proton transport	≤ 40
RS68	1127	AADN01015714.1	*Gallus gallus*	Chromosome 3 genomic contig8.580	Feb. 2004	WGS	1160
		BU372378.1	*Gallus gallus*	cDNA clone ChEST799i10 5'	Nov. 2002	Kidney + adrenal; blast chicken sequences (ESTs): different database	563
		CR352439.1	*Gallus gallus*	Clone EST	Mar. 2004	Heads	≤ 50
RS69	443	XM_420967.1	*Gallus gallus*	Translation repressor	Jul. 2004	similar to Eukaryotic translation initiation factor 4 gamma 2 (eIF-4-gamma 2) (eIF-4G 2) (eIF4G 2) (p97) (Death associated protein 5) (DAP-5); translation initiation	365

						factor act.; regulation of translational initiation	
		AB096098.1	*Gallus gallus*	eIF4G-related protein NAT1	Feb. 2005	Also known as p97, DAP5, EIF4G2; eIF4G-related protein NAT1	365
		AF093110	*Gallus gallus*	Death associated protein 5-like (DAP5)	Mar. 1999	Cardiac neural crest, embryo day 9	327
RS70	1122	AJ721096.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal lymphocytes; nucleic acid binding or transposase act.; DNA transposition	283
		AJ720136.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal lymphocytes; heme binding, metal ion binding, monooxygenase act.; electron transport	276
		AJ720109.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal; GTP binding, GTPase act.; mitochondrial fusion; example: dynamin	204
		AJ719569.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal; protein tyrosine phosphatase act.; intracellular signaling cascade, protein amino acid dephosphorylation	204
RS71	1137	*Homo sapiens, Mus musculus*, etc.					≤ 50
RS73	409	NM_205391.1	*Gallus gallus*	P311 POU (3.1) mRNA	Apr. 2005	Cerebellum, hippocampus, olfactory bulb	636
		NM_004772.1	*Homo sapiens*	Chromosome 5 open reading frame 13	Mar. 2005	Brain; synonyms: P311, PTZ17, D4S114, PRO1873; unknown	163

						function and process	
RS74	1202	AB075383.1	*Tetrao urogallus*	Microsatellite TUD9 sequence	May 2001	Genomic DNA	137
		CR389266.1	*Gallus gallus*	Clone EST	Apr. 2004	Cerebrum	109
RS77	712	BX929571.2	*Gallus gallus*	Clone EST	Mar. 2004	Cerebrum	1025
		AF069771.1	*Gallus gallus*	Lactate dehydrogenase H subunit (LDH-B) mRNA	Jul. 1998	Heart	1019
		NM_204177.1	*Gallus gallus*	Lactate dehydrogenase H subunit (LDH-B) mRNA	Apr. 2005	L-lactate dehydrogenase act., oxidoreductase act.; anaerobic glycolysis, tricarboxylic acid cycle intermediate metabolism	1011
RS80	504	J00850.1/ NM_204305.1	*Gallus gallus*	Gapdh(glyceraldehyde-3-phosphate dehydrogenase)	Apr. 1993/ Apr. 2005	Embryonic cardiac muscle	773
		NM_204305.1	*Gallus gallus*	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Apr. 2005	NAD binding, GAPDH phosphorylating act., oxidoreductase act.; glucose metabolism, glycolysis, nuclearmembrane fusion	765
RS81	1118	L08381.1/ TKYMTCYTBA	*Meleagris gallopavo*	Mitochondrion cytochrome b gene	Jun. 1994	Liver; Oxidation-reduction reactions	1316
RS83	572	AB073301.1	*Coturnix chinensis*	Mitochondrial DNA, 16S ribosomal RNA	Dec. 2003	Liver; general functions	121
RS86	838	XM_416595.1	*Gallus gallus*	Transcription termination factor, RNA polymerase II;	Jul. 2004	GNOMOM; ATP & DNA binding, helicase act.; involved in nuclear mRNA splicing via spliceosome,	1229

				lodestar protein; human factor 2 (LOC418376)		transcription termination, *Hsa*	
		AL391476.20	*Homo sapiens*	DNA sequence from clone RP11-229A19 on chromosome 1	May 2005	gDNA, contains the 3' end of the TTF2 gene for RNA polymerase II transcription termination factor, the TRIM45 gene for tripartite motif-containing45, a ribosomal protein S15a (RPS15A) pseudogene, the gene for immune costimulatory protein B7-H4 and a CpG island	66
RS87	1091	CD527006.1	*Gallus gallus*	ED18 retina library cDNA clone-214_polyTVN	Jun. 2003	Blast chicken sequences (ESTs); same program, but different database	76
		AJ719569.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal Lymphocyte; protein tyrosine phosphatase act.; intracellular signaling cascade, protein a.a. dephosphorylation	66
		AJ632302.1	*Gallus gallus*	Partial TLR7 gene for toll-like receptor 7	Apr. 2005	Innate immunity; transmembrane receptor act.	58
		NM_001007488.2	*Gallus gallus*	Toll-like receptor 1 (TLR1)	Feb. 2005	Genomic DNA	58
RS88	398	NM_204725.1	*Gallus gallus*	Caspase-3, apoptosis-related cysteine peptidase (CASP3)	Oct. 2005	Involved in apoptosis: caspase act., cysteine-type peptidase act.; proteolysis	206
RS90	353	NM_001008479.1/ AJ720221.1	*Gallus gallus*	SUB1 homolog (SUB1)/ Hypothetical protein	Oct. 2005 Jan. 2005	Bursal Lymphocytes; activated RNA polymerase II transcription cofactor 4; DNA binding, transcription coactivator act.; regulation of transcription, DNA-	222

						dependent	
RS91	582	CR391544.1 CR389665.1	*Gallus gallus* *Gallus gallus*	Clone EST Clone EST	Apr. 2004 Apr. 2004	Muscle Limbs	270 258
RS92	1127	XM_853441.1	*Canis familiaris*	similar to Homeodomain-interacting protein kinase 3 (Homolog of protein kinase YAK1) (Fas-interacting serine/threonine-protein kinase) (FIST) (Androgen receptor-interacting nuclear protein kinase) (ANPK), transcript variant 3	Aug. 2005	GNOMOM	115
		HSDJ8L15/ AL122015.17	*Homo sapiens*	DNA sequence from clone RP1-8L15 on chromosome 11p13	Jan. 2006	gDNA; Contains the HIPK3 gene for homeodomain-interacting protein kinase 3; ATP, nucleotide and serine/threonine kinase binding, transferase act.; (anti) or apoptosis, negative regulation of JNK act., peptidyl serine/threonine phosphorylation, regulation of transcription, DNA-dependent	111
RS96	545	NM_001031142.1	*Gallus gallus*	Heterogeneous nuclear ribonucleoprotein D-	Oct. 2005	Bursal lymphocytes; nucleotide and poly (A) binding; RNA	521

				like (HNRPDL)		processing	
RS97	880	XM_421228.1	*Gallus gallus*	RIKEN cDNA D930036F22 gene (LOC423311)	Jul. 2004	GNOMOM; carboxypeptidase A act.; proteolysis	424
RS100	816	XM_424458.1	*Gallus gallus*	Microsomal signal peptidase 21 kDa subunit (SPase 21 kDa subunit) (SPC21) (LOC426850)	Jul. 2004	GNOMOM; serine-type peptidase act.; proteolysis, signal peptidase processing	1170
RS102	770	NM_001006186.1	*Gallus gallus*	Transmembrane emp24 domain trafficking protein 2 (TMED2)	Oct. 2005	Bursal lymphocytes; coated vesicle membrane protein; transport	662
RS103	1106	NM_001004411.1	*Gallus gallus*	Serpin peptidase inhibitor, clade I (Neuroserpin), member 1 (SERPINI1)	Oct. 2005	Serine (or cysteine) protease inhibitor; serine-type endopeptidase inhibitor act.	1497
		NM_005025.2	*Homo sapiens*	Serpin peptidase inhibitor, clade I (Neuroserpin), member 1 (SERPINI1)	Oct. 2005	Synonyms: PI12, neuroserpin; serine-type endopeptidase inhibitor act.; CNS + PNS development	234
RS104	1129	CR390926.1	*Gallus gallus*	Clone EST	Apr. 2004	Cerebrum	617
		XM_414624.1	*Gallus gallus*	F-box/LRR-repeat protein 3B (F-box and leucine-rich repeat protein 3B) (LOC416306)	Jul. 2004	GNOMOM; catalytic act	565
RS106	1101	AB119275.1	*Mus musculus*	GATA6 gene, exon 1	Mar. 2005	Genomic DNA	1051
		AJ720337	*Gallus*	Hypothetical protein	Jan. 2005	Bursal lymphocytes; DNA	1025

		.1	*gallus*			binding, transpoase act.; DNA transposition	
		AJ720136.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal lymphocytes; iron ion binding, monooxygenase act.; electron transport (Cytochrome P450)	882
RS107	365	AC145973.3	*Gallus gallus*	BAC clone CH261-2E2 from chromosome unknown	Dec. 2005	Genomic DNA	466
RS108	331	NM_204395.1	*Gallus gallus*	Regulator of G-protein signaling 2, 24kDa (RGS2)	Apr. 2005	Dorsal root ganglion neurons; alternatively spliced; *Homo sapiens*: GTPase activator act., calmodulin binding, signal transducer act.; cell cycle, regulation of G-protein coupled receptor protein signaling pathway	331
RS110	507	XM_419213.1	*Gallus gallus*	similar to proenkephalin A precursor (LOC421131)	Jul. 2004	Neuropeptide signaling pathway	714
RS111	825	BX936078.1	*Gallus gallus*	Clone EST	Feb. 2004	Cerebrum	650
		BX936226.2		Clone EST	Mar. 2004	Pancreas	646
		BX934467.2		Clone EST	Mar. 2004	Limbs	640
		BX932596.2		Clone EST	Mar. 2004	Cerebellum	591
		AY842452.1	*Mus musculus*	Embryonic development factor 1	Jan. 2005	Nucleoporin	210

				(Ed1)			
RS112	544	BX932259.2	*Gallus gallus*	Clone EST	Apr. 2004	Heart	311
		BX930955.1	*Gallus gallus*	Clone EST	Feb. 2004	Limbs	311
		BX934958.1	*Gallus gallus*	Clone EST	Feb. 2004	Small intestine	303
		BX931890.1	*Gallus gallus*	Clone EST	Feb. 2004	Kidney + Adrenal gland	303
		XM_424963.1	*Gallus gallus*	Autophagy protein 12-like (APG12-like) (LOC427390)	Jul. 2004	GNOMOM; autophagic vacuole formation	276
RS113	1071	XM_420710.1	*Gallus gallus*	RAS-like family 11 member B (LOC422756)	Jul. 2004	GNOMOM; GTP binding; protein transport, small GTPase mediated signal transduction	751
RS114	453	AP003195.2	*Coturnix japonica*	Mitochondrial DNA (NADH 4)	Jul. 2002	Whole Blood; Oxidation-reduction reactions	291
RS118	560	BU284494.1	*Gallus gallus*	cDNA clone ChEST884m16 5'	Nov. 2002	Brain; Blast chicken sequences (ESTs); same program, but different database	82
		AC145975.3	*Gallus gallus*	/BAC clone CH261-4H14 from chromosome unknown	Dec. 2005	Genomic DNA	72
		CR388530.1	*Gallus gallus*	Clone EST	Apr. 2004	Kidney + Adrenal gland	58
		U25026.1	*Gallus gallus*	Clone pNG17 cell division cycle control protein 37 (cdc37)	Feb. 1996	Partial cds	52
Clones RS47, RS105 and RS117 were eliminated from the list because of bad sequencing data.							

APPENDIX B. Identification of Protein Sequences of Clones Belonging to the Reverse cDNA Library from Turkey Pituitary Gland Embryos (ED24) after Using BLASTx (Basic Local Alignment Search Tool for protein) and Blast Chicken Genomic Sequences Search Programs.

Clones	Length (bp)	Accession Number	Species	Protein	Submission	Description of the Protein	Bit (Score)
RS01	504	AADN01 015462.1	*Gallus gallus*	Chromosome 3 genomic contig8.328	Feb. 2004	WGS	809
RS02	378	AADN01 084828.1	*Gallus gallus*	Chrom. 3 genomic contig170.26	Feb. 2004	WGS	347
RS07	1100	AADN01 060104.1	*Gallus gallus*	Chrom. 5 genomic contig44.127	Feb. 2004	WGS	652
RS11	1081	AADN01 053057.1	*Gallus gallus*	Chrom. 15 genomic contig37.30	Feb. 2004	WGS	989
RS12	534	AADN01 007098.1	*Gallus gallus*	Chrom. 4 genomic contig3.617	Feb. 2004	WGS	850
RS14	1022	AADN01 086258.1	*Gallus gallus*	Chrom. 4 genomic contig28371.1	Feb. 2004	WGS	92
RS15	830	AADN01 007392.1	*Gallus gallus*	Chrom. 4 genomic contig3.911	Feb. 2004	WGS	361
RS16	1088	AADN01 042017.1	*Gallus gallus*	Chrom. 1 genomic contig127.136	Feb. 2004	WGS	555
RS28	786	AADN01 012044.1	*Gallus gallus*	Chrom. 1 genomic contig6.65	Feb. 2004	WGS	700
RS29	1155	AADN01 035438.1	*Gallus gallus*	Chrom. 6 genomic contig22.148	Feb. 2004	WGS	773
RS32	763	AADN01 004890.1	*Gallus gallus*	Chrom. 2 genomic contig2.736	Feb. 2004	WGS	519

RS35	665	AADN01 060930.1	*Gallus gallus*	Chrom. 5 genomic contig45.56	Feb. 2004	WGS	515
RS45	982	AADN01 044007.1	*Gallus gallus*	Chrom. 1 genomic contig29.217	Feb. 2004	WGS	741
RS51	584	AADN01 070557.1	*Gallus gallus*	Chrom. 8 genomic contig55.10	Feb. 2004	WGS	339
RS68	1127	AADN01 015714.1	*Gallus gallus*	Chrom. 3 genomic contig8.580	Feb. 2004	WGS	1166
RS76	1084	AADN01 020126.1	*Gallus gallus*	Chrom. 4 genomic contig11.332	Feb. 2004	WGS	864
RS82	321	AADN01 071566.1	*Gallus gallus*	Chrom. 1 genomic contig65.140	Feb. 2004	WGS	295
RS87	1091	AADN01 018642.1	*Gallus gallus*	Chrom. 8 genomic contig10.616	Feb. 2004	WGS	537
RS89	699	AADN01 100359.1	*Gallus gallus*	Chrom. 9 genomic contig86.43	Feb. 2004	WGS	309
RS92	1127	AADN01 084808.1	*Gallus gallus*	Chrom. 3 genomic contig170.6	Feb. 2004	WGS	783
RS96	545	AADN01 106607.1	*Gallus gallus*	Contig13393.1	Feb. 2004	WGS	174
RS104	1129	AADN01 107986.1	*Gallus gallus*	Chrom. 13 genomic contig95.116	Feb. 2004	WGS	617
RS106	1101	AADN01 066049.1	*Gallus gallus*	Contig2950.1	Feb. 2004	WGS	1055
RS107	365	AADN01 013906.1	*Gallus gallus*	Chrom. 1 genomic contig7.374	Feb. 2004	WGS	466
RS109	625	AADN01 103621.1	*Gallus gallus*	Chrom. 11 genomic contig90.49	Feb. 2004	WGS	644
RS112	544	AADN01 005367.1	*Gallus gallus*	Chrom. Z genomic contig102.78	Feb. 2004	WGS	276

RS115 All these clones	635	AADN01 079692.1	*Gallus gallus*	Chrom. 26 genomic contig65.139	Feb. 2004	WGS	329
		Unknown proteins	*Gallus gallus*				≤ 40
RS03	414	BU38913 0.1	*Gallus gallus*	cDNA clone ChEST782h1 5'	Nov. 2002	Brain, ED16; Blast Chicken sequences; different database	557
		XP_42669 8.1	*Gallus gallus*	Glypican-5 precursor	Jul. 2004	Catalytic act.; extracellular matrix	≤ 40
RS04 RS26 RS59 RS94	1084 1116 1235 962	BAD1111 6.1	*Gallus gallus*	Cytochrome oxidase subunit I	Mar. 2004	Whole Blood; Cytochrome C and Quinol oxidase polypeptide I	345-531
		P24984	*Coturnix japonica* (Japanese Quail)	COI	Feb. 2006	Pathway: Respiratory chain; terminal step	343-531
RS05	734	CAG3192 1.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal lymphocytes	305
		NP_06707 2.2 + NP_68941 9.1	*Homo sapiens*	Sorting nexin 6 isoform a + b	Nov. 2005	Intracellular trafficking; tumor necrosis factor receptor-associated factor 4(TRAF4)-associated factor 2	298
		XP_42123 5.1	*Gallus gallus*	Sorting nexin 6 (TRAF4-associated factor 2)	Jul. 2004	GNOMOM; catalytic act., phosphoinositide binding; intracellular signaling cascade	244
RS08 RS24 RS27 RS42 RS56	1062 826 824 1235 744	Unknown proteins	*Gallus gallus*				≤ 40
RS10	1121	AADN01 027748.1	*Gallus gallus*	Chrom. 12 genomic contig16.28	Feb. 2004	WGS	686

		XP_41963 5.1	*Gallus gallus*	similar to G2	Jul. 2004	GNOMOM; catalytic act.	≤ 40
RS17	1108	BAA1923 6.1	*Gallus gallus*	Aminopeptidase H	Feb.1999	Peptidase C1B subfamily	283
		AAH5557 2.1	*Danio rerio*	Bleomycin hydrolase	Jul. 2005	Whole body; cysteine-type endopeptidase act., hydrolase act.; proteolysis	245
RS18 RS20	814 817	AAA4962 9.1	*Meleagris gallopavo*	Pituitary glycoprotein hormone alpha-subunit precursor	Oct. 2001	Pituitary; hormone act.; cell communication, signal transduction	261
		AAL5775 5.1	*Cervus Nippon*	Follicle stimulating hormone alpha-subunit	Jan. 2002	Glycoprotein	223
		AAC6390 0.1	*Trichosurus vulpecula*	Gonadotrophin alpha subunit	Aug. 2001	Pituitary	223
		AAX8543 0.1	*Felis catus*	Thyrotropin alpha	Apr. 2005	Thyroid stimulating hormone alpha	220
		CAA7617 7.1	*Equus burchellii*	Luteinizing hormone alpha subunit	Apr. 2005	Glycoprotein	201
RS36	531	CAG3273 2.1	*Gallus gallus*	Hypothetical protein	Jan. 2005	Bursal Lymphocytes	152
		NP_05805 3.1	*Mus musculus*	Nucleobindin 2	Oct. 2004	DNA and calcium-binding protein; calcium ion homeostasis	≤ 50
RS41	626	BAE1611 4.1	*Gallus gallus*	NADH dehydrogenase subunit 5	Jul. 2005	Whole Blood; KEGG pathway: Oxidative phosphorylation	118
RS44	1220	DR42877 3.1	*Gallus gallus*	Chicken eye (embryo) Unnormalized cDNA clone nax07e07 5'	Jun. 2005	Whole eye ED15; several ESTs giving the same bit score; blast chicken sequences; different database	274
		XP_41498 7.1	*Gallus gallus*	Ankyrin repeat and SOCS box-containing	Jul. 2004	Atrophin-1; catalytic act.; intracellular signaling cascade	≤ 40

				protein 13			
RS52	493	XP_414943.1	*Gallus gallus*	Transcription factor (TFIIIC) alpha chain, partial cds	Jul. 2004	GNOMOM; DNA binding, oxidoreductase act.; metabolism	213
RS55	677	BAB62921.1	*Coturnix japonica*	Cytochrome c oxidase polypeptide III	Jul. 2002	Subunits I, II and III form the functional core of the enzyme complex	310
		CAA36631.1	*Gallus gallus*	Cytochrome oxidase subunit III	Apr. 2005	Cytochrome-c oxidase activity, oxidoreductase activity; electron transport	308
RS57	642	XP_420794.1	*Gallus gallus*	Syntaxin 18	Jul. 2004	GNOMOM; catalytic act., protein transporter act.; intracellular protein transport; Intracellular trafficking and secretion; t-SNARE complex subunit	71
RS62	1117	CAH65302.1	*Gallus gallus*	Hypothetical protein	Apr. 2005	Bursal lymphocytes; Cold shock protein domain	360
		XP_533016.1	*Canis familiaris*	Similar to Cold shock domain protein E1 (UNR protein) N-ras upstream gene protein) isoform 5	Aug. 2005	Similar to Cold shock domain protein E1 (UNR protein) (N-ras upstream gene protein)	359
RS63	1116	XP_417556.1	*Gallus gallus*	Pantothenate kinase 4; hypothetical protein Fang1	Jul. 2004	GNOMOM; protein of unknown function	246
		AAH43496.1	*Homo sapiens*	Pantothenate kinase 4	Jun. 2004	ATP binding, nucleotide binding, transferase act.; coenzyme A biosynthesis	218
RS64	488	AADN01042018.1	*Gallus gallus*	Chrom. 1 genomic contig127.137	Feb. 2004	WGS	145

		XP_41961 6.1	*Gallus gallus*	similar to 1-acyl-sn-glycerol-3-phosphate acyltransferase delta (1-AGP acyltransferase 4) (1-AGPAT 4) Lysophosphatidic acid acyltransferase-delta) (LPAAT-delta) (1-acylglycerol-3-phosphate O-acyltransferase 4) (UNQ499/PRO1016)	Jul. 2004	GNOMOM; acetyltransferase act., catalytic act.; metabolism	≤ 40
RS65	687	XP_41660 5.1	*Gallus gallus*	Translocase of outer mitochondrial membrane 70 homolog A	Jul. 2004	GNOMOM; catalytic act.	86
RS67 RS71	1152 1137	Unknown proteins	*Homo sapiens, Mus musculus, etc.*				≤ 50
RS70	1122	AADN01 066049.1	*Gallus gallus*	Contig2950.1	Feb. 2004	WGS	278
RS73	409	AAF6961 3.1	*Homo sapiens*	PRO1873	May 2001	Fetus, liver; unknown function and process	78
RS75	1196	BU41092 8.1	*Gallus gallus*	cDNA clone ChEST163d24 5'	Nov. 2002	Chicken EST search: muscle	244
		XP_42821 2.1	*Gallus gallus*	Keratin	Jul. 2004	GNOMOM; structural constituent of cytoskeleton	≤ 40
RS77	712	P13743	*Anas*	L-lactate	Feb. 2006	Catalytic act., used as a crystalline;	293

			platyrhynchos/	dehydrogenase B chain (LDH-B) (Epsilon crystalline)		Anaerobic glycolysis; final step	
RS79	1125	AADN01018612.1	*Gallus gallus*	Chrom. 8 genomic contig10.586	Feb. 2004	WGS	551
		XP_424390.1	*Gallus gallus*	similar to putative chromatin modulator	Jul. 2004	Chromatin remodeling protein, contains PhD zinc finger; catalytic act., protein and zinc ion binding; regulation of transcription, DNA-dependent	≤ 40
RS80	504	P00356	*Gallus gallus*	Glyceraldehyde-3-phosphate dehydrogenase	Feb. 2006	Brain; catalytic act., Carbohydrate degradation; glycolysis; pyruvate from D-glyceraldehyde 3-phosphate: step 1	133
RS81	1118	P50663	*Meleagris gallopavo*	Cytochrome b	Feb. 2006	Function: Component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is a respiratory chain that generates an electrochemical potential coupled to ATP synthesis (By similarity) Synonyms: COB, CYTB, MTCYB	369
RS83	572	BU104013.1	*Gallus gallus*	cDNA clone ChEST21l20 5'	Nov. 2002	Chicken EST search: Whole embryo	125
		XP_417218.1	*Gallus gallus*	Ten-m4, partial	Jul. 2004	GNOMOM; catalytic act.	≤ 40
RS86	838	XP_41659	*Gallus*	Transcription	Jul. 2004	GNOMOM; ATP & DNA bidning,	471

		5.1	*gallus*	termination factor RNA polymerase II; lodestar protein; human factor 2		helicase act.	
RS88	398	NP_990056.1	*Gallus gallus*	Caspase 3	Oct. 2005	Involved in apoptosis and apoptosis-related cysteine protease: caspase act., cysteine-type peptidase act.; proteolysis	71
RS97	880	XP_421228.1	*Gallus gallus*	RIKEN cDNA D930036F22 gene	Jul. 2004	GNOMOM; carboxypeptidase A act.; proteolysis	163
		AAH63867.1	*Homo sapiens*	Chrom 14 open reading frame 125 protein	Apr. 2004	GNOMOM; binding	124
RS100	816	XP_424458.1	*Gallus gallus*	Microsomal signal peptidase 21 kDa subunit SPase 21 kDa subunit	Jul. 2004	Intracellular trafficking and secretion; serine-type peptidase act.; proteolysis, signal peptidase processing	356
RS103	1106	NP_001004411.1	*Gallus gallus*	Neuroserpin	Apr. 2005	serine-type endopeptidase inhibitor activity; (serine protease inhibitor that inhibits plasminogen activators)	541
RS108	331	BU421624.1	*Gallus gallus*	cDNA clone ChEST926m9 5'	Nov. 2002	Chicken EST search: Chondrocytes isolated from growth plate cartilage	319
		XP_422860.1	*Gallus gallus*	similar to misato	Jul. 2004	Tubulin (Cytoskeleton), GNOMOM; catalytic act.	≤ 40
RS110	507	XP_419213.1	*Gallus gallus*	Proenkephalin A precursor	Jul. 2004	Neuropeptide signaling pathway	77
RS111	825	XP_417190.1	*Gallus gallus*	Hypothetical protein	Jul. 2004	The function of these proteins is unknown, but they form part of the spliceosome and are thus thought to be involved in mRNA splicing,	282

						GNOMOM	
		AAW32096.1/ CAB96547.1	*Mus musculus*	Embryonic development factor 1/ Hypothetical protein	Jul. 2000	Nucleoporin/ Putative coiled coil region containing nuclear protein	269
RS114	453	CAA36634.1	*Gallus gallus*	NADH dehydrogenase subunit IV	Jul. 2004	Liver; Synonyms: NADH4, ND4	70
RS116	500	AADN01034372.1	*Gallus gallus*	Chrom. 2 genomic contig21.283	Feb. 2004	WGS	353
		NP_001026690.1	*Gallus gallus*	Hypothetical protein	Aug. 2005	Bursa lymphocytes; similar to chromosome 20 open reading frame 6; uncharacterized conserved protein	≤ 40
RS118	560	AADN01092644.1	*Gallus gallus*	Chrom. Z genomic contig78.253	Feb. 2004	WGS	442
		XP_419789.1	*Gallus gallus*	Hypothetical protein	Jul. 2004	GNOMOM	≤ 40
		XP_418212.1	*Gallus gallus*	similar to DNA-binding protein RFX2	Jul. 2004	DNA binding, catalytic act., transcription regulator act.; regulation of transcription, DNA-dependent	≤ 40
		AAC60280.1	*Gallus gallus*	Unknown	Oct. 1997	Retrotransposon CR1; consensus sequence	≤ 40
RS06	471	AADN01015951.1	*Gallus gallus*	Contig1308.4	Feb. 2004	WGS	226
RS19	1122	AADN01034165.1	*Gallus gallus*	Chrom. 2 genomic contig21.76	Feb. 2004	WGS	575
RS38	766	AADN01015439.1	*Gallus gallus*	Chrom. 3 genomic contig8.305	Feb. 2004	WGS	234
RS46	595	AADN01	*Gallus*	Chrom. W genomic	Feb. 2004	WGS	242

		045697.1	*gallus*	contig130.176			
RS58	648	AADN01 052270.1	*Gallus gallus*	Chrom. 1 genomic contig36.222	Feb. 2004	WGS	283
RS61	260	AADN01 047640.1	*Gallus gallus*	Chrom. 4 genomic contig32.99	Feb. 2004	WGS	170
RS66	754	AADN01 013743.1	*Gallus gallus*	Chrom. 1 genomic contig7.211	Feb. 2004	WGS	611
RS69	443	AADN01 074250.1	*Gallus gallus*	Chrom. 5 genomic contig59.81	Feb. 2004	WGS	365
RS74	1202	AADN01 035538.1	*Gallus gallus*	Chrom. 6 genomic contig22.248	Feb. 2004	WGS	133
RS84	1172	AADN01 072398.1	*Gallus gallus*	Chrom. 1 genomic contig57.41	Feb. 2004	WGS	559
RS90	353	AADN01 081169.1	*Gallus gallus*	Chrom. Z genomic contig166.190	Feb. 2004	WGS	222
RS91	582	AADN01 105527.1	*Gallus gallus*	Contig1592.4	Feb. 2004	WGS	272
RS93	632	AADN01 002803.1	*Gallus gallus*	Chrom. 5 genomic contig1.667	Feb. 2004	WGS	307
RS102	770	AADN01 053203.1	*Gallus gallus*	Chrom. 15 genomic contig37.176	Feb. 2004	WGS	636
RS113	1071	AADN01 047641.1	*Gallus gallus*	Chrom. 4 genomic contig32.100	Feb. 2004	WGS	886
Clones RS47, RS105 and RS117 were eliminated from the list because of bad sequencing data.							